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PRECLINICAL STUDIES

4-methylumbelliferone and imatinib combination enhances senescence induction in chronic myeloid leukemia cell lines

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Summary Chronic myeloid leukemia (CML) is a myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome which encodes a constitutively activated tyrosine kinase (*BCR-ABL*). The first line treatment for CML consists on *BCR-ABL* inhibitors such as Imatinib. Nevertheless, such treatment may lead to the selection of resistant cells. Therefore, it is of great value to find molecules that enhance the anti-proliferative effect of first-line drugs. Hyaluronan is the main glycosaminglican of the extracellular matrix which is involved in tumor progression and multidrug resistance. We have previously demonstrated that the inhibition of hyaluronan synthesis by 4-methylumbelliferone (4MU) induces senescence and can revert Vincristine resistance in CML cell lines. However, the effect of 4MU on Imatinib therapy remains unknown. The aim of this work was to determine whether the combination of 4MU with Imatinib is able to modulate the proliferation as well as apoptosis and senescence induction in human CML cell lines. For this purpose the ATCC cell line K562, and its multidrug resistant derivate, Kv562 were used. Cells were exposed to 4MU, Imatinib or a combination of both. We demonstrated that 4MU and Imatinib co-treatment abrogated the proliferation of both cell lines. However, such co-treatment did not increase the levels of apoptosis when compared with the treatment with Imatinib alone. For both cell lines the mechanisms of tumor suppression involved was senescence, since the combination of 4MU and Imatinib arrested the cell cycle and increased senescence associated β-galactosidase activity and senescence associated heterochromatin foci presence when compared to each drug alone. Moreover, 4MU, Imatinib and 4MU + Imatinib decreased pAkt/Akt ratio in both cell lines and reduced the pERK/ERK ratio only in K562 cells. These findings highlight the potential use of 4MU together with Imatinib for CML therapy.

Keywords Chronic myeloid leukemia · Imatinib · 4-methylumbelliferone · Senescence

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome which encodes a constitutively activated tyrosine kinase (Breakpoint Cluster Region - Abelson murine leukemia viral oncogene homolog 1, BCR-ABL) responsible of the leukemogenic transformation [1–3]. The first line treatment for CML consists on BCR-ABL inhibitors such as Imatinib, Nilotinib and Dasatinib. Without adequate therapeutic management, this disease has a median survival of four years [1].

One of the most commonly used in vitro CML model is the human cell line K562 [4, 5]. In these cells, the anti-proliferative effect of Imatinib is mediated by apoptosis and senescence induction [6]. Such biological processes are two of the most important mechanisms of tumor suppression. Apoptosis is a type of cell death [7] while senescence is a terminal differentiation stage associated with ageing and stress conditions [8–11]. Apoptosis is characterized by morphological and biochemical changes, such as reduction of cell size, plasma membrane blebbing, chromatin condensation, DNA fragmentation, reduction of mitochondrial membrane potential and phosphatidyl serine exposure [7]. Senescence is characterized by an irreversible arrest of cell cycle with high activity of senescence associated β-galactosidase (SA-β-gal) and presence of senescence associated heterochromatin foci (SAHF) [8–11]. SA-β-gal differentiates senescent from quiescent cells and it is the main marker of cellular senescence, while SAHF is a consequence of the DNA damage.

Imatinib is a highly effective drug for the treatment of CML; however, the selection pressure that it exerts favors the development of resistant cells [5, 12, 13]. Therefore, it is of great value to find new molecular targets for enhancing the anti-proliferative effect of Imatinib. Considering that multiple factors may contribute to chemoresistance, the impact of tumoral microenvironment on this process has become of great interest. This microenvironment comprises tumor-associated cells and extracellular matrix which plays an active role in cell proliferation, cell death evasion, angiogenesis, invasion, immune suppression and MDR [14–16].

Hyaluronan (HA) is the main glycosaminglycan of the extracellular matrix. HA levels depend on its synthesis mediated by HA synthases (Has), its degradation mediated by hyaluronidases (Hyals) and its internalization mediated by receptors [17–21]. The interaction of HA with its receptors, CD44 and RHAMM, stimulates signaling pathways such as PI3K/Akt and MAPK, which are involved in cell proliferation and MDR [17–19, 22].

In the case of several tumors, high levels of HA correlate with poor prognosis [23–31]. Therefore, many strategies aimed at mitigating the effect of signals triggered by HA have been studied. One of such strategies involves the use of hyaluronan oligomers (oHA), which block the interaction of HA with its receptors. Recently, we have demonstrated that oHA sensitize human leukemia cell lines to the pro-apoptotic and senescent effect of Imatinib [32]. Another promising strategy is the use of 4-methylumbelliferone (4MU), which inhibits HA synthesis through the depletion of Has substrate (UDP-glucuronic acid) and the Has-2 and Has-3 mRNA [33, 34]. It has been demonstrated that 4MU abrogates the proliferation of several tumor cell lines in vitro and in vivo [22, 35–39]. Moreover, 4MU is a non-toxic dietary supplement, which has been approved to be used in Europe and Asia due to its choleretic and biliary antispasmodic activities [34, 35].

Nevertheless, little is it known about the effect of 4MU on hematological malignancies. We have previously demonstrated that 4MU abrogates the proliferation of human chronic myeloid leukemia (CML) cell lines by the induction of senescence [22]. Moreover, 4MU sensitizes the Vincristine-resistant Kv562 cell line to the effect of Vincristine (VCR) through the inhibition of the P-glycoprotein (Pgp) and the PI3K pathway [22]. Besides, it has been reported that 4MU can induce apoptosis in several carcinoma cell lines [35–39]. However, the effects of 4MU on Imatinib therapy remain to be elucidated.

