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Research Article

**GLOBAL METHYLATION PATTERN CHANGES IN BREAST  
AND COLORECTAL CANCER CELLS TREATED WITH  
DIFFERENT CHEMOTHERAPEUTIC DRUGS****Sara S. Al-Taweel<sup>1</sup>, Hussein Sabit<sup>2\*</sup>, Hala Eissa<sup>1</sup>, Shaimaa E. Abdel-Ghany<sup>1</sup>, Ghada M. Nasr<sup>3</sup>, and Mokhtar M. El-Zawahri<sup>1&4</sup>**

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**Abstract:**

*Cancer is a global threat as it is considered the primary cause of death worldwide. Breast cancer is the most common cancer in female worldwide. In the present study we evaluated the role of temozolomide, carboplatin, sodium phenylbutyrate, and cyclophosphamide in changing the methylation landscape of four tumor cell lines; breast, colorectal, lung, and cervical. Cells were treated with 5  $\mu$ M of each drug and the cells were incubated with the drugs for 48 and 96 h before reading the changes in methylation patterns. Global methylation quantification was measured in cells after being treated with the drugs. Data obtained indicated that sodium phenylbutyrate, followed by temozolomide were the drugs most efficient in hypermethylation of the DNA, while carboplatin followed by cyclophosphamide were able to reduce the concentration of 5-mC in the DNA. It has been concluded that using carboplatin in combination with sodium phenylbutyrate (PBA) might induce cell cycle arrest of malignant cells. Further studies are needed to highlight the mechanism of action of these drugs when combined in treatment of cancer.*

**Keywords:** methylation; breast; colon; lung; cervical; epigenetics.

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## INTRODUCTION:

Cancer is a group of diseases characterized by the uncontrolled growth and spread of abnormal cells [1], although the molecular basis underlying the disease remain unknown for many cancers [2]. There are many known cancer causes, including lifestyle factors, such as tobacco use and excess body weight [3], and non-modifiable factors, such as inherited genetic mutations [4] and epigenetic mutations such as hypermethylation of tumor suppressor genes, histone modifications, and miRNA dysregulation [5].

Breast cancer (BC) is the most common cancer and the leading cause of cancer deaths in females worldwide. Annually, about 1.38 million new cases of BC are recognized, of which about 35% females die [6]. Colorectal cancer (CRC) is a third most common epithelial carcinoma. CRC is known to develop from the early precancerous lesion to full-blown malignancy *via* definite phases due to cumulative mutations and aberrant methylation of number of genes [7]. CRC is a multifactorial disease that arises due to the cumulative accumulation of genetic as well as epigenetic alterations in a number of tumor suppressor [8].

Cervical cancer accounts for almost 12% of all cancers in women, representing the second most frequent gynecological malignancy and the major cause of deaths in women worldwide [9]. It is difficult to foresee a dramatic increase in cure rate even with the most optimal combination of cytotoxic drugs, surgery, and radiation; therefore, testing of molecular targeted therapies against this malignancy is highly desirable [10-12]. Lung cancer is the leading cause of cancer death among men and women [13, 14]. Epigenetic alterations, including DNA methylation, histone modifications, and changes in non-coding RNA expression levels, widely reported in the literature, have been determined to play a major role in the genesis of lung cancer [15, 16].

Epigenetic therapy is a novel tumor therapeutic method and refers to targeting of the aberrant epigenetic modifications presumably at cancer-related genes by chemicals, which are epigenetic targeting drugs [17-19]. As a cytotoxic alkylating agent, Temozolomide is converted at physiologic pH to the short-lived active compound, monomethyl triazeno imidazole carboxamide (MTIC).

The cytotoxicity of MTIC is primarily due to methylation of DNA at the O6 and N7 positions of guanine, resulting in inhibition of DNA replication in ovarian, lung, head and neck, brain cancer, and neuroblastoma. Alkylating agents, like carboplatin, substitute alkyl groups for hydrogen atoms on DNA, resulting in the formation of cross links within the DNA chain and thereby resulting in cytotoxic, mutagenic, and carcinogenic effects [20-23]. Cyclophosphamide is an anti-cancer chemotherapy drug. It belongs to a group of drugs called alkylating agents. It works by sticking to one of the

DNA strands and thus hindering cell replication. Cancer cells are among the most affected because they are among the most rapidly dividing cells, however other rapidly dividing but normal cells like hematopoietic cells, intestinal mucosa, hair follicle, reproductive cells and endothelial cells are also affected by the drug [24-27].

