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SYNERGISTIC EFFECTS OF COMBINATORIAL THERAPY WITH ERL AND SAHA INDUCED UPREGULATION OF P21 & PARP GENES EXPRESSION IN MDA-MB-231 BREAST CANCER CELL LINE

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ABSTRACT

Cancer, which is a global threat, is one of the main causes of death worldwide. It is also considered a global public health problem. Breast cancer is a complicated disease and therefore the second most popular type. Currently, more efforts are being made to research new drugs and combination therapies to achieve a synergistic effect in cancer therapy. Herein, MDA-MB-231 breast cancer cells were treated with SAHA, ERL, and their combination for 24 h, and the cell cytotoxicity was evaluated using MTT-assay. Cell cycle distribution and apoptosis level were also investigated. It was shown that the combination of ERL and SAHA synergistically induce cell cytotoxicity and strongly induce apoptosis, cell cycle arrest, and cause upregulation in the level of P21 and PARP at the selected concentration compared with the drugs alone after 24 h. In conclusion, there is a synergistic effect between ERL and SAHA with increasing PARP and P21 gene-level, thereby playing a therapeutic role in killing breast cancer cells.

Keywords: MDA-MB-231; P21; PARP; SAHA; ERL

INTRODUCTION

Cancer is one of the most important motives for mortality worldwide. However, the burden of this lethal disorder is more well-known in some countries, where >80 % of the world population lives, and the growing trend is striking. Meanwhile, breast cancer (BC) considers the most common malignancy among females worldwide, being the second most frequent cause of death in women after lung cancer **[1]**, which is predicted to increase by 78% in the 2030 **[2]**. Considering the high social significance of BC in most scientific centers, development is being made to improve the early diagnostics and search for new methods of BC treatment **[3]**.

Therefore, effective therapy of BC requires maximum therapeutic efficacy, with minimal undesirable effects to ensure a good quality of life for the patients **[4]**. Strategies used against BC include surgical removal of the tumor, chemotherapy, radiotherapy, and hormone-based therapies. Despite the availability of many chemotherapeutic medication today, the power of cancer cells to develop resistance to those drugs poses a serious challenge for the treatment of cancer **[5]**. Chemotherapy is a vital option for cancer treatment; however, its therapeutic outcomes are limited by dose-dependent toxicity and chemoresistance occurrence **[6]**. Meanwhile, none of the available treatment options are curative in patients with advanced cancer **[7]**, which should be spotlighted by the scientific community in the upcoming studies.

One of the latest and promising strategies for the improvement of cancer treatment is the application of combination therapy, which allowed the synergistic effect in the treatment **[8]**. Currently, monotherapy has shown to be inadequate since it may present chemoresistance and tumor recurrence. The application of combination medical therapy would possibly exert a synergistic or additive result and improve antitumor efficaciousness compared with one chemical drug-based therapy **[9]**. Due to these advantages, combination chemotherapy has now become the conventionally applied strategy in clinical practice **[10]**.

It has been reported that histone deacetylases (HDACs) play a key role in the development and progression of various cancers **[11]**. Several HDAC inhibitors (HDACi) are currently in clinical development as anticancer agents; these compounds may be active when used alone, particularly in hematologic malignancies, and are perhaps even more active in combination with other chemotherapy agents **[12]**. HDAC inhibitors

can modulate a variety of genes and pathways in tumor cells, and thus, exhibit strong anti-tumor effects in-vitro and in-vivo by regulating epigenetic enzymes. On the other hand, studies have shown that suberoylanilide hydroxamic (SAHA) exerts a synergistic effect when used in combination with other drugs or radiotherapy for treating cutaneous T-cell lymphoma **[13]**. Erlotinib (ERL), which is a kind of tyrosine kinase domain, could also target cancer cells with some specificity, whereas doing less harm to normal cells, however, resistance to erlotinib can occur which could reduce its efficacy **[14]**.

