Imatinib increases apoptosis index through modulation of survivin subcellular localization in the blast phase of CML cells

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Using MTT, Annexin V/flow cytometry, immunocytochemistry, subcellular fractionation, and Western blotting assays we analyzed the effect of imatinib in two blast phase of chronic myeloid leukemia (CML) cell lines: K562 P-glycoprotein (Pgp)-negative, and Lucena, Pgp-positive. In K562 cell line, the high apoptosis index induced by imatinib was associated with the survivin predominantly in the nucleus. In the Lucena cell line, the low apoptosis index induced by imatinib was associated with a cytoplasmatic survivin localization. Pgp and survivin might be subject to the same molecular regulation, and therefore represent a therapeutic target in the blast phase of CML.

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1. Introduction

Patients in the chronic myeloid leukemia (CML) blast phase are highly refractory to treatment [1,2]. One of the most important imatinib resistance mechanisms in the CML is the inability of the Bcr-Abl CML cells to undergo apoptosis, which is caused by the BCR-ABL mutations decreasing the affinity of imatinib binding [3]. CML cells may also acquire resistance to imatinib by other mechanisms, such as P-glycoprotein (Pgp) overexpression [4–6]. Pgp is a protein encoded by the ABCB1 gene that acts as a drug efflux pump [7]. Another common resistance mechanism in neoplasms is the survivin overexpression, an inhibitor apoptosis protein (IAP) that exerts a central role in cell division and in apoptosis inhibition by blocking the activation of caspasases [6,8]. Our group showed that the modulation of reactive oxygen species enhances imatinib mediated cell death through survivin downregulation, pointing out this IAP as a putative therapeutic target [9]. Survivin has a diversified subcellular localization, the nuclear pool is associated with its function in mitosis progression, while the cytoplasmatic pool is related to apoptosis inhibition [10]. Despite the absence of a clear consensus in the literature, some studies show that differences in nuclear or cytoplasmic compartmentalization may have an influence on the prognosis of different types of cancer patients [11]. However, the prognostic value of survivin subcellular localization in CML is completely unknown.

An interesting and recent object of speculation is the interaction between Pgp or ABCB1 and some IAPs during the process of apoptosis resistance induced by various stimuli. The downregulation of Pgp suppressed the survivin mRNA expression, whereas the reverse impact was not observed in MCF-7 and MCF-7/Adr cell lines, suggesting that survivin might play a key role in the multidrug resistance (MDR) phenotype in the presence of Pgp [12]. This group recently demonstrated that survivin transcription was associated with Pgp/ABCB1 overexpression through the PI3k/Akt pathway in the same cell lines [13]. Moreover, a recent work of our group showed strong positive correlation between survivin and Pgp expressions in late CML patients [14]. The purpose of the present study was to verify if imatinib and/or ara-C would be capable of modifying the apoptosis index through modulation of the survivin subcellular localization in blast phase CML cell lines exhibiting distinct Pgp status.

We analyzed the survivin subcellular localization in two CML cell lines, the K562 Pgp-negative and its vincristine-resistant counterpart K562-Lucena Pgp-positive [15] and evaluated survivin as having a role on chemotherapeutic resistance when exposed to imatinib and/or ara-C. We demonstrated that imatinib was capable of inducing a high rate of apoptosis in the sensitive K562 Pgp-negative CML cell line through survivin modulation from cytoplasmic to nuclear subcellular localization. On the other hand,
survivin modulation from nuclear pool to cytoplasmatic localization was related to the low rate of apoptosis observed in the resistant K562-Lucena cell line overexpressing Pgp. These findings suggest a molecular pathway of connection between survivin and Pgp.

2. Materials and methods

2.1. Cell lines

The human blast CML K562 cell line and its vincristine-resistant derivative K562-Lucena were cultured in RPMI 1640 medium supplemented with fetal bovine serum (FBS) 10%. Besides displaying Pgp/ABC1, Lucena cell line is resistant to oxidative stress, ultraviolet-A radiation and has high catalase content [15]. Proteomic analysis showed that Lucena presents 36 proteins differentially expressed, 14 downregulated and 22 upregulated, in comparison to K562 cell line [16].

2.2. Drugs

Imatinib mesylate (imatinib) and cyclosporine A (CSA, Atoxic®) were provided by Novartis Brazil and diluted in RPMI-1640 prior to use. 1-β-D-arabinofuranosylcytosine (cytarabine, ara-C) was provided by Meizler Biopharma, Brazil. Stock solutions were stored at 4°C.

