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POTENTIAL BREAST CANCER INHIBITION BY TARGETING CANCER STEM CELLS-AN IN VITRO INVESTIGATION

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Abstract

OBJECTIVE
More than 15% of mortality worldwide is due to cancer, a malignant disease caused by the uncontrolled division of abnormal cells. Breast cancer (BC) is the most common type of cancer and the second leading cause of cancer-related death after lung cancer. BC recurrence is mainly attributed to the presence of cancer stem cells (CSCs). This study aims to examine CSCs in vitro and determine their inhibition by anti-cancer agents doxorubicin (DOX) and paclitaxel (PAX) based on assessment of the expression of tumor suppressors SURVIVIN, BAX, BCL2, and p53.
DESIGN
MCF-7 cells, originally derived from human breast adenocarcinoma, were cultured in Dulbecco's modified Eagle's medium. The floating cell populations containing viable cells with CSC characteristics were analyzed by BD FACSAria III to assess the percentage of CSCs. The MCF-7 cells were treated with various doses of DOX and PAX for 48 h.
RESULTS
Changes in cell morphology, proliferation, and high apoptosis were analyzed in treated and control cells. AnnexinV-APC analysis showed the percentage of apoptotic cells following treatment with PAX 300 pM was 66.6%, while treatment with PAX 1 nM led to 68.2% apoptotic cells. DOX 10 nM and DOX 30 nM led to apoptosis rates of 66.7% and 56.9%, respectively. The total RNA was extracted, and quantitative RT-PCR was used to measure the expression of oncogenes and tumor suppressors in the control and treated groups. QRT-PCR showed downregulation of BCl2 and P53 and increased expression in SURVIVIN and BAX. We also characterized the CSCs from MCF-7 cells and evaluated the inhibitory effects of different concentrations of PAX and DOX on the CSCs. PCR results showed downregulation of BCl2 and p53 and upregulation of SURVIVIN and BAX.
CONCLUSIONS
Chemotherapy with anticancer drugs was effective in the MCF-7 cell line. CSCs from MCF-7 cells could be differentiated, and the inhibitory effects of PAX and DOC on CSCs were detected at different concentrations.

Keywords: MCF-7; Gene expression; Doxorubicin (DOX); Paclitaxel (PAX); Breast cancer (BC); Cancer stem cells (CSCs)

1. Introduction

Cancer has become increasingly prevalent worldwide during the past decade [1], with 12.7 million individuals newly diagnosed per year [1, 2]. Cancer causes more than 15% of mortality around the globe, and the numbers are steadily increasing, especially in developing countries [3]. Breast cancer (BC) has a substantial impact on women's health globally, with about 1.7 million new cancer cases diagnosed in 2012, accounting for about 12% of all new malignant cases and 25% of all malignancies in women. It is the fifth most common cause of death from cancer in women worldwide [4].

Causative factors for BC include genetic and epigenetic factors, while others remain unknown. Most cases of BC are associated with an array of factors such as obesity, lifestyle, and hormonal imbalance [5, 6]. However, about 5%-10% of BC cases arise from gene mutations in tumor suppressors and regulatory genes involved in tumor attenuation [7].

The poor survival rate of BC patients has remained almost constant over the past 30 years despite scientific advancements, and better understanding of the disease at the cellular and molecular levels is clearly needed to enable early diagnosis and develop novel therapeutics [8, 9]. Currently, BC treatment plans are based on the type of BC and its stage, with surgery such as lumpectomy, mastectomy, and lymph node dissection and chemotherapy being primary approaches [10, 11]. However, BC recurrence is mainly attributed to the presence of cancer stem cells (CSCs) [12].

CSCs are capable of both initiating tumors and sustaining their growth [13]. Two basic arguments underlie the hypothesis that CSCs are generated from normal stem cells. First, tumors are the result of sequential and progressive accumulation of genetic abnormalities, and adult stem cells are likely sites for malignant transformation due to their long lifespans. Second, CSCs have many properties in common with normal stem cells, including the capacity of self-renewal and the ability to differentiate [14, 15].

Cancer treatment is complicated, and the anti-cancer drugs in current use affect both healthy human cells and cancer cells. Indeed, because cancer cells are similar to healthy human cells, anticancer agents are generally toxic to healthy cells and can cause numerous adverse effects, some of which are life-threatening [16]. However, certain anticancer drugs can differentiate to some degree between healthy cells and cancer cells, and the rate at which cancer cells proliferate may play a role in the apparent selectivity of these agents [17, 18]. This specificity can reduce the severity of adverse effects associated with the drugs' use [19].

Such agents include paclitaxel (PAX) and the anthracycline doxorubicin (DOX). As a chemotherapeutic agent used against cancer, PAX has a broad spectrum of activity [20]. DOX has antimitotic and cytotoxic activity, and it is effective against a broad range of cancers, including hematological cancers, carcinomas, and sarcomas [21].

The MCF-7 BC cell line is an epithelial cancer cell line derived from breast adenocarcinoma [22]. It has been widely used in BC research because the cells retain several ideal features, such as

maintaining a mammary epithelial structure and having low metastatic and non-invasive properties [23]. Anti-cancer drugs such as PAX and DOX can inhibit CSCs in vitro, which suggests how they might lead to attenuation or abolishment of BC in vivo [24, 25]. The mechanism of BC cell inhibition/death may be initiated through the expression of tumor suppressors, reduction of oncogenes, or by the release of soluble anti-tumorigenic factors [26]. Our study was designed to explore the tumor-inhibiting properties of PAX and DOC in vitro, using the MCF-7 BC cell line. In addition, we evaluated whether the cancer cell inhibition was mediated by inhibition of the CSCs through direct cell-cell contact or secreted soluble factors based on assessment of the expression of tumor suppressors SURVIVIN, BAX, BCL2, and p53.

2. Materials and Methods

2.1 Cancer Cells

MCF-7 (ATCC HTB-22) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% penicillin/streptomycin and maintained in a humidified incubator at 37°C at 5% CO_2. The cells were incubated overnight in 24-well tissue culture plates at 2×10^4 cells/well until a confluent monolayer was formed.

