Low concentrations of 5-aza-2′-deoxycytidine induce breast cancer stem cell differentiation by triggering tumor suppressor gene expression

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Background: Breast cancer stem cells (BCSCs) are considered the cause of tumor growth, multidrug resistance, metastasis, and recurrence. Therefore, differentiation therapy to reduce self-renewal of BCSCs is a promising approach. We have examined the effects of 5-aza-2′-deoxycytidine (DAC) on BCSC differentiation.

Materials and methods: BCSCs were treated with a range of DAC concentrations from 0.625 to 100 µM. The differentiation status of DAC-treated BCSCs was graded by changes in cell proliferation, CD44+/CD24− phenotype, expression of tumor suppressor genes, including BRCA1, BRCA2, p15, p16, p53, and PTEN, and antitumor drug resistance.

Results: DAC treatment caused significant BCSC differentiation. BCSCs showed a 15%–23% reduction in proliferation capacity, 3.0%–21.3% decrease in the expression of BCSC marker CD44+/CD24−, activation of p53 expression, and increased p15, p16, BRCA1, and BRCA2 expression. Concentrations of DAC ranging from 0.625 to 40 µM efficiently induce cell cycle arrest in S-phase. ABCG2, highly expressed in BCSCs, also decreased with DAC exposure. Of particular note, drug-sensitivity of BCSCs to doxorubicin, verapamil, and tamoxifen also increased 1.5-, 2.0-, and 3.7-fold, respectively, after pretreatment with DAC.

Conclusion: DAC reduced breast cancer cell survival and induced differentiation through reexpression of tumor suppressor genes. These results indicate the potential of DAC in targeting specific chemotherapy-resistant cells within a tumor.

Keywords: breast cancer, breast cancer stem cells, differentiation, epigenetics, 5-aza-2′-deoxycytidine

Introduction

Breast cancer stem cells (BCSCs) were discovered in 2003 by Al-Hajj et al.1 BCSCs are recognized as a subpopulation expressing CD44+/CD24−/low ESA+ and Lin− markers. Another candidate marker that fits the CSC concept is aldehyde dehydrogenase 1 (ALDH1).2 A number of cancer cell lines also express CD44+, such as colon cancer,3 liver cancer,4 renal cancer,5 bladder cancer,6 gall-bladder cancer,7 hepatocellular carcinoma,8 and human nasopharyngeal carcinoma.9 However a combination of CD44+ and CD24− was found in a BCSC subpopulation within a breast tumor, and is responsible for initiation, progression, chemotherapy resistance, and metastasis.10–12 Therefore, targeting BCSCs is a promising therapeutic approach, and the best strategy is differentiation therapy to reduce the stemness of BCSCs.

Differentiation therapy could be used to differentiate CSCs terminally and make them lose their self-renewal property, a hallmark of the CSC phenotype. Inducing
differentiation also reduces their drug resistance. To date, there are different strategies to induce differentiation of BCSCs using antitumor drugs, signaling pathway inhibitors, or gene knockdowns.

Some drugs such as acetaminophen, cisplatin, and retinoic acid induce differentiation of BCSCs; for example, Takehara et al\textsuperscript{16} reduced the tumorigenic ability of MDA-MB-231 cells using acetaminophen treatment in nude mice. Similarly, cisplatin treatment at 10 and 20 µM also reduced BCSC viability by 36%–51%, proliferation capacity by 36%–67%, and stem cell markers (CD49f, SSEA4) by 12%–67%, while upregulating the differentiation markers, CK18, SMA, and β-tubulin, by 10%–130%.\textsuperscript{17} Exposure to retinoic acid (2 µM) or vorinostat combined with 6 Gy irradiation also reduced by 30% and 70%, respectively, mammosphere survival compared to the irradiated control. In combination with paclitaxel (0.5 µM), retinoic acid and vorinostat decreased by 70% and 60%, respectively, mammosphere survival compared to paclitaxel alone.\textsuperscript{18}

