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Original article

Effects of Salinomycin and Everolimus on Breast Cancer Stem Cells in Hypoxia

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Abstract

Cancer stem cells (CSCs) are a collection of small numbers of cells that have the potential to induce all cell types within the tumor mass and have self-renewal capacity. Today, the reasons for the failure of conventional cancer therapies lie in the fact that they are unable to target cancer stem cells. Targeting the cancer stem cell is thought to provide a very important and revolutionary advance in cancer cell targeting and therapy. Tumor hypoxia is a characteristic of solid tumors and has been associated with poor prognosis and resistance to radiotherapy and chemotherapy. HIF-1 (Hypoxia Inducible Factor-1) is the major transcription factor activated in hypoxic conditions and allows transcriptional activation of various genes that are effective for the adaptation of the cell to the hypoxic condition. Experimental studies have provided evidence that also hypoxia and HIF-1α promote the cancer stem cell phenotype and targeting of HIF-1α may reduce or eliminate cancer stem cells. Breast cancer is the most common form of cancer in women worldwide and affects 10% of the world's female population. 25% to 30% of patients with invasive breast cancer still die from this disease. The recurrence frequency of the disease varies between 60% and 80% within the first 3 years after treatment. In order to target breast cancer stem cells more effectively, in this study we aimed to reveal whether the hypoxic conditions in the tumor, which act as the stem cell production area, at the same time creates resistance to therapy. Thus, we evaluated effect of CSCs targeting agent Salinomycin alone or in combination with Everolimus which is an m-TOR and HIF-1α inhibitor on parental MCF-7 and MDA-231 breast cancer cells and their isolated CSCs in hypoxic conditions. Here it is presented that starting with 2 μM, increased concentrations of salinomycin significantly inhibits proliferation and induce apoptosis in hypoxia, in both parental MCF-7 and MDA-231 breast cancer cells and in their isolated CSCs. The most effective concentration of salinomycin was 10 μM and induced around 35% and 45% of apoptosis in both parental MCF-7 and MDA-231 and their isolated CSCs, respectively. Whereas everolimus alone was not as effective as salinomycin, as 25 μM everolimus induced 30% and 15% of growth inhibition or apoptosis in both parental and CSCs of MCF-7 and MDA-231 cellsin hypoxia, respectively. When lower concentrations of salinomycin (2μM) and everolimus (5μM) was used in combination they show synergistic effect and able to inhibit proliferation at least 35% and 45% in both parental and CSCs of MCF-7 and MDA-231 cells in hypoxia, respectively. Similar results were also obtained for induction of apoptosis in response to salinomycin + everolimus treatment in hypoxia in both parental and CSCs of MCF-7 and MDA-231 cells. Hence using lower concentrations of salinomycin and everolimus together may provide an effective targeting strategy for hypoxic CSCs and may contribute to the development of novel strategies for therapeutic intervention in breast cancer.

Keywords: MCF-7,MDA-231, cancer stem cell, Salinomycin, Everolimus, hypoxia.

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INTRODUCTION

Recent studies suggest that tumor masses should be considered as 'heterogeneous cancer cells' that come together in a 'hierarchy'. This 'hierarchy hypothesis' tells us that in a tumor mass there is a subpopulation of cells representing different features than the general population. These cells are found in small numbers in the tumor mass, have a 'self-renewal capacity' similar to that of normal stem cells, and show a great tendency to form tumors, now called 'cancer stem cells' or 'cancer stem-like cells' or 'tumor-initiating cells'. This situation, also known as "cancer stem cell theory", briefly showing tumors arise from a subpopulation of cancer cells that display stem cell characteristics (Gil et al., 2008). Today, the reasons for the failure of conventional cancer therapies lie in the fact that they are unable to target cancer stem cells, which have a slower proliferation capacity and are over-resistant to chemotherapy or radiotherapy (Alison et al., 2008; Hu et., al 2008).

Breast cancer is the most common form of cancer in women worldwide and affects 10% of the world's female population. 25% to 30% of patients with invasive breast cancer still die from this disease. The recurrence frequency of the disease varies between 60% and 80% within the first 3 years after treatment. Typically, recurrent cancer is less responsive to chemotherapy regimens, possibly because the cancer cells develop resistance mechanisms to the drugs through changes in their genetic and acquired natures (Ercan et al., 2011). The development of novel strategies overcoming chemoresistance and providing chemosensitization is an important area of research. The first evidence for cancer stem cells in solid tumors was shown by Al-Haji et al. in breast cancer in 2003. In this study, a very small cancer cell subpopulation isolated as CD44 + and CD24- from mammary tumors was able to form in vitro mamosphere while re-producing tumors in NOD / SCID (immunocompetent) mice (Al-Hajj et al., 2003). Breast cancer stem cells have been extensively studied by a large number of researchers to find a specific marker. As a result, it has been found that the CD44 (+) CD24 (-) cells, which have an increase in expression of ALDH1 (aldehyde dehydrogenase) in particular, have high tumorigenic activity and are capable of forming mammosphere in vitro (Liu and Wicha, 2010; Klonisch et al., 2008).

