The Role of Sodium Phenylbutyrate in Modifying the Methylome of Breast Cancer Cells

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ABSTRACT: Sodium phenylbutyrate is one of the histone deacetylase inhibitors that play a role as an anti-cancer agent. One of the diseases that could be challenged with sodium phenylbutyrate is breast cancer, which is the most common cancer and the leading cause of cancer death in females worldwide. In the present study, the role of sodium phenylbutyrate was investigated along with different chemotherapeutic drugs in controlling breast cancer cells (MCF-7). Two concentrations were used in the current study; 3µM and 5µM. Trypan blue test was employed to assess the viability of the cells after treatment. On the epigenetic level, global methylation has been quantified in the treated cells and compared to the control. Results obtained indicated that the number of viable cells have generally decreased significantly ($p<0.05$) after treating the cells with drug combinations composed of sodium phenylbutyrate, sodium phenylbutyrate combined with procaine, sodium phenylbutyrate combined with carboplatin, sodium phenylbutyrate combined with cyclophosphamide, and sodium phenylbutyrate combined with temozolomide. The global methylation pattern was also assessed, and all the combinations applied have modified the methylation pattern of the cells. Different combinations have increased/decreased the concentration of 5-MethylCytidine significantly compared to control. This study conclude that the most effective treatment was sodium phenylbutyrate when applied solely at a concentration of 3µM and sodium phenylbutyrate combined with cyclophosphamide at 3µM for further in vivo investigations.

Index Terms: Breast cancer, Sodium phenylbutyrate, Combination therapy, HDACi, Methylation.

1. INTRODUCTION

Cancer represents a major public health problem worldwide [1]. It is a class of diseases characterized by out-of-control cell growth [2]. The cancer cells affect the body when it divides uncontrollably forming lumps or masses of tissue called tumors [3]. Progressive and aggressive forms of breast cancer are notably found in developing countries compared to western countries [4]. DNA methylation, histone modifications and non-coding RNAs are considered underlying epigenetic mechanisms by through which cancer could develop, and might provide potential new therapeutic targets [5], [6].

Between Egyptian women, breast cancer is the most common cancer type, representing 18.9% of total cancer cases, with an age-adjusted rate of 49.6 / 100,000 population [7], [8].

Breast cancer is caused by progressive genetic alterations including different mutations in tumor suppressor genes, oncogenes, and other abnormalities, as well as by epigenetic alterations [9], [10]. Epigenetic alterations result in abnormal transcription regulation and change in the expression profiles affecting apoptosis, cellular proliferation, survival, and differentiation [11].

The development of potent novel anti-cancer drugs selective for tumor cells is, therefore, still required [12].

Histone deacetylase inhibitors (HDACi) are epigenetically-acting agents that inhibit the HDAC enzymatic activity and cause the acetylation of histones, therefore, they are thought to facilitate gene expression [13], [14]. More than 100 chemotherapeutic drugs are used in many combinations, as single chemotherapeutic drug can be used to treat cancer, but often multiple drugs in a certain order or in certain combinations could be used [15], [16]. One of these chemotherapeutic drugs that has been used as a potential treatment of breast cancer, and other types of cancer, is sodium phenylbutyrate, an aromatic fatty acid, which serves as an HDAC inhibitor, affecting the chromatin structure and reprogramming gene expression [17], and also as a chemical chaperone [18].

The present study aims at evaluating the role of the HDAC inhibitor, sodium phenylbutyrate, in combination with other chemotherapeutic drugs in treating breast cancer.

2. MATERIALS AND METHODS

2.1. Cell lines maintenance

Breast adenocarcinoma cell line (MCF-7) was purchased from the Holding Company for Vaccines and Biological Products (VACSERA), Cairo, Egypt. Cells were cultured in RPMI 1640 media, supplemented with 10% v / v FBS and 1% antibacterial / antimycotic mix (penicillin, streptomycin,

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Amphotericin B) in humidified condition with 5% CO2 at 37°C.