Sodium phenylbutyrate (PBA) is a histone deacetylase inhibitor that possesses a broad spectrum of molecular functions. The compound can modulate the structure of chromatin and contributes to the regulation of cell cycle and apoptosis-related genes [28-32].

The present study aimed at evaluating the role of four chemotherapeutic epigenetic drugs on modulating the methylation of different cancer cell lines.

## MATERIALS AND METHODS:

### Cell line maintenance

Breast adenocarcinoma cell line (MCF-7), colorectal cancer cell line (CaCo-2), cervical cancer cell line (HeLa), and lung cancer cell line (A549) were 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, Amphotericin B) in humidified conditions with 5% CO<sub>2</sub> at 37°C.

### Chemotherapeutic drugs

Four chemotherapeutic/epigenetic drugs: sodium phenylbutyrate, cyclophosphamide, carboplatin, and temozolomide were purchased from Santa Cruz Biotechnology (USA).

### Drug preparation and doses

Different concentrations of the drugs were prepared, and combinations including sodium phenylbutyrate with other drugs were also prepared. After several trials, the final concentration of 5µM was chosen to treat cells for two incubation periods (48 and 96 h).

### DNA extraction

Genomic DNA was extracted from untreated (control) and treated cancer cells using DNA extraction kit (Cell Biolabs, USA) following the kit's instructions.

### Global methylation quantification

After being extracted, DNA was subjected to mechanical shearing (by vigorous vortexing 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 enzymatic shearing of the DNA molecules. Anti 5-

Methylcytidine antibody was added to the samples and incubated for 2 h at room temperature. Secondary anti-conjugate antibody was added, and the samples were left for 60 min. After that time substrate solution was added to the reaction mixture followed by the addition of stop solution. The plates were then read by a plate reader at 450 nm.

#### Cell viability assay

The dye exclusion test is used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue dye whereas dead cells do not. The test was performed before and after treatment [33].

#### MTT assay

The MTT assay is a colorimetric assay used for assessing cell metabolic activity. NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color [34].

#### Statistical analysis

Statistical analyses were performed by the SPSS 25 software package (SPSS, Inc, Chicago, IL). All values were expressed as mean  $\pm$ SD. Analysis of variance with t test and analysis of variance (ANOVA) test were used to determine the significance of the difference in a multiple comparison. If the

ANOVA was significant, the Tukey's procedure was used as a post hoc test. Differences with a P value of less than 0.05 were considered statistically significant.

#### RESULTS:

##### Cell count

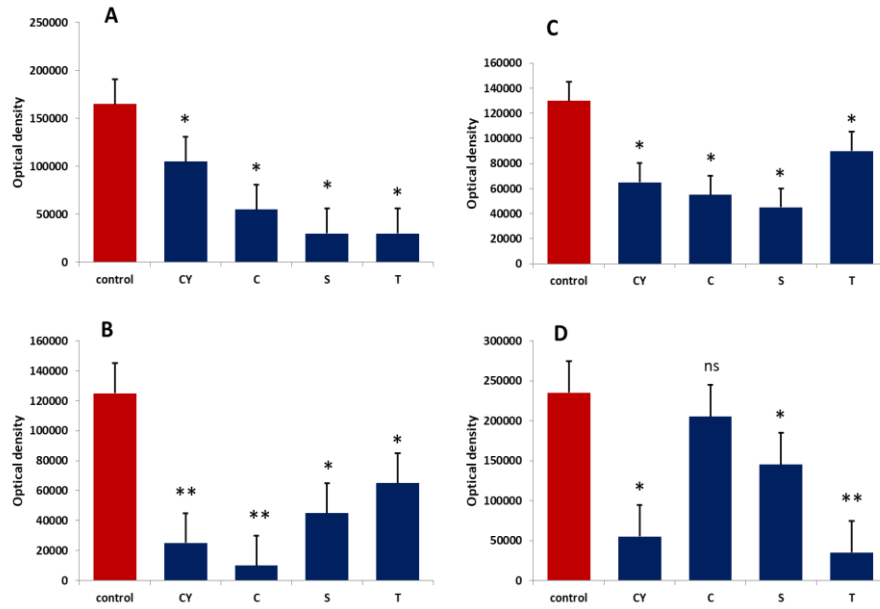
In the present study, cells were treated with different chemotherapeutic drugs for two incubation periods (48 and 96 h.). Cell count was performed before and after treatment as an indicator on the cytotoxic effect of the drugs. Results are shown in Figure 1, Figure 2, and Table 1.