Human solid tumors are invariably less well-oxygenated than the normal tissues from which they arose. This so-called tumor hypoxia leads to resistance to radiotherapy and chemotherapy as well as predisposing for increased tumor metastases. Adaptation of cancer cells to hypoxic condition increases glycosylation and glucose uptake, promotes angiogenesis, regulates apoptosis and leads to invasion and metastasis. HIF-1α (Hypoxia Inducible Factor-1 alpha) is the major transcription factor activated in hypoxia and provides transcriptional activation of various genes that are effective in enabling the adaptation of cancer cells to these conditions. HIF-1 has an alpha (μ) subunit activated by oxygen deficiency and a beta (ß, also known as ARNT) subunit which is constitutively active. Under normoxic conditions, HIF-lα is rapidly hydroxylated by prolyl hydroxylases and is bound to the VHL (von Hippel Lindau) complex and targeted to proteosomal degradation by incorporation into the E-3 ubiquitin ligase

complex. In hypoxic conditions, HIF-1α stabilizes and translocates to the nucleus, where it interacts with HIF-1ß and transcriptional coactivators p300/CBP, and specifically induce the transcription of a large number of genes such as angiogenic factors (eg. vascular endothelial growth factor), growth factors (growth factor-II, IGF-II), glycolytic enzymes (Aldolase A/C), anti-apoptotic proteins such as Bcl-2 (Kilic et al. 2007,Kilic et al. 2013, Semenza, 2009; Semenza 2015) .Thus, HIF-1α provides protection against hypoxia in solid tumors, by promoting angiogenesis, by increasing expression of growth factors, by inhibiting apoptosis and by increasing anaerobic metabolism.

Recent experimental studies have demonstrated that hypoxia and HIFs regulate cancer stem cells and support the stem cell phenotype. For example, in cancer stem cells cultured under hypoxic conditions, an increase in the CD133 (cancer stem cell marker) positive cell population was detected due to the activation of HIF-lα (Iida et al., 2011; Schwab et al., 2012). In hypoxic conditions, an increase was also found in the CD44 + population, which is a cancer stem cell marker. Furthermore, the hypoxic conditions or the HIFs have not only increased the CD133 + fraction in cancer cell populations but also stimulated gene expressions (such as Notch, Sox2, Oct4) to acquire stem cell properties such as proliferation, self-renewal and multipotency (Conley et al. 2012, Liang et al., 2012, Hambardzumyan et al., 2008). Accordingly, hypoxic tumor regions appear to be a complete production area for cancer stem cells. Hence, this study aims to develop a novel strategy targeting breast cancer stem cells under hypoxic conditions.

Salinomycin, a polyether ionophore antibiotic isolated from Streptomyces albus, and recently has been identified as a CSC targeting drug in different types of human cancers including breast cancer (Gupta et al., 2009; Al Dhaheri et al., 2013; Dewangan et al., 2017). On the other hand everolimus (rad001, Afinitor®, 4a) is an mTOR inhibitor, also used to target HIF-1 via inhibition of the growth factor signaling pathway involving PI3K/Akt/mTOR which represents a major route of HIF-1α translation in over 70% of human cancer cell lines (Semenza, 2009). In the present study, we evaluated whether salinomycin when combined with everolimus eliminates breast cancer stem cell population under hypoxic conditions. Our findings shed light on the potential effectiveness of treatment that combines salinomycin with HIF-inhibitor based chemotherapy.

MATERIALS and METHODS

Cell Culture and Hypoxia incubation

Human breast cancer cell lines (MCF-7) and (MDA-231) were obtained from American Type Culture Collection (Manassas, VA) and were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Eggenstein, Germany) containing 10% heat inactivated fetal calf serum (Biochrom, Berlin, Germany), 100 IU/ml penicillin, 100 µg/ml streptomycin (Biochrom), 10 mM glutamine (Biochrom, Germany) in a humidified atmosphere at 37°C with 5% CO2 unless otherwise specified.

Hypoxic conditions were achieved by incubation in an anaerobic chamber (Billups-Rothenberg, Del Mar, California) flushed with 5% CO2, and 95% N2. Because the culture flasks contained ambient oxygen at the beginning of the experiments, the final oxygen content in the hypoxia chamber was <1% (0.1% to 0.5%) after achieving air equilibrium. Salinomycin and everolimus were from (Sigma, germany).

Isolation of CD24– CD44+ breast cancer stem cells was achieved by using the MagCellect Isolation Kit

A small population of CD24low/–CD44+ cells was isolated from MCF-7 human breast adenocarcinoma cells using the MagCellect CD24– CD44+ Breast Cancer Stem Cell Isolation Kit (MAGH111, R&D, USA) according to the manufacturer's instruciton.

For CSC isolation 5x106 cells were suspended in Mag cellect plus buffer (Mag Cellect Buffer Running Buffer;R&D) and incubated with 25 µl for Human CD24 Biotinylated Antibody for 15 min at 4°C. The cells were washed by cold 1X MagCellect Plus Buffer and centrifuged at 300 x g for 8 minutes. The supernatant was removed and the cell pellet was resuspended by 1X MagCellect Plus Buffer. After addition of 50 µL of Streptavidin Ferrofluid, the cell suspensions were incubated for 15 minutes at 4 °C. At the end of the incubation period, the cell suspension was washed by cold 1X MagCellect Plus Buffer and centrifuged at 300 x g for 8 minutes. The supernatant was removed and the cell pellet was resuspended in cold 1X MagCellect Plus Buffer into the tube. The reaction tube was then positioned in the MagCellect magnet (or equivalent) and incubate for 6 minutes at RT. Magnetically tagged CD24+ cells migrated toward the magnet, leaving the untagged CD24low/- cells in suspension in the supernatant. While the tube was firmly held in the magnet, the wanted CD24low/- cells were collected by slowly and carefully aspirating the supernatant and placed in a new tube. Concentrate CD24low/- cells obtained in Step 1 were then concentrated by centrifuging at 300 x g for 8 minutes and the pellet was resuspended in 0.5 mL of 1X MagCellect Plus Buffer. After addition of 10 µL of Human CD44 biotinylated antibody the cell/antibody suspension, was incubated for 15 minutes at 4°C and washed by cold 1X MagCellect Plus Buffer and centrifuge at 300 x g for 8 minutes t the end of the incubation period, The supernatant was removed and the cell pellet was resuspend in 0.5 mL of cold 1X MagCellect Plus Buffer and suplemented with 50 µL of Streptavidin Ferrofluid, the cell suspension was incubate for 15 minutes at 4 °C again. At the end of the incubation period, the cell suspension was washed by cold 1X MagCellect Plus Buffer and centrifuged at 300 x g for 8 minutes. The supernatant was removed and the cell pellet was resuspended in cold 1X MagCellect Plus Buffer into the tube. The reaction tube was then positioned in the MagCellect magnet (or equivalent) and incubated for 6 minutes at RT. Magnetically tagged CD44+ cells will migrate toward the magnet leaving the untagged CD44- cells in suspension in the supernatant. The supernatant was then removed and CD44+ cells were washed from magnetic beads. This final magnetically isolated fraction contained the desired isolated CD24low/-