2.3. Analysis of cell viability

The growth inhibition of the CML K562 and Lucena cell lines was determined by a colorimetric MTT assay (MTT, GE Healthcare, USA) [17]. Briefly, cells were incubated for 24, 48 and 72 h in the presence or absence (control) of various concentrations of imatinib, (0.5, 1.0, 2.5, and 5.0 μM), ara-C (0.1, 0.5, 1.0, 2.5, 5.0, and 10.0 μM), and the drugs in association. The plates were processed as described previously [18]. All of the assays were performed in triplicate.

2.4. Analysis of apoptosis rate

The CML cell lines were cultured for 24 h in the presence of 0.5 μM imatinib, 10 μM ara-C, or the drugs in association. The percentage of apoptotic cells was assessed using the Annexin V assay (Genzyme Diagnostics, USA) as described previously [18]. Propidium iodide (PI) was used to avoid necrotic cell detection (Annexin V®-PI®). The drugs-induced apoptotic rate (Annexin V®-PI® and Annexin V®-PI-®) was compared with the apoptosis in the absence of the drugs used as control (spontaneous apoptosis).

2.5. Determination of efflux pump activity modulation

The CML Lucena cell line was pre-incubated with CSA 5 μg/mL (4.2 μM) or maintained without CSA for 24 h as described previously [19]. After that, cells with CSA were re-incubated with CSA alone or in association with 0.5 μM imatinib, while the cells without CSA were divided in two more groups, 0.5 μM imatinib and without any drug (control). All cells were maintained for an additional 24 h and processed to analyze apoptosis rate by flow cytometry as described above.

2.6. Immunocytochemistry assay

The CML K562 and Lucena cell lines were incubated with 0.5 μM imatinib, 10 μM ara-C, the drugs in association, and without drugs (control) for 24 h. The slides were prepared by centrifugation of the cultured cells in a cytopsin centrifuge. The slides were fixed and permeabilized with acetone and methanol for 10 min. Then, the slides were incubated with 2% H2O2 to block the endogenous peroxidase. Non-specific staining was blocked using blocking solution [25% nonfat dry milk, 2.5% BSA (bovine serum albumin), and 8% PBS q.s.p. 100 mL tris–buffered saline (TBS)] for 1 h, and primary polyclonal antibody against survivin (1:900 dilution, Sigma–Aldrich®, USA). The cell slides were incubated at 4°C overnight in a moist chamber. After washing step, secondary antibodies provided by DAKO LSAB® System-HRP Kit were applied as manufactures instructions. The cells were exposed to 2.4-diaminobenzidine (DAKO Liquid DAB + Substrate Chromogen System Kit), counterstained with Harris hematoxylin, dehydrated, and mounted. Omission of the primary antibody was used as a negative control. The immunostaining was analyzed with a Nikon Eclipse E200 microscope. A 100-fold increase in immersion was used for the visualization [20].

2.7. Subcellular fractionation

The leukemic cell lines were incubated as described above. The cultured cells were washed twice with cold PBS (pH 7.4) and centrifuged at 200 × g for 5 min at 4°C. The pellet was resuspended in cold PBS and centrifuged at 800 x g for 5 min. The supernatant was removed, and the recipient with dry pellet was placed in nitrogen vapor for 1 min to promote cell lysis. The pellet was resuspended in 150 μL of hypotonic buffer A (1.5 mM MgCl2, 10 mM KCl, and 10 mM Tris–HCl, pH 7.9) supplemented with protease inhibitors, 1 μL aprotinin (1 mg/mL) and pepstatin (1 mg/mL), 0.4 mM PMSF, and 3 mM DTT. Then 0.05% Triton X-100 was added. The samples were centrifuged at 2500 × g for 15 min. The supernatant was collected as cytoplasmatic-enriched fraction, and the pellet was resuspended in 150 μL of hypotonic buffer B (20% glycerol, 1.5 mM MgCl2, 10 mM KCl, and 20 mM Tris–HCl; pH 7.9) and 100 μL of hypotonic buffer C (20% glycerol, 1.5 mM MgCl2, 1.25 M KCl, and 20 mM Tris–HCl; pH 7.9). Both buffers were supplemented with protease inhibitors, 0.4 mM PMSF, and 3 mM DTT. The samples were maintained under agitation for 45 min at 4°C and centrifuged at 16,000 × g for 90 min. The supernatant was collected as nuclear-enriched fraction and the pellet removed. All the procedures were realized at 4°C, and samples were maintained in an ice bath. The nuclear and cytoplasmatic-enriched fractions were stored at −20°C (adapted from Connell et al. [21]).