2.2 Cell Culture

A hemocytometer (Neubauer chamber) was used to count the number of cells for subculturing and to assess cell viability using trypan blue exclusion. A 50-µL aliquot of the cell suspension was mixed with 50 µL of trypan blue (1:1) in clean 1.5-mL Eppendorf tubes and allowed to stand at room temperature for 1-2 min to facilitate staining. The hemocytometer was covered with a precision-ground coverslip. Nonviable cells were easily identified due to their blue color, while viable cells appeared translucent and were counted per field area of the hemocytometer.

The percentage of live cells was calculated using the following formula:

$$\frac{live\ cells}{total\ number\ of\ cells} \times 100$$

The percentage of dead cells was calculated using the following formula:

$$\frac{dead\ cells}{total\ number\ of\ cells} \times 100$$

The total number of cells in the given sample was calculated as follows:

$$\frac{total\ number\ of\ cells}{4} \times 2 \times \text{cell count/ml} \times 10^4$$

2.3 Treatment with PAX and DOX

In this study, MCF-7 cells were treated with PAX and DOX and the responses were compared with control samples (MCF-7 cells without treatment). MCF-7 cells were treated with PAX and DOX using the methods of the previous studies [27, 28].

2.4 Cell morphology, proliferation, and viability assay

The morphology of the cultured MCF-7 from both early and late passages was monitored daily for their growth pattern. Any changes in morphology, in relation to cell numbers, death/stress, or other

essential findings, were photographed using inverted phase contrast optics (Nikon ECLIPSE TS100).

Cell viability and proliferation were evaluated by MTT assay [28]. The absorbance of formazan was measured at wavelength 570 nm using a spectrophotometer (SpectraMax i3), and the MTT assay was also used to evaluate the proliferation of the MCF-7 cells. Two hundred microliters of dimethyl sulfoxide was added to each well to solubilize the insoluble formazan crystals by incubation in the dark for 2 h. The absorbance of this colored solution was quantified at 570 nm with a reference wavelength of 630 nm using a spectrophotometer (SpectraMax i3, Molecular Devices, USA).

2.5 Characterization of isolated cells by flow cytometry

Fluorescent activated cell sorting (FACS) analysis and flow cytometry measure optical and fluorescence characteristics of single cells and their physical properties. Cells were counted, and 1×10^5 viable cells were aliquoted per tube per antibody treatment condition. The apoptosis assay was done by using the CaspGLOW™ Fluorescein Active Caspase-3/7 Staining Kit (Thermo Fisher Scientific) according to the manufacturer's protocol [29]. The apoptosis rate (%) in each group was examined and analyzed by using a flow cytometer (BD, USA) [30, 31]. Estimation of the cluster of differentiation (CD) expression included CSC markers CD44, CD133, CD326, and E-cadherin. The cells were stained with PE-CY7 for mouse anti-human CD44 (BD Pharmingen, Cat. No 560533), APC for mouse anti-human CD133 (BD Pharmingen, Cat. No 566596), PE for mouse anti-human CD326 (BD Pharmingen, Cat. No 566841), and FITC mouse anti-E-cadherin (BD Pharmingen, Cat. No 612130) as previously described [32, 33].

2.6 Aldehyde dehydrogenase (ALDH) assay

The expression and activity of ALDH were determined by using an ALDEFLUOR kit (Stem Cells Technologies, Canada). In brief, 5×10^5 harvested treated MCF-7 cells were suspended in 500 μL of ALDEFLUOR assay buffer. Reagent containing the ALDH substrate was added to the cell suspension, and then half of the suspension was immediately transferred into a control tube with diethyl amino benzaldehyde. The activity of ALDH was recorded using flow cytometry (BD FACSCanto II, Canada) and analyzed using BD FACSDiva Software [34, 35].

2.7 Gene expression

Next, the expression of the specific genes *BAX*, *BLC2*, *SURVIVIN*, and *P53* was assessed **Table 1**. Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. An aliquot (1 μL) of each sample was used to test the quantity and purity of the RNA on a nanodrop (NANODROP 2000c). The purity was determined at a wavelength of A260/A280, with values between 1.8 and 2 being defined as pure. The samples were either frozen at (−80°C) until used or used directly for reverse transcription reaction and gene expression studies. Reverse transcription of RNA to cDNA was prepared using a cDNA reverse transcription kit (cDNA, Promega). RNA was mixed with stock solution 1, transferred to cycler sequencing machine for denaturation of RNA at 70°C for 5 min, and maintained on ice, and then 7.7 μL of stock solution 2 was added. The concentration of cDNA was measured using nanodrop at a wavelength of A260/A280 [36, 37].

Genes	Primer sequences
BAX	5′-TGCTTCAGGGTTTCATCCAG-3′ (F) 5′-GGCGGCAATCATCCTCTG-3′ (R)
BLC2	5′-GGCTGGGATGCCTTTGTG-3′ (F) 5′-CAGCCAGGAGAAATCAAACAGA-3′ (R)
SURVIVIN	5′-ACCAGGTGAGAAGTGAGGGA-3′ (F) 5′-AACAGTAGAGGAGCCAGGGA-3′ (R)
P53	5′-GCCCAACAACACCAGCTCCT-3′ (F) 5′-CCTGGGCATCCTTGAGTTCC-3′ (R)

F: forward primer; R: reverse primer.
Table 1: Gene names and primer sequences used for quantitative real-time PCR.

2.8 Statistical analyses

The raw data from all the experiments were analyzed using GraphPad Prism 7.0 (GraphPad Software, USA) software for the computation of the statistical significance using Student's unpaired *t*-test (2-tailed). The results are expressed as the mean of three replicates. P values ≤ 0.05 were statistically significant.

3. Results

MCF-7 cells were successfully cultured under standard culture conditions. The tissue culture flask was not disturbed for at least 24 h to facilitate cell attachment. The medium was changed gently without disturbing the attached cells. The cells showed good proliferation by day 3 and achieved confluence. The cells showed characteristic epithelial morphology in culture.

Figure 1 shows representative phase-contrast images of normal MCF-7 (control) and PAX-treated MCF-7 cells. The control MCF-7 cells had an epithelial morphology (Fig. 1, arrow A), while those treated with PAX showed visible changes in morphology, including cell shrinkage, loss of cytoskeletal attachment (becoming rounded up and floating), membrane damage, and condensation and detachment from the dishes. These morphological changes are shown by arrow B in Figure 1, and at a higher PAX dose, cell death was evident (arrow C).