The aim of this work was to evaluate whether the combination of 4MU and Imatinib is able to modulate proliferation as well as apoptosis and senescence induction in the human CML cell lines K562 and Kv562. Our results indicate that the combination of such drugs abrogated the proliferation of both cell lines, increasing senescence and maintaining the apoptosis levels induced by Imatinib. These findings highlight the potential use of 4MU together with Imatinib for CML therapy.

Materials and methods

Cell culture

Human CML cell lines K562 (VCR sensitive) and Kv562 (VCR resistant) were grown in suspension cultures at 37 °C in a 5 % CO_2 atmosphere with RPMI-1640 supplemented with 10 % heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 10 mM HEPES buffer, 5×10^{-5} M 2-mercaptoethanol, 100 μg/ml streptomycin and 100 IU/ml penicillin (RPMI-C). The resistant cell line was cultured in the presence of 150 ng/ml (162 nM) VCR [22].

Reagents

4MU, X-gal and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich (USA). Imatinib (Novartis, Switzerland). VCR (Filaxis, Argentine). Antibodies (Ab) against pAkt, Akt, pERK, ERK, β-Actin, anti-rabbit secondary horseradish peroxidase, anti-goat secondary horseradish peroxidase were purchased from Santa Cruz Biotechnology (USA). [3H]TdR was purchased from Perkin-Elmer (Boston, USA). RPMI 1640, L-glutamine, streptomycin and penicillin were purchased from Invitrogen (Argentina). Annexin-V-PE Apoptosis Detection Kit I was purchased from BD Pharmingen™ (BD Bioscience, USA).

Cell proliferation

Cell proliferation was analyzed by the [3H]TdR uptake assay evaluated at 48 h in 96-well microtiter plates [22]. Fifty thousand cells per well (250,000 cells/ml) were used. Cells were

grown at 37 °C in a 5 % CO_2 atmosphere with RPMI-C, 4MU (500 or 100 μM), Imatinib (2; 0.5; 0.25 or 0.125 μM) or a combination of both. After pulsing with 1 μCi [3H]TdR for 6 h, cells were harvested and counted in a liquid scintillation counter (Beckman, MD). Results were calculated from the mean cpm of [3H]TdR incorporated in triplicate cultures. Untreated cells represented 100 % cell survival. Cell viability at the beginning of the experiment was higher than 95 %, as assessed by Trypan blue dye.

Apoptosis evaluation

Cells were treated with RPMI-C, 4MU (500 or 100 μM), Imatinib (2; 0.5; 0.25 or 0.125 μM) or a combination of both for 48 h at 37 °C in a 5 % CO_2 atmosphere. Then, membrane asymmetry, DNA fragmentation and hypodiploid DNA content were evaluated. For membrane asymmetry, the Annexin-V-PE Apoptosis Detection Kit I (BD Biosciences, USA) was used following the manufacturer's instructions. A PAS III flow cytometer (FC) (Partec, Germany) was used to acquire data which was analyzed with the WinMDI 2.8 software (Scripps Institute, La Jolla, USA). For DNA fragmentation and hypodiploid DNA content, cells were fixed with phosphate buffer saline (PBS) plus 2 % *p*-formaldehyde (PFA) or 70 % ethanol, respectively, stained with DAPI and evaluated using fluorescence microscopy (Olympus BX51, America Inc.) and FC (PAS III flow cytometer, Partec Germay), respectively [40, 41].

Senescence evaluation

Cell senescence was analyzed by senescence associated β-galactosidase (SA-β-gal) and senescence associated heterochromatin foci (SAHF) [42]. For SA-β-gal, cells were incubated with RPMI-C, 4MU (500 or 100 μM), Imatinib (2; 0.5; 0.25 or 0.125 μM) or a combination of both at 37 °C in a 5 % CO_2 atmosphere. After 48 h, cells were fixed with PBS plus 2 % PFA and washed with PBS. Each vial was incubated for 24 h at 37 °C with 1 ml of staining solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 150 mM NaCl, 30 mM citric acid/phosphate pH = 6). Cells were washed twice with PBS and the SA-β-gal activity was evaluated using an Olympus BX51 (America Inc.) microscope. Blue cells were considered positive. For each condition, 200 cells were counted and the percentage of SA-β-gal positive cells was calculated [6, 42]. For SAHF and cell cycle evaluation, cells were subjected to a similar procedure. After 48 h, cells were fixed with PBS plus 2 % PFA, washed and incubated with 1 μg/ml DAPI in PBS plus 0.2 % Triton X-100 for 30 min at room temperature. Cells were analyzed in a fluorescence microscopy (Olympus BX51, America Inc.) for SAHF and by FC for cell cycle (PAS III flow cytometer, Partec Germay) [43].

Western blot

Cells were treated with RPMI-C, 500 μM 4MU, Imatinib (2; 0.5; 0.25 or 0.125 μM) or a combination of both for 24 h at 37 °C in a 5 % CO_2 atmosphere. Cells were then lysed with hypotonic buffer. After centrifugation (13,000 rpm 30 min), equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane (Osmonics Inc., Gloucester, MA). The membrane was blocked and incubated with specific antibodies to p-Akt, Akt, p-ERK, ERK or β-Actin overnight at 4 °C followed by incubation with a horseradish peroxidase-labeled secondary antibody for 2 h at 37 °C. The reaction was developed using a chemiluminescent detection system. Gel images obtained with a digital camera were subjected to densitometric analysis using the Image Scion Software (Scion Corporation, USA) [22, 44].