Results indicated that for MCF-7 breast cancer cells, the most efficient treatment was S as it severely affected the overall cell count in the 48-h incubation period. The same was obtained in the 96-h incubation period. For colorectal cancer cells (Caco-2), S also was the drug that negatively affected cell proliferation in the 48-h incubation period, while in the 96-h incubation period, C was the most efficient drug in reducing the cell count compared to control.

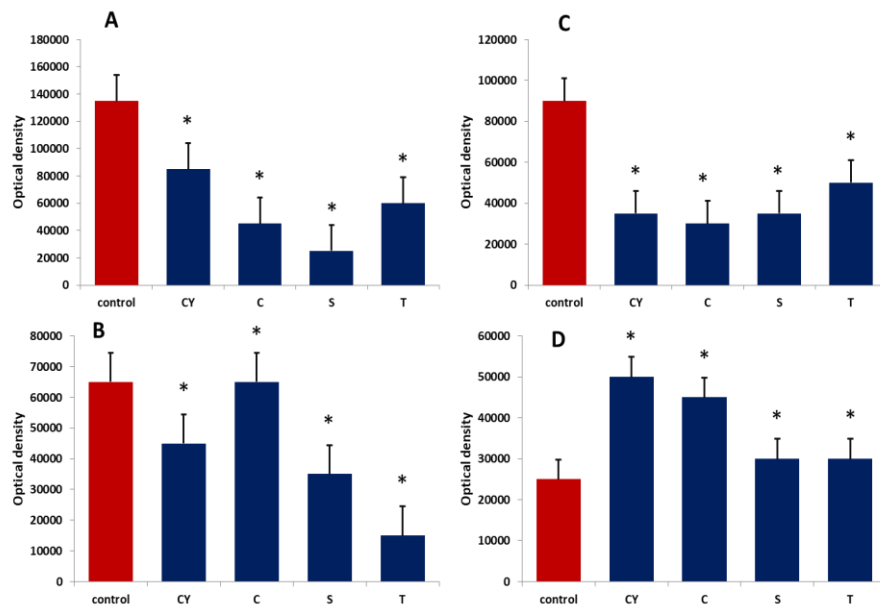
For lung cancer cells (A549), C was the drug that affected cell growth in a severe manner in the short incubation period, while T has affected the cells in the long incubation period. For HeLa cervical cancer cells, T affected the cell proliferation severely in the 48-hours incubation period, while in the 96-hours incubation period, a different profile was obtained. Data obtained showed no significant differences between the two incubation periods of all treatments in all cells.

Table (1): Statistical significance/non-significance between the two

		Group Statistics				
Cell lines/Incubation period		N	Mean	Std. Deviation	t	p
MCF7	T1	15	25667.581	48691.910	<b>0.142</b>	<b>0.888</b>
	T2	15	23334.250	40998.860		
CaCo2	T1	15	25667.579	41741.236	<b>0.754</b>	<b>0.457</b>
	T2	15	16000.910	26872.135		
A549	T1	15	18000.913	35596.455	<b>0.268</b>	<b>0.791</b>
	T2	15	15000.909	24712.043		
HeLa	T1	15	45000.916	81129.862	<b>1.536</b>	<b>0.136</b>
	T2	15	12000.912	18496.470		



**Figure 1:** Viable cells counting by trypan blue test after treating the malignant cells with different types of chemotherapeutic drugs for 48 h. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.



**Figure 2:** Viable cells counting by trypan blue test after treating the malignant cells with different types of chemotherapeutic drugs for 96 h. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.

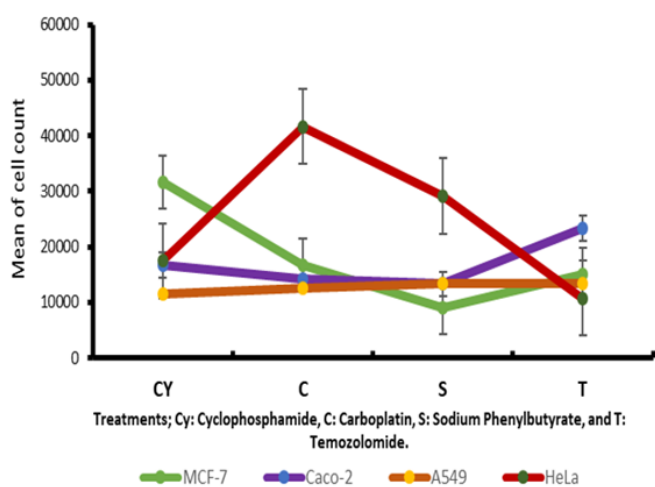
### Cell viability assay

MTT assay was performed to assess the cellular viability after treatment with different types of chemotherapeutic drugs. Results (figure 4 and 5) indicated that S has resulted in lower cell viability in breast cancer cells, colorectal cancer cells, and lung cancer cells. Cervical cancer cells gave a distinctive profile where T has resulted in lowering the cell viability compared to control. These profiles were obtained in the 48-hours incubation period. In the long incubation period, all treatments have caused the cell proliferation to increase compared to control.