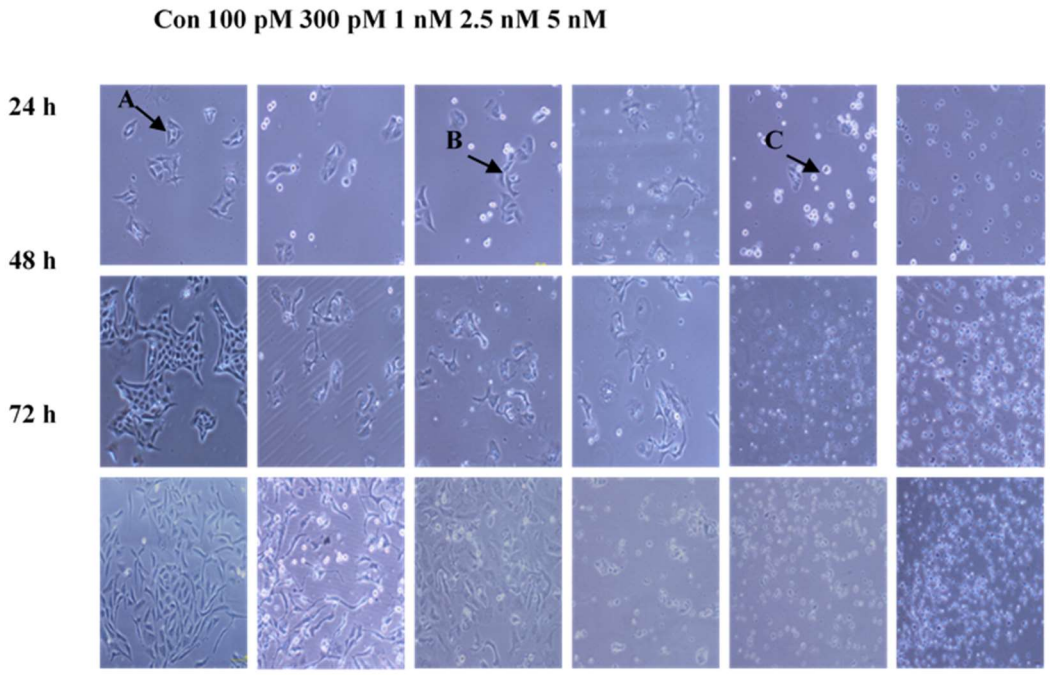

Figure 1: MCF-7 cells treated with PAX at different concentrations (100 pM to 4 nM) showed various morphological changes leading to cell death. In comparison with the control samples, the treated samples had more dead cells especially at 1 nM to 5 nM concentrations. Dead cells appeared as spherical bodies. In addition, the treated cells appeared fragmented at all different doses, and changes in morphology and higher cell death were apparent at all three time points.

Figure 2 displays representative phase-contrast images of normal MCF-7 cells (control) and DOX-treated MCF-7 cells. These images show that control MCF-7 cells had epithelial features, as shown by arrow A, while DOX-treated MCF-7 cells had visible morphological changes, including shrinkage, loss of cytoskeletal attachment (becoming rounded up and floating), membrane damage, and condensation and detachment from the dishes. These morphological changes are shown by arrow B, and arrow C indicates cell death at higher DOX doses.

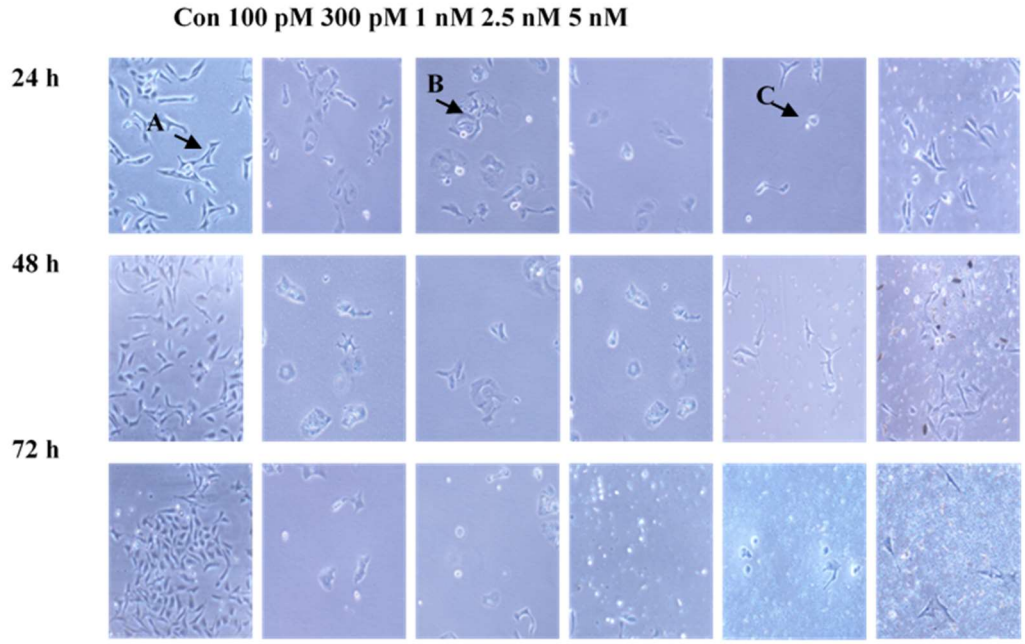

Figure 2. MCF-7 cells treated with DOX showed different shapes and characteristics. MCF-7 cells that were treated with DOX at different concentrations showed changes in morphology and cell death at all three time points.

3.1 MCF-7 proliferation MTT assay

MCF-7 cells that were cultured in vitro were evaluated for proliferation at 48 h using the MTT assay. The results presented in **Figures 3 and 4** show an inverse relationship between cell numbers and anti-cancer drug dose during the culture period. This relationship was statistically significant ($P = 0.001$).

Figure 3 shows the results for the cell proliferation assay of MCF-7 cells following treatment with different concentrations of PAX (100 pM, 300 pM, 1 nM) for 48 h. The mean percentage decreases following PAX treatment were 1.6%, 1.3%, and 1.2% for the doses 100 pM, 300 pM, and 1 nM, respectively, compared with the control. These decreases in cell proliferation were statistically significant ($P < 0.05$).