Statistical analysis

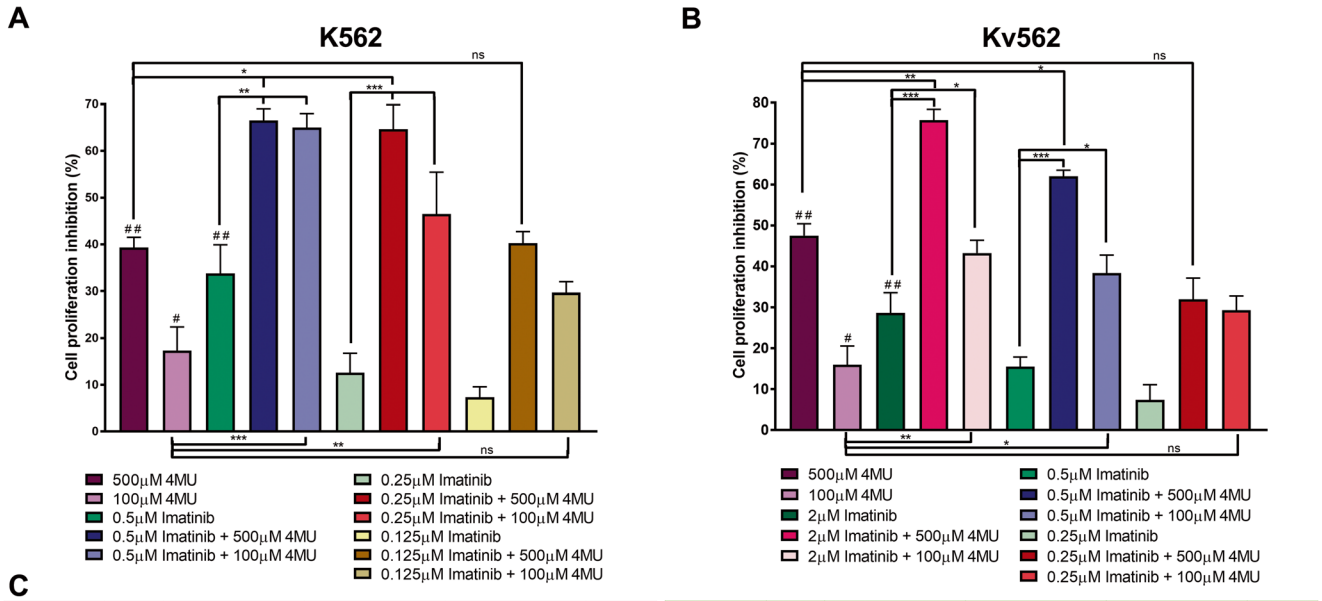
All results were analyzed by one way-ANOVA and Bonferroni's test. The analysis was performed using Prism software (Graph Pad, San Diego, CA, USA). P values <0.05 were regarded as statistically significant [22].

Results

4MU and imatinib combination abrogates the proliferation of K562 and Kv562 cells

To evaluate whether 4MU and Imatinib could modulate cell proliferation, the [3H]TdR uptake was measured. Several concentrations (0.125, 0.25, 0.5, 1, 2 and 4 μM) of Imatinib were tested (data not shown) and three doses for each cell line to perform the experiments were chosen. The highest concentration employed was selected since it was the lowest dose which modulated cell proliferation (0.5 μM for K562 and 2 μM for Kv562). Besides, two lower doses were used to evaluate the ability of 4MU to sensitize the cell lines to the effect of Imatinib (0.125 and 0.25 μM for K562 and 0.25 and 0.5 μM for Kv562). In all cases, 500 and 100 μM 4MU were employed [22].

Figure 1a and c shows that the combination of 500 and 100 μM 4MU with 0.5 or 0.25 μM Imatinib abrogated K562 cells proliferation when compared to each drug alone. However, 0.125 μM Imatinib in combination with 500 and 100 μM 4MU failed to increase cell growth inhibition when compared to 4MU alone.



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K562 cells proliferation inhibition (%)	mean±SD	0.5μM Imatinib +		0.25μM Imatinib +		0.125μM Imatinib +		Untreated
		500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU	
mean±SD		66.5±2.5	65.0±3.0	64.7±5.2	46.5±8.9	40.3±2.5	29.7±2.3	0
500μM 4MU	39.3±2.2	*	--------	*	--------	ns	--------	##
100μM 4MU	17.3±5.0	--------	***	--------	**	--------	ns	#
0.5μM Imatinib	33.8±6.1	**	**	--------	--------	--------	--------	##
0.25μM Imatinib	12.6±4.1	--------	--------	***	***	--------	--------	ns
0.125μM Imatinib	7.3±2.2	--------	--------	--------	--------	**	*	ns

Kv562 cells proliferation inhibition (%)	mean±SD	2μM Imatinib +		0.5μM Imatinib +		0.25μM Imatinib +		Untreated
		500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU	
mean±SD		75.8±2.7	43.3±3.1	62.0±1.6	38.3±4.4	32.0±5.1	29.3±3.5	0
500μM 4MU	47.5±2.9	**	--------	*	--------	ns	--------	##
100μM 4MU	16.0±4.6	--------	**	--------	*	--------	ns	#
2μM Imatinib	28.7±4.9	***	*	--------	--------	--------	--------	##
0.5μM Imatinib	15.5±2.4	--------	--------	***	*	--------	--------	ns
0.25μM Imatinib	7.4±3.7	--------	--------	--------	--------	*	*	ns

Fig. 1 Effect of 4MU and Imatinib combination on cell proliferation. **a** K562 and **b** Kv562 cells were treated with 4MU, Imatinib or a combination of both, cell growth was determined by the [3H]TdR uptake assay for 48 h. Results are expressed as: Cell proliferation inhibition (%) = 100 - (treated cpm × 100 / untreated cpm).cellproliferationpercentage = (treatedcpmxd/untreatedcpm) − 100. Bars represent means ± SD of at least 5 independent experiments # #$p < 0.01$ and #$p < 0.05$ vs. untreated while ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ and ns = no significant ($p > 0.05$) between the different treatments marked. **c** Tables show means ± SD and statistical analyses obtained for cell proliferation inhibition.

