

SRT1720, a potential sensitizer for radiotherapy and cytotoxicity effects of NVB-BEZ235 in metastatic breast cancer cells

Daryoush Fatehi^a, Amin Soltani^b, Mahdi Ghatrehsamani^{b,*}

^a Department of Medical Physics, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, Iran

^b Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, Shahrekord, Iran

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ABSTRACT

Background: Chemo-radio therapy (CRT) resistance is a main barrier in treating the triple negative breast cancer (TNBC). The success of conventional treatment may be ameliorated by elevating the responsiveness of the cancer cells to CRT. NVP-BEZ235 as a PI3K/AKT/mTOR dual inhibitor has been shown promising results in treating breast cancer cells. However, potential radiation-sensitizing effect of NVP-BEZ235 in TNBC remained unclear. In addition, SIRT-1 activation state and environmental cytokine were identified as being responsible for cancer cells responses to CRT. Herein, we investigate the role of interleukin 6 (IL-6) as a tumor environmental cytokine and SIRT1 in the effectiveness of NVP-BEZ235 plus radiotherapy.

Material and methods: TNBC cells were pre-treated with/without IL-6 and were exposed to single and combination of SRT1720 (SIRT1 activator)/EX-527 (SIRT1 inhibitor) and/or NVP-BEZ235 and/or gamma radiation. The effect of our treatments on cellular growth was determined by MTT and the cellular death and CSCs percentage were determined by Flow cytometry. Senescence detection kit was used to assay the effect of our treatments on cellular senescence induction.

Results: Activation of SIRT1 via SRT1720 increased the efficacy of CRT in TNBC cells, especially when IL-6 exists in tumor microenvironment. Additionally, IL-6 pre-treatment followed by exposure to SRT1720 and NVP-BEZ235 significantly increased sensitivity of the cancer stem cells to radiation ($p < 0.05$).

Conclusion: Our result shows that combination of NVP-BEZ235 and SRT1720 may effectively improve late stage breast cancer cells therapeutics approach. Activation of SIRT1 and STAT3 in resistance breast cancer cells improves the in-vitro therapeutic efficacy of CRT.

1. Introduction

Breast cancer (BC) is the major causes of cancer-related death among women. Between the various BC kinds, Triple-negative breast cancer (TNBC) is known to be extremely metastatic and resistance to conventional therapies [1]. Therefore, rate of death and metastasis is higher in women with TNBC [2]. Thus, it is crucial to find new strategies to treat this type of cancer. Several studies have indicated that activation of phosphatidylinositol 3-kinase (PI3K)/AKT/Mammalian target of rapamycin (mTOR) signaling via SIRT1 plays a major role in cancer cells growth and metastasis [3,4]. Activation of PI3K leads to Phosphatidylinositol (3,4,5)-trisphosphate (PIP3) generation which is required for the activation of AKT. AKT acts as a proto-oncogene and mediates mTOR activation, which is common in BC leads to tumor progression, resistance and survival [3]. In addition, many reports

proposed that PI3k/AKT/mTOR activation mediates cancer progression and resistance to CRT [5]. It was shown that activation of PI3k/AKT/mTOR pathway increases cancer stem cells (CSCs) phenotypes [6]. CSCs are major reason for tumor metastasis, progression and resistance to combined treatment regimen including CRT [7]. Further evidence linking SIRT1 expression with PI3k/AKT activation. SIRT1 is a NAD-dependent histone deacetylase that induces deacetylation and activation of AKT as well as PI3k. A number of trials have demonstrated that SIRT1 promotes cancer progression by activating AKT [8,9]. These trials indicated that elevated activation of PI3k/AKT or expression of SIRT1 increase resistance to CRT. Activation of SIRT1 [10], similar to PI3K/AKT, increases tumor metastasis and CSCs phenotype in TNBC [11]. Based on the findings of these trials, application of NVP-BEZ235 in combination with SIRT1 activator/inhibitor can reveal promising outcomes for treatment of patients with TNBC. Despite the PI3k/AKT

Abbreviations: TNBC, triple negative breast cancer; IL-6, interleukin 6; BC, breast cancer; PI3K, phosphatidylinositol 3-kinase; mTOR, Mammalian target of rapamycin; CSC, cancer stem cell; CRT, chemo-radio therapy

* Corresponding author at: Cellular and Molecular Research Center, Shahrekord University of Medical Sciences, PO Box: 88155-571, Shahrekord, Iran.