2.2. Chemotherapeutic drugs

Sodium phenylbutyrate, (representative to HDAC Inhibitor) cyclophosphamide, procaine, carboplatin, and temozolomide (representative to DNMT inhibitor chemotherapeutic agents) were all purchased from Santa Cruz Biotechnology (USA).

2.3. Drug preparation and application

Different concentrations of the drugs were prepared, and combinations included sodium phenylbutyrate with other drugs were also prepared. After several trials, the final concentrations, 3µM and 5µM, were chosen to treat the MCF-7 cells. Table (1) represents the 12-well plate layout indicating the drug combination / concentrations used in the present study.

Different drug combinations were applied to the cultured MCF-7 cells. The combinations were: sodium phenylbutyrate, sodium phenylbutyrate combined with procaine, sodium phenylbutyrate combined with carboplatin, sodium phenylbutyrate combined with cyclophosphamide, and sodium phenylbutyrate combined with temozolomide. These combinations were applied in two different concentrations; 3µM and 5µM.

Table 1. The 12-well plate layout with drug combination / concentrations used in the present study.

Control	S (3µM)	S+P (3µM)	S+C (3µM)
S+CY (3µM)	S+T (3µM)	S (5µM)	S+P (5µM)
S+C (5µM)	S+CY (5µM)	S+T (5µM)	Control

S: sodium phenylbutyrate, P: procaine, C: carboplatin, CY: cyclophosphamide, and T: temozolomide.

2.4. Cell counting

Trypan blue test was used to assess cell viability as well as to count the cells before and after treatment. Briefly, cells were diluted 1:1 with trypan blue dye (Sigma Aldrich, Germany), and the mix was kept for 3 min. at room temperature, then loaded to the hemocytometer slides. Four fields were visualized under inverted microscope (10x). Viable and dead cells count was done according to the following equation:

$$\text{Live cell concentration } (\frac{cells}{ml}) = \frac{live\ cell\ count}{\#\ of\ squares} \times 104$$

$$\text{Viability (\%)} = \frac{live\ cell\ count}{live+dead\ cell\ count} \times 100$$

2.5. DNA extraction

Genomic DNA was extracted from untreated (control) and treated cells using DNA extraction kit (Cell Biolabs, USA). The kit's instructions were followed.

2.6. DNA degradation assay

The extracted genomic DNA from untreated and treated cells was subjected to electrophoretic separation on agarose (1.2%) to elucidate the level of DNA fragmentation. An appropriate aliquot of the extracted DNA was loaded on the gel, and 15V was applied for 5 min. followed by 100 V for 30 min. Gel was photographed after being stained with ethidium bromide.

2.7. Quantification of methylation

After being extracted, DNA was subjected to mechanical sheering (by vigorous vortex for up to 10 min.) before quantifying the global methylation status using MethylFlash methylation quantification kit (Cell Biolabs, USA). Briefly, samples were incubated at 95°C for 5 min. and then chilled immediately on ice. The cooled samples were then treated with S1 nuclease followed by alkaline phosphatase for further sheering of the DNA molecules enzymatically. Anti 5-Methylcytidine was added to the samples and incubated for 2 h. at room temperature. Secondary anti-conjugate was added, and the samples were left for 60 min. followed by adding substrate solution and then stop solution. The plates were then read by plate reader at 450 nm.

2.8. Statistical analysis

Statistical analyses were conducted by SAS9 software (SAS institute, Cary, NC). The analysis was performed according to the following model: $y_{ijk} = \mu + a_i + b_{ij} + e_{ijk}$. Where µ is the population mean, a_i is the effect of each of the five different drugs / drug combinations and b_{ij} is the effect of the concentration within each treatment. The criterion for significance was set at $p<0.05$ for all tests.

