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Original Article

Cancer Biology

Hyaluronan oligomers sensitize chronic myeloid leukemia cell lines to the effect of Imatinib

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Abstract

Chronic myeloid leukemia is a myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome (Ph), generated by a reciprocal translocation occurring between chromosomes 9 and 22 [t(9;22)(q34;q11)]. As a consequence, a fusion gene (*bcr-abl*) encoding a constitutively active kinase is generated. The first-line treatment consists on *BCR-ABL* inhibitors such as Imatinib, Nilotinib and Dasatinib. Nevertheless, such treatment may lead to the selection of resistant cells. Therefore, finding molecules that enhance the anti-proliferative effect of first-line drugs is of value. Hyaluronan oligomers (oHA) are known to be able to sensitize several tumor cells to chemotherapy. We have previously demonstrated that oHA can revert Vincristine resistance in mouse lymphoma and human leukemia cell lines. However, little is known about the role of oHA in hematological malignancies. The aim of this work was to determine whether oHA are able to modulate the anti-proliferative effect of Imatinib in chronic myeloid leukemia (CML) cell lines. The effect on apoptosis and senescence as well as the involvement of signaling pathways were also evaluated. For this purpose, the human CML cell lines K562 and Kv562 (resistant) were used. We demonstrated that oHA sensitized both cell lines to the anti-proliferative effect of Imatinib increasing apoptosis and senescence. Moreover, this effect would be accomplished through the down-regulation of the PI3K signaling pathway. These findings highlight the potential of oHA when used as a co-adjuvant therapy for chronic myeloid leukemia.

Key words: apoptosis, chronic myeloid leukemia, hyaluronan oligomers, Imatinib, senescence

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative syndrome characterized by the presence of the Philadelphia chromosome (Ph), generated by a reciprocal translocation occurring between chromosomes 9 and 22 [t(9;22)(q34;q11)]. As a consequence, a fusion gene (*bcr-abl*) encoding a constitutively active kinase is generated. BCR-ABL is an oncogenic protein responsible of the leukemogenic transformation (Chandraa et al. 2011). For that reason, the first-line treatment for CML consists on *BCR-ABL* inhibitors such as Imatinib, Nilotinib and Dasatinib (Von Bubnoff and Duyster 2010; Wei et al. 2010).

One of the most used models for CML in preclinical studies of Imatinib is the one employing the human cell line K562 (Wetzel et al. 2005; Karimiani et al. 2014). In these cells, the anti-proliferative effect of Imatinib is mediated by the induction of apoptosis as well as senescence (Drullion et al. 2012). Such biological processes are two of the most important mechanisms of tumor suppression. Apoptosis is a type of cell death characterized by morphological and biochemical changes, such as reduction of cell size, cytoplasmic membrane blebbing, DNA fragmentation, exposure of phosphatidylserine and drop of mitochondrial membrane potential (Galluzzi et al. 2012).

Senescence is a terminal differentiation stage associated with ageing, stress conditions and tumor suppression. Senescent cells are characterized by molecular and morphological changes such as (i) irreversible cell cycle arrest, (ii) increase in cellular granulation and size, (iii) the presence of high β-galactosidase activity at suboptimal pH (SA-β-gal), (iv) resistance to apoptosis and (v) the presence of senescence-associated heterochromatin foci (SAHF) (Campisi and d'Adda di Fagagna 2007; Nardella et al. 2011; Rodier and Campisi 2011).

Imatinib is a highly effective drug for the treatment of CML; however, the selection pressure that it exerts favors the development of resistant cells (Wetzel et al. 2005; Mauro 2006; Comert et al. 2013). Therefore, to find new molecular targets for enhancing the anti-proliferative effect of Imatinib is of value.

Over the last years, it has been demonstrated that the extracellular matrix plays an active role in tumor progression and multidrug resistance (MDR) (Bissel and Radisky 2001; Wong and Rustgi 2013). Hyaluronan (HA) is the main glycosaminogycan of the extracellular matrix whose high levels correlate with poor prognosis and malignancy development in several tumors (Boregowda et al. 2006; Tammi et al. 2008; Toole 2009; Sironen et al. 2011; Bourguignon 2012; Auvinen et al. 2013; Provenzano and Hingorani 2013). 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, migration and MDR (Toole 2004, 2009). Many strategies aimed at mitigating the signals triggered by HA have been studied. One of them is the use of hyaluronan oligomers (oHA) which are able to bind HA receptors. However, due to their small size, oHA cannot cross-link receptors, thus attenuating the signaling triggered by HA (Toole 2004, 2009). It is known that oHA can sensitize several tumor cell lines to the effect of chemotherapy (Misra et al. 2003; Alaniz et al. 2006; Cordo-Russo et al. 2008; Slomiany, Dai, Bomar, et al. 2009; Slomiany, Dai, Tolliver, et al. 2009). Nevertheless, little is known about the effect of oHA on CML cell lines. We have previously demonstrated that oHA can sensitize the Vincristine-resistant Kv562 cell line to the effect of Vincristine through the inhibition of the P-glycoprotein (Pgp) and the PI3K pathway (Lompardía et al. 2013). Besides, other authors have reported that oHA can also sensitize the Adryamicin-resistant K562/A02 cells to the effect of Adryamicin (Cui et al. 2009). However, nothing is known about the effect of oHA on Imatinib therapy. Therefore, the aim of this work was to evaluate whether oHA can sensitize CML cell lines to the anti-proliferative effect of Imatinib by analyzing the tumor suppression mechanisms involved. Our results indicate that the combination of oHA with Imatinib enhanced the anti-proliferative effect of Imatinib increasing either apoptotic death or senescence, depending on the concentration of Imatinib employed. When oHA were combined with doses of Imatinib that were able to induce apoptosis by themselves, oHA increased the levels of apoptosis, while the co-incubation of oHA with a lower concentration of Imatinib enhanced senescence induction. Moreover, the mechanism of tumor suppression involved would be regulated by PI3K. These findings highlight the potential of oHA when used as co-adjuvant for first-line therapy against CML.