E-mail address: ghatreh.m@SKUMS.ac.ir (M. Ghatrehsamani).

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and SIRT1 activation, tumor environmental cytokine has major effects on the cellular mechanisms of the action. Interleukin 6 (IL-6) is a well-known tumor environmental cytokine modulates various cellular processes, such as apoptosis via activation of SIRT1. Likewise, Maria et al. have reported that IL-6 directly promote PI3K/AKT activation [12]. In another study, Seyung et al. reported the IL-6 impress CSCs induction and resistance to apoptosis in TNBC cells [12]. These observations suggest that IL-6, SIRT1 and PI3k/AKT/mTOR play a crucial role in TNBC metastasis and resistance to CRT. Although, NVP-BEZ235 as a PI3k/AKT/mTOR inhibitor, has shown promising results; but, efficacy of the drug in combination with radiotherapy and its relation with SIRT1 activation state remains unclear. In the current study, we investigated the effect of NVP-BEZ235 as a survival factor inhibitor and SIRT1 activation/inhibition in the presence of IL-6 plus radiotherapy in TNBC.

2. Material and methods

2.1. Cell culture

The Human breast cancer cell line, MDA-MB231 was purchased from the Pasteur Institute (IRAN) and the cells were cultured in complete RPMI 1640, supplemented with 10% heat-inactivated FBS, 1% pen/strep, in 75 ml flasks at 37 °C and 5% CO₂. The cells were cultured with/without 20 ng/ml IL-6 [13] (eBioscience, San Diego, CA, USA) for 2 weeks. Then, the cultures were exposed to 75 nM NVP-BEZ235 (Novartis Pharma, Basel, Switzerland) and/or 2 μM SRT1720 (SIRT1

activator)/EX-527 (SIRT1 inhibitor) (Selleckchem, Houston, TX, USA) for 24 h (Fig. 1). All mentioned steps were done for gamma irradiation groups separately, but after 24 h of single and combinatorial drugs exposure, the flasks were irradiated with 2 Gy of gamma rays. The cultures were irradiated with 6 MV photons under sterile condition at room temperature. The radiation source was Linac installed in Parsian Hospital, Shahrekord, Iran, with the following parameters: total dose: 2 Gy, dose rate: 1 Gy/min, medium energy: 6 MeV, distance between center of the source and center of the sample containers: 80 cm.

2.2. Cell viability assay

10,000 numbers of IL-6 treated/untreated cells were seeded in the 4 different 96-well plates with 100 μl of culture medium. Afterwards, all defined treatments were done similarly for 24 h. To investigating the effects of our treatment with gamma irradiation, 2 of the 96-well plates (IL-6 treated/untreated) were gamma irradiated. Subsequently, after performing all treatments, 10 μl of MTT reagent (5 mg/ml of PBS) was added to each well, incubated at 37°C for 3 h. For color development, DMSO was added to solubilize formazan crystals. Absorbance at 590 nm was measured using ELISA microplatereader.

2.3. Flow cytometry

Cells were trypsinized with 0.05% trypsin 0.25% EDTA and harvested. To analyzing the percentage of CSCs, phycoerythrin conjugated anti CD44 (ebioscience) and FITC conjugated anti CD24 (ebioscience)

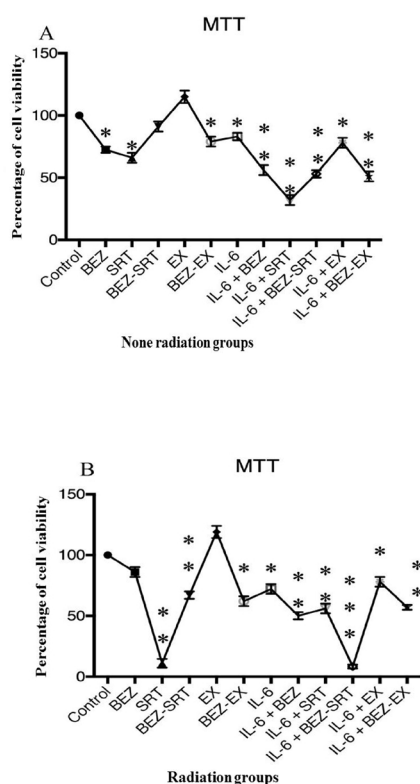


Fig. 1. SRT1720 treatment sensitized MDA-MB231 cells to radiation exposure. MDA-MB231 cells were first treated with/without the IL-6 (for 14 d) and then exposed to SRT1720 or EX-527 and/or NVP-BEZ235 (for 24 h) followed by 2 Gy gamma radiation and then cell viability was assayed. The mean percentage of cell viability \pm SD (n = 3) is shown before radiation (A) and after radiation (B). The table shows the chemical structure of compounds. [Mean \pm SD, * p < 0.05, **p < 0.01, ***p < 0.001; compared to the control group (control A = no treatment, control B = only irradiated culture)]. BEZ; NVP-BEZ235, SRT; SRT1720, EX; EX-527.