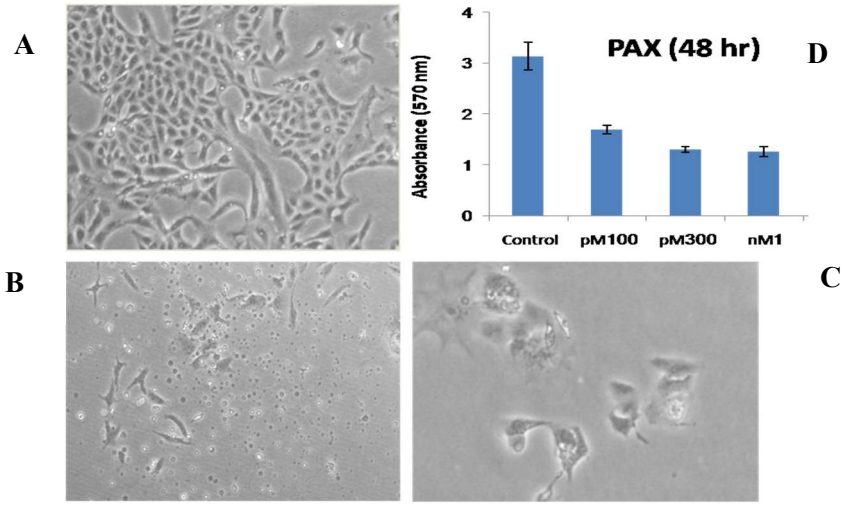

Figure 3. Phase contrast microscopic images of (A) control MCF-7 cells, (B) MCF-7 cells treated with paclitaxel 300 pM, (C) MCF-7 cells treated with paclitaxel 1 nM, and (D) MMT assay of MCF-7 cells treated with paclitaxel for 48 h.

Figure 4 presents the results of the cell proliferation assay of MCF-7 cells following treatment with different concentrations of DOX (3 nM, 10 nM, 30 nM) for 48 h. The mean percentage decreases observed following treatment with DOX (3 nM, 10 nM, 30 nM) were 5%, 1.47%, and 1.44%, respectively, compared with the control. These decreases in cell proliferation were statistically significant ($P < 0.05$).

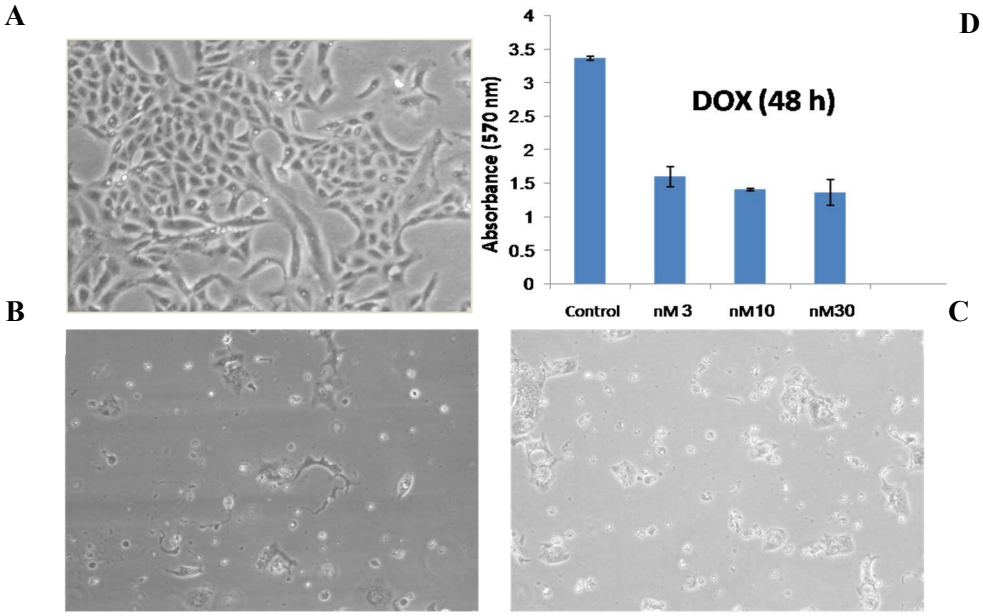

Figure 4. Phase contrast microscopic images of (A) control MCF-7 cells, (B) MCF-7 cells treated with doxorubicin 10 nM, (C) MCF-7 cells treated with doxorubicin 30 nM, and (d) MMT assay of MCF-7 cells treated with doxorubicin for 48 h.

3.2 Apoptotic effects of PAX and DOX on MCF-7 cells

Apoptosis of the MCF-7 cells was evaluated following treatment with PAX (300 pM and 1 nM) and DOX (10 nM and 30 nM) for 48 h **(Figure 5).** The percentage of apoptotic MCF-7 cells treated with PAX 300 pM was 66.6%, while 68.2% of cells treated with PAX 1 nM were apoptotic. Treatment of MCF-7 cells with DOX 10 nM yielded 66.7% apoptotic cells, while DOX 30 nM led to 56.9% apoptosis. Flow cytometric scatter plots showing the percentage of apoptotic cells following treatment with MCF-7.

