Original Article

Disulfiram induces demethylation and reactivates the RASSF1A tumor suppressor gene in breast cancer cells

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ABSTRACT

Breast cancer (BC) is a leading cause of cancer-related deaths globally. Epigenetic alterations, particularly DNA hypermethylation, contribute to BC development by silencing tumor suppressor genes. This study investigated the ability of disulfiram (DSF) to reverse epigenetic silencing of the RASSF1A gene in the MCF-7 breast cancer cell line. Using MTT assays, DSF was found IC50 at doses of 5, 10, 12.5, and 15µM after 72 hours of treatment. Methylation-specific PCR (MSP) and real-time PCR revealed partial demethylation of the RASSF1A gene promoter at a DSF dose of 15µM after 24 hours and all doses (5-15µM) after 72 hours. This demethylation was accompanied by increased RASSF1A gene expression at the highest DSF dose (15µM) after 24 hours and at doses of 10, 12.5, and 15µM after 72 hours. These findings suggest that DSF can effectively induce epigenetic reversion of RASSF1A silencing, restoring its tumor suppressor function. This data highlights the potential of DSF as a novel therapeutic agent for breast cancer, either as a monotherapy or in combination with other therapies.

Keywords: Epigenetic, DNA methylation, *RASSF1A*, Disulfiram, Breast cancer.

Introduction

Breast cancer is still a major worldwide health issue, and it is the second most common and fatal cancer in women, after lung cancer, despite medical research advancements. Unfortunately, there has been a steady rise in breast cancer cases over the last four decades. For instance, in 2020, out of the 2.3 million new cases reported worldwide, an estimated 685,000 deaths occurred (1-3). Regardless of notable progress in cancer treatment, the overall survival rate for these patients remains relatively poor, with the majority surviving for only 2-3 years (4). Breast cancer is a complex disease that involves various cellular and molecular mechanisms. The development of cancer occurs in multiple stages, each of which contributes to different outcomes. Epigenetic mechanisms, in addition to genetics, play a crucial role in cancer development by regulating the activation and suppression of important genes without altering the DNA sequence (5, 6). Epigenetic changes, such as histone modifications, DNA methylation, and miRNA alterations, are linked to the regulation of chromatin and the expression of genes. These changes persist throughout the lifespan of the cell and are transmitted to the next generation through cell division (7-9). Epigenetic changes occur in breast cancer at all stages of the disease, similar to other types of cancer (6, 10). The proper functioning of epi-enzymes helps maintain the normal state. However, when various epi-enzymes are dysregulated, it leads to epigenetic alterations in cancer cells. In breast cancer, hypermethylation of RASSF1A, estrogen receptor α (ER-α), and progesterone receptor (PR) can be used as diagnostic and prognostic markers (11-15).

Hypermethylation silences RASSF1A, a tumor suppressor gene in breast cancer. Thus, it can be a useful diagnostic biomarker in addition to Brca1 and Brca2 genes (12, 16-18). This gene is critical for regulating the cell cycle, maintaining microtubule stability, promoting apoptosis, and enabling autophagy. In cases of breast cancer, this gene is silenced due to a mutation in one allele and methylation in the other allele (19-22). Epigenetic modifications can be modified using epi-drugs like HDAC inhibitors and DNMT inhibitors. Reprogramming of cells is currently being widely investigated in preclinical and clinical

settings (12, 23, 24). The re-expression of RASSF1A via epigenetic reversion increases breast cancer cell sensitivity to chemotherapy (12). Inhibitors of DNMT can be classified into two categories: nucleoside and non-nucleoside agents. Nucleoside analogs can replace cytosine during DNA replication, leading to significant cytotoxicity and major adverse effects (24-28). Hence, the development of safe and effective non-nucleoside DNMT inhibitors is considered a promising approach to overcoming the limitations of nucleosides. The thiol groups present in DNMT enzymes play a significant role in the catalysis of cytosine, suggesting that the reactive thiol component could serve as a non-nucleoside DNMT inhibitor. Disulfiram (DSF) is proposed as a non-nucleoside analog due to its notable characteristics. Interestingly, it is known as a safe drug commonly used in the treatment of alcoholism. Moreover, DSF exhibits an antineoplastic effect and is being investigated as an anti-cancer agent (24, 29-31). This study investigated the impact of DSF on the MCF-7 breast cancer cell line by analyzing the demethylation of the promoter and the subsequent re-expression of the RASSFIA gene.

Materials and Methods

Cell-line and cell culture

MCF-7, a breast cancer cell line, and MRC-5, a normal fibroblast cell line, were obtained from the Pasteur Institute of Iran located in Tehran. MCF-7 cells were cultivated in RPMI 1640 medium, while MRC-5 cells were cultivated in Dulbecco modified Eagle media (DMEM) with high glucose concentration, both obtained from Biowest in France. We added 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin from Sigma in the USA to the culture media of both cell lines. The cells were cultured at a temperature of 37°C in a controlled environment with a 5% concentration of carbon dioxide. When the cells reached 80% confluence, 2×104 cells were seeded in 24-well plates.

