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Vorinostat induces G2/M cell cycle arrest in breast cancer cells *via* upregulation of *PTEN*

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Abstract. – OBJECTIVE: Breast cancer (BC) is the most common type of cancer in females worldwide. Various approaches were proposed to treat the disease, with no sole agent proved efficient. Thus, understanding the molecular mechanisms of different drugs became mandatory. The present study aimed at evaluating the role of erlotinib (ERL) and vorinostat (SAHA) in inducing apoptosis in breast cancer cells. The role of these drugs was assessed also on the expression profile of some cancer-related genes; *PTEN*, *P21*, *TGF*, and *CDH1*.

MATERIALS AND METHODS: In the present study, breast cancer cells (MCF-7) and MDA-MB-231 along with human amniotic cells (WISH) were treated with two concentrations (50, and 100 μM) of erlotinib (ERL) and vorinostat (as known as SAHA) for 24 h. Cells were harvested for downstream analysis. DNA content and apoptosis were analyzed by flow cytometer, and qPCR was performed to assess the expression of different cancer-related genes.

RESULTS: The results indicated that ERL and SAHA arrested both breast cancer cells at the G2/M phase after 24 h compared to normal cells and control. For apoptosis, BC cells showed an elevated level of total apoptosis (early and late) increasing the concentrations of the two applied drugs, with the most effective concentration of ERL at 100 μM in the 24-h treatment. In the control cells, SAHA was proved to be the most effective drug at a concentration of 100 μM with a percentage of apoptosis ranging from 1.7-12% in the 24-h treatment. Necrosis also was dose-dependent in the two breast cancer cell lines used. We further evaluated the expression profiles of *PTEN*, *P21*, *TGF-β*, and *CDH1*. In MCF-7, data indicated that for *TGF-β*, *PTEN*, and *P21*, the most effective treatment was SAHA at a concentration of 100 μM, while for *CDH1*, the most effective concentration was ERL at 100 μM. A similar profile was observed in MDA-MB-232, where for *TGF-β*, *PTEN*, and *P21,* the most effective treatment was SAHA at a concentration of 100 μM, while for *CDH1*, the most effective concentration was SAHA at 50 μM.

CONCLUSIONS: Our results shed some light on the role of ERL and SAHA in regulating the expression of cancer-related genes, though these data need further investigation.

Key Words:
Breast, Cancer, Erlotinib, Vorinostat, PTEN, CDH1, P21, *TGF-β*.

Introduction

Cancer is a life-threatening disease, with 18.1 million new cases diagnosed in 2021 and 9.6 million cancer deaths[1-3]. Breast cancer (BC) is one of the most common cancers in women around the world[4-6]. BC develops in the breast as a result of either genetic or epigenetic alterations[7,8]. Chemotherapy, radiation, advent therapy, and surgery were among the therapeutic choices proposed and offered to patients in a clinical setting[9,10].

The fight against cancer is regarded as the contemporary medicine's greatest challenge. Despite the fact that there are different types of malignancies, breast cancer is still the most commonly diagnosed among females worldwide, and numerous chemotherapeutic medications have been created to treat it. At the moment, significant clinical interest is focused on combination therapy, as it offers the most biological basis[11-13].

Erlotinib, a small-molecule inhibitor of the EGFR tyrosine kinase, showed an antitumor effect in patients with non-small cell lung cancer (NSCLC) who have relapsed following chemotherapy[14,15]. Treatment with erlotinib generated an accumulation of H322 cells in the G1 phase and a decrease in the number of cells in the S and G2-M phases in a time-dependent manner, according to flow cytometric analyses (EGFR). Although many breast cancer cells express EGFR, tyrosine kinases like erlotinib and gefitinib have not been highly effective in the treatment of breast cancer[16-19].