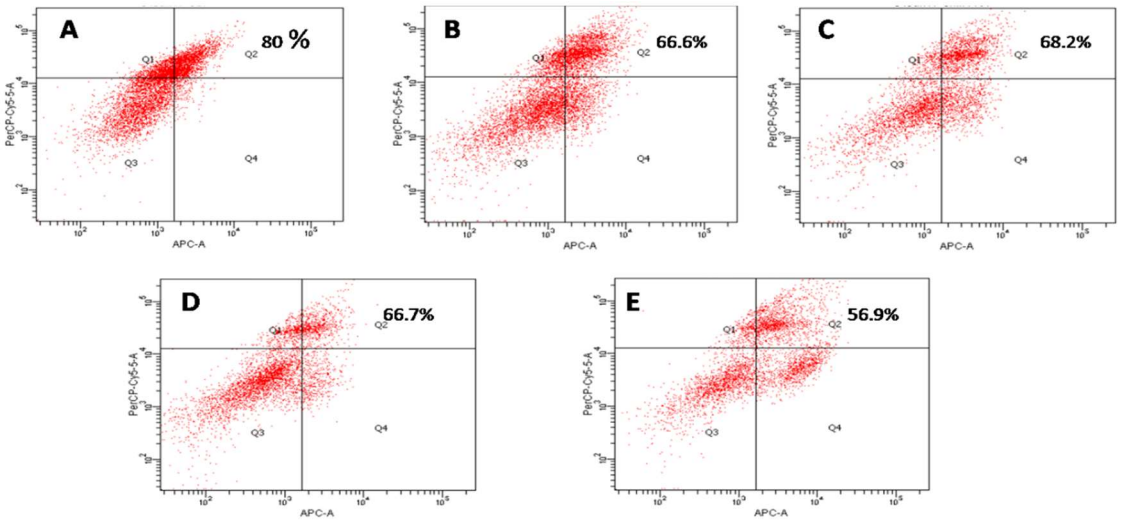

Figure 5. Evaluation of apoptosis by FACS. (A) Untreated control MCF-7 cells, (B) MCF-7 cells treated with PAX (300 pM), (C) MCF-7 cells treated with PAX (1 nM), (D) MCF-7 cells treated with DOX (10 nM), and (E) MCF-7 cells treated with DOX (30 nM).

3.3 CD44 marker expression by flow cytometry

MCF-7 cells were characterized according to CD44 surface markers, using FACS analysis (BD FACS Aria III). MCF-7 cells were positive for the CSC markers CD44 and CD133, but they were negative for CD326 and E-cadherin. The cells were stained with PE-CY7, APC, PE, and Alexa Fluor 488 **(Figures 6-9).**

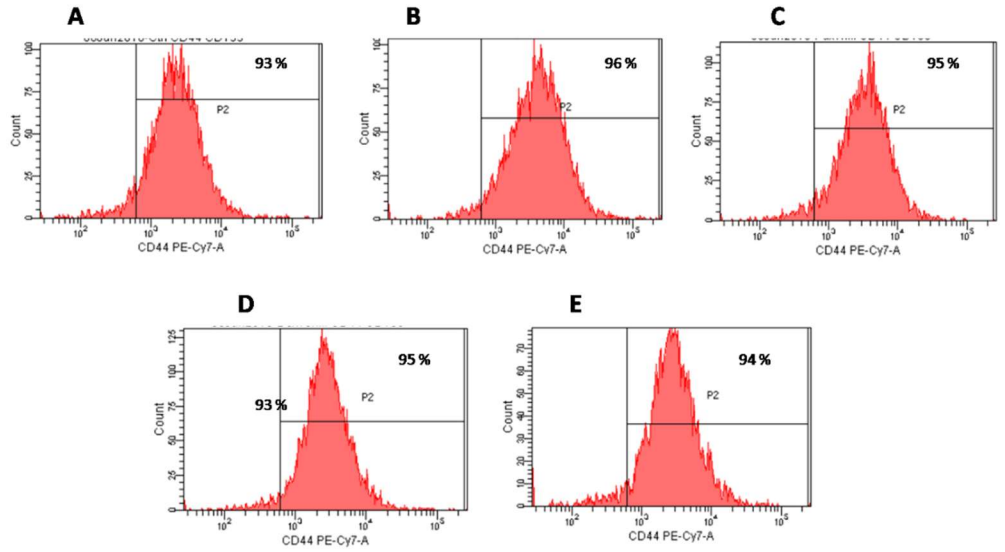

Figure 6. Analysis of CD markers in MCF-7 cells by FACS. Representative histograms from FACS analysis for CD surface-marker expression of control, PAX-treated (1 nM), and DOX-treated (10 nM) MCF-7 cells. The MCF-7 cells were positive for CD44. All positive CSC CD surface markers demonstrated more than 90% expression. (A) Untreated control MCF-7 cells, (B) MCF-7 cells treated with PAX (300 pM), (C) MCF-7 cells treated with PAX (1 nM), (D) MCF-7 cells treated with DOX (10 nM), and (E) MCF-7 cells treated with DOX (30 nM).

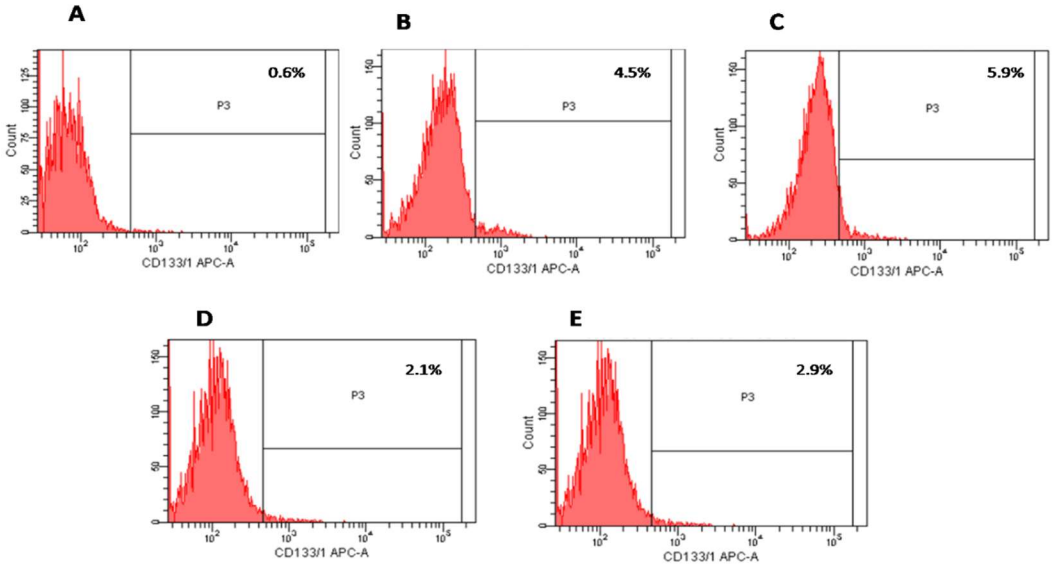

Figure 7. Analysis of CD markers in MCF-7 cells by FACS. Representative histograms from FACS analysis for CD surface-marker expression of MCF-7 cells: control, PAX (1 nM), and DOX (10 nM). The MCF-7 cells were positive for CD133. All positive CSC CD surface markers demonstrated more than 90% expression. (A) Untreated control MCF-7 cells, (B) MCF-7 cells treated with PAX (300 pM), (C) MCF-7 cells treated with PAX (1 nM), (D) MCF-7 cells treated with DOX (10 nM), and (E) MCF-7 cells treated with DOX (30 nM).

