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ORIGINAL PAPER

Everolimus in combination with Imatinib overcomes resistance in Chronic myeloid leukaemia

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Abstract

Although Imatinib and other tyrosine kinase inhibitors (TKIs) have excellent results, the appearance of resistance is a problem in chronic myeloid leukaemia (CML). PI3K/AKT/mTOR pathway is activated by BCR-ABL playing a crucial rule in CML. This study aimed to evaluate the therapeutic potential of Everolimus, in CML models sensitive and resistant to Imatinib. We used one CML cell line sensitive to Imatinib (K562) and two resistant (K562-RC and K56-RD). Cell lines were treated with Everolimus alone and in combination with Imatinib. Cell viability was analysed by resazurin assay. Cell death and cell cycle were analysed by flow cytometry. Additionally, we also studied peripheral blood samples obtained from 52 patients under TKI treatment. Everolimus reduced cell line viability in sensitive (IC_{50} = 20 µM) and resistant models (K562-RC, IC_{50} = 25 µM; K562-RD, IC_{50} = 30 µM). This drug induced cell death by apoptosis and cell cycle arrest in G_0/G_1 phase. Everolimus also reduced cell viability by increasing apoptosis of haematopoietic stem cells ($CD34^+$ cells) with low cytotoxicity to lymphocytes. Everolimus at 25 µM increased apoptotic cells 18.7% in $CD34^+$ cells and only 8% in lymphocytes. The response to Everolimus was influenced by TKI treatment, with a better response in samples from patients under 2nd and 3rd generation TKI and with less toxicity to lymphocytes. Our results reveal that Everolimus induce cell death in CML cells sensitive and resistant to Imatinib, with low cytotoxicity to normal cells, suggesting that Everolimus could be an alternative targeted therapeutic approach in CML patients, even in cases of Imatinib resistance.

Keywords Imatinib · Resistance · mTOR inhibitor · Chronic myeloid leukaemia · Apoptosis

Introduction

Chronic myeloid leukaemia (CML) is a haematological stem cell disorder, characterised by the presence of *BCR-ABL* fusion gene. This unique molecular feature allows the use of specific tyrosine kinase inhibitors (TKIs), like Imatinib, as a therapeutic weapon [1]. The use of TKIs changed the course of the disease entirely but reveal another problem—the resistance to target therapies [2]. In addition to alterations in drug target, like *BCR-ABL* mutations or overexpression, and drug influx/efflux transporters, the activation of multiple signalling pathways that confers tumour advantage could act as a mechanism of resistance in CML [3–5].

PI3K/AKT/mTOR pathway is, after p53, the second most altered signalling pathway in cancer, including in haematologic neoplasias [6]. Cell growth, survival, metabolism, and translation mechanisms are some of the essential processes controlled by PI3K signalling. Once PI3K is activated, it phosphorylates AKT that then activates downstream

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several effector proteins, like mTOR. mTOR is the catalytic subunit of two protein complexes: mTORC1 and mTORC2 [7]. These complexes present different compositions and functions. mTORC1, composed by mTOR, RAPTOR, and mLST6, activates S6K by phosphorylation and inhibits 4E-BP1 proteins, controlling cell cycle (progression from G_1 to S phase), cell proliferation, survival, and angiogenesis. The mTORC2 is composed by mTOR, RICTOR, mLST6, and mSIN1, and participates in cytoskeleton organisation and cell proliferation. The most important function of mTORC2 is the activation of AKT [7, 8].

Since PI3K is one of the multiple BCR-ABL targets, this signalling pathway has been correlated with CML pathogenesis and also with drug resistance acquisition [8, 9]. Some studies refer the compensatory mTOR activation as a BCR-ABL independent resistance mechanism and as an essential pathway for leukaemia stem cells (LSC) survival [10, 11]. Based on these multiple functions, mTOR becomes an attractive target, not only for mature cells but also as a way to target stem cells [12]. Everolimus is one of Rapamycin analogues and acts as an allosteric inhibitor of mTORC1. This inhibition has been associated with apoptosis trigger and cell cycle arrest in cancer cells [13].

In this study, we investigated the effect of Everolimus, in monotherapy and combination with Imatinib, on CML cell lines sensitive and resistant to this TKI. Additionally, the effect of this mTOR inhibitor was also evaluated on CML primary cells. We show the efficacy of Everolimus in target CML cells and synergistic effect with Imatinib, especially in resistant models. The induction of apoptosis was observed in CML cell lines and $CD34^+$ cells from peripheral blood of CML patients.

Methods

In vitro Studies

Cell culture conditions

We used three CML cell lines: K562 cells sensitive to Imatinib, and two Imatinib-resistant models—K562-RC and K562-RD cells. The sensitive cell line was obtained from American Type Culture Collection (ATCC), and the Imatinib-resistant cell lines were developed in our laboratory based on two strategies: a continuous exposure (K562-RC) and a discontinuous exposure to Imatinib (K562-RD), as described in Alves et al. [5]. The mathematical IC_{50} to Imatinib was 75 nM, 605 nM, and 1390 nM to K562, K562-RC, and K562-RD cells, respectively. All the cells were maintained in RPMI-1640 medium supplemented with 10% 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. In the case of resistant cells lines, 250 nM of Imatinib (IMA) (Selleckchem) was added to the medium, according to the scheme of resistance.