The use of combination chemotherapy potentially provides advantages such as chances for better efficacy and dose reduction while increasing and/or maintaining efficacy, decreased toxicity, and reduced or delayed development of drug resistance **[4]**. Thus, we aimed at studying the synergistic effect of combinatorial chemotherapeutic drugs on the MDA-MB-231 breast cancer cell line. Herein, MDA-MB-231 breast cancer cells were, therefore, treated with SAHA, ERL, and their combination for 24 h, and the cell cytotoxicity, cell cycle distribution, and apoptosis levels were also evaluated. We do believe that our findings could aid the scientific community to find a new strategy to treat such kinds of cancers.

MATERIALS AND METHODS

Chemotherapeutic Drugs

Chemotherapeutic drugs, Erlotinib (TKI), and Vorinostat (SAHA) were supplied from Santa Cruz Biotechnology (USA). A stock solution of 16 µM of Erlotinib and 10.5 µM of Vorinostat drugs and a drug combination of both under a concentration (8 µM + 5.25 µM, respectively) was prepared and administered to MDA-MB-231. In a (12-well) tissue culture plate, 1 x 106 cells/well were inoculated and left for 24 h before applying the drug/drug combinations. Combinations of drugs will be mixed separately and added to the wells containing the MDA-MB-231.

Cell Line Culture and Maintenance

The human breast cancer cell line, (MDA-MB-231), was purchased from VACSERA, the holding company for vaccines and biological products, Cairo, Egypt. The cells were preserved at the Roswell Park Memorial Institute (RPMI) 1640 media (Gibco, USA) utilizing a bicarbonate buffering system with variable amounts of vitamins and amino acids supplemented with 10% of fetal bovine serum (HyClone, Logan, UT, USA) and 1% of penicillin\streptomycin mixture (Invitrogen Life Technologies). Cells

were seeded in 12-well U-bottom microplates (Nunc, Denmark) and cultured at 37°C in a 5% CO2 incubator for 24 h.

Cytotoxicity Assay

The cytotoxic/cytostatic effects of both drugs (Erlotinib and Vorinostat) on the MDA-MB-231 breast cancer cell line were tested with a rapid colorimetric assay using MTT-assay and compared with the untreated controls **[15]**. Viable cells with active metabolism convert MTT into formazan while, Dead cells lose this ability and therefore show no signal. The cytotoxicity was calculated as follow:

Cytotoxicity (%) = 1-(mean absorbance of toxicant) / (mean absorbance of negative control) × 100

Apoptosis and Cell Cycle Assay

Apoptosis analysis was performed after fluorescence labeling of the cellular membrane with FITC-Annexin V stain (indicating early apoptosis) cells (2.5 x 105 cells/mL) seeded in a culture dish. It was then treated with Vorinostat (SAHA) and Erlotinib alone and in combination for 24 h, and the cellular DNA with propidium iodide (PI) stain (indicating late apoptosis or necrosis) was done at room temperature for 15 min. The apoptotic analysis was dedicated to differentiating between early and late apoptotic cells. Finally, the apoptosis of the MDA-MB-231 cells was analyzed with a BD FACSanto II Calibur Flow Cytometer (Becton-Dickinson) using a flow cytometer instrument (BD Biosciences, San Jose, CA, USA). The results of flow cytometry were compared with standard cell count and morphology below a fluorescence microscope.

The effect of Erlotinib and Vorinostat (SAHA) on cell proliferation was also evaluated by measuring the distribution of the cells in the three phases of the MDA-MB-231 cell cycle (G0-G1 phase, S phase, G2/M phase) by flow cytometry, In a 6-well plate, (2.5x105 cells/mL) were plated per well for the cell cycle assay. Following treatment with 5 μM Vorinostat (SAHA) and Erlotinib alone and in combination for 24 h. Cells were harvested and fixed in a 70% cold-EtOH at 4˚C for 1 h. After fixation, cells were washed with PBS and incubated with 0.5 mg mL-1 RNase A (Sigma) at 37 °C for 1 h. Nuclear DNA was stained using PI (50 μg mL-1) under subdued light for 30 min at RT. The samples were passed through 200-mesh filters before being analyzed on a BD FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA, USA).

DNA Extraction

Genomic DNA was extracted from treated and non-treated cells for the downstream

analysis and stored at -20 °C until being used. Extraction was performed using the Cell Biolab DNA extraction kit (USA) according to the kit's instructions.