2.8. Western blotting analysis

After subcellular fractionation, the survivin expression was evaluated in the cytoplasmatic and the nuclear fractions. The protein concentration was determined by the Lowry method [22]. 60 μg of protein was subjected to SDS-PAGE on 15% polyacrylamide gels, transferred to Hybond-P membranes (GE Healthcare) and immunostained as described previously [14]. Primary antibodies were against survivin (1:1000 dilution; R&D Systems, USA), lamin B (1:500; Calbiochem, Germany) or HSC70 (1:5000 dilution; Santa Cruz, USA). Secondary antibodies were anti-mouse (1:1000 dilution; Amersham Biosciences, USA), anti-rabbit (1:1000 dilution; Amer- sham Biosciences, USA) or anti-goat (1:1000 dilution; Novus Biologicals, USA). The expression of survivin was normalized with respect to HSC70 for the cytoplasmatic fraction and lamin B for the nuclear fraction.

2.9. Statistical analysis

Statistical and graphical information was determined using the GraphPad Prism software (GraphPad Software Incorporated). ANOVA variance test was used and p values were reported at 95% confidence intervals. The Bonferroni post test was used to compare samples after treatments. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Effect of imatinib and ara-C on the cell viability of the sensitive and the resistant CML cell lines

The sensitive K562 and the resistant Lucena CML cell lines were incubated with various concentrations of imatinib, ara-C and the drugs in association. Imatinib reduced the cell viability in a time-dependent but not dose-dependent manner in the K562 cells. The lowest concentration (0.5 μM) suppressed cell viability by approximately 25% (p < 0.05), 62% (p < 0.05) and 75% (p < 0.01) after 24, 48, and 72 h of incubation, respectively (Fig. 1A). The degree of cell viability suppression was maintained at 1.0, 2.5, and 5.0 μM concentrations, showing statistically significant results after 48 and 72 h of incubation (p < 0.01). Ara-C also caused a time-dependent but not dose-dependent reduction in cell viability. Along the time, all the concentrations tested showed the same picture of the cell viability suppression. Within 24 h of incubation, no alteration in the cell viability was observed. After 48 h, we observed an approximately 20% (p < 0.05) decrease in the cell viability, which increased to 40% (p < 0.05) after 72 h. Nevertheless, the concentration of 0.1 μM was not significant at any of the times tested (p > 0.05) (Fig. 1B). The effect of imatinib combined with ara-C was similar to the viability reduction achieved with the respective concentration of imatinib alone (Fig. 1C). Imatinib 0.5 μM associated with different ara-C concentrations showed a statistically significant decrease in cell viability after 48 h (p < 0.01) and 72 h (p < 0.001), as well the associations with imatinib 1.0 μM (p < 0.001). This result demonstrated that in the sensitive cell line, there was no pharmacological interaction between imatinib and ara-C when these drugs were tested in combination.

The viability of the resistant Lucena cell line was suppressed by 0.5 μM and 1.0 μM imatinib concentrations similarly, by approximately 15% (p > 0.05), 41% (p > 0.05), 70% (p < 0.05) after 24, 48 and 72 h of incubation, respectively (Fig. 1D). Greater concentrations of imatinib (2.5 μM and 5.0 μM) elicited a higher
Fig. 1. Viability of CML K562 and Lucena cell lines induced by imatinib and ara-C determined by MTT assay after 24, 48 and 72 h. Error bars represent ± standard error (*p < 0.05, **p < 0.01, ***p < 0.001).

reduction in the cell viability, with a maximum reduction of 80% within 72 h of incubation (p < 0.01). Ara-C caused a decrease of approximately 40% in the cell viability only after 72 h incubation (p < 0.05), similar to the observation in K562 cells (Fig. 1E). In Lucena cells, imatinib associated to ara-C induced an effect similar to imatinib alone (Fig. 1F). Imatinib (0.5 µM) associated with ara-C 0.5 µM (p < 0.05), 5 µM (p < 0.05) and 10 µM (p < 0.01) showed a statistically significant decrease in cell viability only after 72 h, as well the associations with 1.0 µM of imatinib (p < 0.01).

3.2. Apoptosis induction by imatinib and/or ara-C in the CML cell lines

As shown in Fig. 2A, imatinib was able to induce apoptosis in approximately 60% of the sensitive K562 cells. On the other hand, this cell line was less sensitive to ara-C, which induced apoptosis in 22% of the cells. In the resistant Lucena cell line, the apoptosis index induced by imatinib was found to be of a lesser proportion (12%) than the apoptosis rate observed in the sensitive cell line (60%), and the difference was statistically significant (p < 0.001). A similar apoptosis index was observed with ara-C treatment, which induced 8% apoptosis. When the drugs were tested in combination, the apoptosis index in the Lucena cells (approximately 30%) was higher than the effects of either imatinib (12%) or ara-C (8%) separately, but this enhancement of apoptosis was not statistically significant. In the K562 cell line, no advantage was observed when drugs were associated.