Name	Chemical structure	Target
NVP-BEZ235		Inhibitor of PI3K and mTOR
SRT1720		Selective activator of SIRT1
EX-527		selective inhibitor of SIRT1

as well as corresponding isotope control were added to the cell suspensions and incubated at 4 °C in dark for 30 min. In all cultures the CD44 + /CD24 – cells were identified as a CSC. To investigating cellular death with flow cytometry, the cells were re-suspended in staining buffer and stained with Annexin V-FITC/PI. All process were performed at 4 °C. In the last step, the cells were washed and analyzed with a Partec CyFlow flow cytometer.

2.4. β -Galactosidase staining

Senescence induction was evaluated by a senescence β -Galactosidase staining kit (abcam). Briefly, cells were washed, fixed and subsequently exposed to fresh β -Galactosidase (β -Gal) staining solution at 37 °C overnight. Then the stained senescence cells were evaluated using an Olympus inverted microscope (Japan).

2.5. Statistical analysis

The data were presented as the mean \pm SD for three separated experiments. All results were compared with related control groups using the one-way ANOVA with Dunnett's post hoc test. All analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, San Diego, CA). P values < 0.05 or < 0.01 were taken as a statistically significant.

3. Results

3.1. SRT1720 increases triple negative BC cells response to radiotherapy

As expected, MTT assay showed that NVP-BEZ235 decreased the cell viability of MDA-MB231 cells significantly (Fig. 1A; $P < 0.05$). Surprisingly, IL-6 pretreatment enhanced the efficacy of NVP-BEZ235 (Fig. 1A; $P < 0.001$). Inhibition of SIRT-1 with EX-527 non-significantly enhanced cell proliferation; however, NVP-BEZ235 or IL-6 abolished the proliferation effect induced by EX-527 (Fig. 1A; $P < 0.05$). Interestingly, addition of SRT1720 to the culture medium in order to increase SIRT-1 activity reduced the population of TNBC cells, significantly (Fig. 1A; $P < 0.05$); while NVP-BEZ235 modulated the inhibitory effect of SRT1720 in the combinatorial treatment group. Combinatorial treatment suggesting that IL-6 increased cytotoxic effect induced by SRT1720 (Fig. 1A; $P < 0.01$). Cotreatment with radiotherapy also reflected that SRT1720, sensitized MDA-MB231 cells to radiotherapy (Fig. 1B; $P < 0.001$) and IL-6 or NVP-BEZ235 reduced the effect of SRT1720 on cell sensitization (Fig. 1B; $P < 0.05$). Although, exposure of the cells to IL-6 enhanced cell sensitization to radiotherapy after treatment with NVP-BEZ235 and SRT1720 (Fig. 1B; $P < 0.001$).

3.2. IL-6 treatment has a major role in scaling down the CSC population

To determine the effects of IL-6 in association with CRT treatment on CSCs, we cultured MDA-MB231 cells with/without IL-6 for (14 d); then, the cultures co-treated with NVP-BEZ235 plus SRT1720 or EX-527 and/or radiotherapy. Afterward, the percentage of CD44 + /CD24 – cell populations was investigated by flow cytometry. MDA-MB231 cells revealed a modest decrease in CSCs population (98%–82%) after PI3 K/AKT/mTOR pathways were inhibited with NVP-BEZ235 (Fig. 2A; $P < 0.05$). Surprisingly, combinatorial treatment of cells with NVP-BEZ235 and SRT1720 decreased the percentage of CSCs from 98% to 65% (Fig. 2A; $P < 0.05$). However, similar treatment on IL-6 pretreated cells, decreased CSCs as much as NVP-BEZ235 and/or EX-527 treatment groups (Fig. 2A; $P < 0.05$). We examined the ability of the irradiation in reduction of CSCs population in TNBC cells. Interestingly, all combinatorial treatments that contains SRT1720, significantly decreased CSCs after irradiation (Fig. 2B; $P < 0.05$). Also, IL-6 pretreatment and SRT1720 with/without NVP-BEZ235 reduced CSCs more

greatly than other groups after radiation (85%–59%). Furthermore, coculture of cells with IL-6 and EX-527 showed the highest percentage (95%) of CSCs (Fig. 2B; $P < 0.05$).