3. RESULTS AND DISCUSSION

3.1. Cell viability

The viability of MCF-7 breast cancer cells was assessed using trypan blue test. This test measures the integration / disintegration of the cell membrane as indicator on the viability / death of cells. The obtained results showed that the treatment with sodium phenylbutyrate and other drug combinations in general could reduce the number of viable cells, which suggested that these drugs might activate the apoptotic machinery at the epigenetic level [19]. As illustrated in figure (1). Almost all combinations used have reduced the number of viable cells significantly ($p<0.05$). Meanwhile, the most effective treatment was sodium phenylbutyrate when used alone in a low dose (3µM). While

applying the same drug at a higher concentration i.e., 5µM, has resulted in less decrease in the number of viable cells, when compared to 3 µM concentration, compared to the control. This profile might indicate that sodium phenylbutyrate at high concentrations behaved as a cytostatic agent [20]. Meanwhile, when sodium phenylbutyrate was combined with procaine at a lower concentration (3µM), the number of viable cells have decreased significantly ($p<0.05$). Also when applying the same combination at a higher concentration i.e., 5µM, a less decrease in the cell proliferation was noticed, when compared to 3 µM concentration, compared to the control. This pattern was indicated elsewhere [21]. The same profile was also obtained when combining sodium phenylbutyrate with carboplatin at both low and high concentrations (3µM and 5µM) [22]. On one hand, applying the combinations composed of sodium phenylbutyrate and temozolomide at low concentration (3µM) have resulted in decreasing the number of viable cells significantly ($p<0.05$), Meanwhile as the drug concentration has been raised to 5µM, the number of viable cells continued to decrease. The same result was obtained with sodium phenylbutyrate combined with cyclophosphamide at both concentrations; 3µM and 5µM [23], [24].

The statistical analysis of the data indicated that all the differences in the number of viable cells were significant ($p<0.05$), as illustrated in table (2).

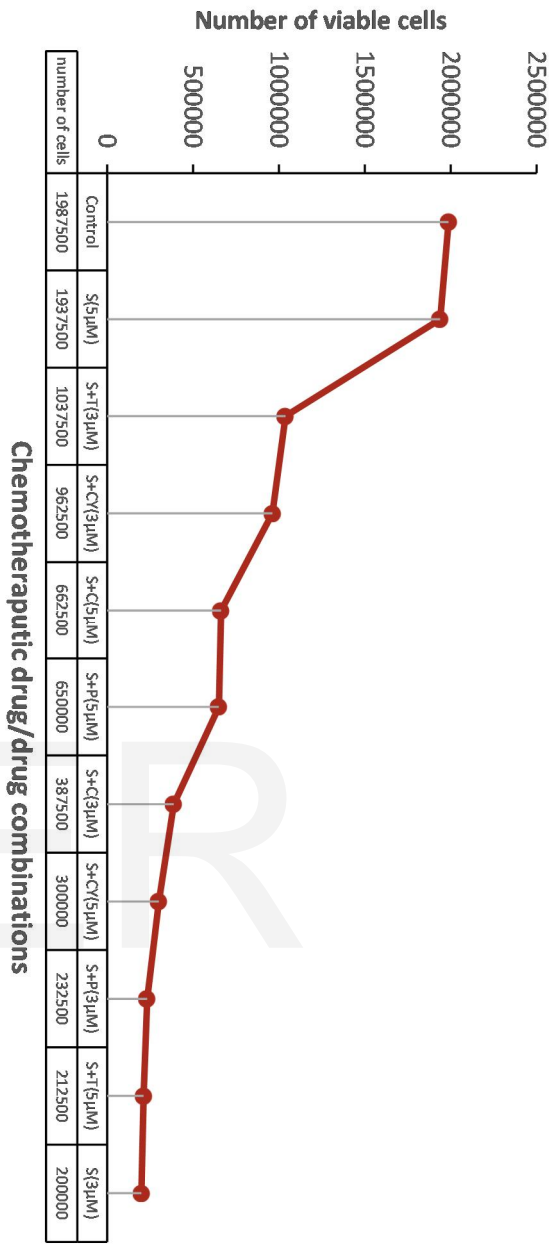

Figure 1. The different chemotherapeutic drug/combinations effect on the viability of breast cancer cells arranged in a descending manner, S: Sodium phenylbutyrate, P: Procaine, C: Carboplatin, CY: Cyclophosphamide, and T: Temozolomide.

Table 2. The mean values of Duncan's multiple range test for cell viability of control and treated cells.