To evaluate if the imatinib resistance profile exhibited by the Lucena cells was provided by the efflux pump activity of Pgp, its efflux activity was modulated and the apoptosis rate measured after imatinib incubation. The modulation promoted an increase of approximately 8% in the apoptosis rate in comparison with imatinib alone (p < 0.05) (Fig. 2B). As observed, the modulation was not able to induce the same apoptosis rate of the sensitive K562 cells, which was around 60%. This result demonstrates that the efflux pump activity is only partially responsible for the resistance phenomenon in Lucena cell line.

Fig. 2. Apoptosis of CML K562 and Lucena cell lines induced by imatinib and ara-C determined by the Annexin V assay. Leukemic cells incubated without the drugs were used as control of spontaneous apoptosis (*p < 0.05, **p < 0.001).
3.3. Effect of imatinib and ara-C in the modulation of the subcellular localization of survivin in the sensitive K562 and the resistant Lucena CML cell lines

Next, we fractionated the cells to investigate survivin subcellular localization after treatment by Western blotting. As shown in Fig. 3, the basal level of the survivin expression was similar between the cytoplasmatic and nuclear pools in the sensitive (A) as well as in the resistant (B) cell lines. After imatinib exposure, there was a predominant nuclear localization of survivin in the K562 cell line promoted by an increased expression in this compartment and with a consequent reduced expression in the cytoplasmatic one. This result is in agreement with the high percentage of apoptotic cells induced by imatinib. On the other hand, there was a predominant cytoplasmatic localization of survivin in the Lucena cell line promoted by a reduced expression in the nuclear compartment ($p < 0.05$). This result is also in agreement with the low apoptosis index induced by imatinib in this cell line. Conversely, ara-C decreased survivin expression in both cell lines, and similar amounts of protein were seen between the compartments. The combination of imatinib with ara-C, in the K562 cell line, promoted a slight reduction of the cytoplasmatic pool, while in the Lucena cell line, there was a predominant cytoplasmatic localization promoted by a reduction in the nuclear pool.

3.4. Survivin subcellular modulation in the sensitive K562 and the resistant Lucena CML after imatinib and/or ara-C

To further address the issue of whether imatinib or ara-C could shift the survivin subcellular localization, we used an immunocytochemistry assay. The K562 (Fig. 4B) and Lucena (Fig. 5B) cells incubated without the drugs (positive control) showed cytoplasmatic and nuclear staining in accordance with the Western blotting data. Incubation of K562 cells with imatinib exhibited a predominant survivin nuclear staining as protein clusters, while in Lucena cells, the staining was predominantly cytoplasmatic, with smaller protein clusters in the nucleus of the cells (Figs. 4C and 5C). Both cell lines incubated with imatinib showed a less intense survivin immunostaining when compared with their controls, mainly in the sensitive cell line. When incubated with ara-C, the sensitive cell line exhibited a survivin pattern staining similar to that of the cells incubated with imatinib, although in a more intense fashion (Fig. 4D). The resistant cell line exhibited a predominant survivin cytoplasmatic staining, which is in disagreement with the Western blotting results (Fig. 5D). Finally, when the drugs were combined, both cell lines showed reduced survivin levels in the nucleus in relation to their positive controls, corroborating the Western blotting data (Figs. 4E and 5E).

The drugs promoted morphologic alterations. The ara-C treatment resulted in bigger cells with a large nucleus (Figs. 4D and 5D). After imatinib and the combined treatments, the sensitive cell line showed a typical morphology of damaged cells, suggesting apoptotic bodies (Fig. 4C and E). This morphologic feature was not seen in the resistant cell line (Fig. 5C and E).