Figure 1b and c shows that both doses of 4MU plus 0.5 or 2 μM Imatinib increased the anti-proliferative effect of each drug alone while the combination of 4MU with the lower dose of Imatinib did not have any effect on Kv562 cell growth when compared to 4MU.

4MU and imatinib co-treatment does not enhance the pro-apoptotic effect of imatinib

It was interesting to evaluate whether 4MU and Imatinib combination abrogated cell proliferation by increasing the apoptosis levels. To this purpose, cells were treated with 4MU (500 and 100 μM), Imatinib (0.5 and 0.25 μM for K562 and 2 and 0.5 μM for Kv562) or a combination of both, since the co-treatment with such concentrations inhibited cell growth. After 48 h of incubation, membrane asymmetry (Annexin-V-PE), hypodiploid content (subG1 peak) and DNA fragmentation were analyzed.

Figure 2a shows that 4MU and Imatinib combination did not modify the percentage of Annexin-V-PE positive K562 and Kv562 cells when compared to Imatinib alone. Moreover, 4MU and Imatinib co-treatment had no effect on the percentage of both cells lines neither in the subG1 peak nor with DNA fragmentation (Fig. 2b and c, respectively).

Taking together, these results allow suggesting that 4MU and Imatinib combination did not modify the levels of apoptosis induced by Imatinib alone.

4MU and imatinib co-treatment causes an arrest in the cell cycle of both cell lines

K562 and Kv562 cell lines were treated with 4MU, Imatinib or a combination of both to study the phases of the cell cycle by FC.

Figure 3a shows that the highest dose of Imatinib tested increased the percentage of cells in G1/0 phase in both cell lines. Imatinib reduced the percentage of Kv562 cells in S (Fig. 3b). 4MU arrested both cell lines in the G2/M phase (Fig. 3c). However, 4MU in combination with Imatinib induced a higher percentage of K562 and Kv562 cells in S phase (Fig. 3b).

4MU and imatinib combination enhances senescence induction

Since 4MU plus Imatinib abrogated both cell lines growth without increasing apoptosis; senescence induction was analyzed. To this end, cells were treated with 4MU, Imatinib or a