Metabolic activity assay

The resazurin assay, methodology based on dehydrogenases enzymes activity, was used to assess the metabolic activity of cells in the absence and presence of Everolimus (EVE) (Selleckchem) and/or Imatinib. For combination studies, we selected the dose of 5 µM of Everolimus, the lower concentration tested in monotherapy, and the dose of 10 nM of IMA that have an inhibitory effect lower than 25% in all models. Both drugs were diluted in DMSO, and we used different stock solutions in order to add the same amount of solvent in each condition tested. The cells were plated at a cell density of 0.5×10^6 cells/mL, and resazurin (Sigma–Aldrich) was prepared as a stock solution of 100 µg/ml in phosphate-buffered saline (PBS). After treatment, resazurin was added to a final concentration of 10 µg/mL to the cells which were then incubated at 37 °C for 2 h. The absorbance at 570 and 600 nm was measured using a Synergy™ HT Multi-Mode Microplate Reader (BioTek Instruments), and metabolic activity was calculated as a percentage of control cells. The results were expressed as a mean ± standard error of the mean (SEM) of at least five independent experiments.

Cell death analysis

Cell death was examined by flow cytometry (FC) through annexin V and propidium iodide double staining and by morphological analysis using optic microscopy. After an incubation period of 48 h, cells were collected and washed with PBS by centrifugation at 400x*g* for 5 min. Then, cells were resuspended in 100 µl of binding buffer and incubated with 5 µl of annexin V-APC (AV) and 2 µl of propidium iodide (PI) staining solution (Biolegend) for 15 min at room temperature in the dark. Cells were analysed in a FACS Calibur (Becton Dickinson) flow cytometer equipped with an argon laser. CellQuest software (Becton Dickinson) was used for the acquisition of data and results were analysed with the Paint-a-Gate software. Results were expressed in percentage of viable cells (AV^-/PI^-), early apoptotic (AV^+/PI^-), late apoptotic/necrotic (AV^+/PI^+), and necrotic cells (AV^-/PI^+), and represent the mean ± SEM of five independent experiments. For morphological analysis, cells from the different conditions were collected and seeded in glass slides. The cells were stained as described in Mendes et al. [14]. Cell morphology was analysed by light microscopy using a Nikon Eclipse 80i microscope equipped with a Nikon Digital Camera DXM 1200 F.

Activated Caspase 3 expression analysis

After 48 h of treatment with Everolimus in monotherapy (5 and 25 µM) and in combination with Imatinib (10 nM IMA + 5 µM EVE), activated caspase-3 expression levels were assessed by FC in all cell lines. For each condition, cells were incubated with a monoclonal antibody anti-activated caspase 3-PE (BD Pharmingen, Becton Dickinson), according to manufacturer's protocol. Briefly, cells were fixed with 100 µl of fix solution (IntraCell, Immunostep) for 15 min and then washed by centrifugation at 300 xg for 5 min. Cells were then permeabilised and stained by incubation for 15 min with 100 µl of permeabilisation solution (IntraCell, Immunostep) and 1 µg of antibody. After washing, cells were analysed by FC, as mentioned before. Results are expressed as a percentage of positive cells for activated caspase 3 and represent the mean ± SEM of five independent experiments.

Cell cycle analysis

Cell cycle analysis was performed in 1×10^6 cells of each conditions after a 48 h of exposure to everolimus (5 and 25 µM) and the combination scheme with Imatinib (10 nM IMA + 5 µM EVE). Cells were washed by centrifugation for 5 min at 300x*g* and fixed by incubation at 4 °C for 30 min with 200 µl of 70% ethanol. Following a wash step, 500 µl of propidium iodide solution with RNase (PI/RNase, Immunostep) was added to the cell pellet, incubated for 15 min at room temperature and analysed by FC. Cell cycle distribution was analysed using the ModFit LT software (Verity Software House). Results were expressed in percentage of cells in the different cell cycle phases (G_0/G_1, S, and G_2/M) according to the PI intensity and denote the mean ± SEM of five independent experiences. A sub-G_1 population was also identified, when present, corresponding to apoptotic cells.

Ex vivo Studies

Patients population and Ethical Statement

Fifty-two patients with chronic myeloid leukaemia were enrolled in the present study. Patients were grouped according to treatment approach and response criteria, established according to European Leukemia Net (ELN). In our cohort, 38 patients were treated with Imatinib and 14 were treated with 2nd or 3rd generation TKI (IMA-resistant group). In this last group were included the patients that fail Imatinib therapy as first line (became Imatinib resistant), and switched for a 2nd or 3rd generation TKI. In our cohort, 49 patients presented molecular response (MR) (35 with MR 4.5; 8 with MR 4.0; 6 with MR 3.0) and only three with a cytogenetic response (CR). The study was conducted according to Helsinki declaration, and the Ethics Committee of Faculty of Medicine of University of Coimbra (Coimbra, Portugal) approved all research procedures (CE-014/2014). All participants provided their informed consent for participation before enrollment.

Cell death analysis in primary cells

Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll sedimentation and cultured in RPMI 1640 medium with 20% of FBS. The cells were plated at 1.0×10^6 cells/mL and incubated in the absence and presence of the Everolimus. After 48 h of incubation, PBMCs were labelled with anti-CD34-APC (Miltenyi Biotec) and annexin V-FITC (Immunostep). Briefly, cells were collected and washed with PBS by centrifugation at 400x*g* for 5 min. Then, cells were resuspended in 100 µl of PBS and incubated with 5 µl of anti-CD34-APC, for 15 min at room temperature in the dark. After washing, cells were resuspended in 100 µl of binding buffer and incubated with 5 µl of annexin V-FITC, for 15 min at room temperature in the dark. Cells were analysed in a FACS Calibur. CellQuest software (Becton Dickinson) was used for the acquisition of data and results were analysed with the Paint-a-Gate software. Percentage of annexin V-positive cells was quantified on the gate of $CD34^+$ and lymphocytes populations and considered as apoptotic cells.