Results

oHA sensitize K562 and Kv562 cells to the anti-proliferative effect of Imatinib

Knowing that oHA were able to sensitize several tumor cell lines to the effect of different chemotherapics (Cordo-Russo et al. 2008; Slomiany, Dai, Bomar, et al. 2009), the effect of oHA in combination with Imatinib on cell growth was assessed. To evaluate whether oHA and Imatinib could modulate cell proliferation, the [^{3}H]-thymidine uptake was measured. PI3K implication was also analyzed. Several concentrations (0.125, 0.25, 0.5, 1, 2 and 4 μM) of Imatinib were tested (data not shown), to choose three doses for each cell line to perform the experiments. 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 oHA 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, 300 μg/mL oHA was employed (Lompardía et al. 2013).

Figure 1A, panel I shows that oHA sensitized K562 cells to the anti-proliferative effect of 0.125 as well as 0.25 μM Imatinib. However, oHA + 0.5 μM Imatinib failed to increase significantly cell growth inhibition showing only a trend. Panel II shows that oHA sensitized Kv562 cells to the anti-proliferative effect of 0.5 as well as 2 μM Imatinib while the combination of oHA with the lower dose of Imatinib did not have any effect on cell growth.

Since oHA are able to decrease the pAkt/Akt ratio in both cell lines (Lompardía et al. 2013), it was interesting to assess whether the inhibition of PI3K signaling pathway could render K562 and Kv562 cells sensitive to Imatinib. As shown in Figure 1B, Ly294002 sensitized both cells lines to the anti-proliferative effect of Imatinib.

oAH enhance the pro-apoptotic effect of Imatinib

It has been reported that Imatinib induces apoptosis in K562 cells (Drullion et al. 2012). Besides, oHA increase the apoptotic effect of several chemotherapeutic drugs (Cordo-Russo et al. 2008; Slomiany, Dai, Bomar, et al. 2009; Quin et al. 2011). Therefore, it was interesting to evaluate whether the oHA sensitization to the anti-proliferative effect of Imatinib was mediated by increased levels of apoptosis. For this purpose, cells were treated with oHA, Imatinib or combinations. After 48 h of incubation, membrane asymmetry (AnnexinV-PE), hypodiploid content (subG1) and fragmentation of DNA were analyzed.

Figure 2A, panel I shows that oHA in combination with 0.25 as well as 0.5 μM Imatinib enhanced the percentage of AnnexinV-PE positive K562 cells while panel II demonstrates that only oHA plus 2 μM Imatinib increased such percentage in Kv562 cells. Moreover, oHA in combination with the highest dose of Imatinib increased the percentage of cells in subG1 peak as well as cells with DNA fragmentation in both cell lines (Figure 2B and C).

Taking together, these results suggest that oHA sensitize both cell lines to the pro-apoptotic effect of Imatinib only at the concentration of Imatinib that is able to induce apoptosis by itself.

Ly294002 sensitizes both cell lines to the pro-apoptotic effect of Imatinib

Since oHA reduce the pAkt/Akt ratio (Lompardía et al. 2013) and sensitize both cell lines to the pro-apoptotic effect of Imatinib, it was of value to evaluate if the inhibition of PI3K favors Imatinib-mediated apoptosis. Therefore, cells were treated with Ly294002, Imatinib or combinations and membrane asymmetry, hypodiploid content of DNA and its fragmentation were assessed.

Figure 3 shows, for both cell lines, that PI3K inhibition with Ly294002 in combination with Imatinib increased the percentage of AnnexinV-PE positive cells, the percentage of cells in subG1 as well as with DNA fragmentation. These results suggest that the inhibition of PI3K enhances the pro-apoptotic effect of Imatinib.