Histone deacetylase inhibitors play an important role in the treatment of lung cancer. These inhibitors are a class of agents that induce epigenetic changes *via* the modulating chromatin structure[20],

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which ultimately results in apoptosis and arresting the cell cycle, and in the inhibition of the angiogenesis process[21]. SAHA is a histone deacetylase inhibitor with antiproliferative properties, and it is currently used as monotherapy for primary T-cell lymphoma[22]. SAHA has also been examined in combination with other chemotherapeutic agents without major limitations[23] to treat breast cancer.

The present study aimed at evaluating the role of ERL and SAHA in inducing apoptosis in breast cancer cells. The role of these drugs was assessed also on the expression profile of some cancer-related genes; *PTEN, P21, TGF*, and *CDH1*. The generated profiles in this study indicated that late apoptosis in the treated MCF-7 cells was induced in a dose-dependent manner when cells were treated with SAHA and ERL.

Materials and Methods

Cell Line Maintenance

Michigan Cancer Foundation-7 (MCF-7) invasive breast ductal carcinoma cells were grown under normal laboratory conditions; 37°C and 5% CO_2. Cells were fed on RPMI-1640 media supplemented with 10% Fetal Bovine Serum (FBS) and 1% ready-made antibiotic mix. Wistar Institute, Susan Hayflick (WISH) normal human amniotic cells were used as control and were mainlined under the standard lab conditions.

Drugs and Treatments

Erlotinib (N-(3-ethynylphenyl)-6,7-bis(2-methoxyethoxy) quinazolin-4-amine; hydrochloride, C22H23N3O4) and Vorinostat (as known as suberanilohydroxamic acid) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A stock solution of each drug was prepared by dissolving 5 mg in 1 mL of Dimethyl sulfoxide (DMSO). MCF-7 and WISH cells were treated with gradient concentrations of both drugs; 10, 25, 50, and 100 µM for 24 and 48 h. Cells were seeded at 104 cells/mL 24 h before treatment.

Harvesting Cells

Twenty-four and 48 hours after treatment, cells were harvested by trypsinization and centrifugation at 2,000 rpm for 20 min. The pellet was divided into three parts for downstream analysis.

DNA Content Assay

We analyzed the changes that took place in the cell cycle upon treating cells with erlotinib, subsequently, the cells were collected in cold 70% ethanol in PBS for 6 h (or overnight). Cells, then, were centrifuged and treated with 50 µg/mL Propidium Iodide (PI) dye, 0.1% Triton X-100 and 50 µg/mL RNAse for 25 min and incubated at room temperature in a dark place. The PI fluorescence was read on a FACScan flow cytometer (BD FACSCalibur™). After adequate gating of cell populations in the FL-2-Area *vs.* FL-2-Width plot of PI fluorescence, the cell cycle distribution was computed. Data were analyzed to show the cell cycle distribution of the treated and untreated cells. To differentiate between apoptotic and necrotic cells, the treated cells were stained also with Annexin V-FITC. Cells were resuspended in 500 µL of 1X Binding Buffer, and 5 µL of Annexin V-FITC was added. Annexin V-FITC was analyzed by flow cytometry (Ex = 488 nm; Em = 530 nm) using FITC signal detector (usually FL1).

Apoptosis Assay

1 × 105 MCF-7 and WISH cells were seeded and treated with both drugs at 10, 25, 50, and 100 µM for 24 and 48 h, leaving untreated cells to serve as a control group. The loss of membrane integrity (high PI fluorescence) after the treatment with the PI solution was used to determine cell death. Flow cytometry was used to evaluate phosphotidylserine exposure using the Annexin V-FITC/PI double labelling kit (BD Biosciences, Franklin Lakes, NJ, USA). Cells can be classified into four groups based on their staining: viable (Annexin V PI), early apoptotic (Annexin V+ PI), late apoptotic (Annexin V+ PI+), and necrotic (Annexin V PI+).

RNA Extraction and cDNA Synthesis

Total RNA was extracted from control and SAHA- and ERL-treated cells along with normal cells using RNA Isolation System (Qiagen, GmbH, Germany). cDNA was synthesized using RT-PCR Kit (Qiagen, GmbH, Hilden, Germany).

