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Vorinostat Combined with DNMTi Epigenetically Controls the Proliferation of Lung Cancer Cells A549

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Abstract: ***Cancer is being considered one of the fatal diseasesin the global population. Lung cancer is one of the most common malignancies in males and inseveral countries also in females.In the presentstudy, we aimed at evaluating the role of different chemotherapeutic drugs belonging to three groups [Histone DeacetylaseInhibitors (HDACi), DNA Methyltransferase Inhibitors (DNMTi), and Alkylating Agents)]. Vorinostat, Carboplatin, Cyclophosphamide, Temozolomide, and Procaine were applied to A549 lung cancer cells in final concentrations of 3µM and 5µM. Drugs were incubated with the cells for 96 h. Cell viability was measured using Trypan blue exclusion test, and the obtained results showed a significant decrease in the cells' viability after being treated. Global methylation, as an essential epigenetic mark, was quantified in the control and treated cells. The results showed a variation in the methylationpatterns, since different combination yielded different methylation profiles. Vorinostat combined with Carboplatinhypomethylated the cells under study, while Vorinostat combined with Cyclophosphamide resulted in hypermethylation of the cells. We concluded that reactivating thehypermethylated genes in the A549 lung cancer cells by combining more than one drug was significantly better than that induced by only one chemotherapeutic agent. However, these results need more confirmatory experiments.***

Keywords: Lung. Cancer Epigenetics, Methylation. Vorinostat

1. Introduction

Cancer is a term used to describea disease in which abnormal cells divide in an uncontrolled fashion, and can invade other tissues **[1,2]**. Over the last century, lung cancer has become one of the biggest death-causing cancer of men worldwide and in some parts of the world also of women**[3]**. Twomajor types of lung cancer are known;small cell lung cancer (SCLC), which representsabout 10-15% of all lung cancers **[4]**,and non-small cell lung cancer (NSCLC) which representsabout 85-90% of all lung cancers **[5,6]**.

Although chemotherapy is considered one of the most effective approaches to treat cancer,it might kill,alongside the malignant cell, any normal cell thatgrows fast, which, in turn, may result in serious damage to patients**[7-9]**. Mostof the chemotherapeutic drugs work by impairing cell division by various mechanisms including damaging DNA and inhibition of the cellular machinery involved in cell division **[10]**.

HDACi's have demonstrated anticancer effects by selectively inducing apoptosis through modulating the expression of pro-apoptotic and anti-apoptotic genes **[11]**. Vorinostat is one of the inhibitors of HDAC family that shows to be efficacious and well-tolerated drug, thus,it has been considered a novel drug in the treatment of various cancers**[12]**.

Epigenetics as a term was first coined by Conrad Waddington in the early 1940s**[13]**. He defined epigenetics as "the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being."**[14]**. Collectively, epigenetics could be viewed as a major molecular mechanism that control gene expression without changing the underlying DNA sequence. Two major epigenetic mechanisms are known; DNA methylation and histone modification **[15,16]**. It represents the programming of the genome to express an appropriate set of genes in certain cells at specific time points in life. It has atrans generational effect as it could affect the recurrence of such disease in the descendants of cells in the individual **[16,17]**.

Methylation of DNA is considered one of the most important epigenetic mechanism in mammals **[18]**. It is a heritable epigenetic mark involving the covalent transfer of a methyl group to the C-5 position of the cytosine ring of DNA by DNA methyltransferases (DNMTs) **[19]**. This can coordinately regulate the chromatin status *via* the interaction of DNMTs with other modifications and with components of the machinery mediating those marks **[20]**.

Histone acetylation is generally associated with elevated transcription, while deacetylated histones are often associated with gene repression **[21,22]**. The balance of histone acetylation and deacetylation is critical in the regulation of gene expression. Aberrant expression of genes that encode HDACs have been linked to tumor development as they dysregulate important cellular functions such as cell proliferation and apoptosis. Thus, HDACs are among the most promising therapeutic targets for cancer treatment **[23,24]**.

The aim of the present investigation is to identify the role of Vorinostatcombined with other chemotherapeutic drugs (DNMTi) in controllingthe proliferation of lung cancer cells A549.

2. Materials and Methods

Cell line maintenance
Lung cancer cells A549 was purchased from the Holding Company for Biological Products and Vaccines (VACSERA, Cairo, Egypt). Cells were maintained in DMEM (Dulbecco's Modified Eagle's Medium, Lonza, USA) supplemented with 10% Fetal Bovine Serum (Sigma, Germany) and 1% antibiotic mix (Sigma, Germany). Cells were grown under the normal laboratory conditions; 5% CO_2 and 37°C.