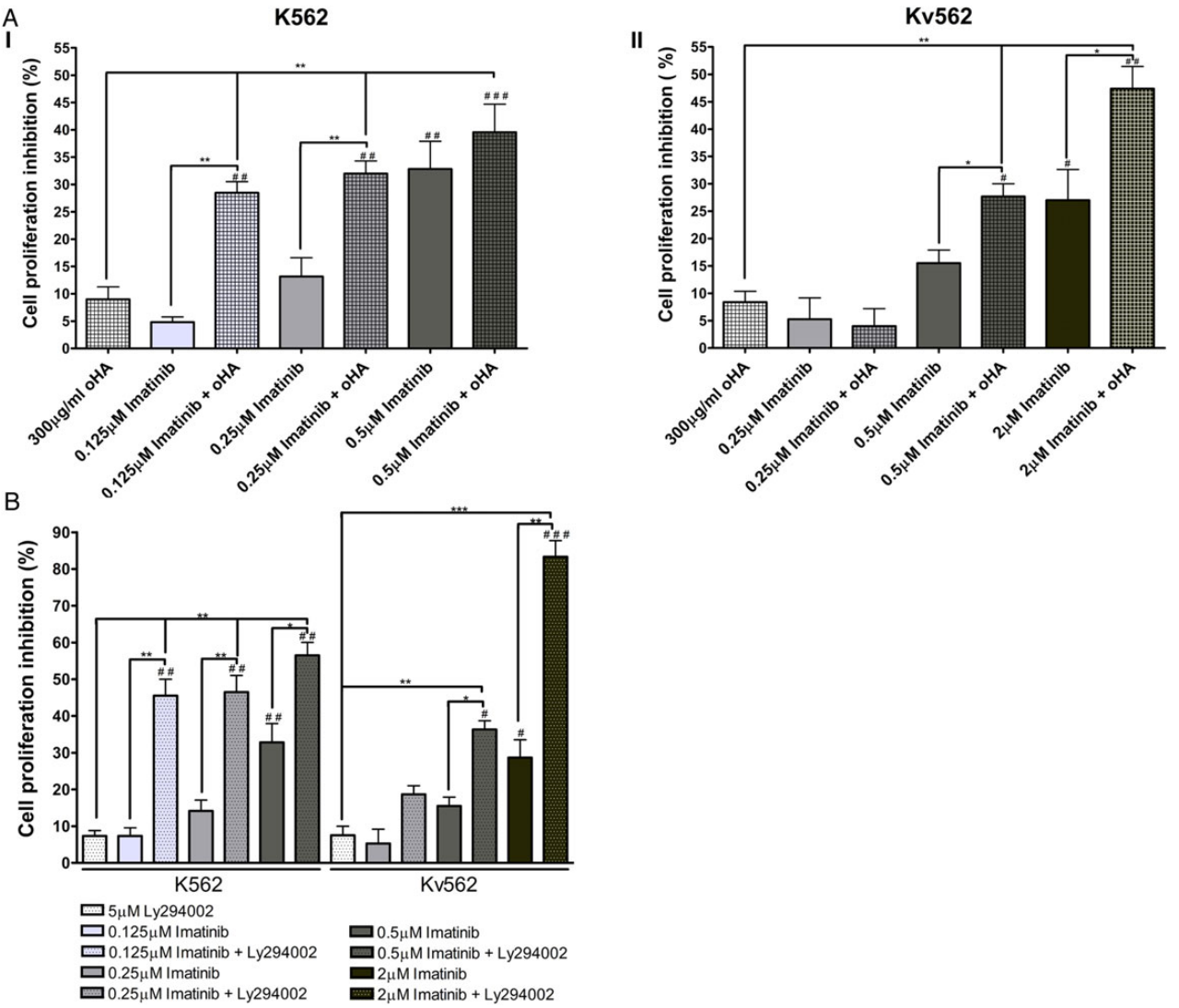

Fig. 1. Effect of oHA and Imatinib on both cell lines proliferation. (**A**) K562 (I) and Kv562 (II) cells were treated with 300 µg/mL oHA, Imatinib or combinations for 48 h. (**B**) 5 µM Ly294002 (PI3K inhibitor) was used to assess if PI3K inhibition was involved in the abrogation of cell growth. Results are expressed as: Cell proliferation percentage = (treated cpm × 100/untreated cpm) − 100. Bars represent means ± SD of at least five 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 as indicated.

oHA enhance senescence induction mediated by Imatinib

Due to the fact that Imatinib induces senescence in K562 cells (Drullion et al. 2012) and oHA sensitize both cell lines to the anti-proliferative effect of lower doses of Imatinib without modulating apoptosis, senescence induction was analyzed. To this end, cells were treated with oHA, Imatinib or combinations. After 48 h, SA-β-gal and SAHF were evaluated. It is worth to note that the increased activity of β-galactosidase (still detectable at suboptimal pH) is the main marker of senescence (SA-β-gal). Furthermore, SAHF are generated due to the characteristic genomic damage that occurs in senescence.

The co-treatment with oHA plus the highest levels, 0.5 or 2 µM Imatinib, did not have any effect neither on SA-β-gal nor on SAHF in K562 and Kv562 cells, respectively (Figure 4). Nevertheless, co-incubation of oHA with lower doses of Imatinib (0.125 and 0.25 µM in K562 and 0.5 µM in Kv562) increased the percentage of SA-β-gal-positive cells as well as the number of cells with SAHF (Figure 4). These results suggest that oHA sensitized both cell lines to the senescent effect of low doses of Imatinib.

Furthermore, the implication of PI3K on senescence induction was analyzed. SA-β-gal and SAHF were evaluated after 48 h of incubation with Ly294002, Imatinib or combinations. The co-treatment with Ly294002 + Imatinib did not have any effect on senescence induction (data not shown).

The co-treatment with oHA and Imatinib decreases the pAkt/Akt ratio

Imatinib as well as oHA abrogate phosphorylation of Akt and ERK in K562 cells (Machado-Neto et al. 2011; Lompardía et al. 2013; Elfineh et al. 2014). Moreover, oHA reduced the pAkt/Akt ratio without affecting the pERK/ERK ratio in Kv562 cells (Lompardía et al. 2013). Therefore, both cell lines were treated with oHA, Imatinib or combinations for 24 h and Akt and ERK phosphorylation was analyzed by western blot.