Disulfiram treatment

DSF was dissolved in dimethyl sulfoxide (DMSO; Sigma, USA) and stored at -20°C as a stock solution with a concentration of 50 mM. The authors prepared serial dilutions at various concentrations, drawing from their prior research (32, 33). Following a single day of seeding the cell line, the cells were subjected to treatment with DSF at concentrations varying from 0 to 35 µM for durations of 24, 48, and 72 hours. The half maximal inhibitory concentration (IC50) of DSF was determined to be 2.5, 10, 12.5, and 15 µM after 72 hours, resulting in a growth inhibition range of 43% to 50%.

MTT assay

To assess the cytotoxic effect of DSF, an MTT assay was conducted on the MCF-7 cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Each well of a 24-well plate was filled with a total of 2×104 cells and left to grow overnight. Following the treatment, we performed the MTT assay according to the described protocol (24).

DNA extraction, bisulfate treatment, and methylation-specific PCR

Genomic DNA was obtained from two groups: MCF-7 cells that were treated and untreated (with untreated cancerous cells serving as a control for methylation), as well as MRC-5 cells (used as a normal and unmethylated control). The PrimePrep™ Genomic DNA Isolation Kit (manufactured by Genet bio, Korea) was used to extract the DNA, following the provided protocols. The DNA concentration was measured using a NanoDrop spectrophotometer. Bisulfite treatment of the genomic DNA was carried out using the EpiTect Bisulfite Kit (Qiagen, Germany) according to the manufacturer's instructions .

The PCR reaction for methylated primers was carried out in a thermal cycler (Thermocycler, Germany) under the following cycling conditions: The procedure begins with an initial denaturation step at a temperature of 95°C for a duration of five minutes. This is followed by 35 cycles of denaturation at a temperature of 94°C for a duration of 30 seconds, annealing at a temperature of 60°C for a duration of 30 seconds, and extension at a temperature of 72°C for a duration of 30 seconds. Finally, there is a final extension step at a temperature of 72°C for a duration of four minutes.

The PCR conditions for unmethylated primers were as follows: an initial denaturation step for 5 minutes at 95°C, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing for 30 seconds at 55.3°C, extension at 72°C for 30 seconds, and a final extension at 72°C for four minutes. Subsequently, we subjected the PCR products to electrophoresis on a 2% agarose gel and observed them by employing ethidium bromide staining (32).

RNA extraction and real-time PCR

We employed the RNeasy Mini Kit from Qiagen, Germany to isolate the total RNA. To remove any genomic DNA, the RNA samples underwent treatment with RNase-free DNase, which was also sourced from Qiagen, Germany. The RNA concentration was quantified using a Biophotometer manufactured by Eppendorf, a company based in Germany. The reverse transcription of the sample (5 µL of total RNA) was performed using the RevertAid TM Kit from Fermentas, EU, following the recommended protocols. Real-time PCR was performed using the Maxima SYBR Green RoxqPCR Master Mix Kit from Fermentase (EU). The RT primers were designed using AlleleID software from Primer Biosoft. The sequences generated by the software for RASSF1A were as follows: forward - TCATCTGGGGCGTCGTG, reverse - CGTTCGTGTCCCGCTCC. For ACTB, the sequences were: forward - GTTGTCGACGACGAGCG, and reverse - GCACAGAGCCTCGCCTT. The RT-PCR reactions were

conducted using the StepOnePlus™ Quantitative RT-PCR System manufactured by Applied Biosystems in the United States. The PCR amplification process consisted of an initial step of 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds, and subsequent annealing and extension for one minute at 60°C, as previously described. The expression level of each target gene was calculated using the 2-ΔΔCt method, as described. To determine the mRNA expression level of RASSF1A, a ratio was calculated by comparing its quantity to that of the endogenous control (ACT B). The melting temperature of specific amplification products and primers was determined via melting curve analysis, conducted in triplicate at 60°C to 95°C increments at 0.3°C/S (32).

Statistical analysis

The mean ± standard deviation (SD) was used to present all quantitative data. To assess statistically significant intergroup differences, a paired-sample t-test was employed. For determining statistically significant intragroup differences, a one-way analysis of variance (ANOVA) with the LSD (least significant difference) post hoc test was performed. The mean difference (mean Diff) was utilized to determine statistical differences at various dosages between the two groups. Data analysis was conducted using SPSS version 21.0. P-values less than $P<0.05$ were considered significant **(Figure 1)**.