CD44+ cells. The cells were then counted, stained, and used in Flow cytometry analysis and in other downstream applications.

Flow Cytometry Analysis of CD24low/-CD44+ cells

In brief, MCF-7 and MDA-231 cells were resuspended 1X MagCellect Plus Buffer and stained with antibodies specific for CD44-Detection (R&D, USA), CD24-Detection (R&D, USA). 1 × 105 cells were incubated with antibodies for 30 min on ice. Unbounded antibody was washed and cells were analyzed on Flow Cytometry (Beckmann Coulture),

WST-1 cell proliferation assay

Cells were reseeded in 96-well plates at a density of 1×104 cells/well, in 200 µL culture medium and incubated with appropriate concentrations of either salinomycin and/or everolimus. 24h post treatment, the cells were washed with PBS and the cell proliferation reagent WST-1 (Roche Molecular Biochemicals, Mannheim, Germany) was added, then samples were incubated for 2 h at 37°C. The absorbance was quantified with a microplate reader (Molecular Devices Corp., CA, USA) at 450 nm.

Determination of apoptosis

Briefly, 1x105 cells were seeded per well in a 24 well plate and treated with different concentrations of salinomycin and everolimus for 24 h. The cells were then analyzed by Muse™ Cell Analyzer (Merck-Millipore, Germany) according to the manufacturer instructions using Muse™ Annexin-V and Dead Cell reagent (Merck-Millipore, Germany). In brief, 1x106 cells were transferred in suspension to a new tube and incubated with 100 µl of Muse Annexin V & Dead Cell reagent)/for 20 minutes at room temperature. Apoptosis was determined by Muse Cell Analyzer and the statistics were shown the percentages of the cells represented by total apoptosis.

RESULTS

Isolation of CD44+/CD24−/low cells

In this study, breast CSCs were isolated from the parental breast cancer cell lines MCF-7 and MDA-231 using commercially available Mag Cellect CD44+/CD24−/low breast cancer stem cell isolation kit. Accordingly, isolated cells were stained with CD44 and CD24 antibodies simultaneously and directed to flow cytometry analysis for CD44 positivity and CD24 negativity. As shown in Figures 1A. and B. parental MCF-7 and MDA-231 cells were negative for CD44 expression. In contrast cancer stem cells isolated from MCF-7 and MDA-231 cells were detected as positive for CD44, (87% and 97%, respectively), whereas both cell lines were low (1% and 4% respectively) for CD 24 expression (Figure 1A and B). Thus these results confirmed the isolation of CSC population from the parental cell lines.

Effect of salinomycin and everolimus on the cell proliferation of parental MCF-7 and MDA-231 and isolated CD44+/CD24−/low CSCs and in hypoxia

Next, the influence of hypoxia on the sensitivity of breast cancer cells and CSCs to chemotherapeutic agents was assessed by comparing the abilities of salinomycin and everolimus to inhibit the proliferation under hypoxia. Salinomycin is a known CSC targeting agent and therefore initially the effects of salinomycin in hypoxia on proliferation of isolated breast CSCs was evaluated via proliferation assay WST-1. We compared the proliferation of CD44+/CD24 low CSCs cells to parental MCF-7 or MDA-231 after salinomycin treatment under hypoxia. Both parental and isolated CSCs from MCF-7 and MDA-231 were incubated in hypoxic culture conditions and treated with different concentrations of either salinomycin or everolimus for 72h. Results from WST-1 assay showed that salinomycin significantly inhibited the proliferation of both parental MCF-7 or MDA-231 cells and isolated CSCs in hypoxia at concentrations with 2, 5, 10 μM. The most significant proliferation inhibitory effect of salinomycin in isolated CSCs from MCF-7 cells was 40% and seen at concentration of 10 μM. However, a slight resistance to salinomycin was seen in parental MCF-7 cells compared to CSCs, as the percentage of the inhibition is around 35% (Figure 2A). Similar results were also obtained for parental MDA-231 cells and in their isolated CSCs. Interestingly salinomycin was more effective in both isolated CSCs and parental MDA-231 when compared to MCF-7 and its CSC counterpart, as the inhibition was around 45%. Salinomycin inhibited the proliferation of both parental MDA-231 cells and CSCs at least 5% more compared to MCF-7 at all concentrations in hypoxia (Figure 2B).