Application of chemotherapeutic drugs
Chemotherapeutic drugs (Vorinostat, Temozolomide, Carboplatin,Procaine, and Cyclophosphamide) were purchased from Santa Cruz Biotechnology (USA). Each drug was dissolved in the appropriate solvent, and a working stock of 5mg/mL was prepared. Two final concentrations of 3µM and 5µM were used in the present study to treat lung cancer cells for 96 h. Vorinostat was applied solely or in combination with other drugs as follows: Vorinostat only, Vorinostat combined with Carboplatin, Vorinostat combined with Cyclophosphamide, Vorinostat combined with Procaine, and Vorinostat combined with Temozolomide.

Harvesting the cells
After 96 h. of incubation, cells were removed from the incubator and the old medium was decanted. Cells were washed twice with warm PBS and trypsinized with 3 mL of 1X Trypsin EDTA (0.025%). Cells were then collected by centrifugation for 10 min. at 10,000 rpm.

Cell viability assay
According to Strober (2001)**[25]**,the number of viable and non-viable cells were counted using Trypan blue exclusion test. Briefly, 50 µL of cell suspension was mixed with an equal volume of Trypan blue dye and left for 3 min. at room temperature.The mix was then loaded on a hemocytometer slide to be read usingan inverted microscope. Clear cells were considered viable, while blue ones were considered dead.

DNA extraction and DNA fragmentation assay
Genomic DNA was extracted from treated and untreated cells using Quick-DNA™ Miniprep Kit (Zymo Research, USA) following the kit's instructions. After extracting DNA, fragmentation assay was performed to assess the degradation pattern of DNA isolated from control and treated lung cancer cells. Briefly, 5µL of the eluted DNA was loaded on agarose gel (0.8%) and subjected to low voltage (15V) for 10 min. and then to 120V for 30 min.

Methylation quantification
Global methylation pattern was identified using MethylFlash DNA methylation kit (Epigentek, USA) following the kit's protocol.

Statistical analysis
Statisticalanalysis (ANOVA) for the obtained data was performed using SPSS 22.0.0.0 program that run under Windows **[26]**.In addition, the differences between means was calculated by using Duncan's MultipleRange test in the same program. Least Significant Difference (LSD) was also used to test the significance between all the treatments and control.

3. Results and Discussion

Cell viability
Trypan blue test was performed to ensure the viability of the cultured cells. This test depends on dye exclusion as the live cells will not allow the dye to enter and, subsequently the live cells will appear clear when being examined under inverted microscope **[27]**. In this study, the initial cell count was $6x10^5$. The results obtained showed a significant decrease in the cell viability after being treated with different concentrations/combinations of the chemotherapeutics compared to control (Figure 1).

Mortality rate ranged from 75% to 89.6% with the most effective drug/combination was Vorinostat at the concentration of 3µM (62,500 viable cells out of $6x10^5$; 89.6% mortality rate) and Vorinostat combined with Carboplatin at the concentration of 3µM (62,500 viable cells, 89.6% mortality rate).**Ramalingam*et al.*(2010)[28]** demonstrated that Vorinostat, as a HDAC inhibitor, enhances the efficacy of Carboplatin in patients with advanced NSCLC. This was observed also by**Owonikoko*et al.*(2010)[29]**who found that Vorinostatat lower concentrations (1µM) inhibited the growth of A549 lung cancer cells by 67%. Vorinostatin addition to Carboplatin led to a significantly greater growth inhibition in A549 lung cancer cells than chemotherapy alone. Ovarian cancer also has been rendered sensitive when treated with Vorinostat combined with Carboplatin **[30]**.

While applying Vorinostat combined with Cyclophosphamideat a concentration of 3µM was the less effective combination, as it yielded the highest number of viable cells (150,000 viable cells out of $6x10^5$; 75% mortality rate).Combination of novel agents with conventional induction and maintenance regimens is a promising strategy for the treatment of several malignancies like lung cancer **[31]**. It was reported earlier that Cyclophosphamide combined with Vorinostatwas feasible and tolerable **[32]**. This wasin accordance with our data,although this conclusion may warrant further study.