Data analysis

Statistical analysis was carried out using GraphPad Prism software, version 7.00 for Windows (GraphPad Software, USA). All values were expressed as mean ± SEM. ANOVA, Tuckey, and Dunnett's post hoc test were used to determine the statistical significance, considering a p value of < 0.05 adjust for the multiple comparison analysis. Tuckey test was used for comparison between association scheme and individual doses of each compound, and the Dunnett's test for comparison with control. The IC_{50} determination was performed by non-linear curve fit dose–response. The effects of drug combinations were determined by the combination index (CI) value (Eq. 1), according to the Chou and Talalay method [15, 16].

$$\mathrm{CI} = \sum_{j=1}^{n} \frac{(D)_j}{(EDx)_j},$$

where D represents the dose of a drug used in combination and EDx the effect of the drug in monotherapy. CI values < 0.9 indicate synergism, CI < 0.7 represent moderate synergism, and CI < 0.3 depict strong synergism. CI values between 0.9 and 1.1 show additive interactions, and CI values > 1.1 denote antagonism.

Results

Everolimus reduce metabolic activity of sensitive and Imatinib-resistant cells

Our results show a decrease in metabolic activity in a time-, dose- and cell type-dependent manner, as represented in Fig. 1. On K562 cells, sensitive to Imatinib, Everolimus revealed a more pronounced effect during the time of exposure, with an IC_{50} of 20 µM at 48 h (Fig. 1a). Comparatively, after the same time of exposure K562-RC cells required a higher concentration of the inhibitor, presenting an IC_{50} of 25 µM (Fig. 1b). For the most Imatinib-resistant model, the K562-RD cells, the IC_{50} was highest approximately 30 µM (Fig. 1c). In both resistant models, we observed a recovery on metabolic activity, after 48 h of exposure for K562-RC cells and after 24 h for K562-RD cell line. Independently of Imatinib sensitivity or resistance, the dose of 50 µM of Everolimus was very toxic with a reduction of 95% in metabolic activity.

To overcome the toxicity associated with targeted therapies and the cases of resistance, we tested the combination scheme between lower doses of Everolimus and Imatinib. In all cell lines, we observed synergism between 5 µM of Everolimus and 10 nM of Imatinib (Fig. 2). On sensitive cells, the combination index (CI) was 0.47 revealing a moderated synergy (Fig. 2a). After 48 h of the combined treatment, K562-RC presented a reduction of 50% on metabolic activity with a CI of 0.22 (Fig. 2b), and the same pattern was observed in K562-RD with a CI of 0.18 (Fig. 2c). For the resistant cell lines, the combination index showed a strong synergism between the drugs.

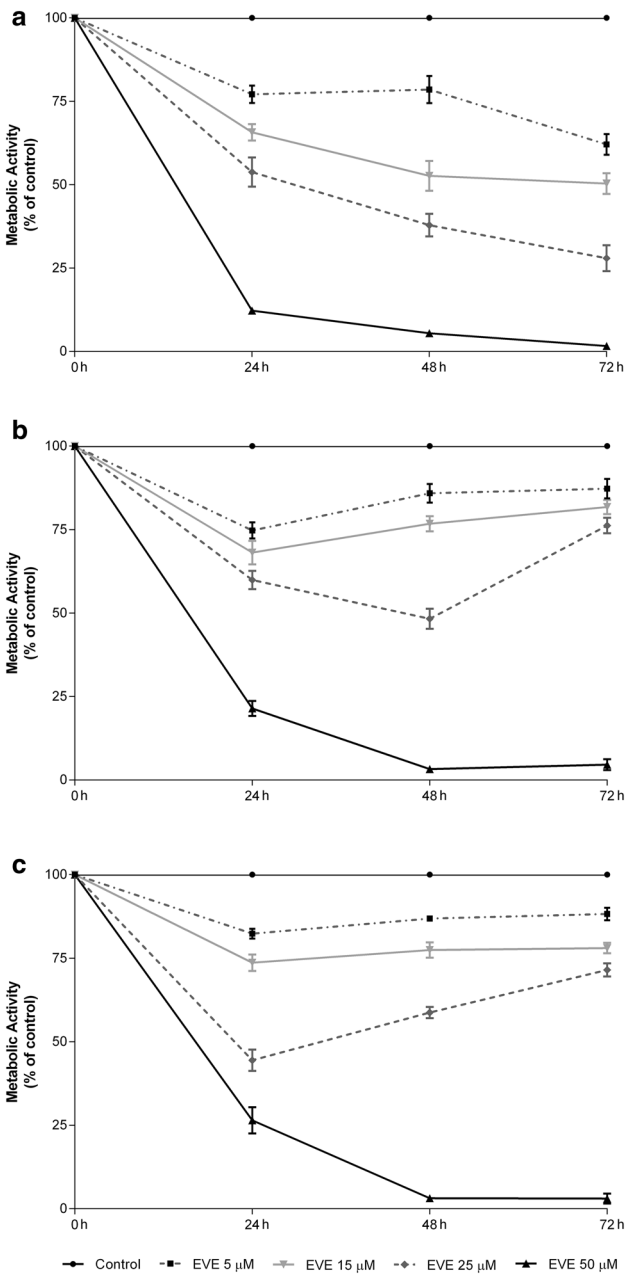

Fig. 1 Dose–response curves of Everolimus on sensitive and Imatinib-resistant CML cell lines. K562 (**a**), K562-RC (**b**), and K562-RD (**c**) cells were incubated an initial density of 0.5×10^6 cells/ml in the absence and presence of different concentrations of EVE, during 72 h. Dose–response curves were established by resazurin reduction at each 24 h, as described in Methods section. Results are expressed in percentage (%) normalised to control. Data are expressed as mean ± SEM obtained from 5 independent experiments