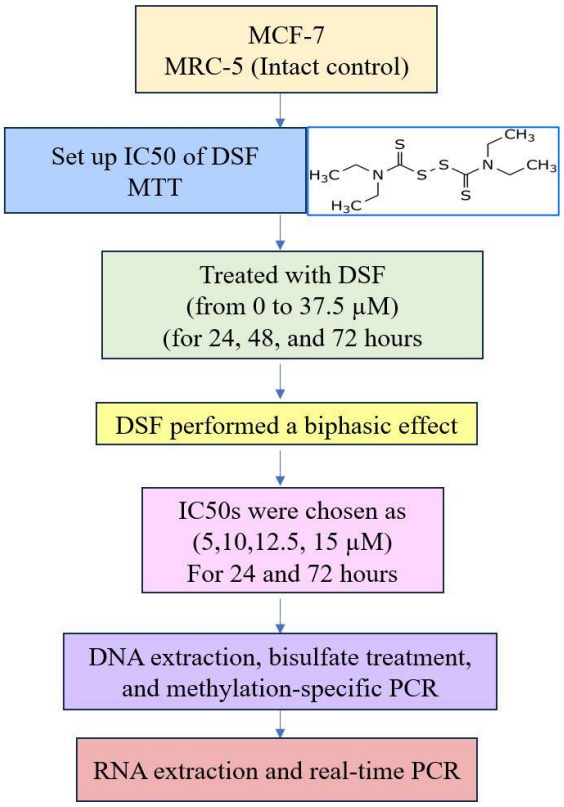

Figure 1. Diagram of the methods

Results and Discussion

MTT results

The IC50 value of DSF was determined by conducting a serial dilution of DSF on MCF-7 cells. The cells were exposed to DSF concentrations that ranged from 2.5 to 37.5 µM for 24, 48, and 72 hours. After 72 hours of treatment, the IC50 values were determined at 5, 10, 12.5, and 15 µM. The results showed a diphasic effect of DSF on the breast cancer cell line **(Figure 2)**.

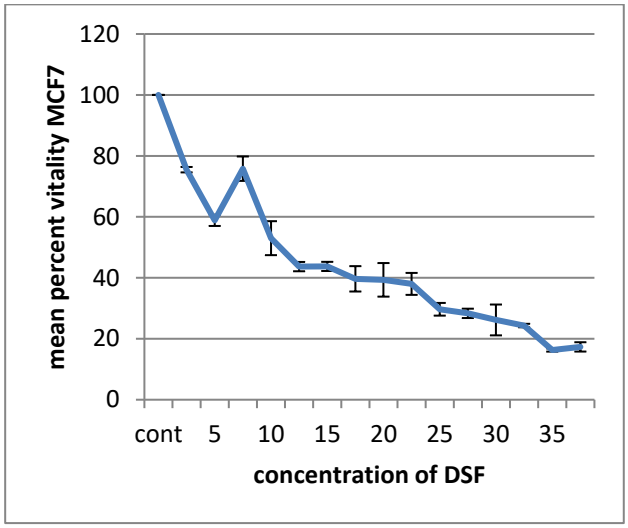

Figure 2. Viability percentage of MCF-7 cells after 72 hours of treatment.

Methyl PCR result

During a study, the changes in the methylation and unmethylation patterns of the RASSF1A promoter were observed in the MCF-7 cancer cell line. After 24 hours of treatment, partial methylation was detected only at the highest dose of treatment (15 µM). However, no unmethylated bands were observed at other treatment doses **(Figures 3a, b)**. On the other hand, methylation-specific PCR (MSP) observed partial demethylation at doses of 5, 10, 12.5, and 15 µM after 72 hours of DSF treatment. This time, both methylated and unmethylated bands were detected **(Figures 3c, d)**.

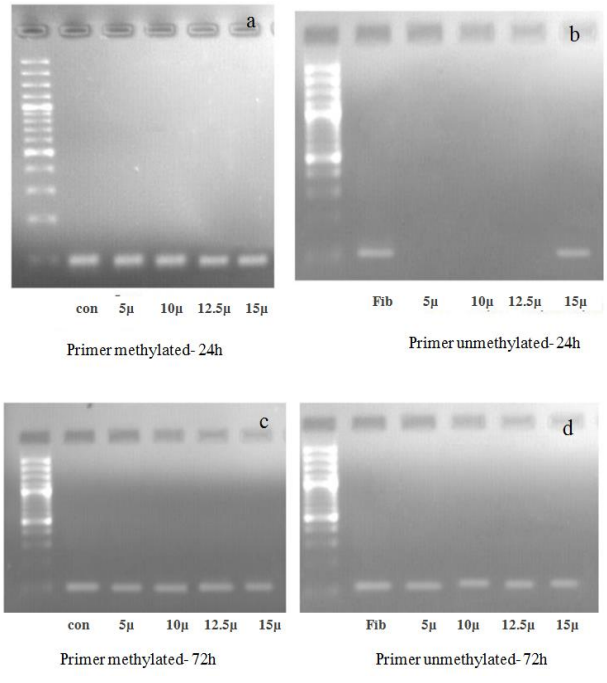

Figure 3. Methylation-specific PCR assay was conducted on MCF-7 cancer cell line to determine RASSF1A promoter methylation/unmethylation after DSF treatment for 24 and 72 hours.

Effects of DSF on gene expression in the MCF-7 cell line

The administration of DSF resulted in an increase in the expression of the RASSF1 gene in MCF-7 cells, which was dependent on the dosage and duration of treatment **(Table 1)**.

Table 1. RASSF1A expression was detected in MRC-5 after 24 and 72 hours of DSF treatment in MCF-7 cells.