IMD-0354, the NF-κB inhibitor, targets to BCSCs in a combination therapy of doxorubicin encapsulated in targeted nanoparticles. IMD-0354 induced differentiation of BCSCs, a decrease in the side-population of cells, inhibiting dye/drug efflux, reducing ABC transporters, reducing colony formation on soft agar, causing low attachment to plates, and decreasing gene expression of stem cell markers, including Oct4, Nanog, and Sox2, and apoptosis resistance.\textsuperscript{19}

Using a different strategy, Pham et al\textsuperscript{20} induced differentiation of BCSCs by knocking down CD44 gene expression with siRNA. CD44 is an important factor contributing to properties of CSC; in association with Wnt, it maintains the immortality of CSC.\textsuperscript{21} Hedgehog and Notch signaling pathway also have a close relationship with CD44 in regulating the self-renewal of CSC.\textsuperscript{22–26} In vitro, CD44 knockdown of BCSCs abolished stemness and increased susceptibility to chemotherapy.\textsuperscript{20,27} In vivo, a combination of CD44 downregulation and doxorubicin strongly suppressed tumor growth, significantly reducing tumor size and weight.\textsuperscript{28}

5-aza-2′-deoxycytidine (DAC) can be used as an epigenetic drug that utilizes a demethylation mechanism; it has been approved for use in malignant disease and cancer treatment by the US Food and Drug Administration.\textsuperscript{29–31} DAC is incorporated into DNA where it inhibits activation of DNA methyltransferase. DAC induces differentiation, apoptosis, and senescence in leukemic cells in vitro\textsuperscript{32–34} and also other cancer cell types.\textsuperscript{35–37} These results show the potential of DAC in treating malignant disease, and thus we have examined the effects of DAC on the differentiation of BCSCs in vitro.

Materials and methods

Cell culture

BCSCs with phenotype CD44−CD24− were isolated as previously reported.\textsuperscript{30} Cells were cultured in T25 culture flasks (Sigma-Aldrich, St Louis, MO, USA) for RNA extraction, flow cytometry, and an E-plate 96 (ACEA Biosciences, Inc., San Diego, CA, USA) for cell proliferation and drug sensitivity assays. The cells were cultured at 37°C in air with 5% CO\textsubscript{2} in Dulbecco’s Modified Eagle’s Medium/F12 (Sigma-Aldrich) supplemented with 10% fetal bovine serum and 1% antibiotic–antimycotic (GeneWorld, Ho Chi Minh City, Vietnam). The medium was replaced every 3 days. When 70%–80% confluence was reached, cells were detached with 0.5% trypsin/0.2% EDTA in Dulbecco’s phosphate-buffered saline (PBS; Sigma-Aldrich). The MCF-7 cell line is used as a control breast cancer cell line. This study was approved by the ethics committee of the Institutional Review Board, Vietnam National University, Vietnam and the ethics committee of Oncology Hospital, Vietnam.

Determination of cell proliferation and drug sensitive by xCELLigence

Cells were seeded on an E-plate 96 (1,000 cells/well) and cultured for 24 hours before adding DAC. Cells were treated with DAC alone or in combination with verapamil, doxorubicin, and tamoxifen (all purchased from Sigma-Aldrich). The drugs were added to the medium every 24 hours. Initially, cells were treated with ten different concentrations of DAC (0.1, 0.625, 1.25, 2.5, 5, 10, 20, 40, 60, 80, and 100 µM) for 114 hours to determine, the most effectively inhibited DAC concentration. Then, the concentration of DAC that most effectively inhibited proliferation was chosen to be combined with verapamil, doxorubicin, and tamoxifen to treat cells for 48 hours. Proliferation in each sample was calculated by comparison with the untreated control, and this was monitored every 15 minutes using the Real-Time Cell Analyzer xCELLigence System (Roche-Applied Science, Indianapolis, IN, USA).