Gene Expression Analysis

Quantitative real-time PCR was used to amplify *P21, PTEN, CDH1*, and *TGF-β* genes. Primers sequences used in this study are presented in Table I. About 100 ng of cDNA was mixed with Cyber Green master mix, forward primer (10 pM), reverse primers (10 pM), and molecular biology-grade water to a final volume of 25 µL. The thermal profile was as follows: pre-PCR heating for 5 min at 95ºC, then 40 cycles of 94ºC for 45 sec, 56-63ºC (depending on each gene) for 30 sec, 72ºC for 45 sec followed by a final extension step

Table I. The primer sequences used in the present study.

Primer name	Forward	Reverse
PTEN	TGAGTTCCCTCAGCCGTTACCT	GAGGTTTCCTCTGGTCCTGGTA
P21	AGGTGGACCTGGAGACTCTCAG	TCCTCTTGGAGAAGATCAGCCG
TGF	TACCTGAACCCGTGTTGCTCTC	GTTGCTGAGGTATCGCCAGGAA
CDH1	GCCTCCTGAAAAGAGAGTGGAAG	TGGCAGTGTCTCTCCAAATCCG
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

of 72ºC for 5 min. All reactions were performed in triplicates on Step One Plus thermal cycler (ABS, UK). $2^{-\Delta\Delta CT}$ method was employed to calculate the fold change in gene expression.

Statistical Analysis

The *t*-test was used in the present study to identify whether the differences between treated and untreated cell counts were significant. Comparisons with *p*-values lower than 0.05 were considered significant.

Results

In the present work, MCF-7, MDA-MB-231 breast cancer cells and WISH normal amniotic cells were treated with ERL and SAHA for 24 h, at final concentrations of 50 and 100 μM. Cell cycle analysis data revealed that for ERL, the higher the concentration applied, the more the arrest of cells at the G2/M phase, and the same phenomenon was observed in the case of SAHA (Figure 1-2).

In the control normal cells, the highest concentration of both SAHA and ERL caused no arrest of the cell cycle at G2/M. Most cells were in the G1 phase, although G2/M was slightly elevated with no significant changes (Figure 3).

For the apoptosis detection, MCF-7 showed an elevated level of total apoptosis (early and late) with increasing concentrations of the two applied drugs. The most effective concentration was SAHA at 100 μM for MCF-7 and ERL at 100 μM for mDA-MB-231 (Figure 4). Necrosis also was dose-dependent in the three cells line used (Figure 5).

In the present study, we evaluated the expression profiles of different cancer-related genes; *PTEN, P21, TGF*, and *CDH1*. Data indicated that, in MCF-7, treatments have resulted in downregulation of *TGF*, while *P21, CDH1*, and *PTEN* were upregulated upon SAHA and ERL treatments. Meanwhile, overall reduction in the expression profile of *TGF* was observed in MDA-MB-231 BC cells. *P21, CDH1*, and *PTEN* were upregulated with the treatments (Figure 6).

Discussion

Breast cancer was the most common malignancy diagnosed worldwide in 2021. Despite major breakthroughs in BC therapy, the biology of the disease remains unknown, and effective treatment remains one of the most difficult tasks in modern oncology[24,25].

Chemotherapy can be given with the intent to cure or to palliate symptoms. All cytotoxic drugs have side effects, and a balance must be struck between toxicity and benefit to the patient. For some tumors, however, treatment with single drugs remains the best option, however, adjuvant and neo-adjuvant therapies are also beneficial in other types[26-28]. For the cytotoxicity effect of the applied drugs, data indicated that ERL and SAHA induced cytotoxicity in MCF-7 and MDA-MB-231 breast cancer cells, and this has been documented by other researchers[11,29].

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKIs) such as ERL can control cancer cells *via* modulation of ER stress mediated apoptosis[30]. ERL is a targeted cancer drug which functions as an inhibitor of the epidermal growth factor receptor (EGFR) tyrosine kinase[29].