Hours Dose	24h	72h	P-value Pair T-test
Control (untreated MCF-7)	1.00	1.00	
5 µmole	1.043 ± 0.296 (P-value= 0.999)	1.685 ± 0.236 (P-value=0.846)	0.136
10 µmole	1.63 ± 0.057 (P-value= 0.840)	3.277 ± 0.117* (P-value= 0.042)	0.005
12.5 µmole	1.46 ± 0.135 (P-value=0.087)	3.367 ± 0.103* (P-value= 0.34)	0.015
15 µmole	2.455 ± 0.159* (P-value<0.001)	9.695 ± 0.886** (P-value<0.001)	0.019
MRC-5 (Normal fibroblast)	236.5 ± 7.847	236.5 ± 7.847	

The RT-PCR analysis results demonstrate that there was no notable re-expression of RASSF1A after 24 hours of treatment, except for the highest dose (15 µM; $P < 0.001$). However, after 72 hours of treatment, the expression of the RASSF1A tumor suppressor increased significantly compared to the control group at doses of 10, 12.5, and 15 µM, but not at 5 µM.

When comparing the two treatment groups, i.e., the 24-hour and 72-hour treatments, it was found that the expression levels of RASSF1A in MCF-7 cells at doses of 10, 12.5, and 15 µM after 72 hours were significantly higher than those in the first group (24-hour treatment; $P < 0.001$). However, there was no significant difference observed in the expression level of RASSF1A at a dose of 5 µM between the two groups ($P = 0.137$).

Descriptive analysis of RASSF1A re-expression between the two groups

The analysis of RASSF1A re-expression showed the highest mean difference at 15 µM between the two groups. However, there were no significant differences in the mean difference of RASSF1A re-expression at 5, 10, and 12.5 µM between the treatment groups of 24-hour and 72 hours. This information is further illustrated in (Figure 4).

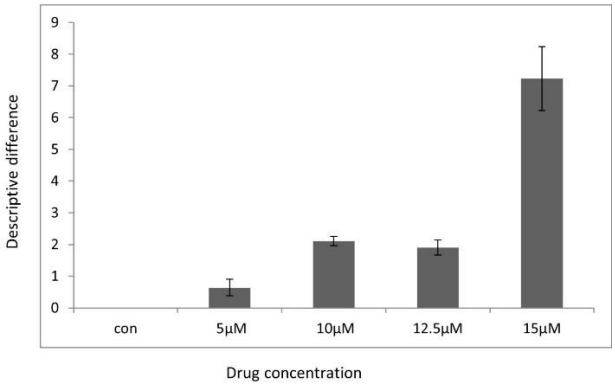

Figure 4. Descriptive difference between two groups of 24h and 72 h treatment.

A group of 303 patients with phase I or II breast cancer were evaluated to determine the effectiveness of DNMT inhibitors, specifically decitabine, a nucleotide analogous. The drugs were administered at the maximum tolerated dosage both as a standalone treatment and in combination with other anti-cancer agents. The results indicated that epi-drug monotherapy had a modest anti-tumor effect. However, in the combination group, it was observed that there was a reduction in drug resistance (34). As mentioned earlier, the use of nucleotide DNMT inhibitors can have harmful effects and may not be appropriate for long-term therapy due to their potential to cause cancer (35). On the other hand, non-nucleotide agents such as DSF have the potential to induce safe epigenetic alterations for prolonged therapy. The anti-tumor activity of DSF is believed to be achieved through epigenetic alterations and other mechanisms (36, 37). Both in vivo and in vitro studies have shown DSF as a selective drug that reduces cell viability in prostate cancer (38). Reversing epigenetic alterations in tumor suppressor genes is a valuable therapeutic approach (39, 40).

In this study, we investigated the effects of different doses (5, 10, 12.5, and 15µM) of DSF on the DNA methylation status of the tumor suppressor gene, RASSF1A, in the MCF-7 breast cancer cell line. We examined the epigenetic changes after 24 and 72 hours of treatment. Our analysis revealed that only the highest dose (15µM) of DSF caused partial demethylation after 24 hours. However, after 72 hours of treatment, all doses of DSF led to significant RASSF1A demethylation. We also found that RASSF1A re-expression occurred at dose 15µM of DSF after 24 hours, while it was significant at doses 10, 12.5, and 15µM of DSF after 72 hours compared to non-treated MCF-7 cells. The findings of our study demonstrated that DSF had an effect that varied depending on the time , with the highest mean difference of RASSF1A re-expression between the two groups observed at a concentration of 15µM. These findings are consistent with the report by Stresemann *et al*., which suggested that epigenetic reversion requires at least 72 hours of treatment (41).