Gene expression analysis

To determine if DAC is effective in DNA demethylation and reactivating silenced genes, real-time polymerase chain reaction (RT-PCR) was used to detect changes in the expression of p15, p16, p53, PTEN, BRCA1, and BRCA2 genes silenced in BCSCs by hypermethylation in their promoters. RNA was extracted using an easy-BLUE TM Total RNA Extraction Kit (Intron Biotechnology, Seongnam, South Korea) after cells were exposed to DAC at inhibitory concentration in 72 hours. A Brilliant III Ultra Fast SYBR
Green QRT-PCR master mix kit (Agilent Technology, Santa Clara, CA, USA) was used for reverse transcription and quantitative RT-PCR. The experiment was monitored using an Eppendorf Mastercycler® RealPlex² (Eppendorf, Hamburg, Germany) and then gene expression was calculated by the 2^ΔΔCT method. The PCR primer sequences used in this study are shown in Table 1.

**Flow cytometry assays**

Differentiation of CSCs was identified by the CD44+/CD24− level. The cells were treated with DAC, trypsinized, and washed in PBS before being stained with monoclonal antibodies anti-CD44 and anti-CD24 (BD Biosciences, Franklin Lakes, NJ, USA). Tubes were incubated in the dark at room temperature for 30 minutes before FACSSort solution (BD Biosciences) was added. Using CellQuest Pro software (BD Biosciences), the CD44+/CD24− level was identified by quadrant analysis.

To analyze ABCG2 expression, BCSCs were fixed with FCM Fixation Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 minutes and washed in PBS. Cold FCM Permeabilization Buffer (Santa Cruz Biotechnology) was added for 5 minutes at room temperature. Approximately 10⁶ cells were labeled with 2 µL ABCG2-FITC antibody (Santa Cruz Biotechnology) at 37°C for 30 minutes. Labeled cells were analyzed using a FACSSort flow cytometer (BD Biosciences).

To analyze cell cycle, BCSCs were harvested by trypsinization, washed with PBS, and resuspended in 0.5 mL PBS. The tubes were gently vortexed, and 4.5 mL ice cold 70% ethanol was added dropwise over 30–60 seconds before incubation for 2 hours at 4°C. Cells were washed and stained with PI staining solution (Sigma-Aldrich) and analyzed using a FACSCalibur flow cytometer (BD Biosciences).

**Table 1** Primer sequences used for reverse transcription polymerase chain reaction

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<tr>
<th>Genes</th>
<th>Primers</th>
<th>T_m (°C)</th>
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<td>p15</td>
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<td></td>
<td>Reverse 5′-GGGTTACGCCCTTGGCCGCT-3′</td>
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<td>p16</td>
<td>Forward 5′-TTGAGGGCGACAGGTGGCA-3′</td>
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<td>Reverse 5′-GACCGTAACTATTGCTGG-3′</td>
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<td>p53</td>
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<tr>
<td></td>
<td>Reverse 5′-GGGGCAGACACGTGCATAT-3′</td>
<td>63</td>
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<tr>
<td>PTEN</td>
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<td></td>
<td>Reverse 5′-AAATTTTTTTCTTGGCAGGA-3′</td>
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<td>BRCA1</td>
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<td></td>
<td>Reverse 5′-TCCAGTGCCACTTCAAGGG-3′</td>
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**Statistical analysis**

All graphs and statistical procedures were done using GraphPad 6 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance and t-tests were used for data analysis, and results are expressed as mean ± standard deviation. Statistical significance was set at P<0.05.

**Results**

**DAC inhibits BCSC proliferation at low concentrations**

DAC inhibited BCSC proliferation (Figure 1A). At the highest concentration (100 µM) of DAC for 114 hours; no cell death was observed, indicating lack of cytotoxicity across the whole range, but there was a decrease in proliferation at all concentrations. The doubling times were significantly greater than the control group, which had a doubling time of 24.9±0.5 hours (Figure 2A). DAC clearly did not inhibit MCF-7 cells (Figure 1B); indeed, proliferation increased in MCF-7 cells treated with 1.25, 2.5, 5, 10, 40, 60, 80, and 100 µM DAC. At 0.625 and 20 µM, DAC increased the doubling times to 26.1±0.5 and 26.5±0.6 hours, respectively, with doubling times in the control group being 25.9±0.4 hours (Figure 2B). The data in Figure 1A indicate that low doses of DAC effectively inhibited BCSC proliferation. Based on these results, we used a low concentration of DAC (<10 µM) for all the future experiments.