It has also been claimed that SAHA inhibits MCF-7 and MDA-MB-231 growth in a dose-dependent manner by lowering HDAC1, 2, 3, 4, and 7 expressions while increasing acetylated histone H3 and H4. Furthermore, SAHA causes MCF-7 and MDA-MB-231 cells to enter the G2/M phase of the cell cycle, and it may cause caspase-independent autophagic cell death rather than apoptotic cell death. The treatment of tamoxifen-resistant human breast cancer with SAHA-mediated autophagic cell death is a potential novel method[31,32].

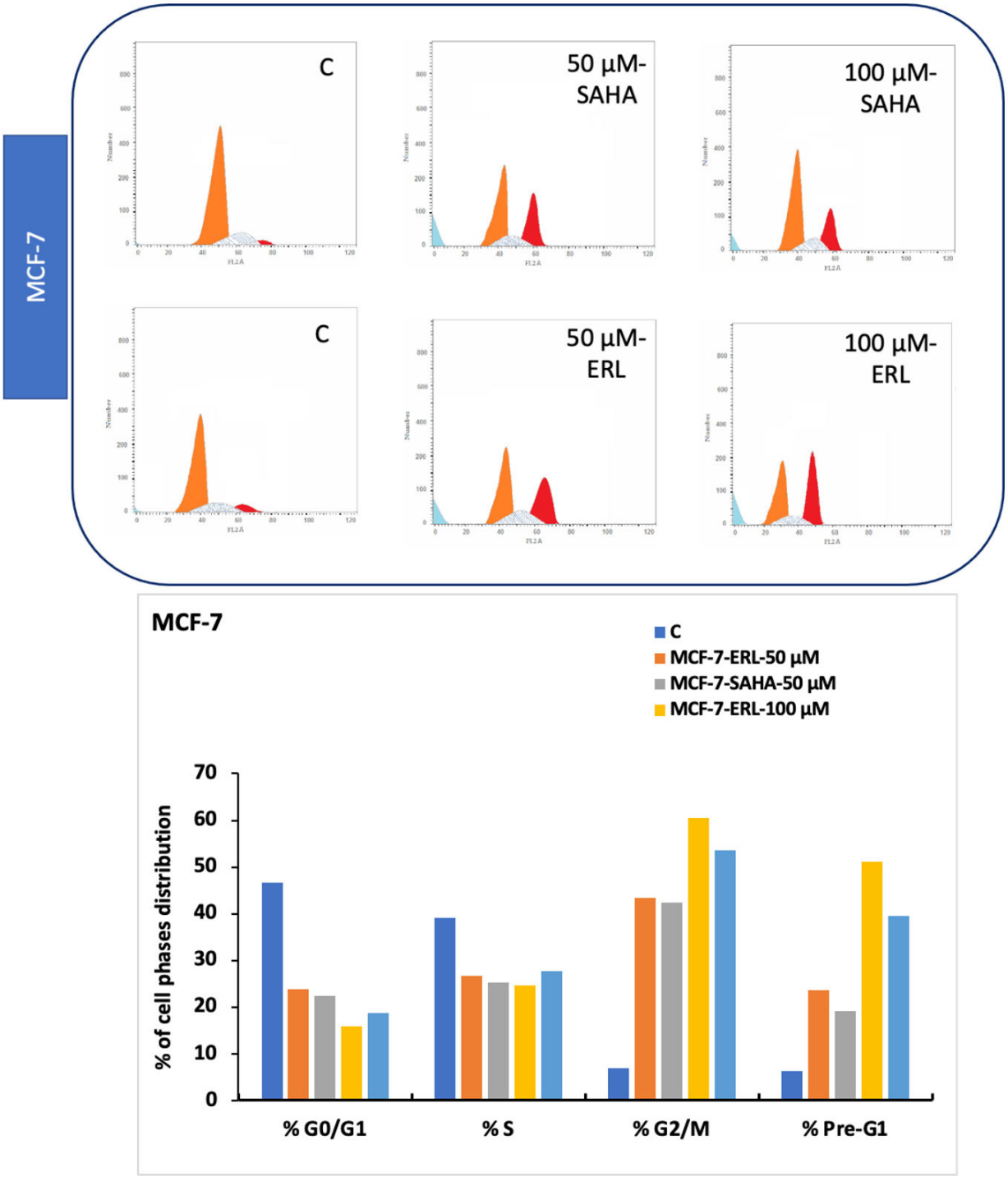

Figure 1. Cell cycle stages distribution in MCF-7 breast cancer cells treated with drugs for 24 h. ERL: Erlotinib, SAHA: Vorinostat.