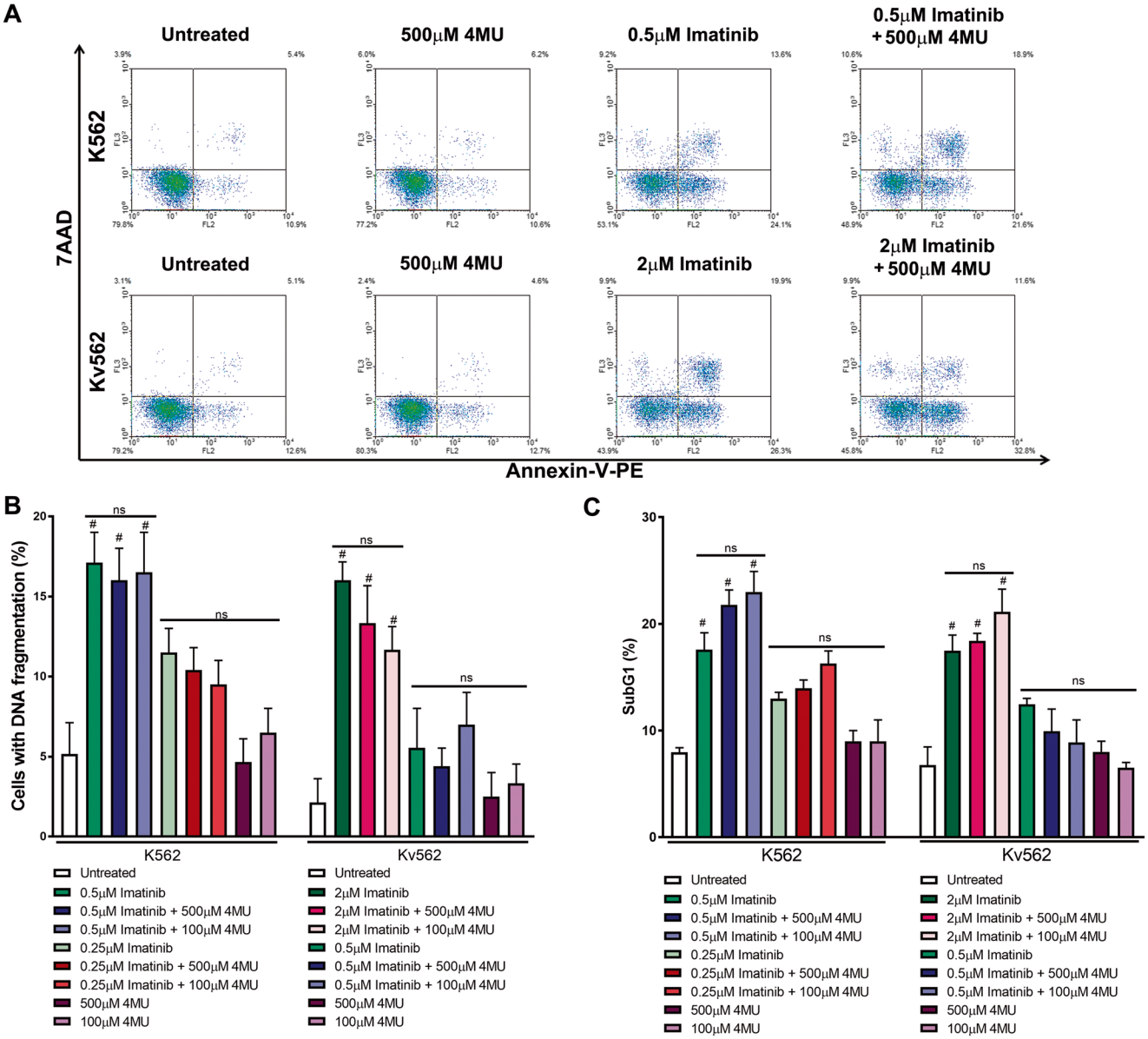


Fig. 2 Effect of 4MU and Imatinib on apoptosis induction in K562 and Kv562 cell lines. To determine if 4MU and Imatinib co-treatment enhances the induction of apoptosis, both cell lines were treated with these compounds alone or in combination for 48 h and then three different parameters were analyzed: **a** Asymmetry membrane was evaluated by FC. After treatments cells were stained with AnnexinV-PE/7AAD. The picture shows a representative experiment of 3 independent determinations. No significant differences were found for total cells stained with Annexin-V-PE between treatments with Imatinib and 4MU + Imatinib. **b** DNA fragmentation was analyzed by fluorescence microscopy. K562 and Kv562 cells were fixed with PFA and stained with DAPI. Bars represent means ± SD of cells with DNA fragmentation of 3 independent experiments #$p < 0.05$ vs. untreated while ns = no significant ($p > 0.05$) between the different treatments marked. **c** Hypodiploid content was evaluated by FC. Cells were fixed with ethanol and stained with DAPI. Bars represent means ± SD of cells in subG1 peak of 3 independent experiments #$p < 0.05$ vs. untreated while ns = no significant ($p > 0.05$) between the different treatments marked.

combination of both. After 48 h, SA-β-gal activity and SAHF were evaluated.

The co-treatment with 4MU plus Imatinib increased the percentage of SA-β-gal positive cells (Fig. 4a and c) as well as the number of cells with SAHF (Fig. 4b), suggesting that the combination of 4MU and Imatinib enhances senescence induction.

The co-treatment with 4MU and imatinib decreases the pAkt/Akt and pERK/ERK ratios similarly to each drug alone

To evaluate the effect of 4MU and Imatinib on Akt and ERK phosphorylation, K562 and Kv562 cell lines were treated with 4MU, Imatinib or a combination of both

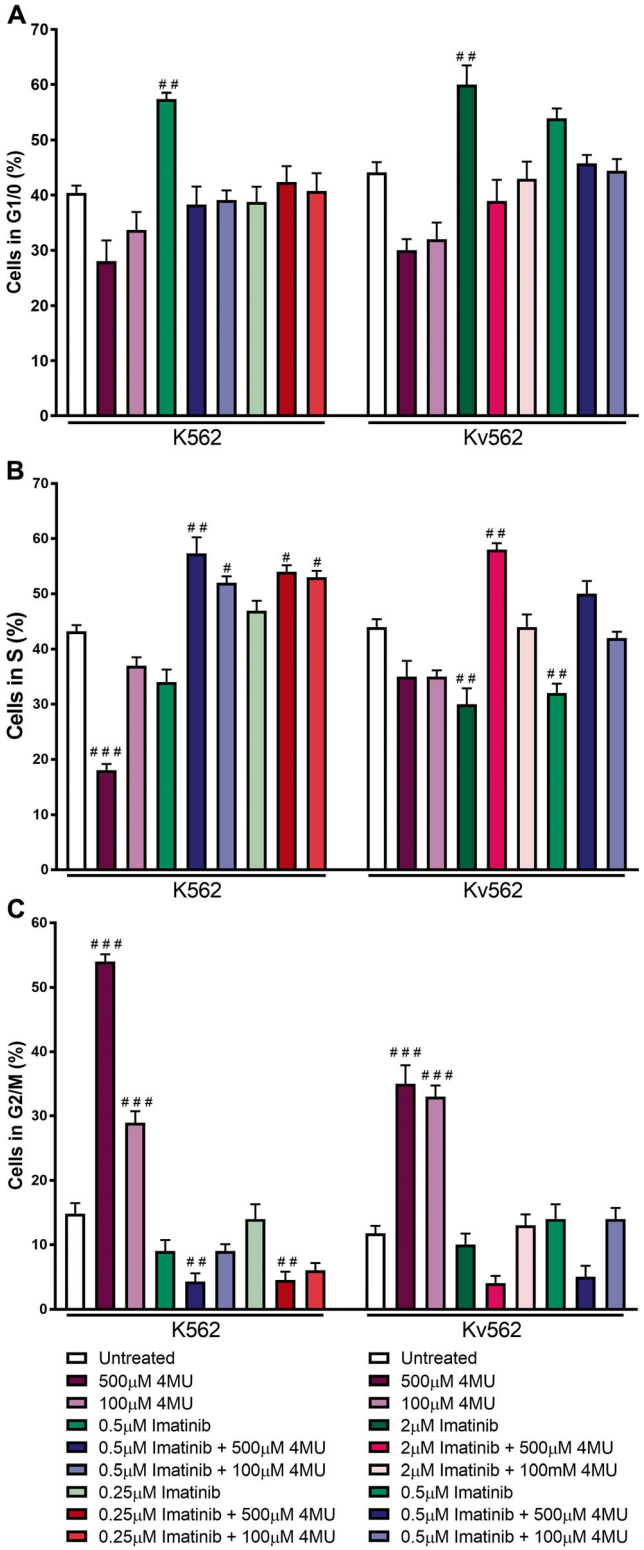


Fig. 3 Cell cycle phases distribution. To determine if 4MU and Imatinib modified the distribution of the phases of cell cycle, both cell lines were treated with such inhibitors or a combination of both for 48 h. Cells were fixed with PFA, stained with DAPI and evaluated by FC. Bars represent means ± SD of percentage of cells in G1/0 (**a**), S (**b**), G2/M (**c**) phases of cell cycle of 3 independent experiments # # #$p < 0.001$, # #$p < 0.01$ and #$p < 0.05$ vs. untreated.

for 24 h analyzing pAkt/Akt and pERK/ERK ratio by Western Blot.