**DAC altered tumor suppressor gene expression**

The effect of DAC exposure at 5 and 10 µM on the expression of p15, p16, p53, BRCA1, and BRCA2 genes was determined and compared to the control group. At both concentrations, DAC reactivated p53 in comparison with control group, which did not express this gene. Otherwise, p15, p16, BRCA1, and BRCA2 genes increased their expression on treatment with DAC. When treated with 5 µM DAC, p15, p16, BRCA1, and BRCA2 gene expression increased 2.95-, 5.00-, 106.30-, and 1.40-fold, respectively, in comparison with the control group. At 10 µM DAC concentration, the results were similar, with corresponding 7.46-, 1.03-, 3.00-, and 1.50-fold increase in gene expression (Figure 3). However, PTEN expression was unchanged when treated with 5 and 10 µM DAC.

**DAC arrests BCSCs in S phase**

The cell cycle of BCSCs changed after DAC exposure (Figure 4); in the control group the distribution of cells was 63.5%±3.2% in the G1 phase, 9.3%±0.47% in the S phase,
Figure 1: Cell proliferation curve after exposure to DAC for 114 hours.
Notes: DAC concentrations range from 0.625 to 100 µM. (A) Bcsc, (B) McF-7.
Abbreviations: Bcsc, breast cancer stem cell; DAC, 5-aza-2′-deoxycytidine.

Figure 2: Relationship between BCSC doubling time and DAC concentration.
Notes: DAC concentration from 0.125 to 100 µM decreases BCSC proliferation in comparison to the control group (A) (*P<0.0006) and MCF-7 (B) (*P<0.0001).
Abbreviations: BCSC, breast cancer stem cell; DAC, 5-aza-2′-deoxycytidine.
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and 21.0%±1.1% in the G/M phase (Figure 4A). This ratio changed after exposure to DAC because of arrest mainly in the S phase in a dose-dependent manner. The distribution of cells in the S phase in the control group was 9.3%±0.47% (Figure 4H). This increased after DAC exposure at 0.625, 1.25, 5, 10, 20, and 40 µM DAC, with cells in the S phase accounting for 11.2%±0.6%, 14.2%±0.8%, 13.2%±0.7%, 19.1%±0.95%, 19.2%±0.96%, and 18.2%±0.91%.

**DAC decreases the BCSC population**

To determine the effect of DAC exposure on the differentiation of BCSCs, we treated BCSCs for 72 hours and assessed the CD44+/CD24− population; the number of cells in the CD44+/CD24− population significantly decreased compared to the control group (Figure 5). The percentage of CD44+/CD24− cells was 52.7% in the untreated group, and 17.8% in the 5 µM DAC group and 25.1% in the 10 µM DAC group.

Figure 3 mRNA expression levels of tumor suppressor genes in normalization to GAPDH.

Notes: p53 was reactivated after treatment with 5 and 10 µM of Dac for 72 hours.

*P<0.00001, **P<0.000001, ***P<0.0000003.

Abbreviation: Dac, 5-aza-2′-deoxycytidine.

Figure 4 Effect of DAC exposure on cell cycle distribution.

Notes: The number of cells in S phase increased after DAC exposure: (A) 9.3% in control, (B) 11.2% at 0.625 µM, (C) 14.2% at 1.25 µM, (D) 13.2% at 5 µM, (E) 19.1% at 10 µM, (F) 19.2% at 20 µM, and (G) 18.2% at 40 µM (P<0.0001). Cell cycle distribution is given in summary (H) *P<0.0001.