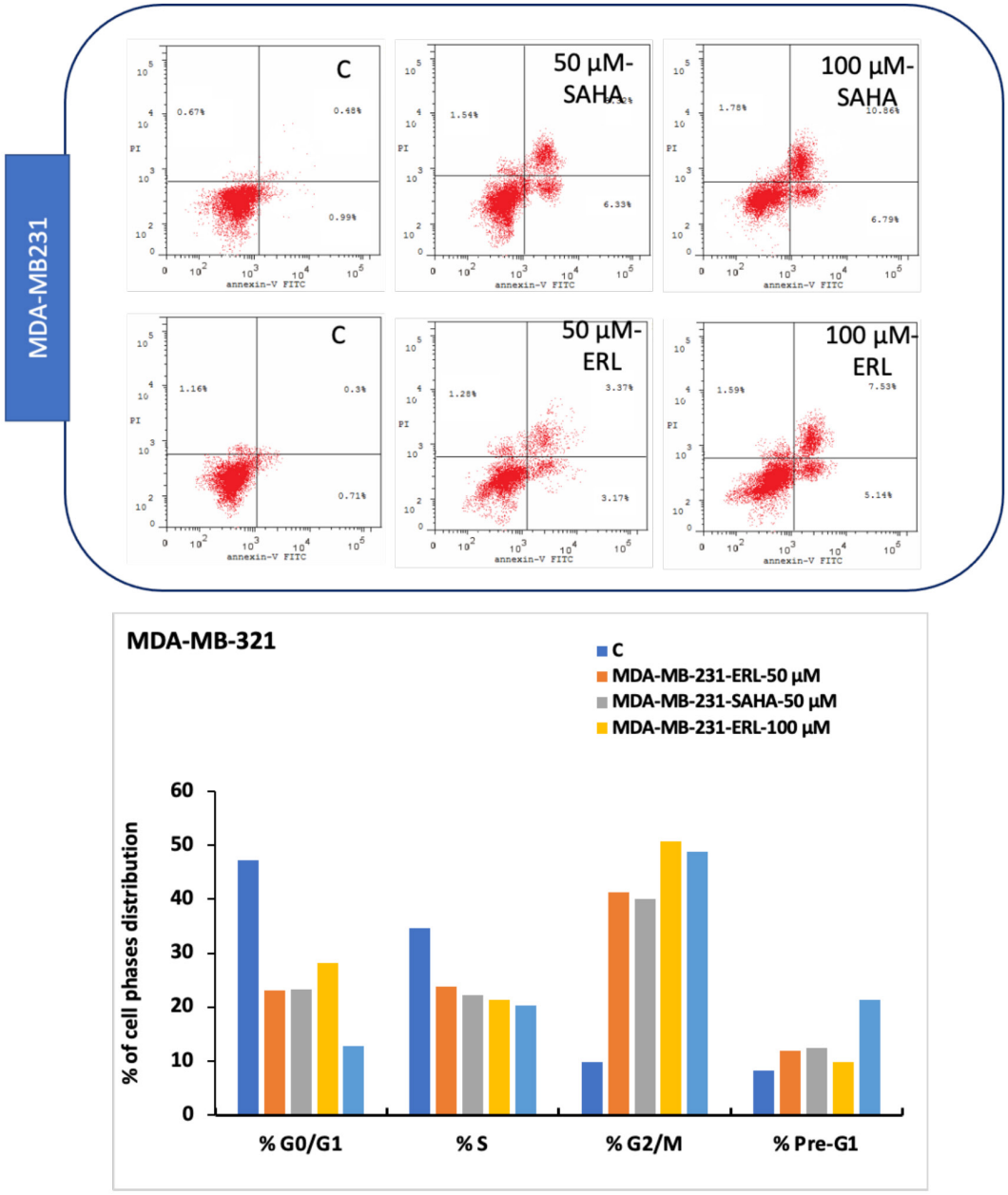

Figure 2. Cell cycle stages distribution in MDA-MB-231 breast cancer cells treated with drugs for 24 h. ERL: Erlotinib, SAHA: Vorinostat.

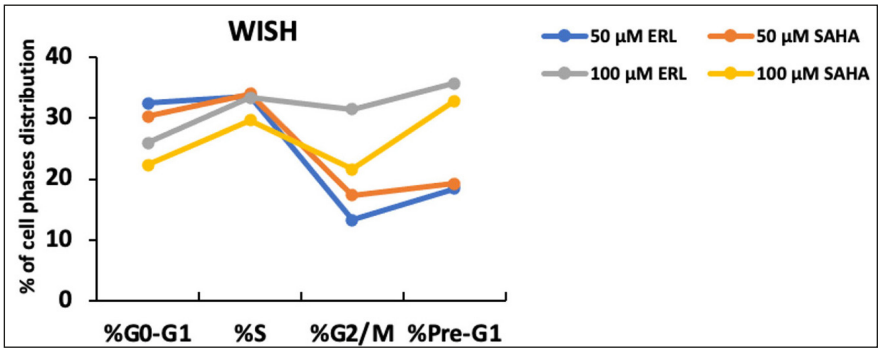

Figure 3. Cell cycle stages distribution in WISH normal cells treated with drugs for 24 h. ERL: Erlotinib, SAHA: Vorinostat.