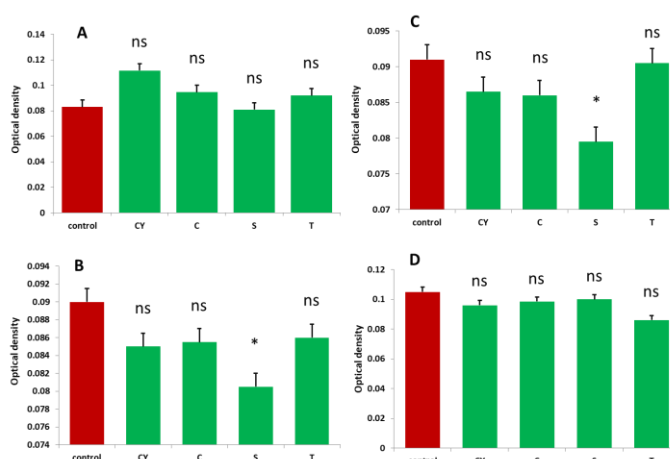
### Global methylation quantification

Global DNA methylation was quantified in cells after being treated with chemotherapeutic/epigenetics drugs. Results (figure 6 and 7) indicated that in the 48-h incubation period, breast cancer cells C has resulted in an increase in global DNA methylation, compared to control. In colorectal cancer cells, T treatment increased the concentration of 5-mC compared to control, while in lung cancer cells, S treatment has increased the global methylation of the cells.

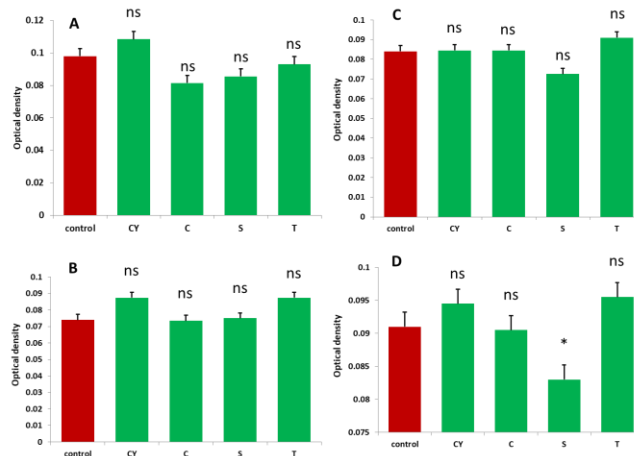
On the other hand, S treatment has caused the DNA global methylation to increase in cervical cancer cells. Furthermore, in the 96-hour incubation period, T treatment had elevated the concentration of 5-mC in breast cancer cells compared to control, while in colorectal cancer cells, lung cancer cells, and cervical cancer cells, S caused the level of global methylation to increase compared to control. Results were shown in Fig. 6 and Fig. 7.



**Figure 3:** The average cell count of all cells treated with different types of chemotherapeutic drugs.



**Figure 4:** Mitochondrial reductase enzyme activity as measured by after treating the malignant cells with different types of chemotherapeutic drugs for 48 h. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.



**Figure 5:** Mitochondrial reductase enzyme activity as measured by after treating the malignant cells with different types of chemotherapeutic drugs for 96 h. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.