In both cell lines, 500 μM 4MU plus 2 μM Imatinib decreased the pAkt/Akt ratio when compared to baseline conditions. However, the inhibition index induced by the co-treatment was similar to that obtained for each drug alone (Fig. 5a). Similar results were found after treatment with 4MU plus Imatinib for the pERK/ERK ratio in K562 cell line. Both, the co-treatment and each drug alone had a similar effect on ERK phosphorylation (Fig. 5b).

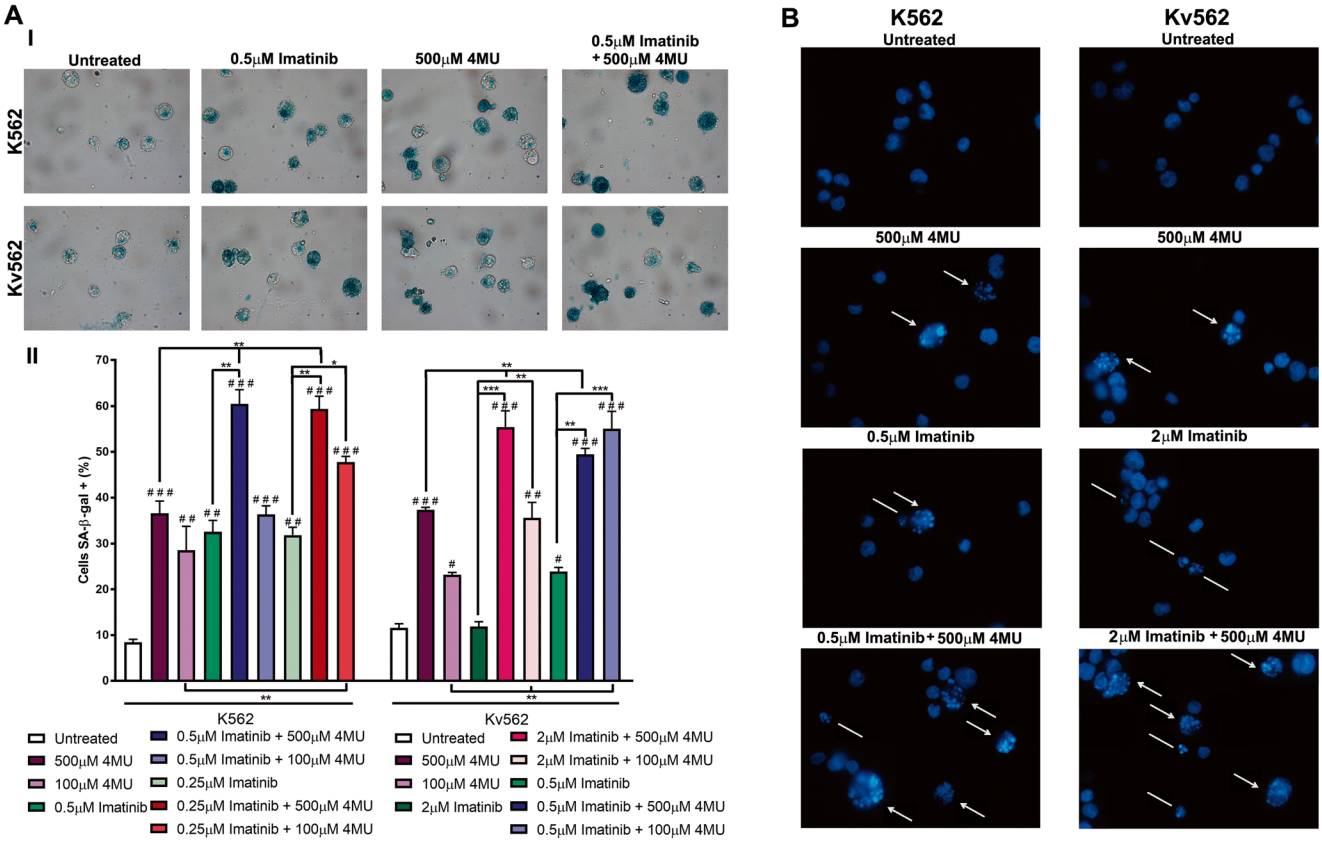
Discussion

CML is a myeloproliferative syndrome which represents the 15 % of all leukemias. Imatinib is a highly effective drug for the treatment of CML; however, the selection pressure that it exerts favors the development of resistant cells [5, 12, 13]. Therefore, to find new molecular targets for enhancing the anti-proliferative effect of Imatinib is of value. In this work, we demonstrated for the first time that 4MU and Imatinib combination enhances senescence induction decreasing cell growth and keeping the apoptosis levels induced by Imatinib on K562 and Kv562 cell lines.

The anti-proliferative effect exerted by Imatinib was mediated by apoptosis and senescence induction with G0/1 arrest in both cell lines. Moreover, Imatinib decreased pAkt/Akt and pERK/ERK ratios in K562 cells while it abrogated Akt phosphorylation in Kv562 cells. These results are in accordance with the effects on K562 cells reported by other authors [6, 45, 46]. It is worth noting that the effect of the same dose of Imatinib was higher in K562 cells than in Kv562 cells since the latter presents Pgp and Imatinib is a substrate of this pump [22, 47–49].

4MU inhibited both cell lines proliferation inducing senescence and G2/M arrest. Moreover, 4MU decreased the pAkt/Akt ratio in both cell lines while it decreased the pERK/ERK ratio only in K562 cells. These results are in accordance with our previous work [22] and with other reports in several carcinoma cell lines [35–39]. Interestingly, in contrast to the anti-proliferative activity of Imatinib, the effect of 4MU was similar in both cell lines, suggesting that 4MU cannot be extruded by Pgp. Moreover, as we previously demonstrated, 4MU was able to inhibit the activity of such pump through the inhibition of HA synthesis [22].