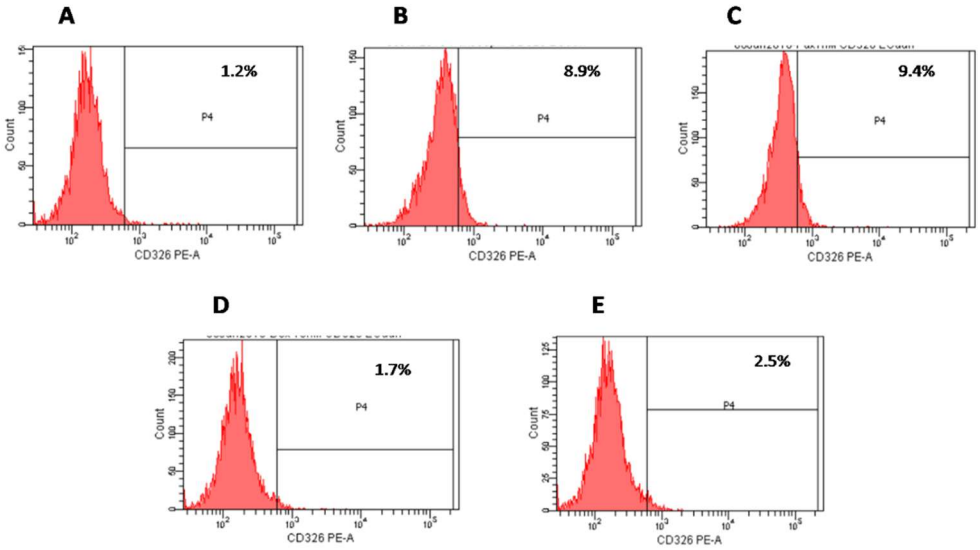

Figure 8. Analysis of CD marker of MCF-7 by FACS. Representative histograms for FACS analysis of CD surface-marker expression of MCF-7 cells: control, PAX (1 nM), and DOX (10 nM). The MCF-7 cells were positive for CD326. All negative CD surface markers demonstrated less than 10% expression. (A) Control MCF-7 cells untreated, (B) MCF-7 cells treated with PAX (300 pM), (C) MCF-7 cells treated with PAX (1 nM), (D) MCF-7 cells treated with DOX (10 nM), and (E) MCF-7 cells treated with DOX (30 nM).

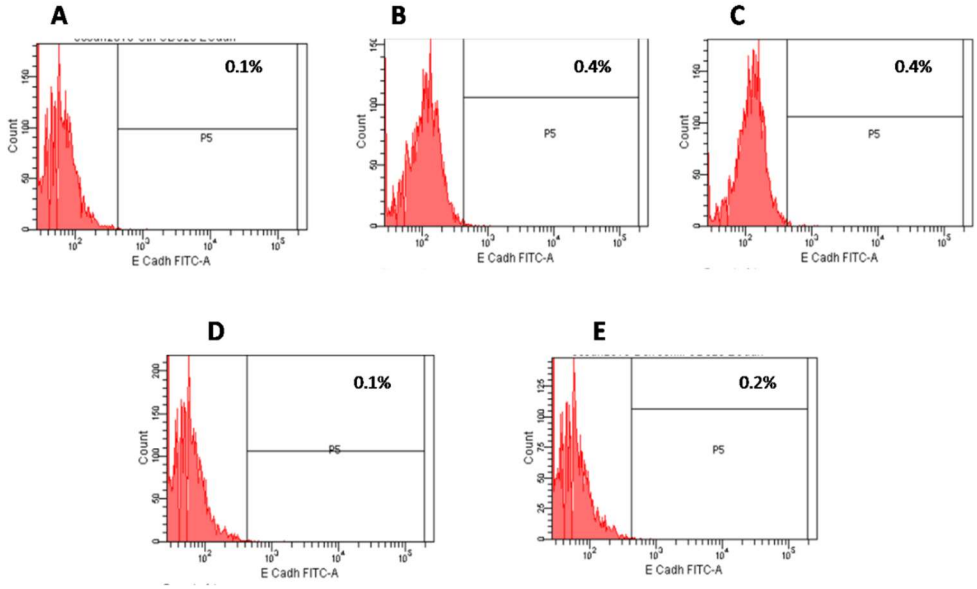

Figure 9. Analysis of CD marker of MCF-7 cells by FACS. Representative histograms of FACS analysis for CD surface-marker expression of MCF-7 cells: control, PAX (1 nM), and DOX (10 nM). The MCF-7 cells were positive for E-cadherin (ECadh). All negative CD surface markers demonstrated less than 10% expression. (A) Control MCF-7 cell untreated, (B) MCF-7 cells

treated with PAX (300 pM), (C) MCF-7 cells treated with PAX (1 nM), (D) MCF-7 cells treated with DOX (10 nM), and (E) MCF-7 cells treated with DOX (30 nM).

3.4 Aldehyde dehydrogenase (ALDH)

The results in **Figure 10** show that ALDH1 expression was higher in untreated control MCF-7 cells than in treated cells.