3.3. Activation of SIRT1 reduced senescence induction and increased radiation induced cellular death

We studied the effect of apoptosis and senescence induction after our treatments. 24 h after EX527 treatment we recognized an increase in senescence where the cellular death rate dropped beneath the control cultures insignificantly (Fig. 3A & C). Activation of SIRT1 via SRT1720 decreased senescence induction (Fig. 3C) but it increased sensitivity of TNBC to cellular death through radiotherapy (Fig. 3B; $p < 0.01$). This sensitivity increased remarkably when cells were pretreated with IL-6 (Fig. 3B; $p < 0.01$) and we combined treatment of SRT1720 and NVP-BEZ235 (Fig. 3B; $p < 0.001$).

4. Discussion

The role of simultaneous CRT in the BC therapy has earlier been verified [14]. The discovery of PI3K/AKT/mTOR inhibitor, NVP-BEZ235 as a novel orally administered drug revealed a new way for cancer treatment. NVP-BEZ235 is capable to induce cytotoxicity and cell cycle arrest, based on the cell type and concentration. The function of this drug may occur via enhancement mitotic inhibitory factors or inhibit the activation of various kinds of pathways. Combinatorial therapies have been improved to reduce NVP-BEZ235 side effects and utilized greater efficacy as well as tolerability in the cancer treatment [15]. IL-6 is an environmental cytokine that increases metastasis of BC cells and has a major role in cancer therapies outcomes. The results of Yin et al. study revealed that effects of IL-6 is extremely depends on activation of SIRT1 [16].

However, the effectiveness of NVP-BEZ235 with IL-6 pretreated cells in combination with SIRT1 activation/inhibition has remained unclear yet. Thus, the purpose of the present study was to evaluate the single and combinatorial effects of NVP-BEZ235 with SIRT1 activation/inhibition states in metastatic BC cells that pretreated with IL-6. In addition, the radiotherapy effects on the treated cell were analyzed. Activation of SIRT1 with SRT1720 induced greater cytotoxic effects in MDA-MB231 cells and incredibly increased sensitization to radiotherapy even more than NVP-BEZ235 single treatment. However, combination of NVP-BEZ235 and SRT1720 reduced cancer stem cells population significantly. Thus, NVP-BEZ235 and SRT1720 combination provide suitable treatment options when CSCs increased in the tumor mass. In addition, combinatorial treatment of NVP-BEZ235 and SRT1720 increased cancer stem cells sensitization to radiotherapy specially when IL-6 presence in tumor environment. To the best of our knowledge, the outcomes of the current study proved, for the first time, the synergistic effect of NVP-BEZ235 and SRT1720 for in vitro TNBC therapy (Fig. 4). Radiotherapy resistance induction via SIRT1 inhibitor (EX-527) can confirm the additive effect of NVP-BEZ235 and SRT1720 on radiation induced cellular death. Moreover, cells treated with EX-527 shown no significant cytotoxic effect; remarkably proliferation was detected in TNBC. Similar to our study, Sonnemann et al. demonstrated that exposure of Ewing's sarcoma cells to SRT1720 increased the efficacy of chemotherapy [17]. We found that only NVP-BEZ235 enhanced cellular death significantly in non-radiation cultures irrespective of SIRT1; however, it did not change cellular death significantly in radiated groups. In contrast, SRT1720 increased radiation induces cellular death and this cytotoxic effect was augmented upon the addition of the NVP-BEZ235. The synergistic effects of SRT1720 and NVP-BEZ235 increased markedly when cells pretreated with IL-6. Thus, combinatorial treatment of NVP-BEZ235 and SRT1720 could be a useful therapy for breast cancer when IL-6 represent in tumor microenvironment. Considerably, IL-6 increase in tumor microenvironment especially after irradiation [18,19]. It has been shown that the cytotoxic