Apoptosis as a mechanism of cell death activated by Everolimus

We evaluate the mechanism of cell death activated by Everolimus, for the doses of 5 and 25 µM using annexin V/PI double staining (Fig. 3a). For sensitive cells, the 5 and 25 µM of EVE showed a reduction of 25 and 50% of viability, respectively. These decreases were accompanied by an increase in the percentage of cells in early apoptosis and late apoptosis/necrosis. All the differences described reveal statistical significance. As observed in K562 cells, in the resistant models the dose of 25 µM of EVE induced a reduction of 50% on cell viability through apoptosis activation. However, no significant cell death was observed in resistant cell lines when exposure to the lowest concentration (5 µM) of EVE.

The morphological evaluation by optical microscopy confirmed apoptosis activation (Fig. 3b). After EVE treatment, the cells showed morphological aspects typical of apoptosis, like blebbing and cellular contraction. Additionally, we observed an increase in vacuolization on exposure cells, proving the toxicity induced by the drug. To support the previous results, we evaluated the percentage

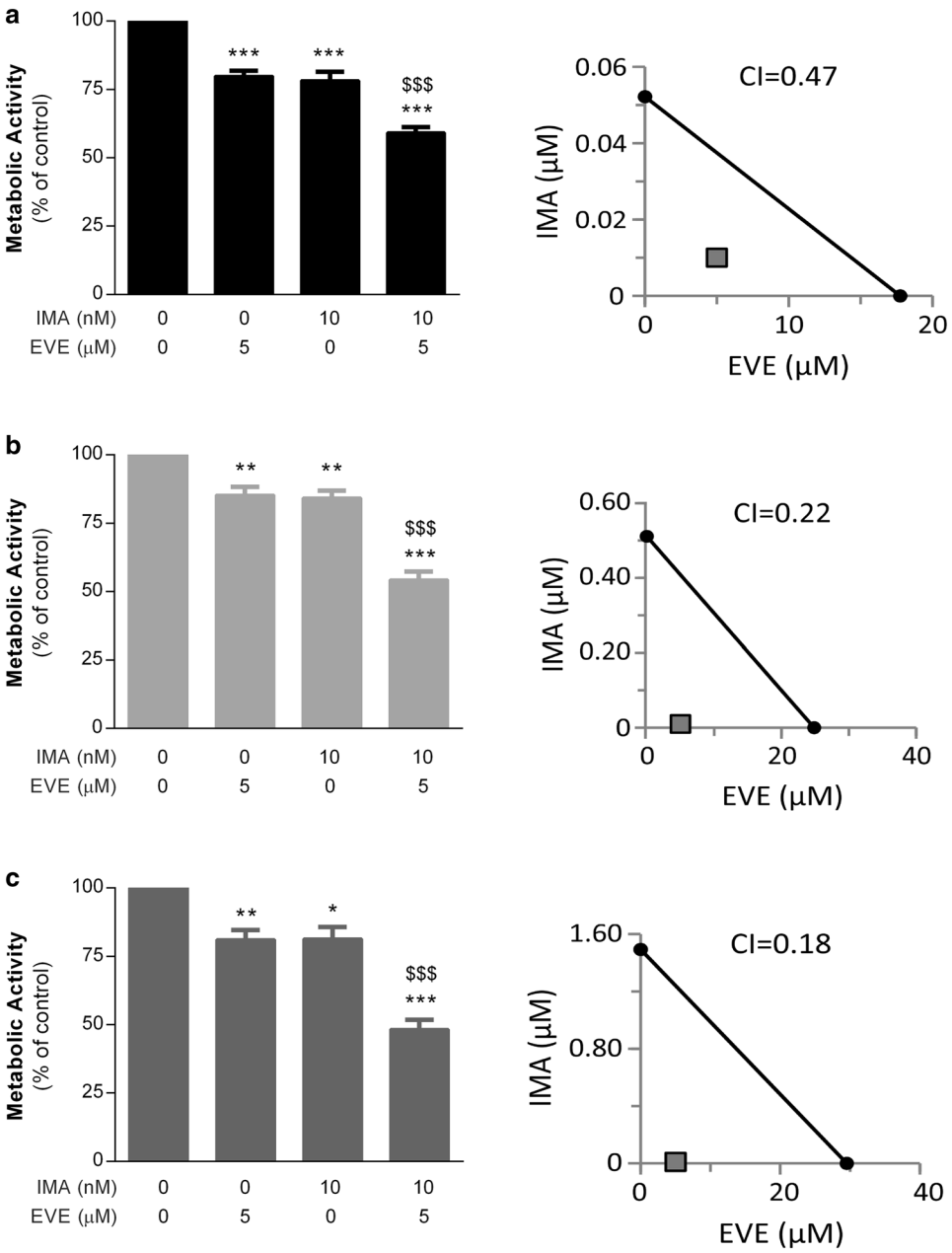

Fig. 2 Effect of Everolimus in combination with Imatinib on metabolic activity of CML cells and respective isobolograms. K562 (**a**), K562-RC (**b**), and K562-RD (**c**) cells were incubated an initial density of 0.5×10^6 cells/ml in the absence and presence of EVE 5 μM plus Imatinib 10 nM during 48 h. The classic isobolograms translate the synergism between the two drugs with respective CI. CI values < 0.9 indicate synergism, CI < 0.7 representing moderate synergism and CI < 0.3 depicts strong synergism. Results are expressed in percentage (%) normalised to control. Data are expressed as mean ± SEM obtained from five independent experiments

of cells with caspase-3 on the activated form (a protease activated in apoptotic cells) (Fig. 3c). In all cell lines, the 25 μM of EVE induces an increase of positive cells to the activated form of caspase-3 comparing with untreated cells ($p < 0.01$). The dose of 5 μM induced a moderate increase of these cells only in sensitive cells.