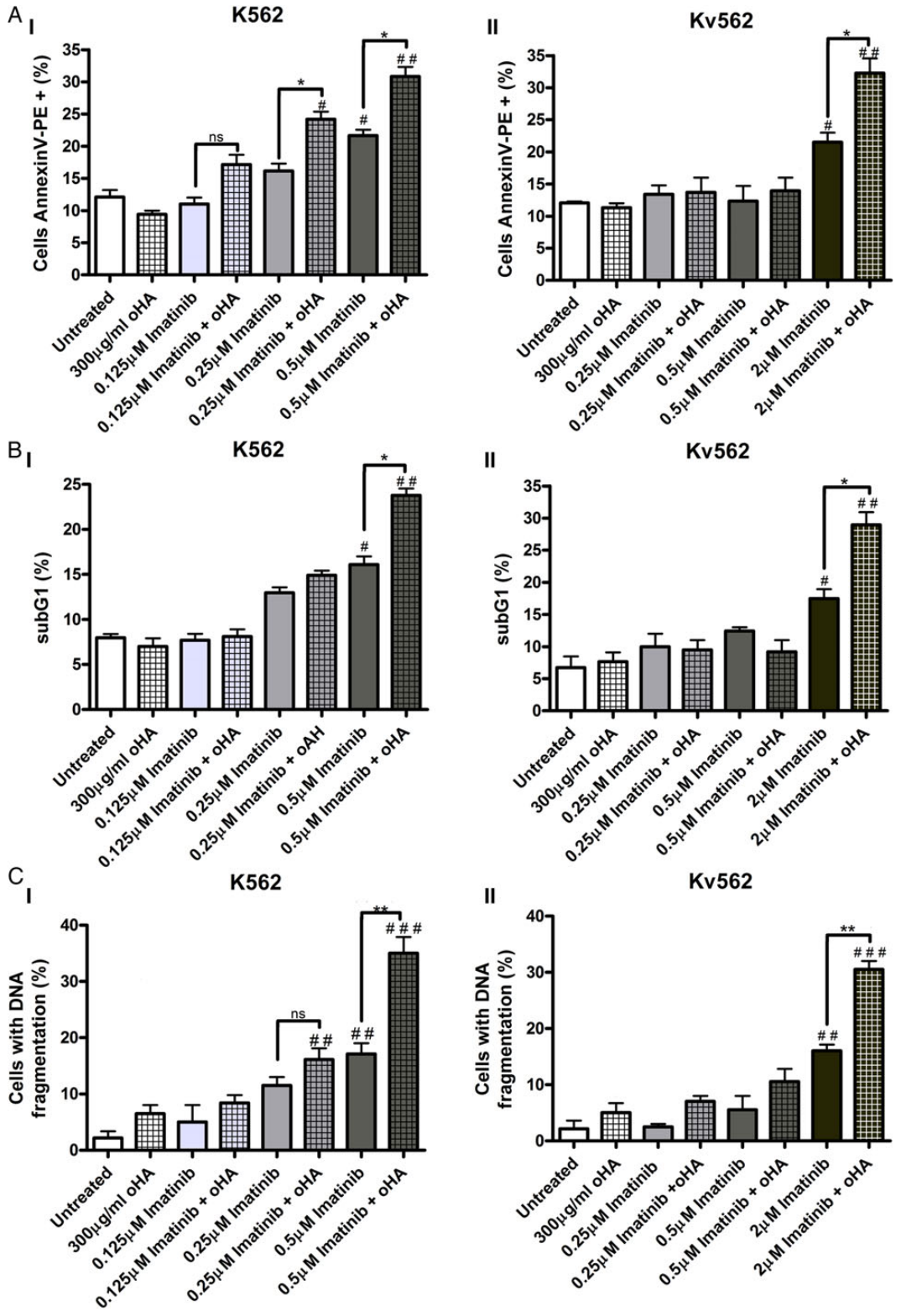

Fig. 2. Effect of oHA and Imatinib on apoptosis induction in K562 and Kv562 cell lines. To determine if oHA and Imatinib co-treatment enhanced the induction of apoptosis, both cell lines were treated with these compounds for 48 h and then three different parameters were analyzed. (**A**) The membrane asymmetry was evaluated by FC. After treatments, cells were stained with Annexin-V-PE/7AAD. Bars represent means ± SD of Annexin-V positive cells of three independent experiments ##$P < 0.01$ and #$P < 0.05$ vs. untreated while *$P < 0.05$ and ns $P > 0.05$ between the different treatments as indicated. (**B**) The 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 three independent experiments ##$P < 0.01$ and #$P < 0.05$ vs. untreated while **$P < 0.01$ and *$P < 0.05$ between the different treatments as indicated. (**C**) DNA fragmentation was analyzed by fluorescence microscopy. K562 (I) and Kv562 (II) cells were fixed with PFA and stained with DAPI. Bars represent means ± SD of cells with DNA fragmentation of three independent experiments ###$P < 0.001$ and ##$P < 0.01$ vs. untreated while **$P < 0.01$ and ns $P > 0.05$ between the different treatments as indicated.