In addition, ongoing studies are investigating the combination of Vorinostat with Temozolomide**[33]**. On the other hand, different drug combinations have resulted in different cell viability profiles indicating that even the lower concentrations were capable to induce cell death[29]. This reduction in the cell number might be attributed to the activation of the internal apoptotic machinery as indicated elsewhere **[34, 35].**

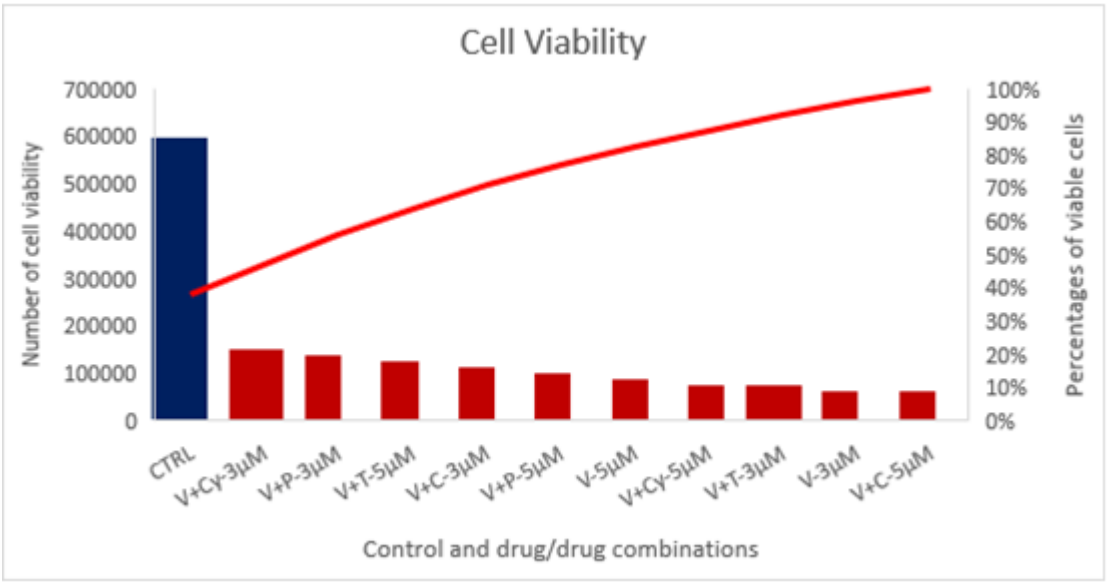

Figure 1: Cell viability test results. CTRL: Control, V: Vorinostat, C: Carboplatin Cy: Cyclophosphamide, P: Procaine, and T: Temozolomide

DNA fragmentation assay

DNA fragmentation is a method used to identify the effect of treating cancer cell lines with chemotherapeutic drugs **[36]**. Here in the present study we assessed DNA fragmentation occurred in lung cancer cells A549 after being treated with different combinations/concentrations of chemotherapeutics. The data obtained showed a severe damage in cellular DNA in all treated cells regardless the drug/drug combination compared to control. The most effective drug combination in inducing DNA fragmentation was Vorinostat combined with Carboplatin at the concentration of 5µM and Vorinostat at concentration 5µM. The rest of the combinations could degrade DNA with less severity as shown in figure (2). Several researches indicated that chemotherapeutic treatment of cancer cell might induce DNA fragmentation, which will lead to apoptosis**[37-40]**.

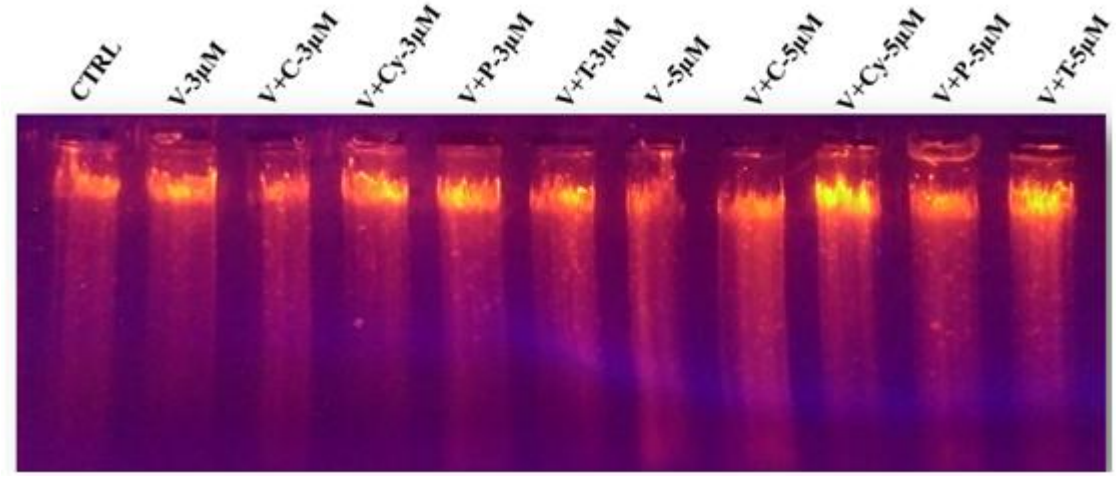

Figure 2: The results of DNA fragmentation assay after treating the lung cancer cells with different chemotherapeutic drugs.CTRL: Control, V: Vorinostat, C: Carboplatin Cy: Cyclophosphamide, P: Procaine, and T: Temozolomide