Abbreviations: CSC, cancer stem cell; Dac, 5-aza-2′-deoxycytidine.
Figure 5 Decrease in the CD44+CD24− population after DAC exposure.

Notes: There were 52.7% CD44+CD24− cells in the control group (A) compared to 17.8% CD44+CD24− cells in the 5 µM DAC group (B), and 25.1% CD44+CD24− cells in the 10 µM DAC group (C) (P<0.001). Decrease in the CD44+CD24− population with respect to the control (D) **P<0.001, ***P<0.0005.

Abbreviation: DAC, 5-aza-2′-deoxycytidine.

DAC reduces ABCG2 expression of BCSCs

To verify the effect of DAC on BCSC drug resistance, the level of ABCG2, a key protein expressed in CSCs, was measured. In the control population (Figure 6), ABCG2 expression was 99.9±5.0%, which changed after exposure to DAC. A concentration of 2.5, 5, 10, and 20 µM DAC strongly reduced the expression by 15.8±0.6%, 63±3.0%, 19.8±1.0%, and 19.5±1.0%, respectively. The results provide a supplementary explanation for the antitumor drug sensitivity of BCSCs treated with DAC.

BCSCs with DAC are sensitized to antitumor drugs

Half-maximal inhibitory concentration (IC50) values in the DAC–doxorubicin, DAC–verapamil, and DAC–tamoxifen groups were reduced in comparison to the control group (Figure 7). The IC50 of doxorubicin was 0.89 and 0.24 µg/mL in groups with and without 10 µM DAC, respectively, resulting in a 3.7-fold reduction. The effectiveness of verapamil was also increased by 10 µM DAC, the IC50 of verapamil with and without 10 µM DAC was reduced 1.97-fold from 35.3 to 17.9 µM, respectively. On the other hand, the IC50 of tamoxifen changed from 150 to 101 µM, a 1.48-fold reduction in the presence of DAC. The results, which were statistically significant, show that DAC had the strongest effect in combination with doxorubicin followed by verapamil and then tamoxifen.

The doubling times of BCSCs in samples pretreated with DAC were longer than those treated with antitumor drugs alone. Doxorubicin at 0.05, 0.1, 0.25, 0.5, and 1 µg/mL resulted in BCSC doubling times of 20.1±0.18, 20.1±0.09, 26.4±0.16, 29.3±0.28, and 29.4±0.31 hours, respectively,
and in combination with 10 µM DAC, the BCSC doubling times were 45.36±0.14, 48.2±0.19, 44.6±0.17, 49.4±0.18, and 54.9±0.01 hours, respectively (Figure 8). Similarly, verapamil at 10, 20, 40, 60, and 80 µM resulted in doubling times of 32.1±0.14, 32.6±0.12, 36.4±0.13, 61.7±0.35, and 54.8±0.44 hours, respectively. Addition of 10 µM DAC resulted in an increase of doubling times to 49.5±0.3, 50.9±0.3, 48.8±0.29, 48.5±0.31, and 66.7±0.95 hours, respectively. Tamoxifen effectively inhibited BCSCs; at 25, 50, 100, 125, and 150 µM DAC, doubling times were 23.4±0.14, 22.8±0.22, 39.9±1, 56.7±1.8, and 75.3±8.2 hours, respectively. At 10 µM DAC, the doubling times changed to 52.4±0.33, 60.0±0.32, and 113.2±11.5 at 25, 50, and 100 µM tamoxifen, respectively. Interestingly, we found that 125 and 150 µM tamoxifen combined with DAC caused BCSC death. These results demonstrate that the combination of antitumor drugs with DAC increased the doubling time and as well as the drug sensitivity of BCSCs.