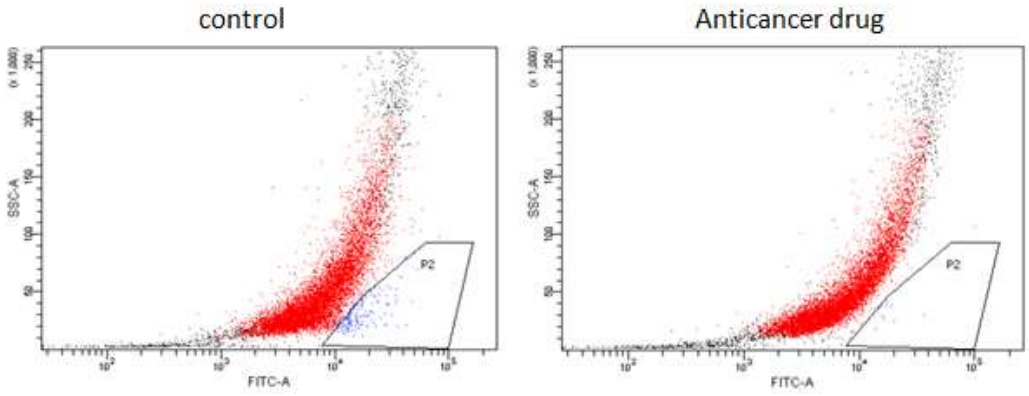

Figure 10. ALDH1 expression in MCF-7 cells by FACS. These results, together with the conservation of high CD44/CD133 ratio and ALDH1$^+$ in both the primary tumor and the metastases, suggested the importance of CSCs during tumor progression and metastasis.

3.5 Real-Time Quantitative Polymerase Chain Reaction Method (RT-QPCR)

The MCF-7 cells treated with PAX (300 pM, 1 nM) and DOX (10 nM, 30 nM) for 48 h were evaluated for expression of genes related to apoptosis, including *SURVIVIN*, *BLC2*, *BAX*, and *P53*. *BAX*, *SURVIVIN*, the tumor suppressor gene P53 showed increased gene expression, indicating that the low concentrations of PAX and DOX induced a high level of gene expression, while higher concentrations were associated with decreased expression.

The results in **Table 2 and Figure 11** show increased expression of the *SURVIVIN* gene following treatment with PAX (300 pm) and PAX (1 nM). The fold increases were 1.98-fold and 1.2-fold for PAX (300 pM) and PAX (1 nM), respectively. An increase in *SURVIVIN* gene expression was also observed with DOX (10 nM) and DOX (30 nM) by 35.4-fold and 34.5-fold, respectively.

BAX gene expression increased by 1.07-fold with PAX 300 pM and by 1.58-fold with PAX 1 nm. While in the DOX group, DOX (10 nM, 30 nM) increased by 1.81-fold and 1.19-fold, respectively. However, we observed lower expression of *BLC2* in the DOX (30 nM) treated MCF-7 cells by -1.2-fold and higher expression in the 10 nM DOX treated by 1-fold. While *BLC2* expression in the cells treated with PAX 300 pM or 1 nM was lower by -1.53 and -1.52-fold. *P53* expression was observed to be high (1.81-fold) with 10 nM DOX and lower (-1.7-fold) with 30 nM DOX. In the PAX-treated group, *P53* was reduced by -1.23 (1 nM) and -1.9 (300 pM) fold compared with the control.

Treatment	*BCL2*	*SURVIVIN*	*BAX*	*P53*
PAX, 1 nM	-1.5262592	1.98618499	1.58008262	-1.2226403
DOX, 10 nM	1.01395948	35.2609637	1.81503831	1.62450479
PAX, 300 pM	-1.5384615	1.18920712	1.07177346	-1.2388090
DOX, 30 pM	-1.2150668	34.535304	1.1974787	-1.9171180

Table 2. Evaluation by qRT-PCR of the expression of *BCL2*, *SURVIVIN*, *BAX*, and *P53* in MCF-7 cells after treatment with PAX and DOX

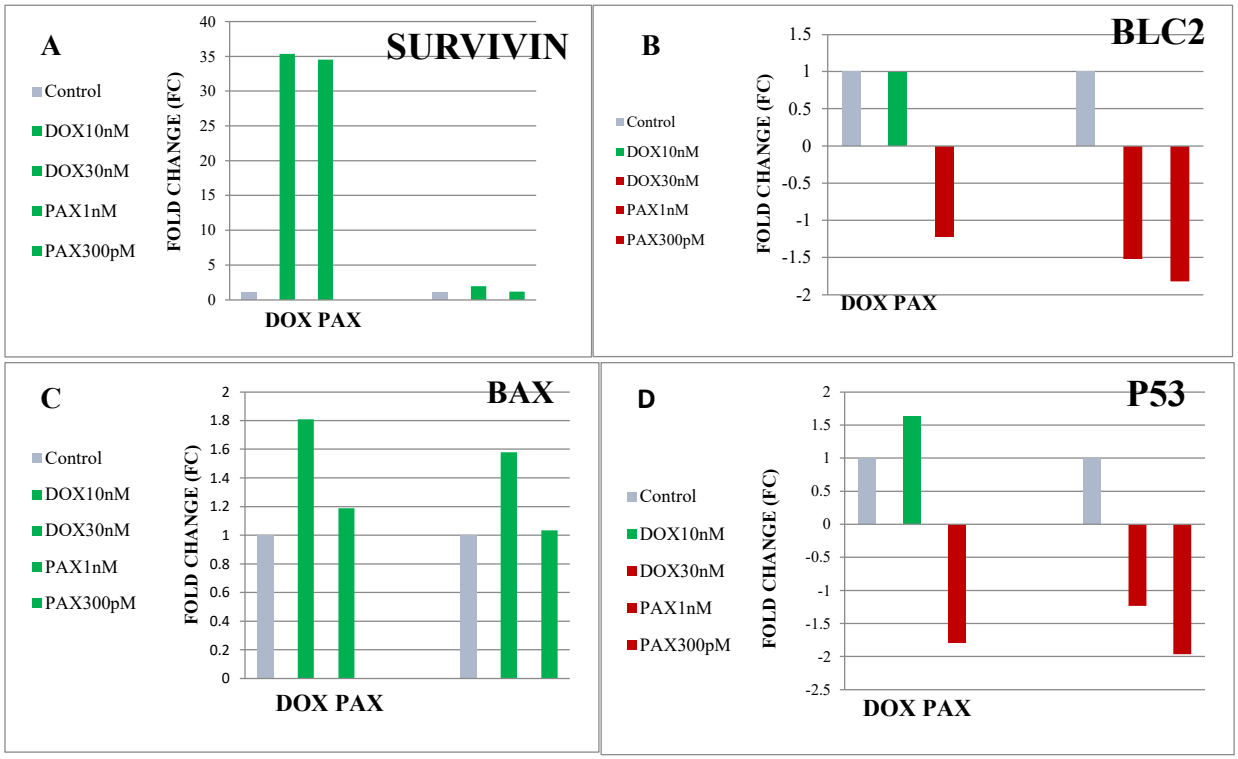

Figure 11. Analysis of apoptotic gene expression of (A) *SURVIVIN*, (B) *BCL2*, (C) *BAX*, and (D) *P53* by qRT-PCR. The MCF-7 cells were treated with PAX and DOX separately. The expression level was normalized with respect to the expression level of GAPDH. Data analysis and relative quantitation were done using the comparative Ct method (ΔΔCt). The results shown are representative of three independent experiments, and the data are presented as mean fold induction in relation to basal levels assigned ($P < 0.05$).