Quantification of DNA methylation

Global methylation pattern was identified using the EZ DNA Methylation™ Kit (Zymo Research, USA) in DNA extracted from both treated and untreated lung cancer cells A549. A standard curve was generated to calculate the corrected 5-MethylCytosine concentrations.

DNAmethylation patterns are largely modified in cancer cells compared to control, and can therefore be used to distinguish cancer cells from normal tissues **[41]**. Results obtained (Figure 3&4) showed a significant variation in the patterns of global methylation between different treatments. Some drugs/combinations (Vorinostat combined with Procaine at concentration 3µM, Vorinostat combined with Temozolomide at the concentration of 5µM, Vorinostat at the concentration of 3µM, Vorinostat combined with Carboplatin at the concentration of 5µM, and Vorinostat combined with Cyclophosphamide at the concentration of 3µM) were able to globally hypomethylate the lung cancer cells A549. Genome-wide studies showed that reactivation of hypermethylated genes by combining more than one drug was significantly better than that induced by only one chemotherapeutic agent**[42]**.

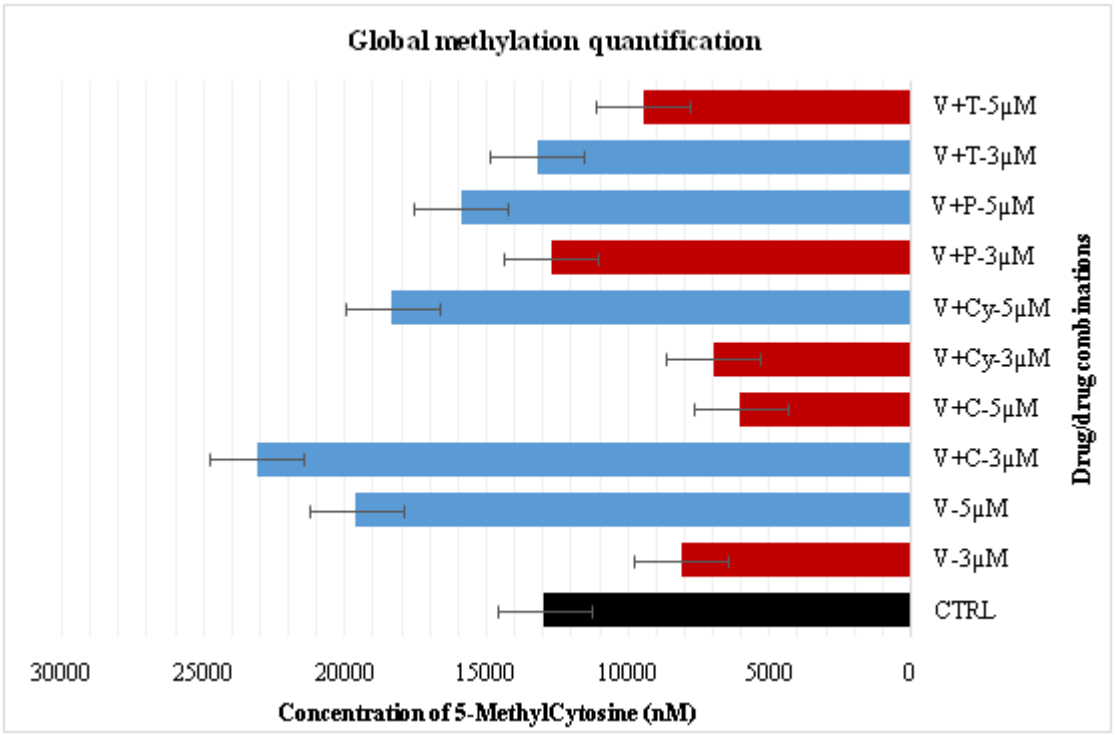

Figure 3: Global quantification of DNA methylation. CTRL: Control, V: Vorinostat, C: Carboplatin Cy: Cyclophosphamide, P: Procaine, and T: Temozolomide.