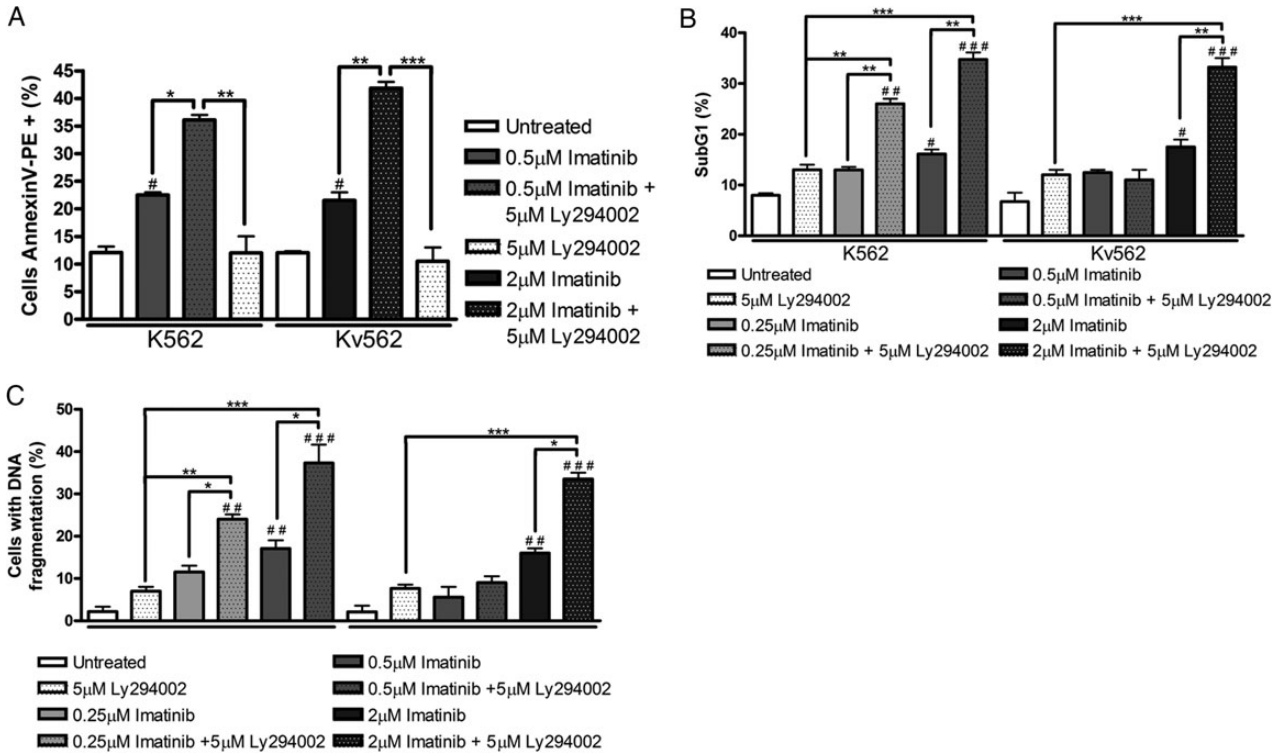

Fig. 3. Implication of PI3K in apoptosis induced by Imatinib. To determine if the inhibition of PI3K enhanced the induction of apoptosis, both cell lines were treated with Ly294002, Imatinib or combinations for 48 h. (**A**) Evaluation of membrane asymmetry. After treatments, cells were stained with Annexin-V-PE/7AAD and analyzed by FC. Bars represent means ± SD of Annexin-V positive cells of three independent experiments ##$P < 0.01$ and #$P < 0.05$ vs. untreated while ***$P < 0.001$, **$P < 0.1$ and *$P < 0.05$ between the different treatments as indicated. (**B**) Evaluation of hypodiploid cells. Cells were fixed with ethanol and stained with DAPI. Twenty thousand events were acquired by FC. Bars represent means ± SD of cells in subG1 peak of three independent experiments ###$P < 0.001$ and ##$P < 0.01$ vs. untreated while ***$P < 0.001$, **$P < 0.01$ and *$P < 0.05$ between the different treatments as indicated. (**C**) Analysis of DNA fragmentation. K562 (I) and Kv562 (II) cells were fixed with PFA and stained with DAPI. For each treatment, 200 cells were counted by fluorescence microscopy. Bars represent means ± SD of cells with DNA fragmentation of three independent experiments ###$P < 0.001$, ##$P < 0.01$ and $P < 0.05$ vs. untreated while ***$P < 0.001$ and $P < 0.01$ between the different treatments as indicated.

In K562 cells, oHA, Imatinib as well as their co-incubation treatment decreased pERK/ERK ratio respect to untreated conditions. However, no significant differences were observed between them. In Kv562 cells, none of the treatments (oHA, Imatinib or their combinations) modulated the phosphorilation of ERK respect to untreated. Therefore, oHA and Imatinib co-treatment did not have any effect on the pERK/ERK ratio when compared with each treatment alone (data not shown). Similar results were found for oHA + 0.25 or 0.5 μM Imatinib for the pAkt/Akt ratio in K562 and Kv562 cells, respectively (Figure 5A). However, the combination of oHA with the highest doses of Imatinib employed (0.5 μM in K562 and 2 μM in Kv562) abrogated the phosphorylation of Akt in both cell lines.

Discussion

CML represents a 15% of all leukemias, with an incidence of 2 cases/100,000 people per year. Although Imatinib is a highly effective drug, after some time, the selection of resistant cells may occur, leading to therapeutic failure. Current studies on the biology of CML are vital to find new molecular targets to improve therapies. Since multiple factors may contribute to chemoresistance, the impact of extracellular matrix components on this process has become of great interest (Bissel and Radisky 2001; Cordo-Russo et al. 2008).

HA is the main glycosaminoglican of the extracellular matrix and its levels are known to correlate with poor prognosis (Toole 2009; Sironen et al. 2011; Auvinen et al. 2013). Contrarily, oHA can block several biological mechanisms mediated by HA. Therefore, it was of value to assess whether oHA could improve the Imatinib effect on CML cell lines. For this purpose, we used the K562 and Kv562 cell lines. The former is sensitive to Imatinib and several chemotherapeutic drugs such as Vincristine and Doxorubicin while the latter is multidrug resistant due to Pgp functionality and PI3K over-activation (Lompardía et al. 2013). In this work, we demonstrated for the first time that oHA enhanced the anti-proliferative effect of Imatinib increasing apoptosis and senescence induction in both cell lines (Figure 6A).

Initially, several concentrations of Imatinib were evaluated showing that Kv562 cells required higher doses than K562 cells to attain a similar effect. This result was expected since Kv562 cells express Pgp and Imatinib can be extruded through this efflux pump (Czyzewski and Styczynski 2009; Silva et al. 2013). Imatinib abrogated the proliferation of K562 and Kv562 cells inducing apoptosis and senescence. Moreover, the BCR-ABL inhibitor reduced pAkt/Akt ratio in both cell lines while it only decreased the pERK/ERK ratio in K562 cells. Our results are in agreement with several studies performed in K562 cells (Machado-Neto et al. 2011; Li et al. 2013; Elfineh et al. 2014). However, this is the first study reporting the effect of Imatinib on Kv562 cells.