Discussion

Previous studies have investigated the ability of DAC to induce expression of tumor suppressor genes in a variety of cell lines. Tumor suppressor genes that are reported to increase their expression in response to DAC exposure include Rb, p53, p21, p27, p15, p16, Apaf-1, PTEN, BRCA1, and BRCA2. In this study, the effect of DAC exposure on p15, p16, p53, PTEN, BRCA1, and BRCA2 expression was similar to previous reports. Interestingly, we found that p53 expression in BCSC was restored by DAC treatment, which raises the hypothesis that the demethylation of p53 caused reexpression and vice versa in BCSC. Moreover, it has been determined that hypermethylation in the promoter region leads to tumorigenesis where there are no p53 gene mutations. Collectively, demethylation of p53 is important in regulating this expression. Reexpression of p53 inhibited cell proliferation and induced proapoptotic gene expression in human cancer cells. In addition to p53, the results also showed that p15, p16, PTEN, BRCA1, and BRCA2 increased their expression in response to DAC exposure. The p15 gene is inactivated in response to promoter region hypermethylation, and treatment with DAC activates p15 mRNA. Hypermethylation of p16 promoters, which are strongly associated with the risk of breast cancer, may be involved in the pathogenesis of breast cancer. Demethylation of BRCA1 and BRCA2 by DAC resulted in reexpression of these genes. PTEN promoter methylation may have decreased the expression of PTEN, since expression of the PTEN gene was associated with low methylation levels.
Expression of tumor suppressor genes, such as $p16$, $p16$, $p53$, PTEN, BRCA1, and BRCA2 are involved in the cell cycle regulation. Increased expression of these genes could induce cell cycle arrest, thereby inhibiting cell proliferation. Our results were similar to that of Hurtubise and Momparler, who showed that DAC affects the S phase of breast cancer cells. Using DAC as a demethylating agent could either reactivate silenced genes or increase the expression of hypermethylated genes. Expression of tumor suppressor genes, such as $p15$, $p16$, $p53$, and PTEN, was increased. It is known that DAC acts as a proliferation inhibitor. Consequently, BCSCs treated with DAC are affected both in proliferation and cell cycle regulation.

Low doses of DAC have inhibitory effects on some cancer cell lines. Singh et al. reported that cell proliferation of the MCF-7 cell line was decreased 65% on treatment with 5 µM DAC. Treating MCF-7 cells with 0.5, 1, 2, and 5 µM DAC resulted in a 7%, 15%, 37%, and 45% decrease in
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Dai et al. used 102.4 µM DAC to decrease cell growth by 58.6%.

An important characteristic of BCSCs is their resistance to chemotherapeutic drugs; resistance is related to high expression of ATP-binding cassette transporters. ABC transporters are membrane transporters that can pump many structurally unrelated small molecules (such as cytotoxic drugs and dyes) out of cells. Therefore, increase in ABC transporters enables CSCs to resist many cancer therapies. ABCG2 is the most notable breast cancer resistance protein of this superfamily; its expression is decreased during the differentiation of BCSCs to non-CSCs. It also helps protect cells from cytotoxic agents. As a result of epigenetic changes, BCSCs treated with DAC have significantly lower ABCG2 levels that increase the antitumor drug sensitivity to drugs such as doxorubicin, verapamil, and tamoxifen. Our results showed that drug sensitivity of BCSCs was increased when antitumor drugs were combined with DAC, giving similar results to those obtained by Mirza et al., who showed that the combination of DAC with doxorubicin, paclitaxel, and 5-fluorouracil resulted in a 15%, 16%, and 13% decrease in cell proliferation, respectively, in MCF-7 and MDA cell lines when compared to the drugs being used alone.

**Conclusion**

Low-dose DAC acts as a demethylating agent causing aberrant gene expression. Its exposure induces epigenetic changes in BCSCs, such as p53 reactivation and increased expression of tumor suppressor genes p15, p16, PTEN, BRCA1, and BRCA2. DAC-treated BCSCs have significantly decreased expression of ABCG2, are arrested in S phase of the cell cycle, and are more sensitive to doxorubicin, tamoxifen, and verapamil. Most importantly, DAC exposure inhibits the BCSC population. These findings suggest that DAC could potentially be used in epigenetic therapies targeting BCSC differentiation.

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Disclosure
The authors report no conflicts of interest in this work.

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