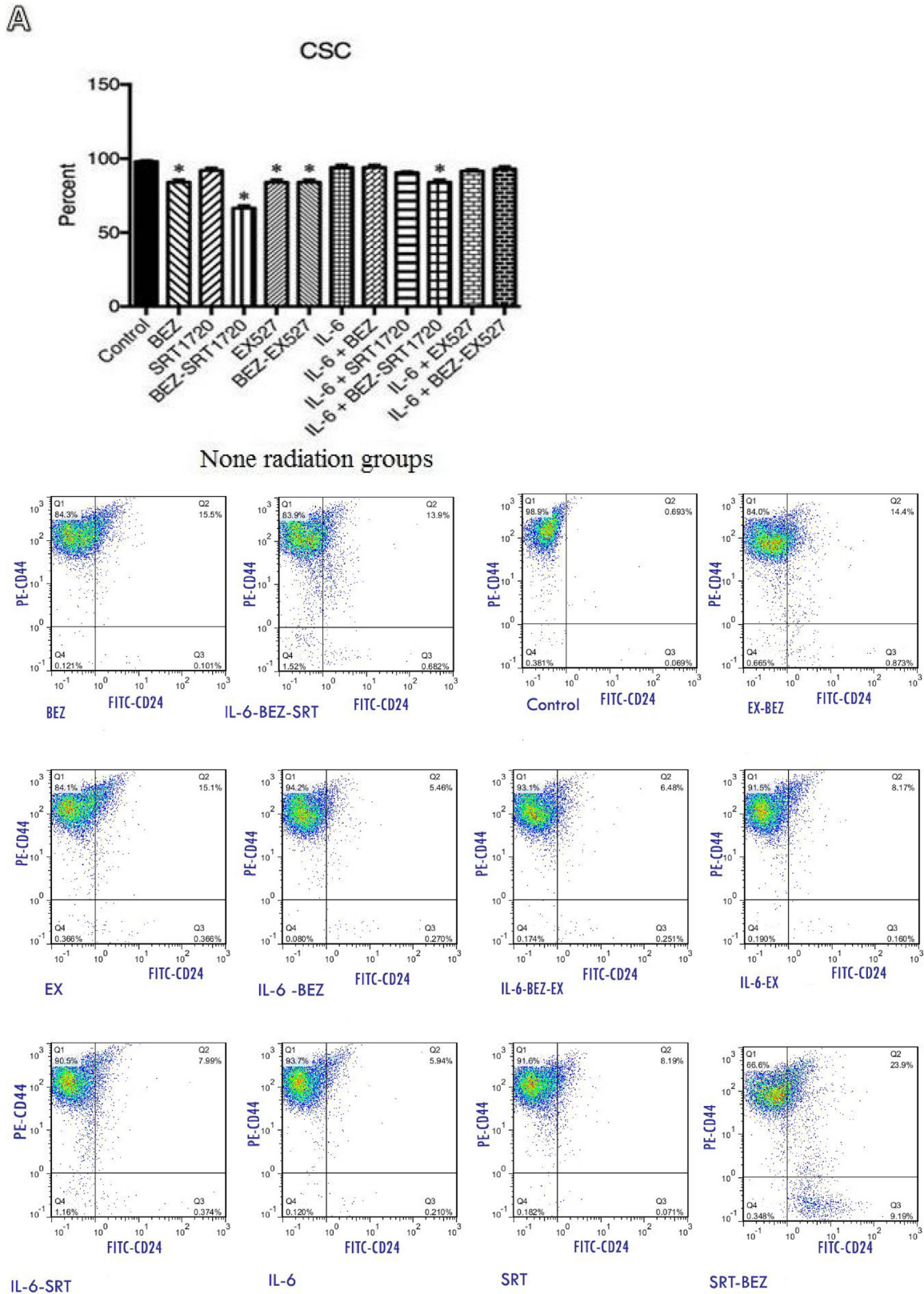


Fig. 2. Flow cytometry analysis of CSCs population that displays CD44 + CD24- phenotype. The percentages of CSCs population in all treated cultures (A) before and after (B) radiotherapy. Plots represent mean of 3 independent repeats for each experiment. [Mean ± SD, * p < 0.05; compared to the control group – (control A = no treatment, control B = only irradiated culture)]. BEZ; NVP-BEZ235, SRT; SRT1720, EX; EX-527.