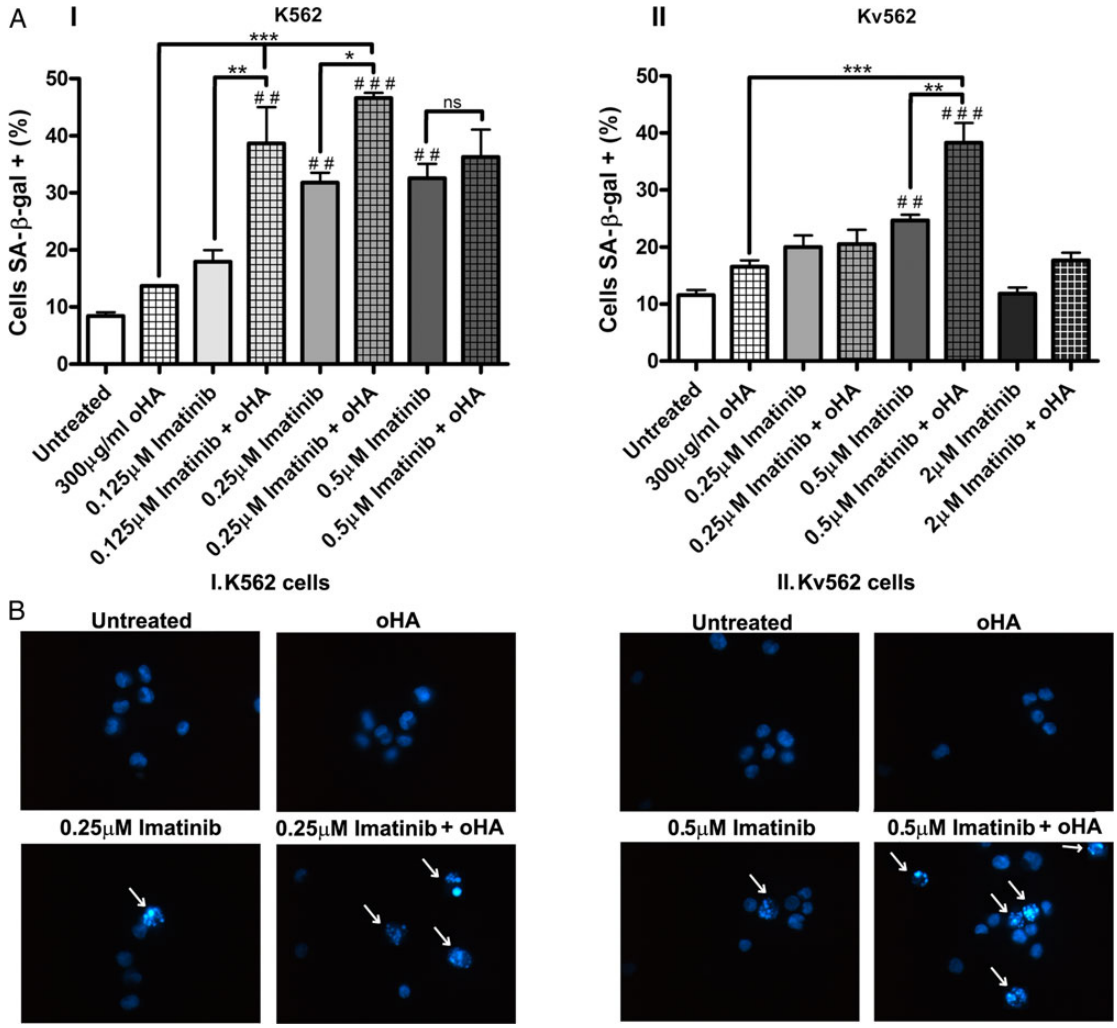

Fig. 4. Evaluation of senescence induction. To determine if oHA enhanced the senescence induced by Imatinib, both cell lines were treated with 300 μg/mL oHA, Imatinib or combinations for 48 h. SA-β-gal activity (**A**) and SAHF (**B**) (magnification 400×) were evaluated. Bars represent means ± SD of three independent experiments ###$P < 0.001$ and ##$P < 0.01$ vs. untreated while ***$P < 0.001$, $P < 0.01$ and *$P < 0.05$ between the different treatments as indicated. Arrows indicate nuclei with SAHF. This figure is available in black and white in print and in color at *Glycobiology* online.

The role of oHA has previously been described (Lompardía et al. 2013). Briefly, in K562 cells, oHA-CD44 interaction partially abrogates PI3K/Akt and MEK/ERK signaling pathways reducing cell growth. In Kv562 cells, oHA are known to reduce MDR by two different mechanisms, the inhibition of Pgp mediated by CD44 and the inhibition of PI3K mediated by RHAMM. We suggest that all such effects of oHA would be mediated by the inhibition of HA interaction with its receptors. Moreover, we previously demonstrated that oHA are able to displace HA in both cell lines (Lompardía et al. 2013).

When combining the highest doses of Imatinib with oHA, a major anti-proliferative effect was obtained. Moreover, this co-treatment induced a dramatic decrease in the pAkt levels with a significant induction of apoptosis in both cell lines. These results are in agreement with Slomiany, Dai, Bomar, et al. (2009) who demonstrated that oHA sensitized malignant peripheral nerve sheath tumors to apoptotic death.

Although the co-incubation of the lowest concentrations of Imatinib with oHA also reduced both cell lines proliferation, this co-treatment did not modify the levels of apoptosis induced by each compound alone. In this case, the mechanism of tumor suppression involved was senescence. These results are in accordance with previous works carried out in our laboratory which demonstrated that oHA sensitize Kv562 cells to the senescent effect of Vincristine (Lompardía et al. 2013). Moreover, the combinations of low doses of Imatinib plus oHA did not show a significant abrogation of the pAkt/Akt ratio compared with Imatinib or oHA alone while the co-treatment with high doses of Imatinib plus oHA prevented the phosphorylation of Akt. Taking into account these results, we postulate that the induction of apoptosis could be mediated by the sharp drop in pAkt levels while senescence would be triggered by partial reductions in pAkt (Figure 6B).

Considering that high doses of Imatinib were able to generate significant levels of apoptosis while low doses increased only the induction of senescence, we hypothesized that Imatinib targets the tumor suppression mechanism while oHA would enhance the pathway affected by Imatinib.

Taking into consideration the hypothesis about the role of Akt phophorilation on apoptosis induction, it was interesting to evaluate whether the inhibition of PI3K enhanced the pro-apoptotic effect of