Everolimus (rad001, Afinitor®) is an mTOR inhibitor, also used to target HIF-1 via inhibition of the growth factor signaling pathway involving PI3K/Akt/mTOR. Next, we examined whether everolimus can inihibit proliferation of both parental and CSCs in hypoxia. As shown in Fig 3A and B everolimus significantly reduced proliferation of parental MCF-7 cells in hypoxia at concentrations of 5, 10 and 25 μM. The highest inhibitory effect was detected as 35 % at a concentration of 25 μM. In contrast, compared to parental MCF-7 cells isolated, CSCs showed resistance to everolimus as the highest concentration (25 μM) of everolimus reduced proliferation not more than 20%. In contrast, in parental MDA-231 cells everolimus reduced the proliferation only 15 % at 2 μM concentration and increased concentrations of everolimus (5, 10 and 25 μM) did not further reduced the proliferation. (Figure 3A). Similarly, MDA-231 CSCs also showed resistance to everolimus. The highest concentration of everolimus (25 μM) reduced the proliferation of CSCs not more than 16%. (Figure 3B). Thus parental MDA-231 and their isolated CSCs show resistance to treatment by everolimus in hypoxia.

Next whether or not salinomycin and everolimus exert synergytic effects when used in combination was examined. Accordingly all cells were incubated either with 2 or 5 μM of salinomycin

plus 5 or 10 μM of everolimus in hypoxia and tested for their ability to inhibit proliferation. As seen in Figure 4A combination treatment of 2 μM salinomycin and 5 μM everolimus inhibited proliferation at least 35% in both isolated CSCs and parental MCF-7 cells. Increasing the concentration of salinomycin and/or everolimus did not change the percentage of the inhibition in parental MCF-7 cells whereas a slight decrease in proliferation was seen in CSCs when 5 μM of salinomycin plus 5 or 10 μM everolimus was used in combination. As seen in Fig. 4B combination use of 2 μM salinomycin and 5 μM everolimus inhibited proliferation at least 45% in isolated CSCs and parental MDA-231 cells. Increasing concentrations of salinomycin or everolimus did not further reduced the proliferation in either parental or CSCs of MDA-231 cells, but a slight increase was detectable in proliferation. Combination use of salinomycin plus everolimus was more effective in both CSCs and parental MDA-231 cells when compared to MCF-7 (Figure 4B). These results showed that salinomycin and everolimus showed synergistic effects when used in combination.

Apoptototic effects of salinomycin and everolimus in parental MCF-7 and MDA-231 and isolated CD44+/CD24−/low CSCs and in hypoxia

Next, combination of salinomycin and everolimus examined for their potential to induce apoptosis in parental MCF-7 and MDA-231 and isolated CD44+/CD24−/low CSCs and in hypoxia. Accordingly all cells were incubated with 2 and 5 μM of salinomycin or 5 and 10 μM of everolimus alone or in combination in hypoxia and examined with Annnexin V staining test for their ability to induce apoptosis. As seen in Figure 4A, 2 and 5 μM of salinomycin alone induced 10% and 17% of apoptosis, respectively, in parental MCF-7 cells whereas a slight increase was detectable in apoptosis in CSCs from MCF-7. Similarly, 5 and 10 μM of everolimus alone induced only 9% and 18% of apoptosis, respectively, in parental MCF-7 cells. However, a slight decrease was detectable in apoptosis in response to everolimus in CSCs from MCF-7. Combination use of 2 μM salinomycin and 5 μM everolimus induced 25% and 35% apoptosis in both parental MCF-7 and isolated CSCs, respectively. Increasing the concentration of salinomycin and/or everolimus did not change the percentage of the inhibition in parental MCF-7 cells whereas a slight decrease was seen in CSCs when 5 μM of salinomycin was used in combination with 5 or 10 μM of everolimus (Figure 4A). Thus, 2 μM salinomycin and 5 μM everolimus showed a synergistic effect in inducing apoptosis in both parental and CSC of MCF-7. As seen in Fig. 4B, in parental MDA-231 cells 2 and 5 μM of salinomycin alone induced 30% and 15% of apoptosis, respectively, whereas a slight decrease was detectable in apoptosis on CSCs from MDA-231. Similarly, on parental MDA-231 cells 5 and 10 μM of everolimus alone induced only 15 % and 18 % of apoptosis, respectively. However, a slight decrease was detectable in apoptosis in response to everolimus in CSCs, as well. Combination use of 2 μM salinomycin and 5 μM everolimus induced 45 % and 53% apoptosis in isolated CSCs and parental MDA-231 cells, respectively. When used together, increasing

concentrations of salinomycin or everolimus did not further increased apoptosis, in contrast a slight decrease was detectable. Combination use of salinomycin and everolimus was more effective in CSCs from MCF-7 when compared to parental cells, however this effect was seen as vice versa in MDA-231 cells (Figure 4B). Hence we show here salinomycin and everolimus induce apoptosis in hypoxia in oth CSCs and parental MCF-7 and MDA-231 cells. Accordingly, when used in lower concentrations combination of salinomycin and everolimus shows synergistic effect and induce significant apoptosis in hypoxia.

DISCUSSION

Cancer stem cells comprise a small population within a tumor mass and have self-renewal capacity, thus can re-initiate tumor. Conventional cancer therapies fail to target cancer stem cells, accordingly the dormant cancerous stem cells re-enter the proliferative phase which often results in relapse.

Hypoxia is a characteristic of solid tumors and associated with poor prognosis and resistance to chemotherapy and radiotherapy. Hypoxic regions in solid tumors recognized as 'the stem cell production area' and create resistance to therapy (Keith et al., 2007). HIF-1α is the major transcription factor activated in response to hypoxia and allows transcription of number of genes involved adaptation and survival of cancer cells in hypoxic conditions. Breast cancer is the most common form of cancer in women worldwide and affects 10% of the world's female population. 25% to 30% of patients with invasive breast cancer still die from this disease. The recurrence frequency of the disease varies between 60% and 80% within the first 3 years after treatment. Hence in this study we aimed to reveal whether tumor hypoxia elicits resistance to therapeutics in the elimination of breast cancer stem cells, and whether this resistance can be reversed by therapeutic agents salinomycin and everolimus targeting both stem cells and HIF-1 α, respectively. Here, it is presented that when used in combination salinomycin and everolimus show synergistic effect and can effectively target breast cancer stem cells by inducing apoptosis under hypoxic conditions.