Duncan Grouping	Mean	N	Treatment
A	1987500	4	Control
B	1937500	4	S (2)
C	1037500	4	S+T (1)

Duncan Grouping	Mean	N	Treatment
D	962500	4	S+CY (1)
E	662500	4	S+C (2)
F	650000	4	S+P (2)
G	387500	4	S+C (1)
H	300000	4	S+CY (2)
I	232500	4	S+P (1)
J	212500	4	S+T (2)
K	200000	4	S (1)

Means with the same letter are not significantly different. Where S: Sodium phenylbutyrate, P: Procaine, C: Carboplatin, CY: Cyclophosphamide, and T: Temozolomide, (1): Concentration of 3µM, (2): Concentration of 5µM.

3.2. DNA fragmentation assay

The DNA fragmentation assay was used to identify the effect of external factors on the normal state of DNA in the target cells [25]. DNA extracted from untreated (control) and treated samples were loaded on agarose gel to visualize the degradation of the DNA after the treatment period. Slight smears were observed in the control (first lane-from the right) as compared to the treated samples that revealed a significant smearing due to the apoptosis-related DNA fragmentation [26]. As illustrated in figure (2).

Severe DNA degradation obtained after treating cells with different drug combinations might be occurred due to the activation of CAD (caspase activated DNase) which, in turn, carried out the degradation process of the whole genome [27]. This also suggests that the drugs applied might behaved as inducers of CAD as they could disassociate CAD from its inhibitor [28].

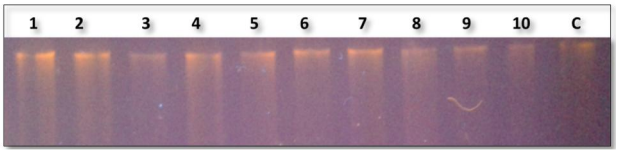

Figure 2. DNA degredation assay. Lanes 1-10 represent different combination and concentration of the drugs. 1: Sodium phenylbutyrate 3µM, 2: Sodium phenylbutyrate combined with Procaine 3µM, 3: Sodium phenylbutyrate combined with Carboplatin 3µM, 4: Sodium phenylbutyrate combined with Cyclophosphamide 3µM, 5: Sodium phenylbutyrate combined with Temozolomide 3µM, 6: Sodium phenylbutyrate 5µM, 7: Sodium phenylbutyrate combined with Procaine 5µM, 8: Sodium phenylbutyrate combined with Carboplatin 5µM, 9: Sodium phenylbutyrate combined with Cyclophosphamide µM, 10: Sodium phenylbutyrate combined with Temozolomide 5µM, and C: control.

3.3. Quantification of DNA methylation

DNA methylation is a crucial epigenetic event that modulates chromatin structure and gene expression. Aberrant DNA methylation patterns are frequently found in human malignancies [29], [30]. DNMTs are the enzyme family that catalyzes the transfer of methyl groups to cytosine in the genomic DNA [31]. In the present study, global methylation quantification was measured (MethylFlash kit, Cell Biolabs, USA). The differences of the methylation patterns between the drugs combinations applied were also assessed and were illustrated in table (3).

Table 3. The mean values of Duncan's multiple range test for quantification of global DNA methylation.

Duncan Grouping			Mean	N	Treatment
	A		19196	4	S (2)
	B		15289	4	S+T (2)
C	B		12980	4	S+P (2)
C	B		12625	4	S (1)
C	D		11204	4	S+T (1)
E	D		9724	4	S+CY (2)
E	F		8185	4	S+C (1)
E	F	G	7061	4	S+P (1)
	F	G	6173	4	S+C(2)
		G	5285	4	Control
	H		-101	4	S+CY (1)

Means with the same letter are not significantly different. Where S: Sodium phenylbutyrate, P: Procaine, C: Carboplatin, CY: Cyclophosphamide, and T: Temozolomide, (1): Concentration of 3µM, (2): Concentration of 5µM.

Methylation quantification revealed that all drug/drug combinations have resulted in increasing the level of 5-MethylCytidine in comparison to control. The only exception of this profile was the drug combination composed of sodium phenylbutyrate and cyclophosphamide at a final concentration of 3µM, where a hypomethylation pattern has been noticed, figure (3 and 4). The data obtained in the present study was in agreement with several studies that showed the same profile either using MCF-7 cell line or the same drug/drug combinations [32]-[34].