## DISCUSSION:

### Cell count after treatment

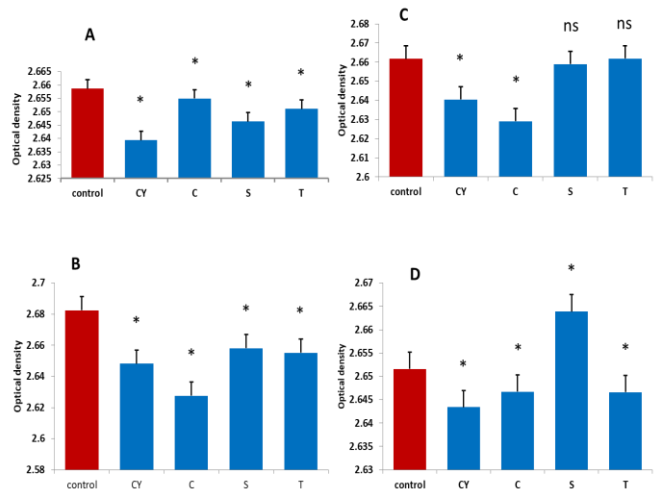
It has been indicated earlier that treating malignant cancer cells with epigenetic drugs causes cell proliferation to decrease [2, 35]. In the present study, breast, colorectal, lung, and cervical cancer cells were treated with different types of chemotherapeutic drugs, and the obtained results indicated that for the 48-h incubation period, PBA was the most effecting drug in reducing the cell count in breast, colorectal, and lung cancer cells. Low concentrations (15 mmol/L) of PBA inhibited cell growth and proliferation mainly by causing prominent changes in cell morphology and promoting S- and G2/M-dependent cell cycle arrest [31, 36]. Temozolomide was the drug the affected the cell growth severely, when compared to control. This profile was obtained in several studies [37-41]. In the second incubation period, PBA was the efficient drug in reducing cell proliferation of breast cancer cells [42], while in colorectal cancer cells carboplatin decreased the cell count compared to control [43]. Temozolomide also has negatively affected the lung cancer cell proliferation [44]. In cervical cancer cells, the overall growth of treated cells was higher than that of control cells, and this might be due to the cytostatic effect of the studied drugs on cervical cancer cells when exposed to long periods (96 h.) [45].

### Cell viability assay

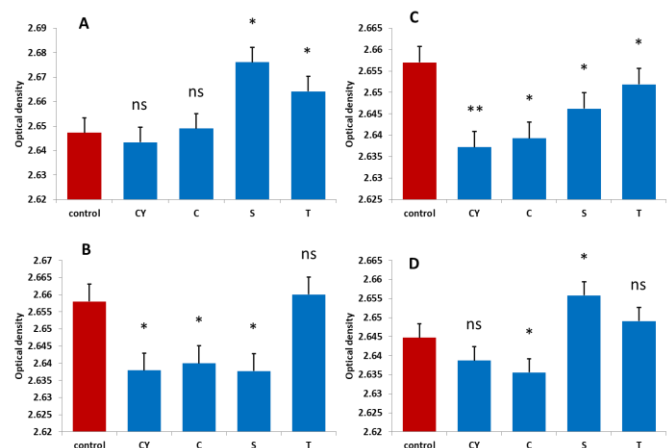
MTT was performed to assess the overall cell viability through measuring the mitochondrial reductase activity in the cells treated with different types of chemotherapeutic/epigenetic drugs [46]. Results obtained indicated almost the same profile of cell proliferation with exceptions, where all the drugs have caused the cell viability to increase in comparison to control cells. This profile was obtained in breast, colorectal, lung, and cervical cancer cells [47, 48].

### Global methylation quantification

The quantification of global DNA methylation was used to underlie the effects of treating malignant cells with different types of epigenetic drugs [49-51]. Here, data obtained showed that in the first incubation period (48 h) cyclophosphamide was found to hypomethylate breast and cervical cancer cells [26, 52], while carboplatin was found to hypomethylate colon and lung cancer cells [43, 53]. PBA was found to increase the methylation levels in lung and cervical cancer cells [42], while breast cancer cells and colon cancer cells were hypermethylated after being treated with carboplatin and temozolomide, respectively [39]. In the second incubation period (96 h.) PBA has caused a significant hypermethylation in colon [28], lung [54], and cervical cancer cells [42], while temozolomide has elevated the global methylation level in breast cancer cells [37, 55, 56].



**Figure 6:** DNA global methylation assay after treatment of malignant cells by the chemotherapeutic drugs for 48 hours. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.



**Figure 7:** DNA global methylation assay after treatment of malignant cells by the chemotherapeutic drugs for 96 hours. A: breast cancer cells, B: colon cancer, C cells lung cancer cells, and D: cervical cancer cells. CY: Cyclophosphamide, C: carboplatin, S: Sodium phenylbutyrate, and T: Temozolomide. ns: Non-significant differences, \*: significant, and \*\*: very significant.

**CONCLUSIONS:**

In the present study, the role of different types of chemotherapeutic/epigenetic drugs in modulating the methylation of breast, colorectal, lung, and cervical cancer cells was assessed. Cells were treated with a final concentration of 5  $\mu\text{m}$  of each drug for 48 and 96 h before assigning the changes. Data obtained indicated that temozolomide, carboplatin, PBA, and cyclophosphamide were able to change the methylation patterns of the studied cells. We can conclude that using a combination of PBA and carboplatin could be useful in controlling malignant cell proliferation *in vitro*.

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**Conflict of interests**

The authors declare no conflict of interests.

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