For the combination scheme, the cells were treated with 5 μM of Everolimus plus 10 nM of Imatinib. On K562 cells, we observed a higher percentage of cell death with combination scheme comparing not only with untreated cells but also with each inhibitor in monotherapy. The effect on resistant models was different between the two cell lines. K562-RC showed a reduction to 70% of cell viability with an increase of cells in early apoptosis, while in K562-RD cells no significant cell death was induced by two drugs (Fig. 3a). The findings on annexin V/PI experiments were supported by the morphological evaluation and caspase 3 activation. For the combination scheme, only K562 and K562-RC cells reveal a significant activation of apoptosis (Fig. 3c).

Cell cycle arrest on G_0/G_1 phase promoted by Everolimus

Since a drug could present not only a cytotoxic effect but also a cytostatic one, we evaluated the cell cycle distribution of our cells after Everolimus exposure. As described in

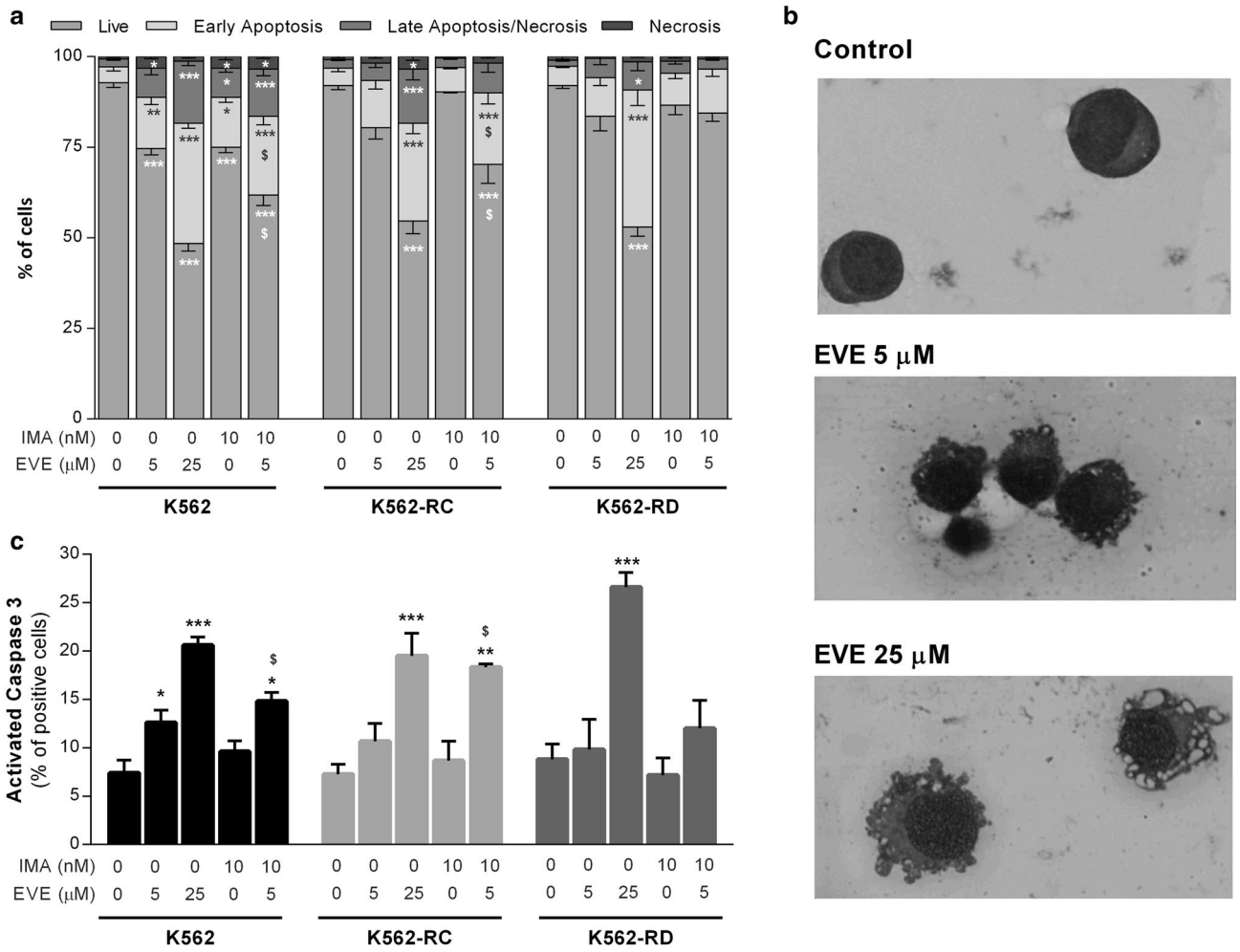

Fig. 3 Analysis of cell death induced by Everolimus in CML cell lines. **a** Cell death was detected by annexin V/propidium iodide staining and analysed by flow cytometry (FC); data are expressed as a percentage (%) of live, early apoptotic, late apoptotic/necrotic, and necrotic cells. **b** Cell smears were stained with May-Grünwald-Giemsa (amplification: 500x). K562 cells were used as representative cell line since the results were similar. **c** The activated caspase 3 expression levels were analysed by FC. Results were obtained after 48 h of incubation and represent mean ± SEM of 5 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (comparison with control); $ $p < 0.05$ (comparison with lower dose of correspondent inhibitor)