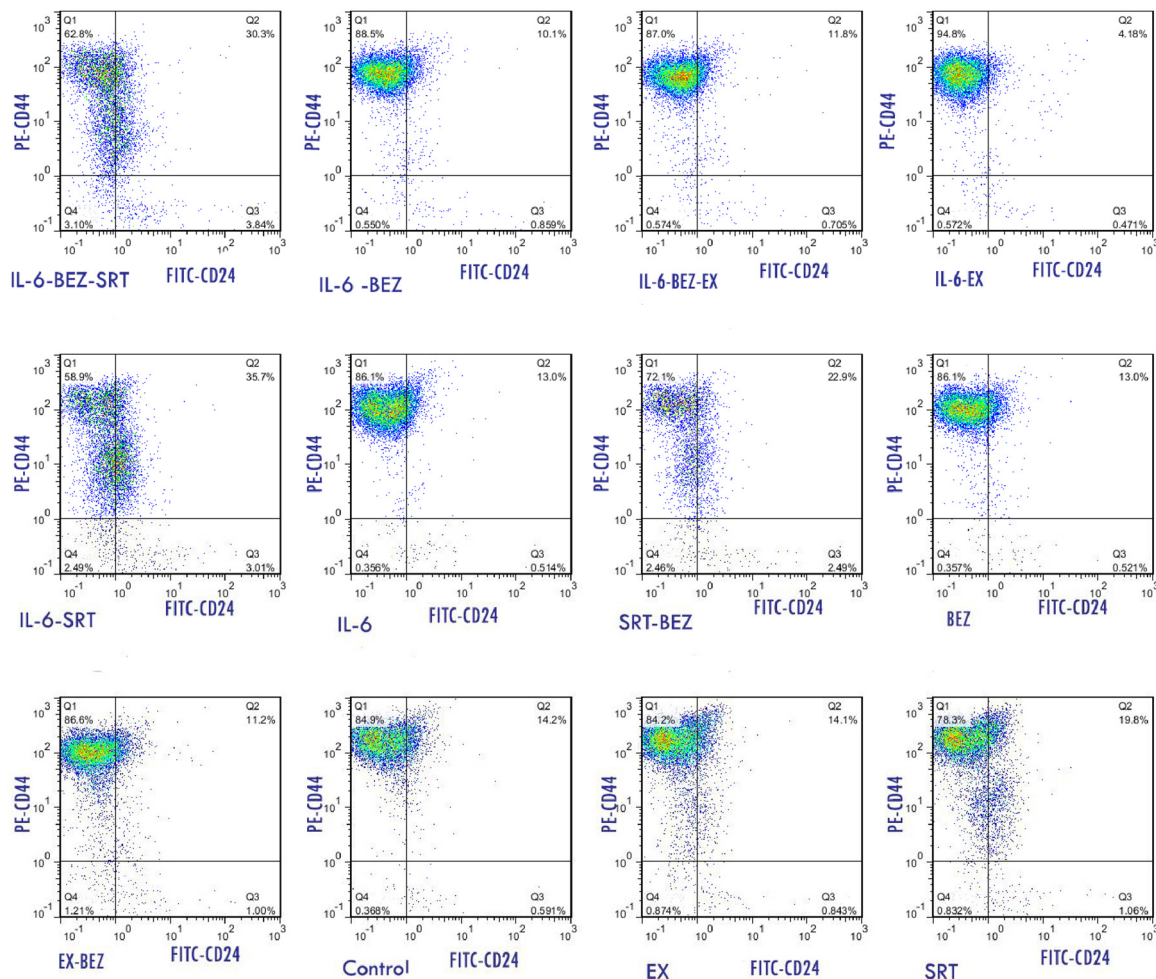
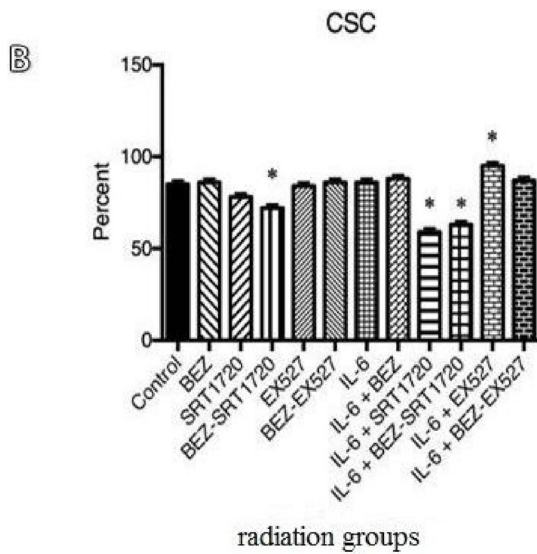