Quantitative Real-Time PCR

The qPCR was performed to measures the expression profile of certain genes from a sample under specific biological conditions. This measurement is expressed in the Cycles Threshold (CT) of PCR. The target genes used in this study were p21 and PARP, where β-actin were used as a housekeeping gene.

Statistical Analysis

The results were analyzed using the student's t-test. The data is presented as a mean with standard deviation (SEM). Statistical significance was determined by $p< 0.05$ values. The main impacts of treatments and replicates were incorporated in the model.

RESULTS

MDA-MB-231 Breast Cancer and WISH Cells Cytotoxicity

Our result revealed that the cytotoxicity of the MDA-MB-231 cell line was increased after using a concentration of 10.5 µL of vorinostat (SAHA) (D1) compared to the control cells. While treated cells with 16 µL of Erlotinib showed a significant increase in cell cytotoxicity that raised from 0.711 to 0.7655 after treating. Furthermore, the combined treatments drastically increased the cell cytotoxicity compared with the control cells from 0.711 to 0.8715, meaning that this concentration of both drugs enhances the cell cytotoxicity of the MDA-MB-231 cell line (**Figure 1**).

P21 and PARP Gene Expression in MDA-MB 231 Breast Cancer Cell Line

As portrayed in (**Figure 2**), P21 gene expression was upregulated in MDA-MB 231 cells treated with SAHA, Erlotinib, and dual drugs compared to control cells while it is downregulated when compared to wish cells. On the other side, we noticed that PARP gene expression was significantly up-regulated in MDA-MB 231 cells treated with both drugs compared to control more than cells treated with a single drug alone (SAHA) or (ERL). Accumulating evidence has shown that p21 is a modulator of apoptotic pathways that can induce either pro-apoptotic or anti-apoptotic responses **[16]**. These results indicated that in AML cells p21 was an important factor that blocked the apoptotic mechanism induced by SAHA. Several mechanisms may contribute to the anti-apoptotic effect of p21, including inhibition of caspase-3, caspase-9, and cyclin A/Cdk2 complex, a requisite effector of apoptotic death **[16]**. P21 directly bound and inactivated procaspase-3, resulting in resistance to Fas-mediated apoptosis **[17]**. IR

(ionizing radiation) induced caspase-9 activation was abrogated by p21 in colon cancer cells **[18]**.

These results suggested that reduced ability to rapidly cleaved caspase-8 and PARP was closely correlated with up-regulated p21 expression in those cells, indicating that p21 exerted an anti-apoptotic effect in AML cell lines via blockage of caspase-8 dependent extrinsic pathway. Caspase-8 is an important downstream effector of the TRAIL apoptotic pathway **[16]**.

Effect of SAHA (Vorinostat), Erlotinib, and Combination of Both Chemotherapeutic Drugs on the Cell Cycle

We evaluated the effects of SAHA or erlotinib alone and in combination on the cell cycle dispersion of MDA-MB-231 breast cancer cells. Our result revealed that The MDA-MB-231 cell line underwent major changes in cell viability when treated with SAHA and showed an important change in the percentages of cells in different cell cycle phases. After 24h SAHA caused cell cycle arrest at G2/M in the MDA-MB-231 breast cancer cell line (**Figure 3 A**). The percentage of cells arrested in G2/M raised from 22.54 % in the absence of SAHA to 39.98 % in SAHA presence with a decrease in the percentage of cells on S phase and G0/G1 phase in treated cells (**Figure 3 D**). While treatment with 16µl of erlotinib demonstrated a cell cycle arrest of MDA MB-231 at G0/G1 phase with an increased number in S phase and decreased number in G2/M phase compared with MDA MB-231 cells alone that showed a significant increase in G0/G1 phase and decreased in s G2/M phase respectively (**Figure 3 B**). When MDA MB-231 cells were treated with the combination of SAHA and Erlotinib, the cells showed significantly increased cell cycle arrest at the G2/M phase than Erlotinib or SAHA alone. As shown in (**Figure 3 C**) consecutively, decrease in S and G0/G1 phase.