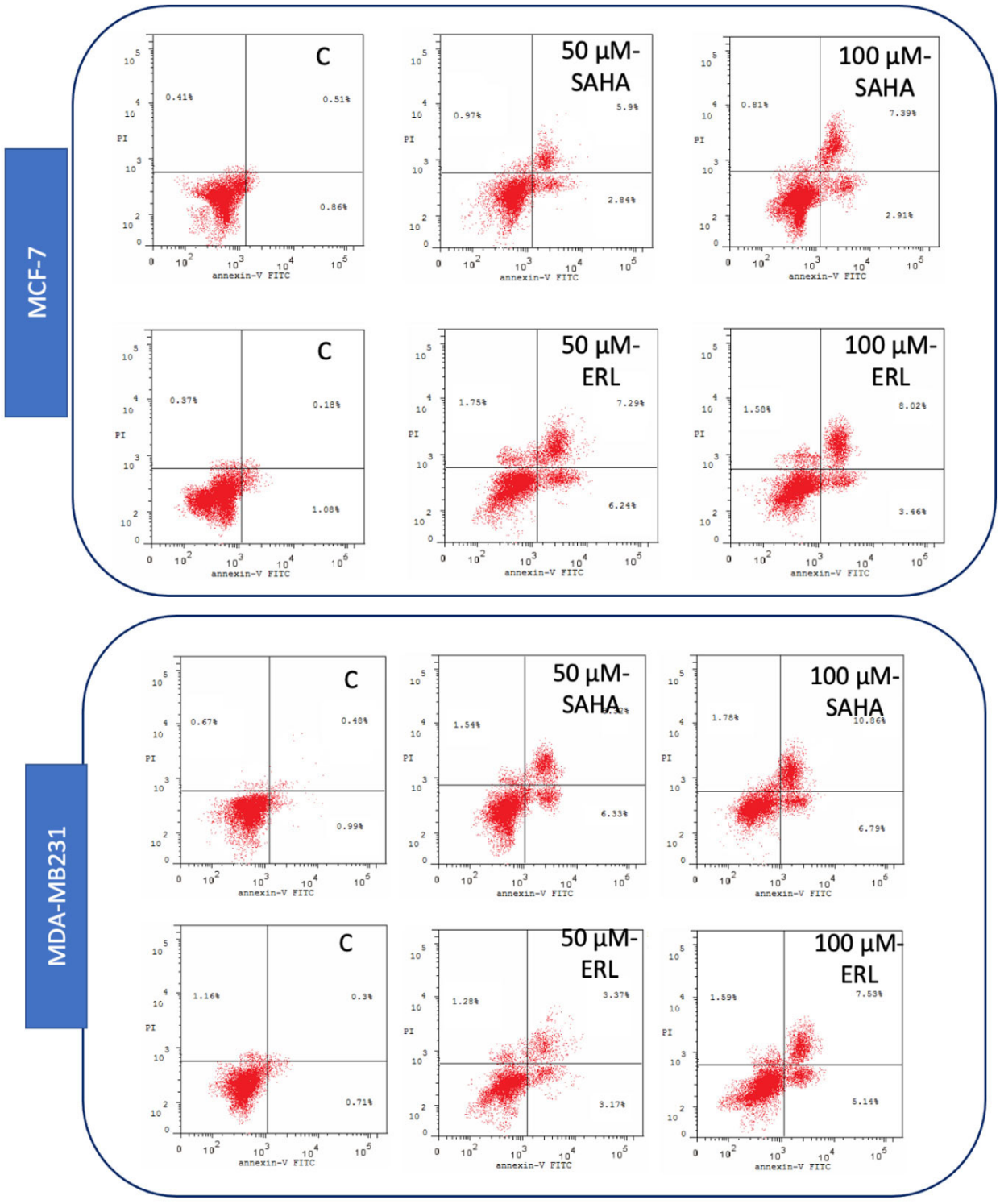

Figure 4. Apoptosis fractions. Percentages of apoptosis in MCF-7 and MDA-MB-231 breast cancer cells