Wiggins *et al*. reported a biphasic cytotoxic effect of DSF on the MCF-7 cell line. They found that at 1µM, DSF reduced the cell viability of MCF-7, but at 10µM, cell viability increased and was

comparable to the control group. However, when the concentration was increased to 100μM, the cell toxicity effect of DSF reappeared (42). Furthermore, DSF has been shown to have a significant toxic effect on GC cells, which is characterized by biphasic toxicity. This effect is highly dependent on the presence of copper (Cu). When DSF is combined with Cu, it induces both apoptotic and autophagic cell death. The mechanism underlying these effects involves the downregulation of S6K1, c-Myc, and their downstream molecules, including GLUT1, PKM2, and LDHA (43). In our cytotoxicity study (as shown in Fig 1), we observed an unexpected result where treatment with 7.5μM showed higher viability compared to both lower and higher concentrations. However, we did detect cytotoxicity at concentrations of 5μM, 10μM, 12.5μM, and 15μM of DSF after 72 hours of treatment. In contrast, a study conducted by Dastjerdi and colleagues reported that the effective dose of DSF required to attain the IC50 in PANC-1 cells was 13μM after 24 hours and did not show biphasic characteristics (32). The reason why our report differs from their result is due to the use of different cell lines in both studies, which have varying reactions towards DSF. DSF has a biphasic cytotoxicity profile in cancer cell lines such as MCF-7, Hela, HePG2, and OVCAR-3, while a monophasic effect was observed in ACH, H69AR, PRE, and PANC-1 cell lines. This discrepancy is a result of the different responses of various cancer cells to DSF exposure (33, 42, 44).

Silencing of the RASSF1A gene via promoter hypermethylation is reported in numerous cancer types such as BC, lung, and hepatocellular carcinoma, leading to reduced expression of this tumor suppressor gene, which is associated with tumor progression (45-49). In 2018, Jabir and Hamzah conducted a study on the cell-free DNA of plasma samples from breast cancer patients. They investigated whether this could be a non-invasive tool for diagnostic and prognostic purposes. The study revealed that different methylation patterns of the RASSF1A gene were dependent on cancer stages, age, and menopause status. Moreover, the study found that hypermethylation levels were observed to increase with tumor development (50). The results of the study showed that higher levels of RASSF1A were negatively correlated with tumor grade, tumor size, TNM stage, and lymph node metastasis in HER2+ breast cancer patients. In addition, it was found that patients with higher levels of RASSF1A had a better chance of five-year survival. These findings suggest that RASSF1A plays an important role as a tumor suppressor in HER2+ breast cancer and that LV-5HH-RASSF1A could be a promising gene therapy for this type of cancer, which confirms our studies (51).

A family of enzymes known as DNA methyltransferases catalyze DNA methylation, one of the key processes involved in gene expression regulation (52). It is commonly known that tumor growth can be reduced by inhibiting the activity of DNA methyltransferase. Therefore, DNA methyltransferase inhibitors could be effective agents for reversing DNA hypermethylation. As a result, modulation of this epigenetic alteration of tumor suppressor genes is considered a promising therapeutic approach for treating cancers (53-55). DSF is a type of non-nucleoside DNMT1 inhibitor that can decrease global DNA methylation. This process can help to reactivate genes that have been silenced through epigenetic mechanisms. In particular, when tested on cultured prostate cancer cells, DSF was found to reduce DNMT1 activity in a dose-dependent manner when applied to a hemimethylated DNA substrate. This decrease in activity resulted in the demethylation of the promoters of APC and RARB genes, ultimately leading to the re-expression of these genes (24). In separate research, it has been suggested that global DNA demethylation of peripheral blood mononuclear cells (PBMC) occurs in prostate cancer patients who were given varying doses (250 and 500 mg/day) of DSF. The study was conducted on patients and did not report any significant differences between the doses (56). In our research, we observed that DSF caused demethylation of the RASSAF1A gene in MCF-7 cell culture. This effect was more prominent at higher doses after 24 hours and at all doses after 72 hours. It's worth noting that Dastjerdi *et al*. found that DSF did not induce re-expression of the RASSAF1A gene in the PANC1 cell line. However, it did significantly upregulate other genes like Bax and P21 after 24 hours (32). It appears that our results differ from the ones previously reported, which could be attributed to the use of different cell lines in our respective studies. A previous study also found that applying DSF/copper (Cu) to breast cancer cell lines caused them to die and made it harder for them to form colonies in the lab after 24 hours (57). Wiggins *et al*. demonstrated DSF's anti-cancer effect on MCF-7 and BT474 breast cancer cell lines by disrupting intracellular zinc levels (42). We did not utilize copper (Cu) or zinc and did not investigate the mechanisms of DSF. However, our study demonstrated that administering DSF inhibited the growth of MCF-7 cells at doses of 5, 10, 12.5, and 15μM after 72 hours of treatment. Interestingly, the DSF treatment for 24 and 48 hours did not result in significant cell death, which is consistent with previous findings by Noorirad *et al* (58). Nucleoside analogs of DNMT inhibitors, including 5-azaC, 5-aza-dC, and decitabine, work by incorporating into the DNA molecule during duplication. They are generally considered cytotoxic epi-drugs in cancer cells. However, there is a growing interest in developing safer non-nucleoside inhibitors of DNMTs (59-61). After considering the available evidence, it seems that DSF, which is a non-toxic drug with mild side effects, might be a promising candidate for cancer treatment using the epigenetic approach (62, 63).

Conclusion

In conclusion, our study indicates that DSF has the potential to be a DNA demethylation drug. It can reactivate the epigenetically silenced tumor suppressor gene such as RASSF1A in the MCF-7 breast cancer cells, which induces cell cytotoxicity. However, further research is needed to investigate the precise mechanisms of action of DSF and its effectiveness in different cell lines of breast cancer.