Induction of Apoptosis in MDA-MB-231 Cells

MDA-MB-231 breast cancer cells which subjected to SAHA monotherapy showed increasing in early apoptosis with 3.99% and 8.83% late apoptosis (**Figure 4 A**), while Erlotinib promote apoptosis in the MDA-MB-231 breast cancer cell line increasing from 0.29% in untreated cells to 5.8 % in ERL treated cells as shown in (**Figure 4 B**). On the other hand, the combination of both chemotherapeutic drugs Vorinostat (SAHA) and Erlotinib induced synergistic cell death (**Figure 4 C**), as apoptotic cells were markedly increased in MDA-MB-231with 10.61% late apoptosis compared to normal (untreated) cells of 0.29% (**Figure 4 D**).

We next determined the mechanism by which ERL and SAHA kill MDA-MB-231 breast cancerous cells. We observed enhanced apoptosis of MDA-MB-231 cells treated with ERL+SAHA compared with ERL and SAHA (**Figure 3**). In MDA-MB-231cells, we found that the combination therapy of ERL +SAHA induces 7.26% early apoptotic cell death compared to 5.8% early apoptotic cell death upon ERL treatment and 3.99% early apoptotic cell death upon SAHA treatment, compared to the control untreated cells.

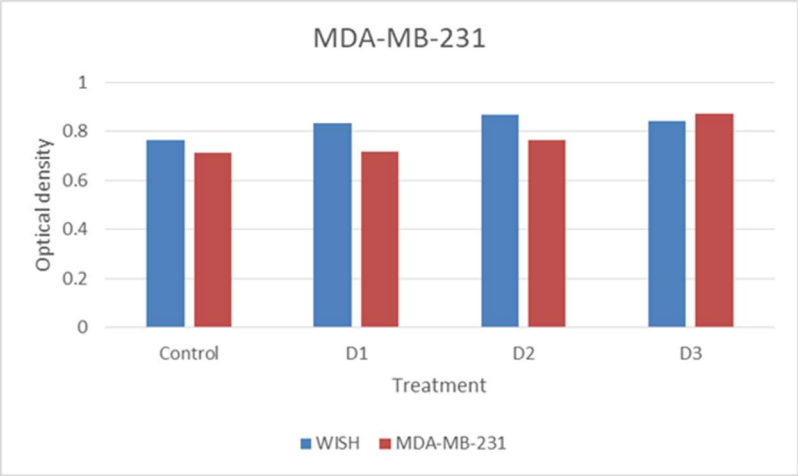

Figure 1: The effect of treatment with D1 (SAHA), D2 (erlotinib), and D3 (combination) on MDA-MB-231 breast cancer and WISH cells

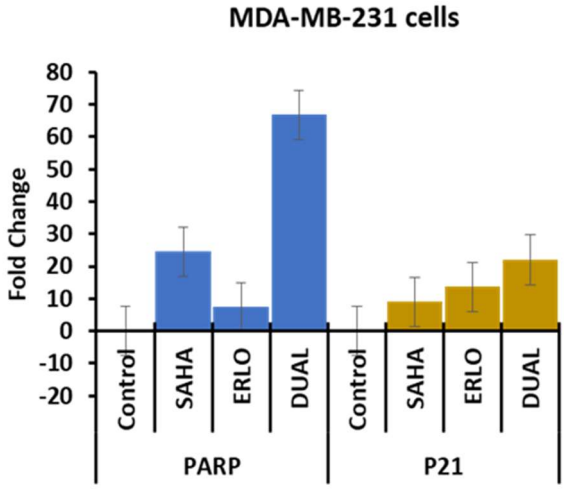

Figure 2: Effect of SAHA (vorinostat), erlotinib, and combination of both chemotherapeutic drugs on the expression level of P21 and PARP genes in MDA-MB 231 breast cancer cell line