Fig. 2. (continued)

effects of SIRT1720 can be mediated through activation of pATM [20]. Furthermore, IL-6 enhance transcription and phosphorylation of ATM [20]. Storozhuk et al. have shown that activation of ATM and inhibition of AKT/mTOR via metformin increases radiation efficacy in none small

cell lung cancer [21]. Thus, we hypothesized that vigorous activation of ATM through SRT1720 and IL-6 administration is essential for efficient CRT. Therefore, administration of SRT1720 enhanced cytotoxicity effects of CRT in comparison with single treatment for BC cells, as

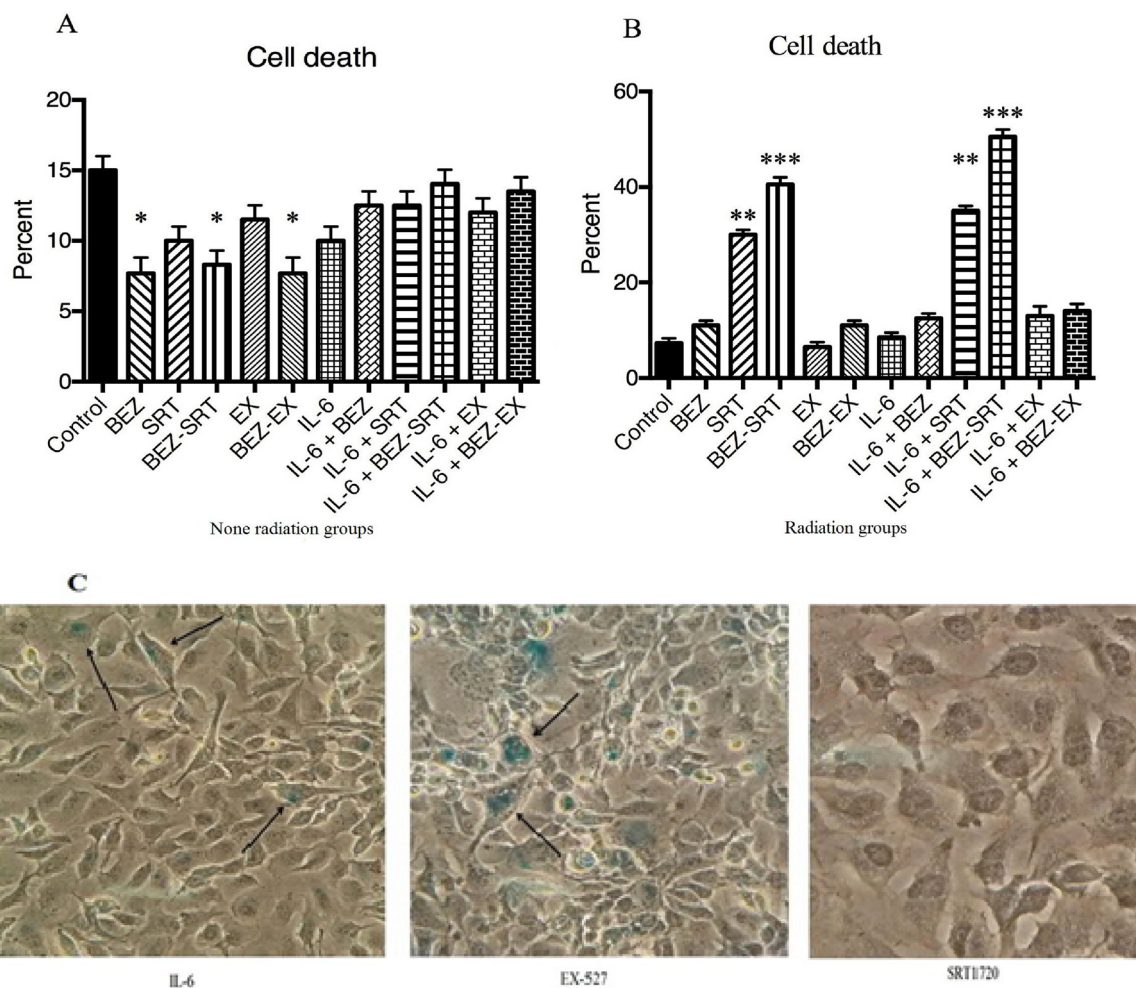


Fig. 3. Flow cytometry analysis of cellular death. The percentages of cellular death in all treated cultures (A) before and (B) after radiotherapy. Plots represent mean of 3 independent repeats for each experiment. (C) β -Galactosidase Staining was used for senescence detection. [Mean \pm SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared to the control group (control A = no treatment, control B = only irradiated culture)]. BEZ; NVP-BEZ235, SRT; SRT1720, EX; EX-527.