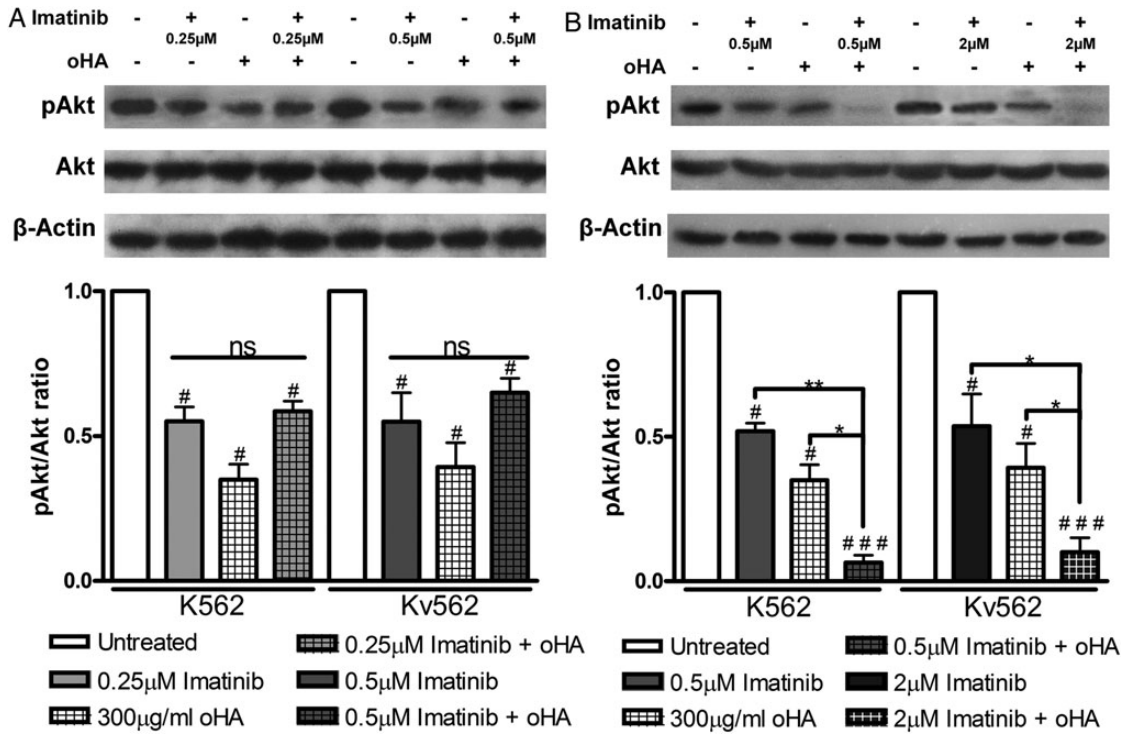

Fig. 5. Modulation of the pAkt/Akt ratio. (**A**) K562 and Kv562 cells were treated with 300 μg/mL oHA, 0.25 or 0.5 μM Imatinib or combinations for 24 h. Akt phosphorylation was evaluated by WB. Results are expressed as: pAkt/Akt ratio = $[(\text{pAkt}/\beta\text{-actin})/(\text{Akt}/\beta\text{-actin})]_{\text{treated}}/[(\text{pAkt}/\beta\text{-actin})/(\text{Akt}/\beta\text{-actin})]_{\text{untreated}}$. Bars represent means ± SD of at least three independent experiments #$P < 0.05$ and ns = no significant ($P > 0.05$) vs. untreated. (**B**) Both cell lines were treated with 300 μg/mL oHA, 0.5 or 2 μM Imatinib or combinations for 24 h. The phosphorylation of Akt was evaluated by WB. Results are expressed as pAkt/Akt ratio (calculated as in A). Bars represent means ± SD of at least three independent experiments, ###$P < 0.001$ and #$P < 0.05$ vs. untreated while **$P < 0.01$ and *$P < 0.05$ between the different treatments as indicated.

Imatinib. We demonstrated that Ly294002 sensitized both cell lines to the anti-proliferative and pro-apoptotic effect of Imatinib. These results correlate with previous reports showing an improvement of the Imatinib effect achieved by the inhibition of PI3K (Ciarcia et al. 2013). Moreover, several reports indicate that an over-activation of PI3K is involved in apoptosis evasion and Imatinib resistance (Chang et al. 2003; McCubrey et al. 2006; Quentmeier et al. 2011). Hence, abrogating this signaling pathway would be crucial for an optimal effect of Imatinib. Considering that HA increased PI3K activity in both cell lines (Lompardía et al. 2013) and it is has been demonstrated that the bone marrow presents high levels of HA (Goncharova et al. 2012; Solis et al. 2012) (Figure 6AI), preventing the signaling triggered by HA would be a useful strategy to improve the effect of Imatinib (Figure 6AII). In this sense, oHA represent a valuable tool to be used as a potential co-adjuvant in the therapy against CML.

Conclusion

Combination of Imatinib with oHA enhanced the anti-proliferative effect of the BCR-ABL inhibitor in both cell lines. This effect was mediated by sensitization either to apoptotic death or senescence depending on the concentration of Imatinib employed. When oHA were combined with doses of Imatinib that induced apoptosis itself, oHA increased the levels of apoptosis, while co-incubation of oHA with lower concentrations of Imatinib enhanced the induction of senescence. Moreover, the mechanism of tumor suppression involved would be regulated by PI3K activation. These findings highlight the use of oHA as a potential co-adjuvant for first-line therapy against CML.

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 (Lompardía et al. 2013).

Reagents

Recombinant low-molecular weight (LMW; $2.5–3 \times 10^5$ Da) HA (CPN spol.s.r.o Czech Republic) was supplied by Farmatrade (Argentina). Hyaluronidase Bovine testes (#H3884, Type IV-S, lyophilized powder, essentially salt-free), X-gal, LY294002 and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma-Aldrich. Imatinib was kindly provided by Dr Irene Larripa. Antibodies (Ab) against pAkt, Akt, pERK, ERK, β-Actin, anti-rabbit secondary horseradish peroxidase and anti-goat secondary horseradish peroxidase Abs were purchased from Santa Cruz Biotechnology. [^{3}H]-Thymidine was purchased from Perkin-Elmer (Boston). RPMI 1640, L-glutamine, streptomycin and penicillin were purchased from Invitrogen (Argentina). PE Annexin V Apoptosis Detection Kit I was purchased from BD Pharmingen™ (BD Bioscience).