The current study provides a number of evidences supporting this conclusion. First, we demonstrated that starting with 2 μM, increased concentations of salinomycin significantly inhibits proliferation and induce apoptosis in hypoxia, in both MCF-7 and MDA-231 breast cancer cells and in their isolated CSCs. The most effective concentation of salinomycin (10 μM) alone induced around 45% of apoptosis in all cell types. Lower concentrations such as 2 μM induced only around 20% and 30% of apoptosis in all types of MCF-7 and MDA-231 cells, respectively. Thus our findings are in line with studies suggesting 10 μM of salinomycin is effective in both MDA-231 and MCF-7 parental and isolated CSCs (Oak et al., 2012; Skog et al., 2004). Second, we showed that 5 μM of everolimus alone does not inhibit proliferation or induce apoptosis in all types of MCF-7 cells and it show not more than 15 % of

inhibitory effect or apoptosis indcution in MDA-231 cell types in hypoxia. Third we show that when lower concentrations of salinomycin (2μM) and everolimus (5μM) used in combination they show synergystic effect and able to inhibit proliferation at least 35 % and 45% in both parental and CSCs of MCF-7 and MDA-231 cells, respectively. Similar results were also obtained for induction of apoptosis in response to salinomycin + everolimus treatment in hypoxia in both parental and CSCs of MCF-7 and MDA-231 cells. Interestingly combination use of salinomycin and everolimus was more effective in both CSCs and parental MDA-231 cells when compared to MCF-7. MDA-MB-231 cells are triple negative (ER; Estrogen Receptor, PR; Progesteron Receptor and HER2; human epidermal growth factor receptor 2 negative), whereas MCF7 is ER and PR positive breast cancer cell line (Oak et al. 2012; Skog et al., 2004). More importantly our results suggest that salinomycin everolimus combination treatment may target triple negative cells more effectively but this needs to be further elucidated. Thus, here we showed everolimus an m-TOR and HIF-1α inhibitor may sensitize breast cancer stem cells in response to salinomycin in hypoxia.

Using everolimus together with salinomycin may provide an effective targeting strategy for hypoxic CSCs and may contribute to the development of novel strategies for therapeutic intervention in breast cancer. It will be the object of our further investigations to elucidate the exact mechanism behind the sensitization under hypoxic conditions in CSCs and in hypoxic activation of HIF-1. Also it will be of our future interest to identify the molecules mediating the sensitization effect of everolimus.

Acknowledgement

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FIGURES AND LEGENDS

Figure 1.

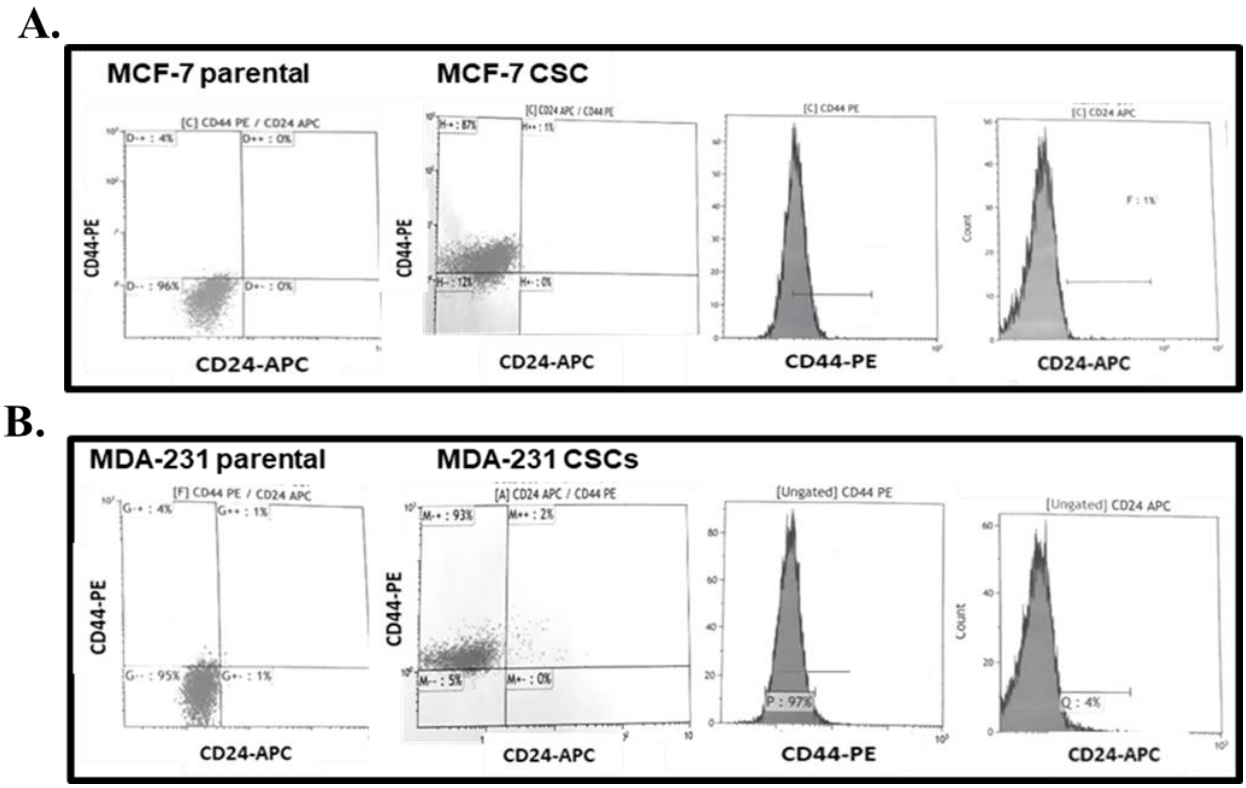

Figure 1. Cancer stem cells were isolated from parental MCF-7 and MDA-231 cells, stained with CD44-PE and CD24-APC antibodies and subjected to flow cytometry analysis for CD44 and CD24 expression.