Table 1, Everolimus presented a cytostatic effect by inducing a cell cycle arrest in G_0/G_1 phase (Fig. 4). On sensitive cells, we observed for the highest dose of EVE a significant increase in the percentage of cells in G_0/G_1 phase. This cytostatic effect was more pronounced in resistant models, especially to K562-RD cells. The untreated K562-RC cells present approximately 44% of cells on the G_0/G_1 phase, wherein the presence of treatment this value raised 15% ($p < 0.001$). On K562-RD cells, the cytostatic effect was even higher with an increase of 22% of cells in G_0/G_1 phase ($p < 0.001$) with Everolimus treatment (Table 1). In all cell lines when cells were treated with 25 μM of EVE, we observed an increase on Sub-G_1 peak (confirming the apoptosis induction). The combination strategy induced a significant cytostatic effect, with arrest in G_0/G_1 phase, on resistant models (Table 1).

Everolimus triggered apoptosis on CD34$^+$ CML population

Since Everolimus was capable of inducing apoptosis in resistant cell lines, we hypothesised that the same effect should also occur in haematopoietic stem cells (HSC) (CD34$^+$ cells) from CML patients under TKI treatment. This cell population includes the healthy HSC and the leukaemia stem cells (LSC), being the last associated with the resistance acquisition. We tested two doses of Everolimus (5 μM and 25 μM) in PBMCs from 52 patients and observed activation of apoptosis with an increase of annexin V-positive populations (Fig. 5). On CD34$^+$ cells, the small dose of everolimus induced an increase of 10% in apoptotic cells in comparison with controls ($p < 0.001$) (Fig. 5a). With higher

Table 1 Effects of Everolimus in cell cycle of sensitive and Imatinib-resistant cells

		Sub-G_1 (%)	G_0/G_1 (%)	*S* (%)	G_2/M (%)
K562 cells					
	Control	0.6 ± 0.4	35.8 ± 1.2	56.2 ± 1.7	8.0 ± 0.9
	EVE 5 μM	3.6 ± 1.2	39.6 ± 1.7	51.6 ± 1.5	9.2 ± 0.7
	EVE 25 μM	12.8 ± 0.9 ***	44.0 ± 1.7 **	46.2 ± 2.1 **	9.8 ± 0.5
	IMA 10 ηM	3.8 ± 0.6	32.2 ± 1.4	61.0 ± 2.4	6.8 ± 1.4
	IMA 10 ηM + EVE 5 μM	5.0 ± 1.0 **	38.8 ± 1.3	49.0 ± 1.8 *	12.2 ± 0.8 *
K562-RC Cells					
	Control	0.2 ± 0.2	43.8 ± 1.0	42.6 ± 2.1	13.6 ± 1.4
	EVE 5 μM	2.4 ± 1.2	58.8 ± 2.0 ***	31.8 ± 2.6 *	9.6 ± 1.5
	EVE 25 μM	30.8 ± 1.0 ***	57.8 ± 2.5 ***	38.4 ± 3.5	4.0 ± 1.7 ***
	IMA 10 ηM	1.0 ± 0.7	40.8 ± 2.4	50.2 ± 3.8	9.2 ± 1.6
	IMA 10 ηM + EVE 5 μM	2.8 ± 1.5	59.8 ± 0.6 ***	31.0 ± 1.1 *	9.4 ± 0.5
K562-RD cells					
	Control	0.6 ± 0.3	44.6 ± 2.0	43.8 ± 3.7	11.4 ± 2.0
	EVE 5 μM	0.8 ± 0.5	67.3 ± 1.7 ***	22.5 ± 0.9 ***	10.0 ± 0.7
	EVE 25 μM	10.0 ± 1.9 ***	65.2 ± 2.2 ***	26.4 ± 1.7 ***	8.2 ± 1.0
	IMA 10 ηM	1.8 ± 0.5	45.0 ± 1.0	41.3 ± 2.1	13.8 ± 1.6
	IMA 10 ηM + EVE 5 μM	0.8 ± 0.5	65.5 ± 2.0 ***	25.0 ± 2.1 ***	9.5 ± 0.5