4. Discussion

Imatinib as a single drug has been shown to be ineffective, as it produces a transient response in blast phase CML [23]. Thus, in the present study we selected ara-C for the evaluation of a possible enhancement in cytotoxicity when combined with imatinib in the treatment of two blast-phase CML cell lines. Besides, ara-C was chosen due to the fact that its export from cells through Pgp, as a drug substrate for this transporter protein, has not been completely established [24,25]. The combined use of imatinib and ara-C did not cause a decrease in the cell viability of both vincristine-sensitive and -resistant cell lines. However, when we evaluated the induction of apoptosis by treatments with imatinib and/or ara-C through the Annexin V/PI assay we observed significantly different apoptosis indexes between the two cell lines. Imatinib was able to induce a markedly higher apoptosis index in the CML K562 cell line, which was more sensitive than CML Lucena cell line. This finding suggests a cross-resistance between vincristine and imatinib, which can be explained by the fact that Lucena cells overexpress Pgp. Moreover, previous studies have shown that imatinib is a substrate for this drug transporter protein [4,7,26]. Ara-C induced a low apoptosis index in both cell lines, but when the drugs were combined, the apoptosis index in the sensitive cells was similar to that found with imatinib alone. However, the same combination of drugs in the resistant cells elicited a slight increase in apoptotic cells when compared to the cells exposed to single treatments.

![Fig. 3. Survivin levels in K562 and Lucena cells after subcellular fractionation when exposed to imatinib and ara-C. HSC70 and lamin B controls ensure protein loading and purity of the fractions. Data are from one of the three experiments with reproducible results ($^*p < 0.05$).](image-url)
was not statistically significant, this finding is in accordance with previous works [27–29].

Here, we demonstrated that modulation of efflux pump activity by CSA was able to promote only a little increase of apoptosis in Lucena cells. This finding suggests that the efflux pump activity is partially responsible for the drug resistance profile in these cells giving support to the theory of a relationship between Pgp and survivin as shown previously [12,14,19]. Some properties of this biological phenomenon have become clear in the last years. A study conducted by Liu and collaborators [13] showed that survivin transcription was associated with Pgp/ABCB1 overexpression through the PI3k/Akt pathway in breast cancer cell lines, suggesting that survivin might play a key role in the MDR phenotype in the presence of Pgp. Indeed, our group recently demonstrated that survivin and Pgp are associated and highly expressed in late but not in early chronic phase of CML, suggesting that this association may play a biological role in the disease progression [14]. Additionally, we observed that the sensitive K562 (Pgp-negative) as well as the resistant Lucena (Pgp-positive) cell lines displayed nuclear and cytoplasmatic survivin expression. Imatinib was capable of modulating the survivin subcellular localization given that, after treatment with this drug, the sensitive cell line exhibited survivin predominantly in the nucleus compared to predominantly in the cytoplasm in the resistant cell line. Furthermore, the induction of a high percentage of apoptosis in the sensitive cell line was associated with the survivin modulation to the nucleus and the reduction of its cytoplasmatic expression. This is in agreement with the data in the literature, which suggest that nuclear survivin is a better prognostic factor in some neoplasms, such as colorectal [30], breast [31], lung [32], and pancreatic [33]. On the other hand, in the Lucena cell line, the survivin cytoplasmatic localization was associated with imatinib resistance, as confirmed by the low apoptosis rate. This finding is also in accordance with some studies in the literature that showed cytoplasmatic survivin as an unfavorable prognostic factor in pancreatic cancer [33], glioblastoma multiforme [34], and colorectal carcinoma [35].

Moreover, alternative splicing of the survivin gene can give rise to five different mRNA isoforms (survivin-standard or wild type, survivin-ΔEx3, survivin-2a, survivin-2B and survivin-3B), which can present differential subcellular localization and, therefore,
targeting this common pathway is warranted. Therefore, it becomes necessary new methodologies and more studies using specific monoclonal antibodies to discriminate survivin isoforms in different cancer cells [42]. Moreover, most studies analyze mRNA might suffer post-translational changes, herein does not reflect survivin protein levels.

Despite the limited number of cell lines analyzed in the present study, it is important to emphasize that these cell lines are undoubtedly different. In addition to Pgp expression, Lucena cells present high expression of the Oct-4, ABCG2, ABCB1, BCR-ABL and OCT1 genes as compared to K562 cells [16,43].

In conclusion, we showed that imatinib was able to modulate the subcellular localization of survivin and increase the apoptotic rate in the sensitive K562 cell line. In the resistant Lucena cell line, the cytoplasmatic localization of survivin was associated with its antiapoptotic activity, this being in agreement with the smaller apoptosis index observed in these cells. Since there was a different mobilization of survivin in the cell compartments triggered by imatinib in Pgp-negative and Pgp-positive cells, our results suggest a relationship between survivin and Pgp. However, as our study is the first to evaluate the subcellular localization of survivin after treatment with imatinib and ara-C in CML cells, it is not possible to compare our findings with those of other studies. Further exploration to confirm these findings is warranted. Therefore, we speculate whether Pgp and survivin are subject to the same mechanisms as compared to K562 cells [16,43].

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