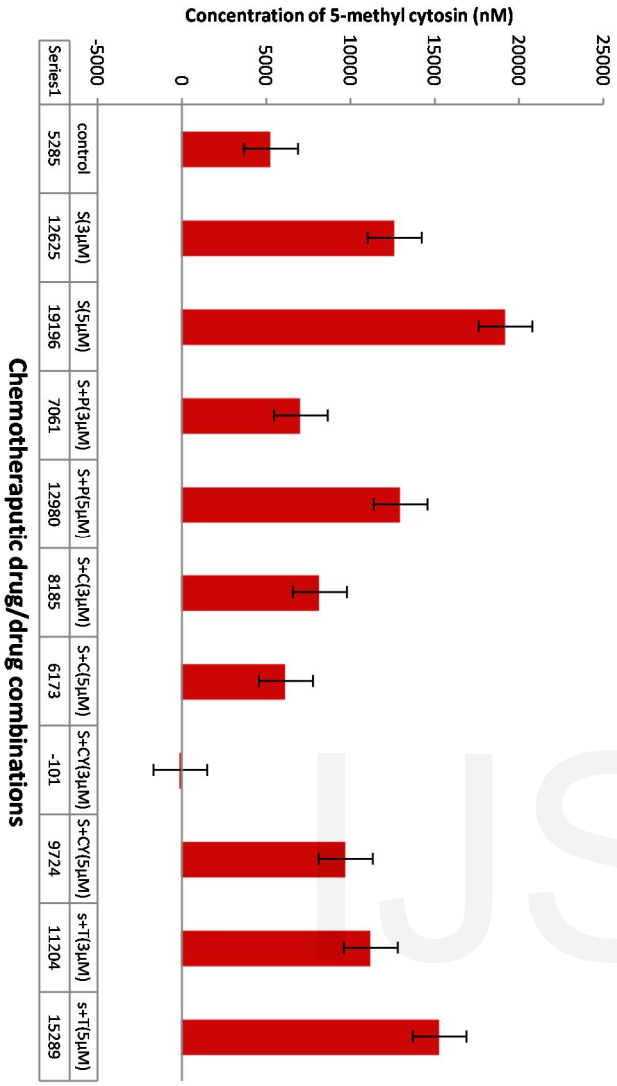

Figure 3. The concentrations of 5-MethylCytidine in the cells after being treated with chemotherapeutic drugs. S: Sodium phenylbutyrate, P: Procaine, C: Carboplatin, CY: Cyclophosphamide, and T: Temozolomide.

Meanwhile, the highest level of 5-MethylCytidine has been obtained when the cells were treated with sodium phenylbutyrate (5µM), and that might suggest that sodium phenylbutyrate in this concentration behaved as a cytostatic agent via arresting the cell cycle in G1/G2 phase, as it maintained the cell viability along with hypermethylation pattern [35], [36].

Interestingly, a decreased level of 5-MethylCytidine has been obtained when the malignant breast cancer cells were exposed to sodium phenylbutyrate combined with cyclophosphamide at a final concentration of 3µM drug combination. That might indicate that this drug combination induced cell apoptosis via hypomethylating several cell death-related genes, as this profile has been noticed in the number of viable/dead cells obtained after this treatment. It has been indicated elsewhere [37], [38] that lower concentrations of cyclophosphamide combined with sodium phenylbutyrate might induce apoptosis in malignant cell lines.

On the other hand, drug concentrations also affected both cell proliferation and/or 5-MethylCytidine methylation level. The resulted profile indicated that the methylation level was increased proportionally with increasing the drug/drug combination concentration in all the tested drugs. Meanwhile, the only drug combination that escaped this profile was sodium phenylbutyrate combined with carboplatin where the level of 5-MethylCytidine was decreased with the increasing of the drug combination concentration up to 5µM. These data were in agreement with other studies that indicated the same profile but using different cell lines [39], [40].