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ comparing with Control

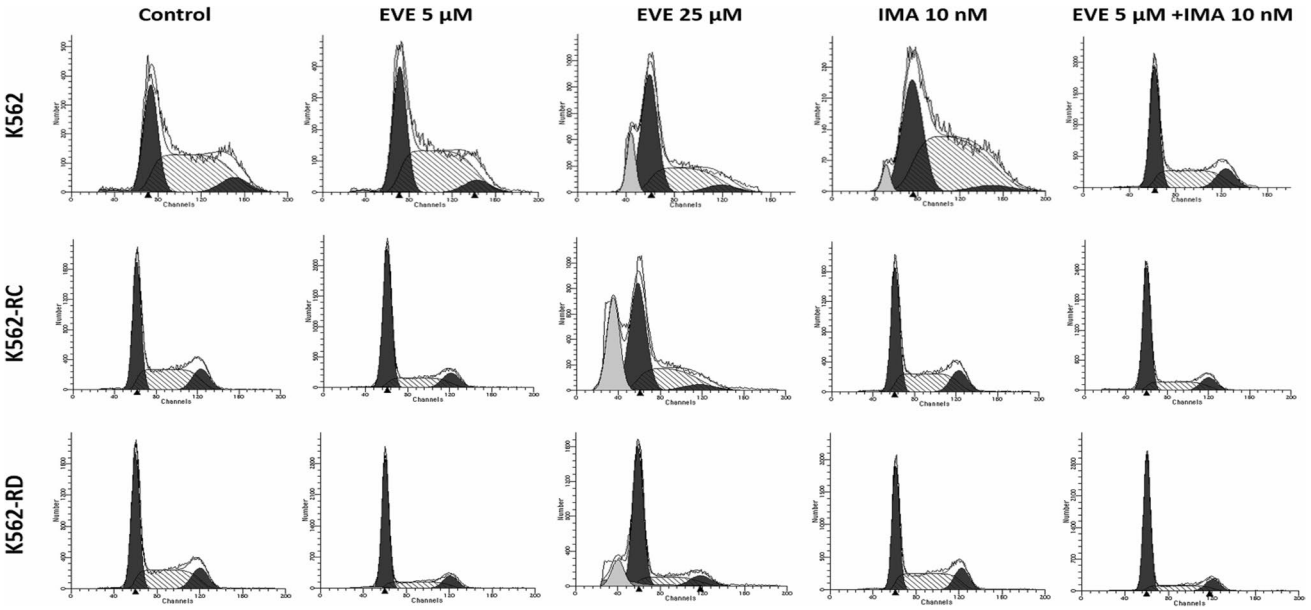

Fig. 4 DNA representative histograms from cell cycle analysis after Everolimus treatment in CML cell lines. Cell cycle analysis was performed with propidium iodide/RNAse staining and analysed by flow cytometry (FC). The initial and the last dark grey peaks correspond to G_0/G_1 and G_2/M, respectively; between them is S phase. Sub-G_1 when present is represented in light grey peak

dose, very similar to the IC_{50} of resistant cell lines, this value increased to 18.7% ($p < 0.001$). The same study was also performed on Lymphocytes populations, as a control to the toxicity of Everolimus in the other cell populations (Fig. 5b). Lymphocytes also undergo apoptosis, however in values very different from $CD34^+$ cells, with a maximum of 8% of apoptosis comparing with the untreated cells. Comparing the two populations, Everolimus effect was at least two times higher on $CD34^+$ cells comparing to lymphocytes.

In our cohort, 38 patients are under Imatinib treatment (first-line TKI), and 14 are under a 2nd or 3rd generation TKI (IMA Resistant). We assess if TKI treatment influenced

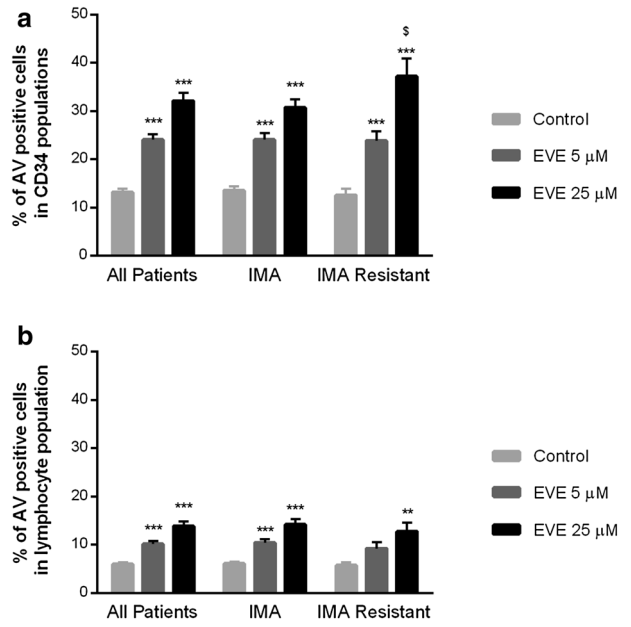

Fig. 5 Apoptosis induced on $CD34^+$ cells and Lymphocytes of CML patients by Everolimus. The percentage of apoptotic cells in $CD34^+$ (**a**) and lymphocytes (**b**) populations from primary CML cultures induced by EVE, detected by annexin V using FC. The data represent the total of patients analysed ($n=52$, All patients) that were subdivided by treatment: 38 under Imatinib (IMA) and 14 taken 2nd generation TKI (IMA Resistant). Results were obtained after 48 h of incubation and represent mean ± SEM. *** $p<0.001$ (comparison with respective control); $ $p<0.05$ (comparison with Imatinib group)

the effect of Everolimus on $CD34^+$ cells and lymphocytes. When exposed to 25 μM of EVE, we observed a higher percentage of apoptotic cells on IMA-resistant patients (Fig. 5). On this group, the rate of apoptotic cells increased 24.6% compared with control, while on Imatinib-treated group the increased was 17.1%. Regarding response, the effect of Everolimus seems to be more effective in patients with CR than in patients with MR; however, statistical analysis was not possible because of the small group size ($n=3$ to CR).

Discussion

The emergence of CML resistance to TKI treatment requires the investment in other potential targets to overcome this resistant phenotype. In our work, we demonstrated that Everolimus, a mTOR inhibitor, can trigger apoptosis and induce cell cycle arrest in CML cells, even in cases of Imatinib resistance. The efficacy of Everolimus in monotherapy was observed in all models, being more pronounced in sensitive K562 cells. On resistant models, we saw a reversion on the effect of Everolimus, indicating the need to adjust the administration scheme. A combination scheme allows the use of several drugs at the same time, with the purpose to magnify the results targeting multiple pathways and also to minimise side effects using lower doses than in monotherapy. We tested the combination of lower doses of Imatinib and Everolimus, to check the possible synergisms between drugs. In the three models, we demonstrated a synergistic effect by the association of two drugs. Although in monotherapy scheme K562-RD cell required more dose of the mTOR inhibitor (30 μM), in combination strategy this was the cell line with most robust synergism (CI 0.18). The combination index of 0.22 of K562-RC also reveals a strong association in this model.