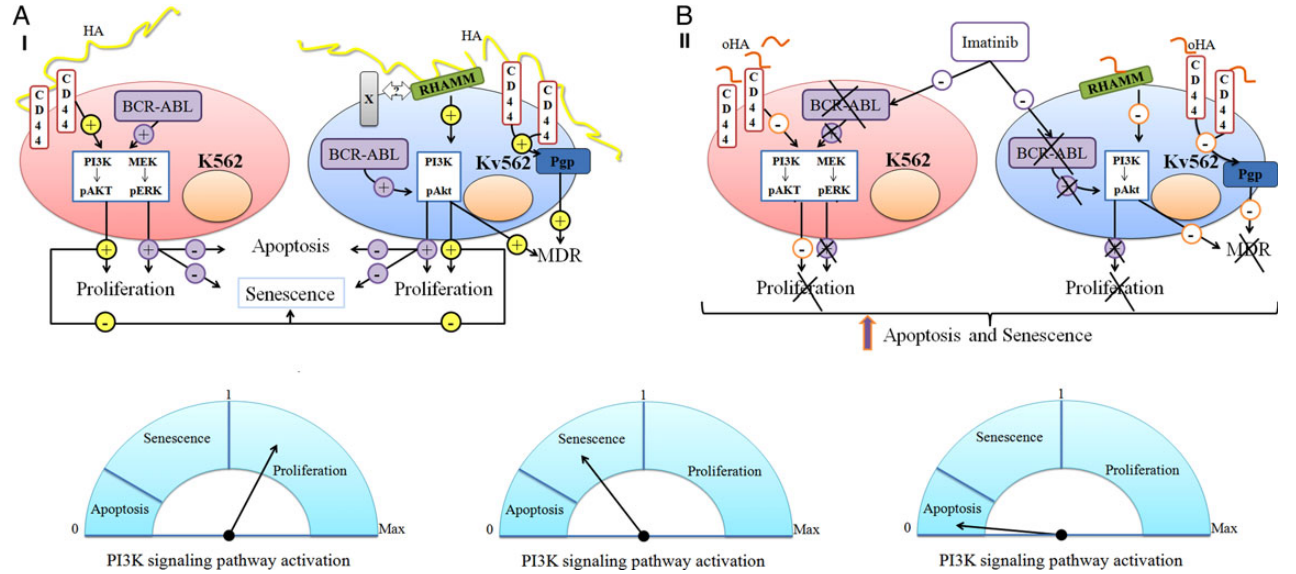

Fig. 6. Suggested mechanism of action of oHA in combination with Imatinib in K562 and Kv562 cells. (**A**) (I) Proposed role of HA and BCR-ABL in the induction of cell proliferation. In K562 cells, the HA-CD44 interaction as well as BCR-ABL might activate PI3K/Akt and MEK/ERK signalling pathways inducing cell proliferation. In Kv562 cells, the HA-RHAMM interaction would activate CD44 or other cell surface receptors triggering PI3K/Akt pathway enhancing cell proliferation. BCR-ABL also activates PI3K/Akt increasing Kv562 cells growth. Moreover, HA abrogates senescence induction while BCR-ABL prevents apoptosis and senescence induction in both cell lines. (II) Proposed mechanism of action of oHA and Imatinib. oHA would block the effect of HA while Imatinib inhibits BCR-ABL activity. Consequently, the combination of both compounds decreases K562 cells proliferation due to down-regulation of PI3K/Akt and MEK/ERK pathways. Moreover, the co-treatment with oHA plus Imatinib enhances apoptosis and senescence induction. Furthermore, oHA revert multidrug resistance of Kv562 cells through Pgp inhibition and the decrease of pAkt. Therefore, oHA sensitized Kv562 cells to the anti-proliferative effect of Imatinib inducing apoptosis and senescence. (**B**) We suggest that a high activity of PI3K would favor cell proliferation, while partial or total inhibition of this pathway might induce senescence or apoptosis, respectively. This figure is available in black and white in print and in color at *Glycobiology* online.

Preparation of oHA

The size distribution of the oHA was from HA4 tetrasaccharides to HA14 oligosaccharides. They were generated as described previously (Cordo-Russo et al. 2008). Briefly, oligosaccharides were obtained after enzymatic digestion of LMW-HA (5 mg/mL) with Hyaluronidase bovine testes employing 500 U/mg of HA during 24 h at 37°C. The reaction was stopped by heat denaturation for 5 min. The suspension was then filtered through 0.22 µm filter and then hyaulonidase and oHA were separated using Centricon™ with 10 kDa pore size. The size of oHA was determined by HPAEC-PAD.

Cell proliferation

Cell proliferation was analyzed by the [^{3}H]-thymidine uptake assay evaluated at 48 h in 96 wells microtiter plates (Lompardía et al. 2013). Fifty thousand cells per well (250,000 cells/mL) were used. Cells were grown at 37°C in a 5% CO2 atmosphere with RPMI-C, 300 µg/mL oHA, 5 µM Ly294002, Imatinib (2; 0.5; 0.25 or 0.125 µM) or combinations. After pulsing with 1 µCi [^{3}H]-thymidine for 6 h, cells were harvested and counted in a liquid scintillation counter (Beckman, MD). Results were calculated from the mean cpm of [^{3}H]-thymidine incorporated in triplicate cultures. Untreated cells represented 100% cell survival. Cell viability at the beginning of the experiment was >95%, as assessed by the Trypan blue dye.

Evaluation of apoptosis

Cells were treated with RPMI-C, 300 µg/mL oHA, 5 µM Ly294002, Imatinib (2; 0.5; 0.25 or 0.125 µM) or combinations 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 PE Annexin V Apoptosis Detection Kit I (BD Biosciences) was used following the manufacturer's instructions. A Pas III flow cytometer (Partec, Germany) was used to acquire data which were analyzed with theWinMDI 2.8 software (Scripps Institute, La Jolla). For DNA fragmentation and hypodiploid DNA content, cells were stained with DAPI and evaluated using fluorescence microscopy (Olympus BX51, America Inc.) and flow cytometry (Pas III flow cytometer, Partec, Germay), respectively (Cavaliere et al. 2009; Papademetrio et al. 2014).

Evaluation of senescence

Cell senescence was analyzed by SA-β-gal and SAHF (Debacq-Chainiaux et al. 2009). For SA-β-gal, cells were incubated with RPMI-C, 300 µg/mL oHA, 5 µM Ly294002, Imatinib (2; 0.5; 0.25 or 0.125 µM) or combinations 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 (Debacq-Chainiaux et al. 2009; Drullion et al. 2012). For SAHF, cells were subjected to a similar procedure. After 48 h, cells were fixed, 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 by fluorescence microscopy (Olympus BX51, America Inc.) for SAHF (Yang et al. 2012).

Western blot

Cells were treated with RPMI-C, 300 μg/mL oHA, Imatinib (2; 0.5; 0.25 or 0.125 μM) or combinations for 24 h at 37°C in a 5% CO_2 atmosphere. Cells were then lysed with hypotonic buffer. After centrifugation, 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) (Lompardía et al. 2013).

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). *P*-values <0.05 were regarded as statistically significant (Lompardía et al. 2013).

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Conflict of interest statement

None declared.

Abbreviations

oHA, hyaluronan oligomers; HA, hyaluronan; MDR, multidrug resistance; CD44, cluster differentiation 44; RHAMM, receptor for hyaluronan-mediated motility; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; pAkt, phosphorylated Akt; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; pERK, phosphorylated ERK; Pgp, P-glycoprotein; CML, chronic myeloid leukemia; Ph, Philadelphia chromosome; SA-β-gal, senescence associated β-galactosidase; SAHF senescence-associated heterochromatin foci; FBS fetal bovine serum; LMW, low-molecular weight; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 7AAD, 7-amino-actinomycin; PBS, phosphate-buffered saline; FC, flow cytometry; WB, western blot; PFA, paraformaldehyde; DAPI, 4′,6-diamidino-2-phenylindole; SD, standard deviation.

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