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Conflict of interest: Our project was approved by the Ethical Committee of Babol University of Medical Sciences (1394/3535).

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Ethics statement: None

References

1. Ye F, Dewanjee S, Li Y, Jha NK, Chen Z-S, Kumar A, et al. Advancements in clinical aspects of targeted therapy and immunotherapy in breast cancer. Molecular cancer. 2023;22(1):105.
2. Giaquinto AN, Sung H, Miller KD, Kramer JL, Newman LA, Minihan A, et al. Breast cancer statistics, 2022. CA: a cancer journal for clinicians. 2022;72(6):524-41.
3. Cuthrell KM, Tzenios N. Breast Cancer: Updated and Deep Insights. International Research Journal of Oncology. 2023;6(1):104-18.
4. Kulis M, Esteller M. DNA methylation and cancer. Advances in genetics. 2010;70:27-56.
5. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. Nature reviews genetics. 2002;3(6):415-28.
6. Zhang W, Wang H, Qi Y, Li S, Geng C. Epigenetic study of early breast cancer (EBC) based on DNA methylation and gene integration analysis. Scientific Reports. 2022;12(1):1989.
7. Lee JE, Kim M-Y, editors. Cancer epigenetics: Past, present and future. Seminars in cancer biology; 2022: Elsevier.
8. Baylin SB, Jones PA. Epigenetic determinants of cancer. Cold Spring Harbor perspectives in biology. 2016;8(9):a019505.
9. Obeagu EI, Obeagu GU. Breast cancer: A review of risk factors and diagnosis. Medicine. 2024;103(3):e36905.
10. Montenegro M, González-Guerrero R, Sánchez-del-Campo L, Piñero-Madrona A, Cabezas-Herrera J, Rodríguez-López J. Targeting the epigenetics of the DNA damage response in breast cancer. Cell death & disease. 2016;7(4):e2180-e.
11. Chen S, Duan H, Zhang D, Sun G. Correlation between RASSF1A Methylation in Cell-Free DNA and the Prognosis of Cancer Patients: A Systematic Review and Meta-Analysis. Journal of Oncology. 2022;2022.
12. Falahi F, van Kruchten M, Martinet N, Hospers G, Rots MG. Current and upcoming approaches to exploit the reversibility of epigenetic mutations in breast cancer. Breast Cancer Research. 2014;16(4):1-11.
13. Sukocheva OA, Lukina E, Friedemann M, Menschikowski M, Hagelgans A, Aliev G, editors. The crucial role of epigenetic regulation in breast cancer anti-estrogen resistance: Current findings and future perspectives. Seminars in cancer biology; 2022: Elsevier.
14. Li Z, Wei H, Li S, Wu P, Mao X. The role of progesterone receptors in breast cancer. Drug Design, Development and Therapy. 2022:305-14.
15. Kanaan Y. Methylation of ESRα Promoters in Benign Breast Tumors Could Be a Signature for Progression into Breast Cancer in African American Women. 2024.
16. Kioulafa M, Kaklamanis L, Mavroudis D, Georgoulias V, Lianidou ES. Prognostic significance of RASSF1A promoter methylation in operable breast cancer. Clinical biochemistry. 2009;42(10-11):970-5.
17. Spitzwieser M, Holzweber E, Pfeiler G, Hacker S, Cichna-Markl M. Applicability of HIN-1, MGMT and RASSF1A promoter methylation as biomarkers for detecting field cancerization in breast cancer. Breast Cancer Research. 2015;17(1):1-13.
18. Abiola SA, Ben-Chioma AE, Fidelis BG, Aloy SC, Elekima I. Epigenetic Modulation in Breast Cancer: From Mechanisms to Therapeutic Interventions. International Research Journal of Oncology. 2024;7(1):1-13.
19. Lao TD, Thieu HH, Nguyen DH, Le TAH. Hypermethylation of the RASSF1A gene promoter as the tumor DNA marker for nasopharyngeal carcinoma. The International Journal of Biological Markers. 2022;37(1):31-9.
20. Sadzeviciene I, Snipaitiene K, Scesnaite-Jerdiakova A, Daniunaite K, Sabaliauskaite R, Laurinaviciene A, et al. Analysis of intrinsic breast cancer subtypes: the clinical utility of epigenetic biomarkers and TP53 mutation status in triple-negative cases. International Journal of Molecular Sciences. 2022;23(23):15429.
21. Vuong LD, Nguyen QN. ABERRANT METHYLATION OF CANCER-RELATED GENES IN VIETNAMESE BREAST CANCER PATIENTS: ASSOCIATIONS WITH CLINICOPATHOLOGICAL FEATURES. Experimental Oncology. 2023;45(2):195-202.
22. Jiang Y, Cui L, Chen W-d, Shen S-h, Ding L-d. The prognostic role of RASSF1A promoter methylation in breast cancer: a meta-analysis of published data. PloS one. 2012;7(5):e36780.
23. Kim A, Mo K, Kwon H, Choe S, Park M, Kwak W, et al. Epigenetic Regulation in Breast Cancer: Insights on Epidrugs. Epigenomes. 2023;7(1):6.
24. Lin J, Haffner MC, Zhang Y, Lee BH, Brennen WN, Britton J, et al. Disulfiram is a DNA demethylating agent and inhibits prostate cancer cell growth. The Prostate. 2011;71(4):333-43.
25. Medina-Franco JL, Caulfield T. Advances in the computational development of DNA methyltransferase inhibitors. Drug discovery today. 2011;16(9-10):418-25.