It was suggested that the malignant cells under study lost their viability due to one of two mechanisms; either hypermethylation of IAP (inhibitor of apoptosis proteins genes), mainly with sodium phenylbutyrate at 3 µM concentration, which directs the cells machinery to commit apoptosis, or hypomethylation of TSG (tumor suppressor genes), mainly with sodium phenylbutyrate combined with cyclophosphamide at 3µM concentration; which, in turn, have led to the progression of apoptosis too [41]-[43].

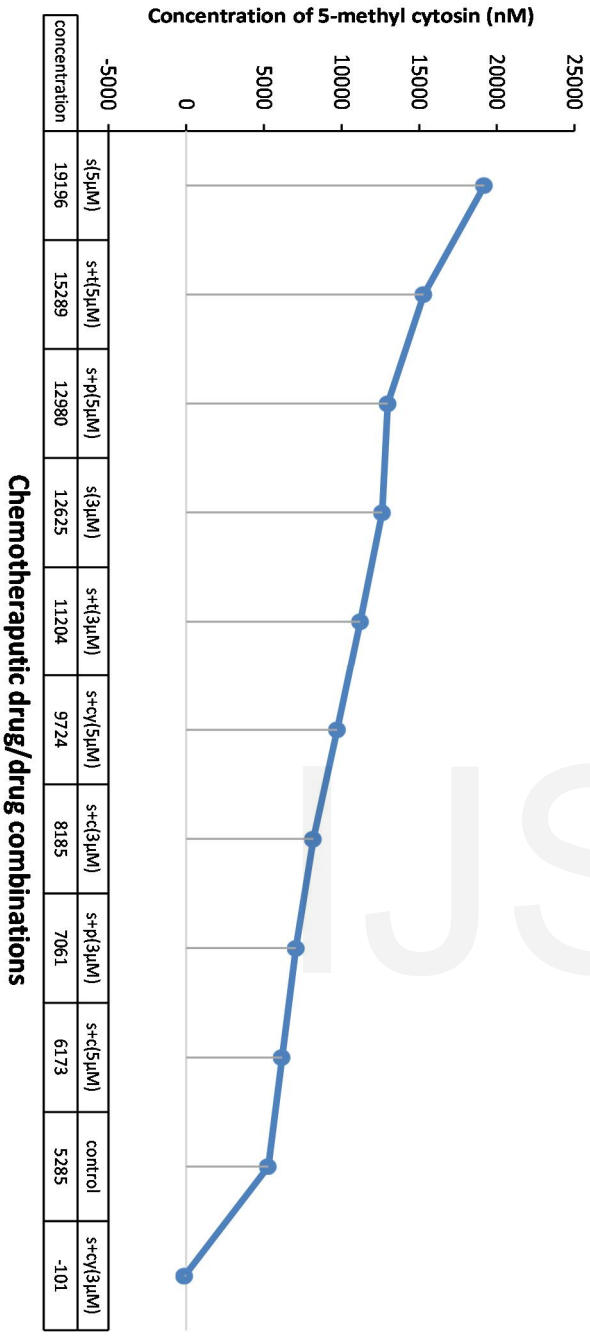

Figure 4. The different chemotherapeutic drug/combinations effect on the concentrations of the global 5-methyl cytidine arranged in a descending manner, S: Sodium phenylbutyrate, P: Procaine, C: Carboplatin, CY: Cyclophosphamide, and T: Temozolomide.

4. CONCLUSION

This study shed some light on the role chemotherapeutic drugs/combinations play in controlling malignancies. Here, in the present study, both hyper- and hypomethylation patterns obtained are in favor of controlling breast cancer cells proliferation, as it resulted in suppressing the overall cell proliferation, although in vitro applications must be followed by clinical investigations to help assign a specific drug/drug combination. As reported in this study we could consider the combination composed of sodium phenylbutyrate and cyclophosphamide at final concentration of 3µM, and sodium phenylbutyrate solely at its low concentrations, i.e., 3µM a potential treatment to withhold breast cancer cells.

5. CONFLICT OF INTEREST

The authors of this work declare no conflict of interest.

6. ACKNOWLEDGMENT

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