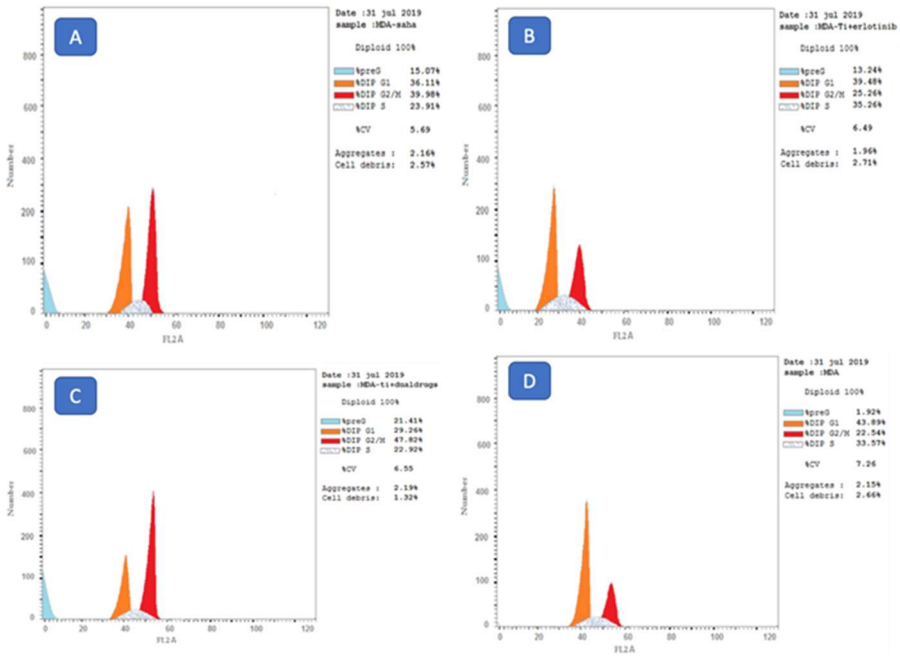

Figure 3: Effect of (A) SAHA (vorinostat), (B) erlotinib, and (C) in a combination of both on the (D) basal cell cycle of MDA-MB-231 breast cancer cell line

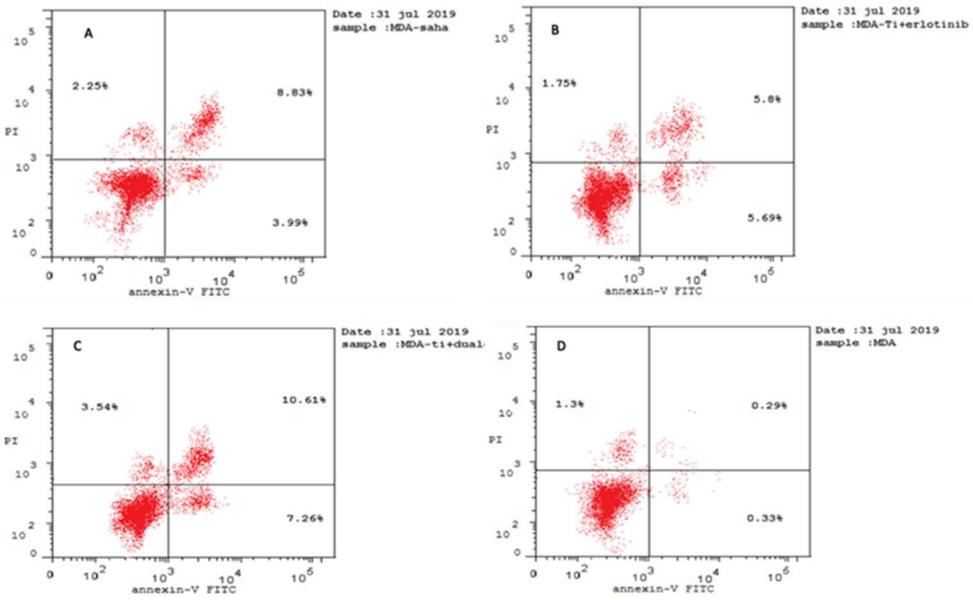

Figure 4: Effect of (A) SAHA (vorinostat), or (B) erlotinib, and (C) their combination, (D) control on the apoptosis analysis using annexin V and PI biomarkers for MDA-MB-231 breast cancer cell

DISCUSSION

In the imminent years, new advances in cancer remedies will depend upon combining drugs to target cancer cells, thereby improving patient survival outcomes **[19]**.