The co-incubation of several doses of 4MU and Imatinib significantly abrogated the proliferation of K562 and Kv562 cells, keeping the apoptosis levels induced by Imatinib. The anti-proliferative effect of the co-treatments was mediated by the induction of senescence. It is worth noting that Imatinib arrested both cell lines in G1/0 phase while 4MU caused an arrest in G2/M phase; however, after the combination of such inhibitors, an accumulation of cells in S phase was induced in



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Cell SA-β-gal positives (%)	K562 cells						Kv562 cells					
	mean±SD	Untreated	0.5μM Imatinib +		0.25μM Imatinib +		mean±SD	Untreated	2μM Imatinib +		0.5μM Imatinib +	
			500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU			500μM 4MU	100μM 4MU	500μM 4MU	100μM 4MU
mean±SD		8.4±0.6	60.5±3.1	36.3±1.9	59.4±2.8	47.8±1.2		11.6±0.9	55.4±3.6	35.6±3.4	49.5±1.3	55.1±3.8
500μM 4MU	36.6±2.6	###	**	--------	**	--------	37.3±0.5	###	**	--------	**	--------
100μM 4MU	28.6±5.1	##	--------	ns	--------	**	23.2±0.5	#	--------	**	--------	**
2μM Imatinib	--------	--------	--------	--------	--------	--------	11.8±1.1	ns	***	**	--------	--------
0.5μM Imatinib	32.5±2.5	##	**	ns	--------	--------	23.9±0.9	#	--------	--------	**	***
0.25μM Imatinib	31.8±1.7	##	--------	--------	**	*	--------	--------	--------	--------	--------	--------

Fig. 4 Evaluation of senescence induction. To determine if 4MU and Imatinib combination enhances senescence induction, both cell lines were treated either with such inhibitors or a combination of both for 48 h and SA-β-gal and SAHF were evaluated **a** Determination of SA-β-gal activity. (I) Pictures show a representative experiment (II) Bars represent means ± SD of 3 independent experiments # # #$p < 0.001$, #$p < 0.01$ and #$p < 0.05$ vs. untreated while ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ between the different treatments marked. **b** SAHF, magnification 400X. Arrows indicate nuclei with SAHF while lines indicate nuclei with DNA fragmentation characteristic of senescent and apoptotic cells, respectively. **c** Tables show means ± SD and statistical analyses obtained for determination of SA-β-gal activity.

both cell lines. Although the co-incubation of Imatinib and 4MU did not reduce pAkt and pERK levels when compared to each inhibitor alone, these co-treatments decreased the phosphorilation of ERK in K562 cells and the phosphorylation of Akt in both cell lines.

We suggest that under baseline conditions, BCR-ABL and HA activate survival pathways involved in cell proliferation and MDR (Fig. 6a). Imatinib inhibits BCR-ABL kinase activity inducing different tumors suppression mechanism such as senescence and apoptosis (Fig. 6b). 4MU inhibits HA synthesis abrogating cell proliferation and MDR and favoring senescence (Fig. 6c). However, the combination of 4MU and Imatinib is the treatment that exerts the strongest anti-proliferative effect through apoptosis induction and through the increase in the levels of senescence (Fig. 6d).

Taking into account these results, we suggest that 4MU would be a potential novel drug for CML therapy, since it showed a significant anti-proliferative effect, and combined with Imatinib, it would enhance senescence induction. Moreover, bone marrow presents high amounts of HA [50, 51] which would activate survival pathways and Pgp thus reducing the effect of Imatinib. Therefore, 4MU would be useful since it decreases HA levels and mitigates its survival signaling. In addition, 4MU would not be extruded by Pgp, since it inhibits such pump activity and therefore contributes to hinder the development of multidrug resistance [22]. It is worth noting that 4MU was