26. Copeland RA, Olhava EJ, Scott MP. Targeting epigenetic enzymes for drug discovery. Current opinion in chemical biology. 2010;14(4):505-10.
27. Su Y, Hopfinger NR, Nguyen TD, Pogash TJ, Santucci-Pereira J, Russo J. Epigenetic reprogramming of epithelial mesenchymal transition in triple negative breast cancer cells with DNA methyltransferase and histone deacetylase inhibitors. Journal of Experimental & Clinical Cancer Research. 2018;37:1-18.
28. Wong KK, editor DNMT1: A key drug target in triple-negative breast cancer. Seminars in cancer biology; 2021: Elsevier.
29. Zheng X, Liu Z, Mi M, Wen Q, Wu G, Zhang L. Disulfiram improves the anti-PD-1 therapy efficacy by regulating PD-L1 expression via epigenetically reactivation of IRF7 in triple negative breast cancer. Frontiers in Oncology. 2021;11:734853.
30. Hamidi N, Feizi F, Azadmehr A, Zabihi E, Khafri S, Zarei-Behjani Z, et al. Disulfiram ameliorates bleomycin induced pulmonary inflammation and fibrosis in rats. Biotechnic & Histochemistry. 2023;98(8):584-92.
31. Zeng M, Wu B, Wei W, Jiang Z, Li P, Quan Y, et al. Disulfiram: A novel repurposed drug for cancer therapy. Chinese Medical Journal. 2024;137(12):1389-98.
32. Dastjerdi MN, Babazadeh Z, Rabbani M, Gharagozloo M, Esmaeili A, Narimani M. Effects of disulfiram on apoptosis in PANC-1 human pancreatic cancer cell line. Research in Pharmaceutical Sciences. 2014;9(4):287.
33. Wickström M, Danielsson K, Rickardson L, Gullbo J, Nygren P, Isaksson A, et al. Pharmacological profiling of disulfiram using human tumor cell lines and human tumor cells from patients. Biochemical pharmacology. 2007;73(1):25-33.
34. Stewart DJ, Issa J-P, Kurzrock R, Nunez MI, Jelinek J, Hong D, et al. Decitabine effect on tumor global DNA methylation and other parameters in a phase I trial in refractory solid tumors and lymphomas. Clinical Cancer Research. 2009;15(11):3881-8.
35. Chen T, Mahdadi S, Vidal M, Desbène-Finck S. Non-Nucleoside Inhibitors of DNMT1 and DNMT3 for Targeted Cancer Therapy. Pharmacological Research. 2024:107328.
36. Fugger K, Bajrami I, Silva Dos Santos M, Young SJ, Kunzelmann S, Kelly G, et al. Targeting the nucleotide salvage factor DNPH1 sensitizes BRCA-deficient cells to PARP inhibitors. Science. 2021;372(6538):156-65.
37. Wu H-l, Gong Y, Ji P, Xie Y-f, Jiang Y-Z, Liu G-y. Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy. Journal of Hematology & Oncology. 2022;15(1):1-21.
38. Iljin K, Ketola K, Vainio P, Halonen P, Kohonen P, Fey V, et al. High-throughput cell-based screening of 4910 known drugs and drug-like small molecules identifies disulfiram as an inhibitor of prostate cancer cell growth. Clinical Cancer Research. 2009;15(19):6070-8.
39. Castro-Muñoz LJ, Ulloa EV, Sahlgren C, Lizano M, La Cruz-Hernández D, Contreras-Paredes A. Modulating epigenetic modifications for cancer therapy. Oncology Reports. 2023;49(3):1-23.
40. Fatma H, Maurya SK, Siddique HR, editors. Epigenetic modifications of c-MYC: Role in cancer cell reprogramming, progression and chemoresistance. Seminars in Cancer Biology; 2022: Elsevier.
41. Stresemann C, Brueckner B, Musch T, Stopper H, Lyko F. Functional diversity of DNA methyltransferase inhibitors in human cancer cell lines. Cancer research. 2006;66(5):2794-800.
42. Wiggins HL, Wymant JM, Solfa F, Hiscox SE, Taylor KM, Westwell AD, et al. Disulfiram-induced cytotoxicity and endo-lysosomal sequestration of zinc in breast cancer cells. Biochemical pharmacology. 2015;93(3):332-42.
43. Du C, Guan X, Liu Y, Xu Z, Du X, Li B, et al. Disulfiram/copper induces antitumor activity against gastric cancer cells in vitro and in vivo by inhibiting S6K1 and c-Myc. Cancer Chemotherapy and Pharmacology. 2022;89(4):451-8.
44. Zhang L, Tian B, Li Y, Lei T, Meng J, Yang L, et al. A copper-mediated disulfiram-loaded pH-triggered PEG-shedding TAT peptide-modified lipid nanocapsules for use in tumor therapy. ACS applied materials & interfaces. 2015;7(45):25147-61.
45. Tahoon A, El-Khateeb D, Mosbeh A, Tantawy El Sayed I, Khalil A. Significance of promoter methylation of multiple tumor suppressor genes in hepatocellular carcinoma. Egyptian Journal of Medical Human Genetics. 2022;23(1):1-9.
46. Xie B, Peng F, He F, Cheng Y, Cheng J, Zhou Z, et al. DNA methylation influences the CTCF-modulated transcription of RASSF1A in lung cancer cells. Cell Biology International. 2022;46(11):1900-14.
47. Motawi TK, El-Maraghy SA, Sabry D, Nady OM, Senousy MA. Cromolyn chitosan nanoparticles reverse the DNA methylation of RASSF1A and p16 genes and mitigate DNMT1 and METTL3 expression in breast cancer cell line and tumor xenograft model in mice. Chemico-Biological Interactions. 2022;365:110094.
48. Yadav P, Masroor M, Nandi K, Kaza R, Jain S, Khurana N, et al. Promoter methylation of BRCA1, DAPK1 and RASSF1A is associated with increased mortality among indian women with breast cancer. Asian Pacific Journal of Cancer Prevention: APJCP. 2018;19(2):443.
49. Alshammari E, Zhang Y, Sobota J, Yang Z. Aberrant DNA methylation of tumor suppressor genes and oncogenes as cancer biomarkers. Genomic and Epigenomic Biomarkers of Toxicology and Disease: Clinical and Therapeutic Actions. 2022:251-71.
50. Jabir FA, Hamzah SK. SOX17 and RASSAF1A promoters methylation in circulation tumor cell and cell free DNA isolated from plasma in breast cancer Iraqi women patients. Research Journal of Pharmacy and Technology. 2018;11(5):2000-5.
51. He S, Hou Y, Hou L, Chen N, Yang X, Wang H, et al. Targeted RASSF1A expression inhibits proliferation of