Some authors suggest a relation between the PI3K/AKT/mTOR pathway and drug resistance acquisition. For example, resistances to retinoic acid (ATRA—*All-trans* Retinoic Acid), vincristine, and TRAIL have been associated with activation of mTOR signalling pathway [17]. Burchert et al. demonstrated that activation of this cell signalling as a compensatory mechanism after Imatinib treatment [10], while others categorised the re-activation of signalling pathways, crucial for CML pathogenesis, as a BCR-ABL-independent resistance mechanism [11, 18]. In a clinical trial, only 27% of patients without BCR-ABL mutations achieved major molecular response when treated with Ponatinib (the strongest TKI) [19]. These data highlight the necessity of new targeted therapies, as everolimus, for CML patients. In agreement with our results, the use of rapamycin or other rapalogs has been described as capable of inducing a pro-apoptotic effect and also an antiproliferative effect in multiple neoplasias. The suppression of PI3K/AKT/mTOR pathway leads to nuclear accumulation of p27, and consequently a cell cycle arrest at the G_1 phase and activation of apoptosis in response to DNA damage [6, 20]. According to Yang et al., mTOR inhibitors could not only abolish the aberrant activation of mTOR by Imatinib treatment but also decrease the risk of Imatinib resistance [21].

For some authors, the use of Everolimus and other similar drugs is not sufficiency to achieve higher results, since this is an allosteric mTORC1 inhibitor and the mTO RC2 complex is described as rapamycin insensitive. The function of the last complex is crucial for full activation of AKT by phosphorylation [7]. However, rapamycin and rapalogs present an indirect effect on mTORC2 in cases of prolonged exposure (over 24 h). These drugs may sequester the newly synthesised mTOR molecules interfering with mTORC2 assembly, as demonstrated in acute myeloid leukaemia (AML) cells [22, 23]. As a consequence, this mechanism prevents the late re-activation of AKT, blocking the compensatory mechanism. In CML, the BCR-ABL affects the PI3K/AKT/mTOR pathway by two ways: directly by the phosphorylation of PI3K; and indirectly by the downregulation and/or impairment of the function of the natural inhibitor of this pathway, the PTEN [24, 25]. This indirect mechanism is particularly important in leukemic stem cells [24, 25].

Furthermore, some studies suggest that PI3K/AKT/mTOR pathway acts as a pro-survival factor for LSCs, in addition, to be crucial for maintaining self-renewal [26–28]. In AML patients, mTOR downstream effectors were found constitutively phosphorylated on LSCs in opposition to the observed in normal $CD34^+$ cells [29]. The authors hypothesised that this effect might be due to PTEN phosphorylation or down-regulation [24, 29].

Among many characteristics, LSCs are described as expressing drug efflux transporters, as BCRP, to avoid the effect of chemotherapy agents [30]. Another link between PI3K/AKT/mTOR pathway and drug resistance is the expression/regulation of BCRP expression/function. In glioblastoma stem-like cells, AKT was responsible for regulating BCRP activity conducting to drug resistance [31]. In AML and acute lymphoblastic leukaemia (ALL), Huang et al. observed that the activated PI3K upregulates BCRP expression and elevates the percentage of cancer stem-like cells [32]. Additionally, in CML, the acquisition of resistance is also correlated with the increased function of BCRP, and this is at least in part related to AKT activation [33]. According to Hegedüs et al., rapamycin interacts with BCRP inhibiting its function without disturber its plasma membrane distribution [34]. In most of the studies, the resistant CML models present alterations on *BCR-ABL*, more frequently point mutations. In this study, we used two Imatinib-resistant CML cell lines without *BCR-ABL* mutations but with modifications on drug influx and efflux transporters. Both resistant models present higher levels of BCRP and PgP transporters comparing with K562 sensitive cells, as a mechanism to avoid Imatinib action [5]. The relation between PI3K/AKT/mTOR signalling and BCRP transporter could justify the strong combination index observed in K562-RC and K562-RD cells. All this knowledge on drugs resistance mechanism, on activation of this cell signalling and stem cell characteristics, suggest that this axis could act as therapeutically target to reach LSC [35]. In our study, we access the use of Everolimus in $CD34^+$ cell population from CML treated patients. On this population of primary progenitor cells, the mTOR inhibitor induces apoptosis with low toxicity to lymphocytes. As described for different mTOR inhibitors [11], we also observed a higher effect of Everolimus in the $CD34^+$ cells from patients that were Imatinib resistant and required the use of a 2nd or 3rd generation TKI.

Our results suggest the importance of the PI3K/AKT/mTOR pathway for the CML-resistant phenotype and the possible role as a therapeutic target in CML. Everolimus inhibition, in monotherapy and especially in combination with Imatinib, seems an advantageous strategy in cases of TKI resistance without the *BCR-ABL* mutation.

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Authors' contributions RA, AA, and ABSR designed the experiments. RA and ACG drafted the manuscript. RA, ACG, and JJ performed the experiments. JA and AAS executed the statistical analyses. PFT recruited and provided the clinical information of the participants. JNC, AA, and ABSR revised the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interests.

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