Figure 2.

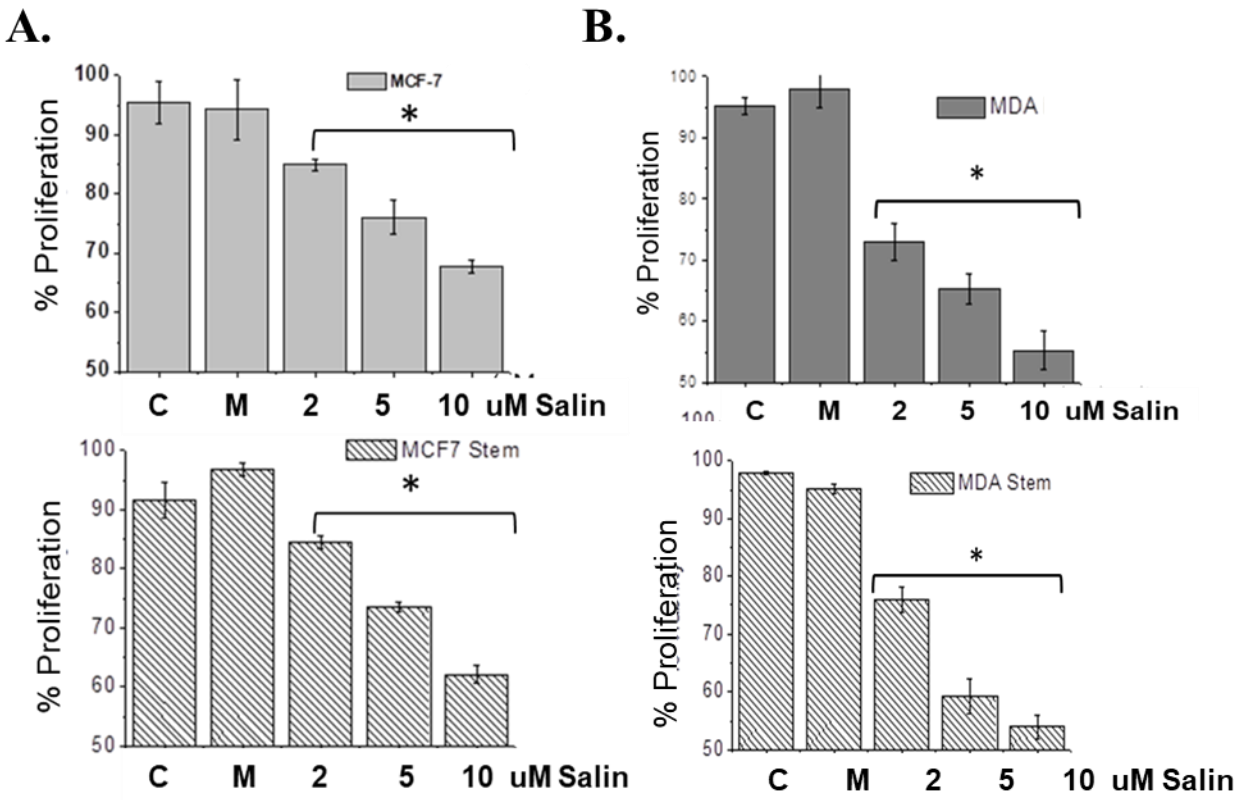

Figure 2. Inhibition of the proliferation in parental MCF-7 and MDA-231 and their isolated CSCs by of different concentrations of salinomycin (S) treatment for 72h in hypoxia. C, control nontreated, M, methanol treated. *Columns,* mean of three independent experiments done in duplicate; *bars,* SD. For statistical analysis student's t test was performed comparing the percentage of the proliferation inhibition by salinomycin (2,5-10 μM) treatment vs by methanol treatment in hypoxia (*$p<0.01$).

Figure 3.