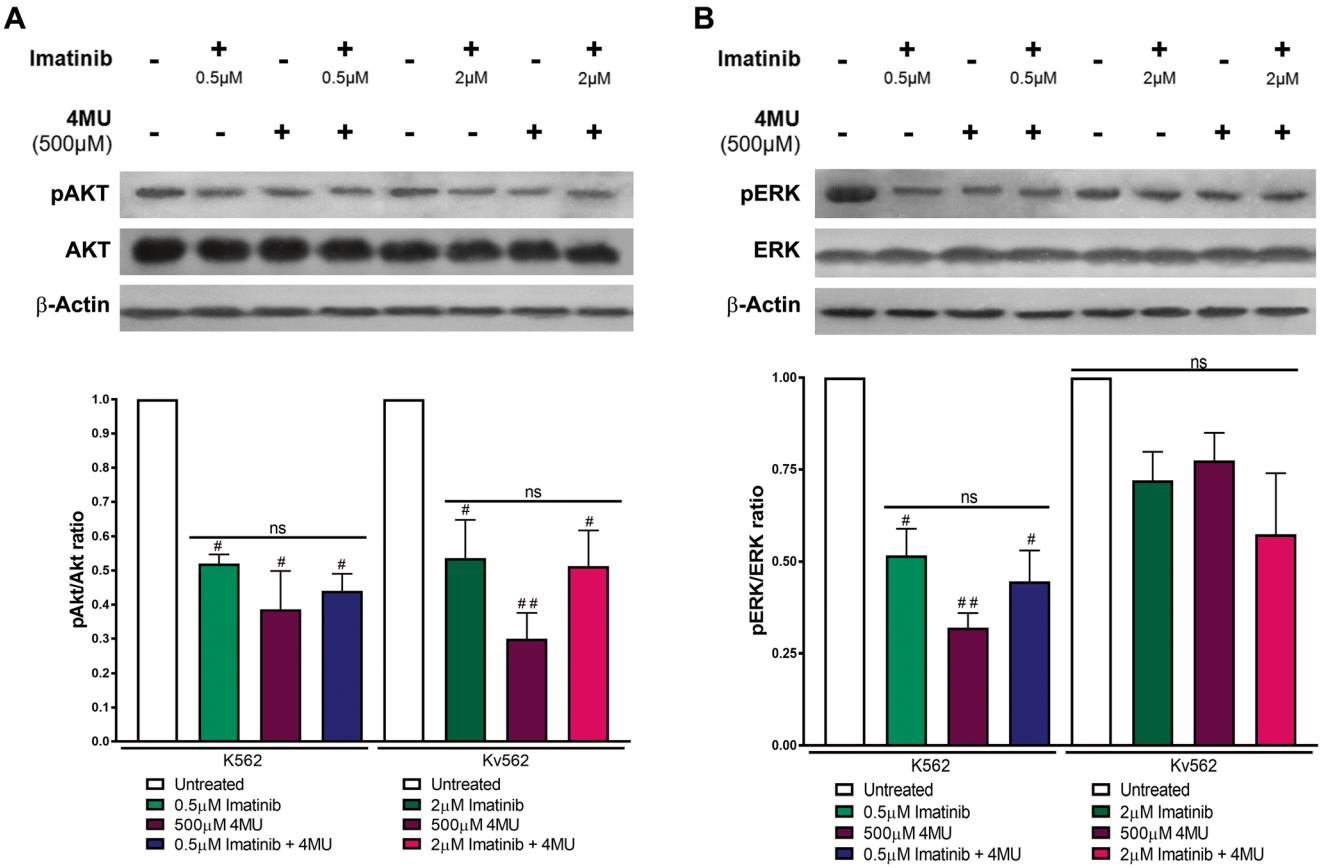

Fig. 5 Modulation of pAkt/Akt and pERK/ERK ratios. **a** K562 and Kv562 cells were treated either with 4MU, Imatinib or a combination of both for 24 h. The phosphorilation of Akt was evaluated by WB. Results are expressed as pAkt/Akt ratio = $[(pAkt/\beta\text{-actin}) / (Akt/\beta\text{-actin})]_{treated}$ / $[(pAkt/\beta\text{-actin}) / (Akt/\beta\text{-actin})]_{untreated.}$ Bars represent means ± SD of at least 3 independent experiments # #p < 0.01, #p < 0.05 vs. untreated and ns = no significant (p > 0.05) between the different treatments marked. **b** Both cell lines were treated either with 4MU, Imatinib or a combination of both for 24 h. The phosphorilation of ERK was evaluated by WB. Results are expressed as pERK/ERK ratio (calculated likewise). Bars represent means ± SD of at least 3 independent experiments, # #p < 0.01 and #p < 0.05 vs. untreated while ns = no significant (p > 0.05) between the different treatments marked.

approved for human use in Asia and in Europe due to its choleretic and biliary antispasmodic activities [34]. Besides, in several clinical trials, no serious adverse effects have been reported [34]. As additional advantages, 4MU can be administered orally and is unexpensive. For all these reasons, 4MU can be considered a promising new drug to treat CML either as monotherapy or in combination with Imatinib.

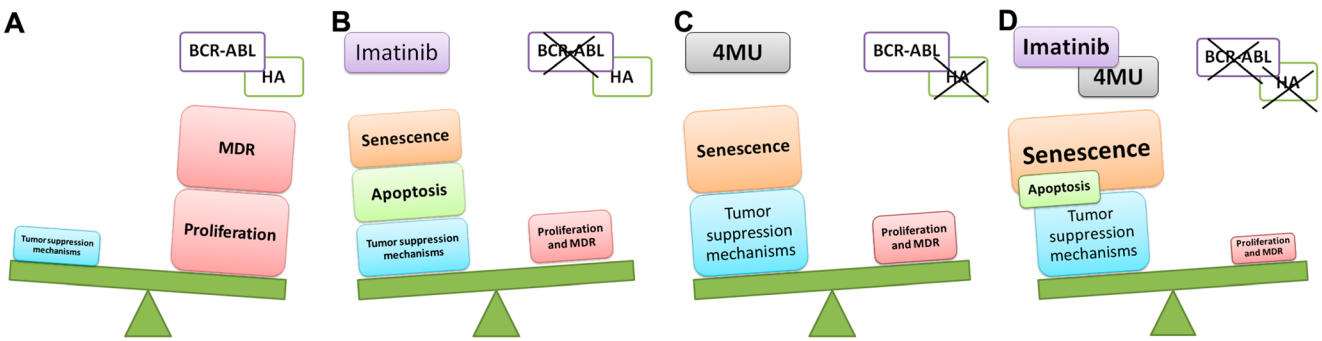

Fig. 6 Suggested role of BCR-ABL, HA, 4MU and Imatinib on K562 and Kv562 cells proliferation. **a** In baseline conditions, BCR-ABL and HA activates several survival pathways involved in cell proliferation and multidrug resistance. **b** Imatinib inhibits BCR-ABL kinase activity inducing senescence and apoptosis, two of the most important tumor suppression mechanisms. **c** 4MU inhibits HA synthesis abrogating cell proliferation as well as multidrug resistance and favoring senescence. **d** The combination of 4MU and Imatinib induce the strongest anti-proliferative effect due to apoptosis and increasing levels of senescence.

Compliance with ethical standards

Conflict of interest Author SLL declares that she has no conflict of interest. Author MD declares that she has no conflict of interest. Author DLP declares that she has no conflict of interest. Author MP declares that he has no conflict of interest. Author EA declares that she has no conflict of interest. Author SEH declares that she has no conflict of interest.

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Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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