4. Discussion

The incidence of BC continues to increase despite significant advancements in the current treatment regime. The underlying cause leading to cancer relapses is attributed to the presence of CSCs. These cells are known to reside within the core of the tumor tissue, where a hypoxic environment prevails [38]. Furthermore, drugs may fail to reach the core and are thus unable to

fully ablate CSCs and prevent relapses. A basic understanding of the expression level of CSCs in cancer tissues will, therefore, be helpful. Although studying the primary tissues from different cancers would provide a meaningful representation of the CSCs in individual cases, there are practical limitations to obtaining samples and subsequently working with primary cultures derived from them. Therefore, in vitro investigations such as the current study rely on the use of commercial cancer cell lines [39, 40].

These investigations will provide opportunities for assessing the presence of CSCs and their increase or decrease following treatment with standard anticancer agents.

The determination of anti-proliferative actions of the anticancer drugs PAX and DOX on MCF-7 cells at various concentrations and their effects on the numbers of CSCs, thus confirm whether MCF-7 cells treated with PAX or DOX the recovered by CSCs of cancer cells, this may due to the increases of DOX affecting turnover of nucleosomes surrounding promoters of active genes, or to the formation of DOX–DNA complexes activating the DNA damage response pathway [41-43]. For the cell viability assay, the reduction of MCF-7 cell viability by PAX confirmed that long-term exposure of PAX in MCF-7 cells led to changes in the cell morphology and cell death; these results agreed with previously reported findings [44, 45]. However, the use of PAX is limited because the precise mechanisms underlying its antitumor effects are not entirely understood, while DOX remains one of the most active and widely used chemotherapeutic agents in the treatment of early and advanced BC [46].

The MCF-7 cells treated with anticancer agents showed highly significant inhibition of proliferation during different time points of culture, compared with the untreated controls. Our findings are similar to other studies in which BC cells were also found to be inhibited following treatment with DOX [38-39, 47]. The results also supported the idea that PAX induces apoptosis and demonstrated the efficacy of chemotherapeutic agents [48].

Apoptosis is the programmed cell death that maintains the healthy survival/death balance in metazoan cells [49]. The capability of anticancer drugs to induce selective apoptosis of cancer cells while having minimal effects on normal cells is highly advantageous for therapeutic purposes [50].

Following the initial evaluation of the anti-cancer drugs in the current study, cells were then characterized for the presence of CSC-related CD surface markers using FACS. The phenotype $CD44^{+}/CD24^{-low}$ represents vital breast CSCs and has been utilized to enrich CSCs in cell lines [51]. The phenotypic examination by flow cytometry to identify cells with common CSC-related cell surface markers showed expression of CD44 and CD133 and a lack of expression of CD326 and E-cadherin. These findings accord with previously reported results [52]. Our findings are also similar to an earlier report that used CSCs derived from human BC tumor [53]. The CSC markers including CD44, CD133, and ALDH have been proved to be useful targets for defining the CSC population in solid tumors. The conservation of a high CD44/CD133 ratio and ALDH1 in both the primary tumor and the metastases highlights the importance of CSCs during tumor progression and metastasis [54].

In this study, we observed that ALDH1 expression was higher in control (1.6%) than in treated MCF-7 cells (0.1%). This confirmed that the treatment of MCF-7 cells with PAX reduced the number of CSCs. This finding is in line with an earlier report [55]. Although reduction of ALDH1 levels can be used to delineate the presence CSCs, more evidence is needed to unequivocally accept ALDH1 as a biomarker for understanding the efficacy of anticancer agents and for subsequent therapeutic follow-up. Moreover, given that ALDH1 is a detoxification enzyme that is overexpressed in CSCs, its presence may be associated with therapy resistance in various cancers, including chemotherapy and radiation therapy [56].

The MCF-7 cells treated with PAX/DOX for 48 h were evaluated for expression of apoptosis-related genes, namely *SURVIVIN*, *BLC2*, *BAX*, and *P53*. We observed increased expression of *SURVIVIN* following treatment with both PAX and DOX. *SURVIVIN* is a new member of the inhibitor of apoptosis protein family that is selectively over-expressed in common human cancers but not in normal adult tissues. It is also expressed in cancer cell lines. Chemotherapeutic drugs exert adverse effects on cell survival [57]. *BCL2* family genes have been found to play a central regulatory role in apoptosis induction. Activation of the *BCL2* family of genes must occur to inhibit apoptosis and provoke carcinogenesis in a wide variety of cancers [58]. The mildly upregulated of pro-apoptotic *BAX* gene expression compared with control may be because of the gene and protein expression of *BAX* in BC cells increase sensitivity to apoptotic stimuli and decrease tumor growth [59]. Treatment with Taxol had an up-regulating effect on both *BCL2* and *BAX* transcript levels after 48 h of incubation in previous studies [60-62], which agreed with our results.

5. Conclusion

In the present study, we evaluated the anticancer properties of PAX and DOX against the well-established MCF-7 cell line derived from human breast adenocarcinoma cells. The putative anti-tumor mechanism(s) of these drugs was identified by measuring the differential gene expression of oncogenes and tumor suppressors. This study confirms that targeting CSCs is an effective strategy to achieve tumor inhibition. Determination of the dosage levels that may be required to ablate the CSCs within the core of the tumor remains a significant challenge. Improved methods to enable drug delivery, such as using nanocarriers, will further the attempts towards control or complete elimination of tumors and tumor relapses. Further work is needed to determine whether similar results will be observed in vivo, and combination therapy may be especially useful in appropriately selected patients.

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