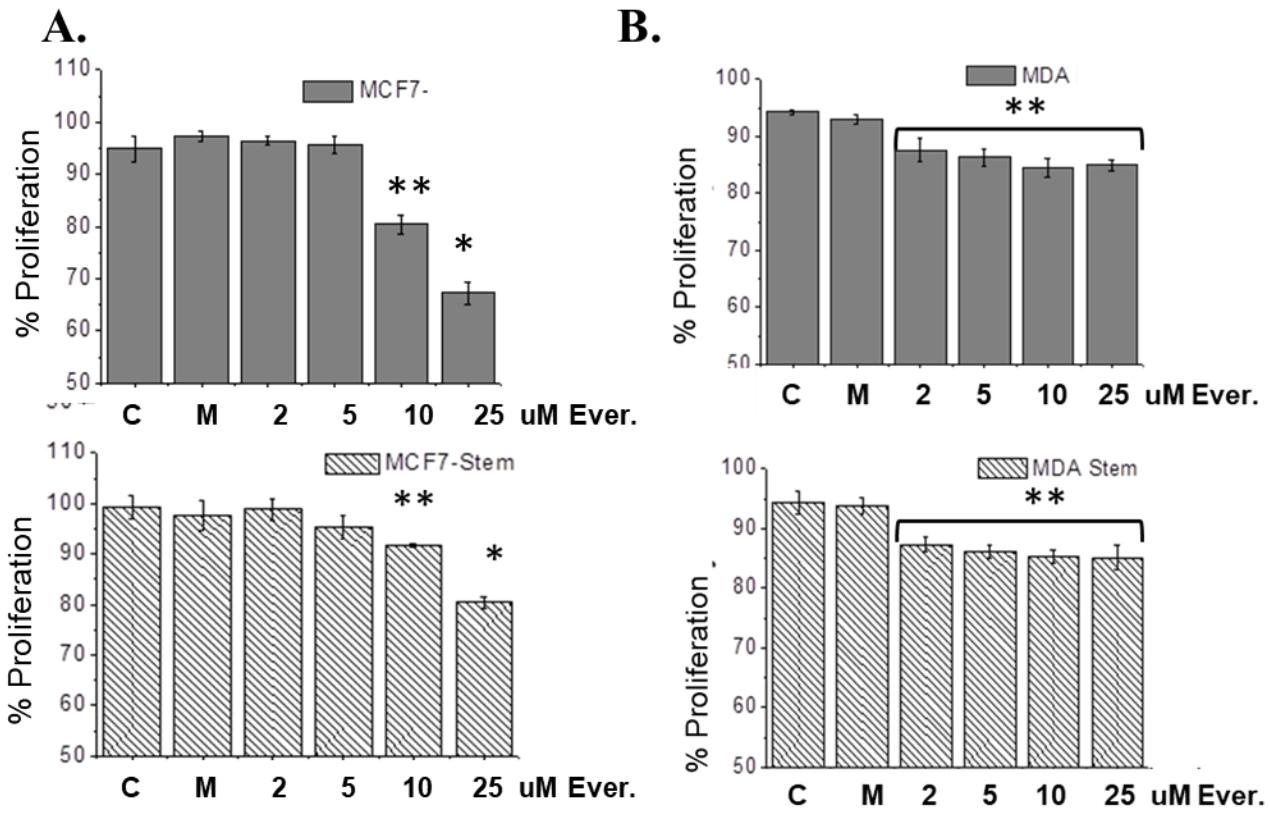

Figure 3. Inhibition of the proliferation in parental MCF-7 and MDA-231 and their isolated CSCs by different concentrations of everolimus (E) treatment for 72h in hypoxia. C, control nontreated, M, methanol treated. *Columns*, mean of three independent experiments done in duplicate; *bars*, SD. For statistical analysis student's t test was performed comparing the percentage of the proliferation inhibition by everolimus (2,5,10 and 25 μM) treatment vs by methanol treatment in hypoxia (*$p<0.01$). **$p<0.05$).

Figure 4.