HER2-positive breast cancer cells in vitro. Experimental and Therapeutic Medicine. 2023;25(6):1-8.

52. Cui D, Xu X. DNA methyltransferases, DNA methylation, and age-associated cognitive function. International journal of molecular sciences. 2018;19(5):1315.

53. Del Castillo Falconi VM, Torres-Arciga K, Matus-Ortega G, Díaz-Chávez J, Herrera LA. DNA Methyltransferases: From Evolution to Clinical Applications. International Journal of Molecular Sciences. 2022;23(16):8994.

54. Feng S, De Carvalho DD. Clinical advances in targeting epigenetics for cancer therapy. The FEBS journal. 2022;289(5):1214-39.

55. Sheng J, Shi W, Guo H, Long W, Wang Y, Qi J, et al. The inhibitory effect of (−)-epigallocatechin-3-gallate on breast cancer progression via reducing SCUBE2 methylation and DNMT activity. Molecules. 2019;24(16):2899.

56. Schweizer MT, Lin J, Blackford A, Bardia A, King S, Armstrong AJ, et al. Pharmacodynamic study of disulfiram in men with non-metastatic recurrent prostate cancer. Prostate cancer and prostatic diseases. 2013;16(4):357-61.

57. Yip N, Fombon I, Liu P, Brown S, Kannappan V, Armesilla A, et al. Disulfiram modulated ROS–MAPK and NFκB pathways and targeted breast cancer cells with cancer stem cell-like properties. British journal of cancer. 2011;104(10):1564-74.

58. Noorirad SN, Pourghasem M, Feizi F, Abedian Z, Ghasemi M, Babazadeh Z, et al. Time dependent of epigenetic effect of disulfiram on tumor suppressor gene of RASSF1A in Hela cancer cell line. Journal of Basic Research in Medical Science. 2018;5:8-13.

59. Tao L, Zhou Y, Luo Y, Qiu J, Xiao Y, Zou J, et al. Epigenetic regulation in cancer therapy: From mechanisms to clinical advances. MedComm–Oncology. 2024;3(1):e59.

60. Kantarjian HM, Roboz GJ, Kropf PL, Yee KW, O'Connell CL, Tibes R, et al. Guadecitabine (SGI-110) in treatment-naive patients with acute myeloid leukaemia: phase 2 results from a multicentre, randomised, phase 1/2 trial. The Lancet Oncology. 2017;18(10):1317-26.

61. Gallimore F, Fandy TE. Therapeutic Applications of Azanucleoside Analogs as DNA Demethylating Agents. Epigenomes. 2023;7(3):12.

62. Li Z, Gao Y, Zhang J, Han L, Zhao H. DNA methylation meningioma biomarkers: attributes and limitations. Frontiers in Molecular Neuroscience. 2023;16.

63. Zhang Z, Wang G, Li Y, Lei D, Xiang J, Ouyang L, et al. Recent progress in DNA methyltransferase inhibitors as anticancer agents. Frontiers in Pharmacology. 2022;13:1072651.