indicated by the existing cytotoxicity assays. Furthermore, we found that EX-527 enhanced premature senescence and resistance to radiotherapy in TNBC cells. Indeed, activation of SIRT1 through SRT1720 reduces cellular senescence induction and increases sensitivity of TNBC

cells to NVP-BEZ235 and radiotherapy, especially when IL-6 exists in the tumor microenvironment. On the other hand, Yao et al. [22] reported that SIRT1 reduced senescence induction through FOXO3a activation. IL-6 is another factor that can activate FOXO3a activation

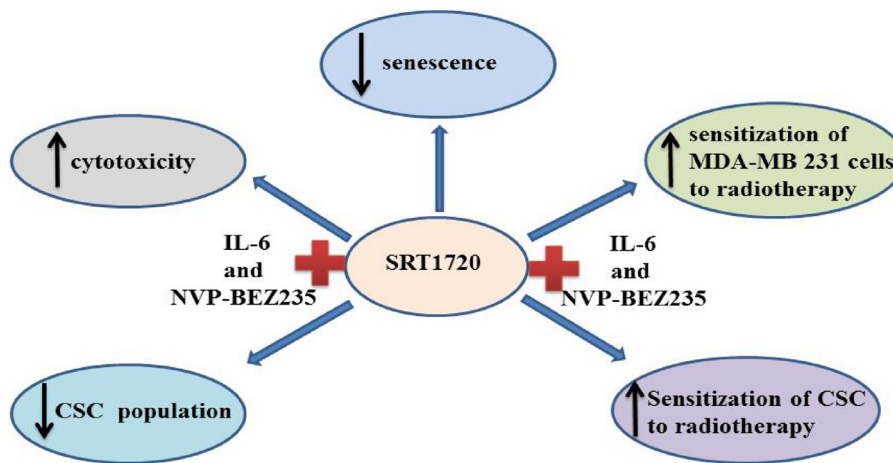


Fig. 4. SRT1720 increase the efficacy of NVP-BEZ235 in the presence of IL-6. SRT1720 increase the cytotoxic effect of NVP-BEZ235 in the presence of IL-6 and sensitized cancer cells to radiation. SRT1720 can decrease the CSCs population and senescence cells.

[23]. Moreover, Shiota et al. showed that expression of FOXO3a increases sensitivity of cancer cells to chemotherapy [24]. Therefore, it is possible to assume that IL-6 and SRT1720 increase sensitivity of cancer cells to CRT via FOXO3a activation.

NVP-BE2235 is in phase I/II clinical trials for different kinds of cancers [25]. To improve NVP-BE2235 effectiveness, we investigated the effect of this drug in various situations of tumor cells. Tumor microenvironment and status of the tumor cells have main role in cancer cells fate. Actually, activation of many signaling pathway may increase resistance of cancer cells to CRT. To overcome these difficulties and to develop the efficacy of CRT, cellular signaling may be adjusted with special kinds of the drugs. In our current study, we extended the effectiveness of NVP-BE2235 by activating of SIRT1 via SRT1720. Although, recent literature suggest that IL-6 is a tumor promoting factors but our study demonstrated that IL-6 can increase synergistic effect of NVP-BE2235 and SRT1720. The difference between the studies could be because of the discrepancy between the cell lines or duration of IL-6 treatment. Thus, chronic presence of IL-6 in tumor microenvironment, especially at the later stage of BC, combinatorial treatment of NVP-BE2235 and SRT1720 could be a useful choice.

5. Conclusion

The results of this study suggest that NVP-BE2235 in combination with IL-6 and SRT1720 acts as a chemo/radio-sensitizer and reduces tumor resistance to CRT by modulating the CSCs ratio and increasing cellular death in response to a low dose of radiotherapy. Furthermore, activation of SIRT1 (by SRT1720) and STAT3 via IL-6 increase efficacy of NVP-BE2235 in TNBC cells, which are highly metastatic and aggressive. This study demonstrates a novel combinatorial treatment strategy that by future in vivo study could be implemented in the late stage- and metastatic tumors of animal models and clinical trials, for the efficient treatment of CRT resistant breast cancers.

Conflicts of interest

None.

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