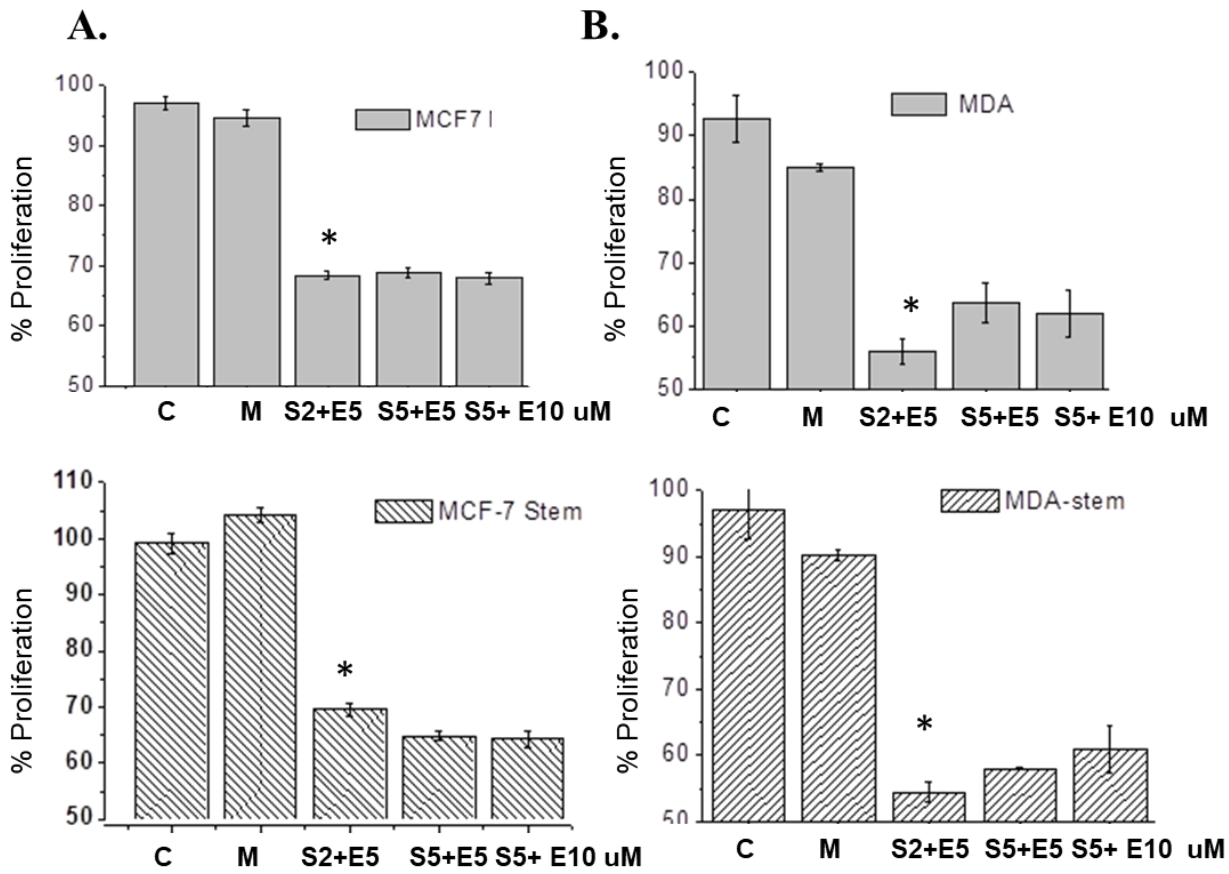

Figure 4. Inhibition of the proliferation in parental MCF-7 and MDA-231 and their isolated CSCs by of different concentrations of salinomycin (S) or everolimus (E) treatment either alone or together. *Columns,* mean of three independent experiments done in duplicate; *bars,* SD. For statistical analysis student's t test was performed comparing the percentage of the proliferation inhibition by salinomycin (2 and 5 μM,) plus everolimus (5 and 10 μM) treatment *vs* by salinomycin treatment alone added with that of everolimus alone under hypoxia (*$p<0.01$).

Figure 5.

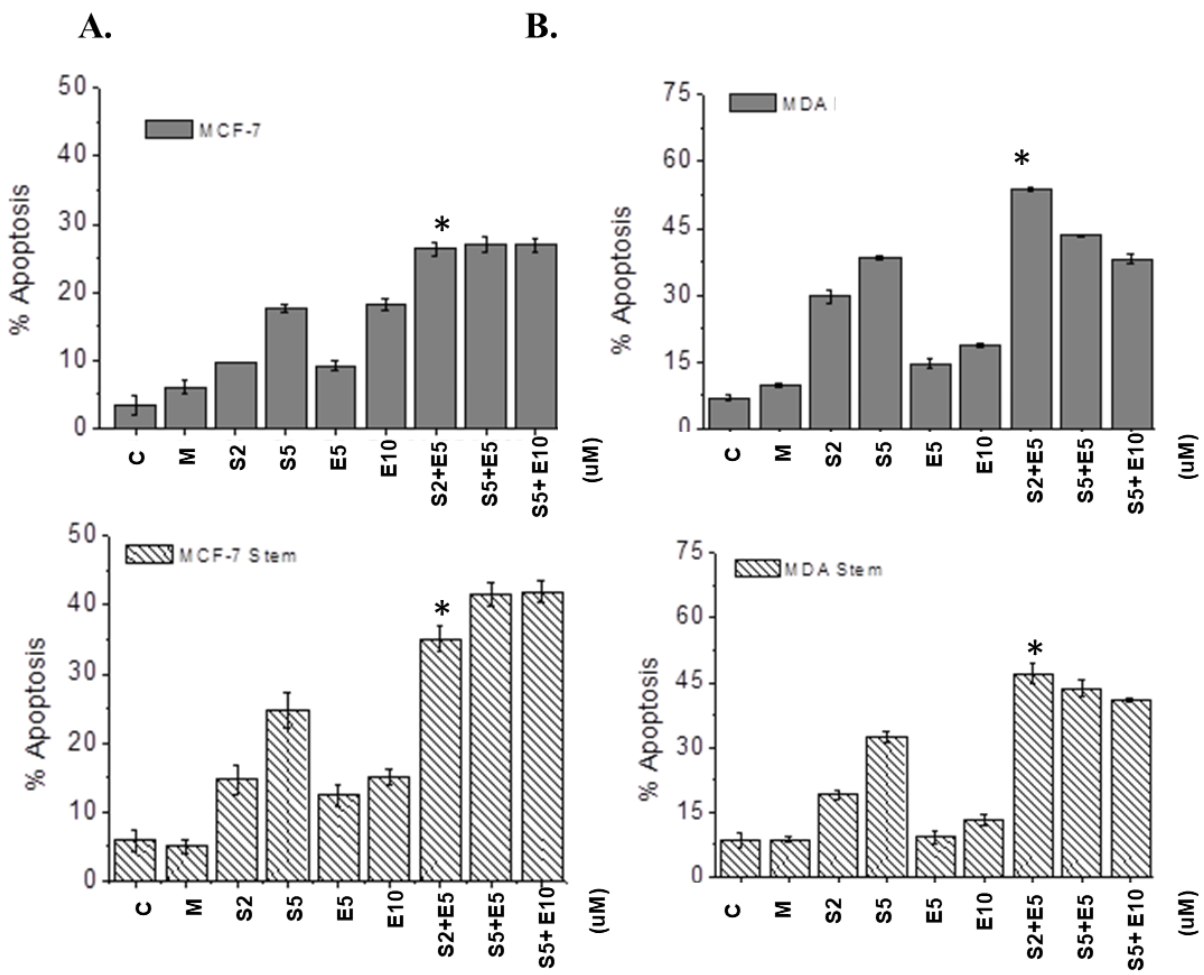

Figure 5. Induction of apoptosis in parental MCF-7 and MDA-231 and their isolated CSCs by different concentrations of salinomycin (S) or everolimus (E) treatment either alone or together. *Columns,* mean of three independent experiments done in duplicate; *bars,* SD. For statistical analysis student's t test was performed comparing specific apoptosis of combined use of salinomycin (2 and 5 μM,) plus everolimus (5 and 10 μM) *vs* specific apoptosis of salinomycin alone added with specific apoptosis of everolimus alone under hypoxia (*p<0.01)

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