Cytotoxic Effect of SAHA, Erlotinib, and Combination on MDAMB231

Data indicated no significant difference in cell toxicity between control and MDAMB231 breast cancer cells treated with SAHA **[20; 21]**. Additionally, **[22]** reported that single-agent therapy with HDACis has only limited clinical benefit in solid tumors. Also, it has been reported that HDIs, unlike other cytostatic-type compounds, have much lower toxicity in normal cells as opposed to cancer cells **[23]**. In addition, the results showed no significant differences in cytotoxicity when MDAMB231 cells were treated with erlotinib between control and treated cells. These results in conflict with **[24; 25]** who reported that breast cancer patients did not respond well to EGFR inhibitors alone. However, the development of resistance to tyrosine kinase inhibitors is a significant clinical problem, as it occurs in almost all patients receiving these drugs for long periods. Therefore, strategies to overcome secondary resistance to tyrosine kinase inhibitors have been explored. This includes the use of epigenetically active agents such as HDACs **[23; 26]**. Meanwhile, in MDAMB231 breast cancer cells, results indicated that cells treated with combination therapy exerted synergistic antitumor activity on MDAMB231 cells **[27]**.

Specific therapeutic combinations can be optimized for specific and personalized results **[28]**.

Our data show that targeting HDACs not alone but in combination with EGFR is a promising therapeutic strategy. Additionally, **[29]** found that the multi-target EGFR/HDAC inhibitor also showed promise in preclinical cancer models, including breast cancer cell lines and EGFR-resistant NSCLC TKI and breast cancer cell lines.

Effect of Chemotherapeutic Drugs on P21, PARP Gene Expression

In early-stage breast cancer, gene expression has emerged as a method of classification into biologically and clinically relevant subtypes, defining prognosis and selection of patients for treatment **[30]**. Previous studies have shown the role of p21 protein in growth arrest and apoptosis induction through p53-dependent or independent activation pathways and indicated that it is up-regulation in cell lines tumors is probably a consequence of cancer treatment **[31]**. The synergistic effect of inducing apoptosis resulting from the treatment of MDAMB231

cells with the ERL and SAHA combination could be explained by the increase in apoptotic molecules released or, on the contrary, by the decrease in anti-apoptotic released. Some of these key apoptotic molecules, which are used as markers of apoptosis in BC, are p21 as cell-cycle promoters. **[32]**.

It was revealed that PARP overexpression was found to be significantly increased with dual treatment more than any treatment alone. These results are consistent with previous studies **[33]**. Therefore, our data support the hypothesis that the combination with different anticancer therapies (chemo, radiotherapy, or anti-hormonal therapy and TKI), has greater efficacy in different settings **[26]**. Our results revealed that the combination treatment has synergistic effects on cell viability and poly ADP-ribose polymerase (PARP) activations (markers of apoptosis) **[14]**.

We hypothesized that elevated PARP 1 expression may be related to insensitivity to chemotherapy, especially DNA-damaging agents **[34]**. In addition, the proteolytic cleavage of PARP was induced by SAHA **[35]**.

Effect of SAHA, Erlotinib, and Combination on Cell Cycle Progression and Level of Apoptosis

Our results showed that vorinostat induced G2/M arrest, while erlotinib induced G1 arrest in the MDAMB231cell line. Interestingly, the combination of vorinostat and erlotinib resulted in an accumulation of cells in the G2/M phase to the detriment of the G1 phase. These results are consistent with **[36]** who suggested that vorinostat and dasatinib (TKI) might be useful in controlling tumor cell growth, as different tumor cell lines exhibit defects in cell cycle checkpoints. The cytotoxic effects of SAHA on breast cancer cells were manifested by G1 and G2/M cell cycle arrest and eventual apoptosis **[37].**

Almost all results in the current literature support the synergistic effect of combination therapy and how it affects future research on increasing treatment efficacy and decreasing morbidity compared to conventional chemotherapy **[24; 38]**.

CONCLUSION

Our results endorse that SAHA, an effective chemotherapeutic agent combined with tyrosine kinase inhibitor (erlotinib), may produce a synergistic anti-tumor impact in BC (MDA-MB-231) as compared to their monotherapy. These findings have a significant impact withinside the search for more effective treatment for human breast cancer.

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