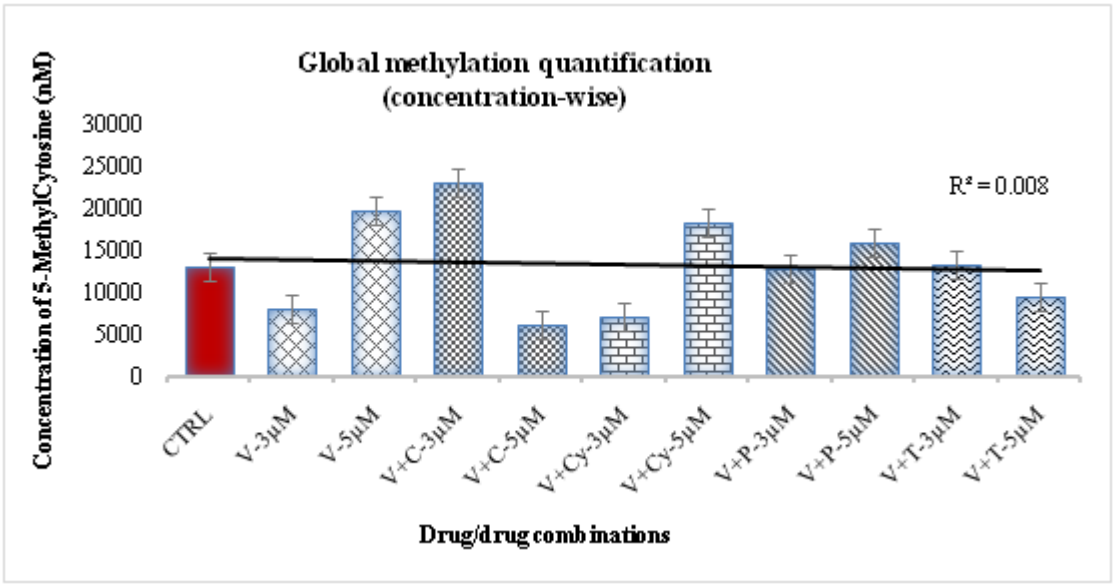

Figure 4: Global quantification of DNA methylation. Data were arranged in concentration-based manner. CTRL: Control, V: Vorinostat, C: Carboplatin Cy: Cyclophosphamide, P: Procaine, and T: Temozolomide

It was indicated earlier that histone and DNA methylation patterns in several malignant cells could be partially modified by the clinically promising HDAC inhibitor, Vorinostat**[43]**.Temozolomide shows its anti-cancer activity by methylating the DNA, as the interaction between DNA and Temozolomide causes localized distortion of DNA away from the normal B-form, resulting in a wider major groove and greater steric accessibility of functional groups in the base of the groove**[44]**. The ability of Temozolomide to methylate histones confers to its potentially unique mechanism of action **[45]**.

Meanwhile, other drugs/combinations (Vorinostat combined with Procaine at the concentration of 5µM, Vorinostat combined with Cyclophosphamide at the concentration of 5µM, Vorinostat combined with Carboplatin at the concentration of 3µM, Vorinostat at the concentration of 5µM and Vorinostat combined with Temozolomide at the concentration of 3µM) were able to globally hypermethylate the cells under study.Procaine also has growth-inhibitory effects, as it causes mitotic arrest. Thus, Procaine could be considered as a promising candidate for future epigenetics-based cancer therapies **[46]**.

Chemotherapeutic drugs have different mechanisms in inducing cell death **[47]**. HDAC inhibitor, like Vorinostat tends to hypomethylate lung cancer cells when applied solely in low concentration **[48,49]**, while applying a combination contained DNMT inhibitor and HDAC

inhibitor might hypermethylate the cells, and hence down regulates several tumor suppression-related genes **[11,50]**.

4. Conclusion

In the present study, the role of Vorinostat and other chemotherapeutic drugs (Carboplatin, Cyclophosphamide, Procaine, and Temozolomide) were evaluated as controller of the proliferation of lung cancer cells A549. Two final concentration of the drugs were applied (3µM and 5µM) to the cells, incubated for 96 hand cells viability were measured using the Trypan blue test. The global methylation pattern was assessed, and the results obtained indicated that different drug combinations were capable to hypo/hypermethylated the lung cancer cells A549. The present investigation needs more confirmatory studies to identify the actual mechanism(s) by which these drugs could control the malignant cells proliferation.

5. Conflict of Interests

The authors declare no conflict of interests.

6. Author Contribution

HS: put the idea and wrote the manuscript, MF: conducted the experiments, SEA: performed the statistical analysis and formattedthe references, OAMS: participated in writing the manuscript and editing the references, and MME: critically revised the final manuscript.

7. Acknowledgement

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