treated with drugs for 24 h.

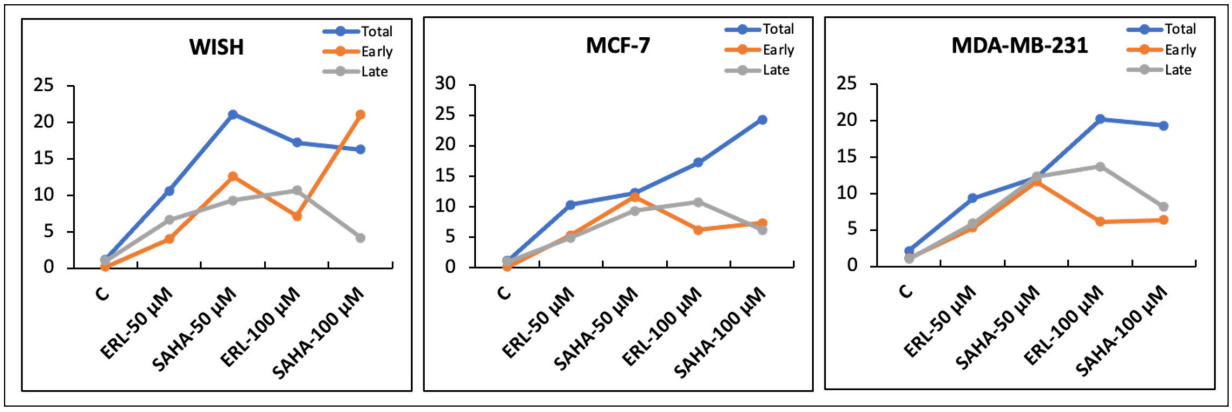

Figure 5. Necrosis in normal cells. ERL: Erlotinib, SAHA: Vorinostat.

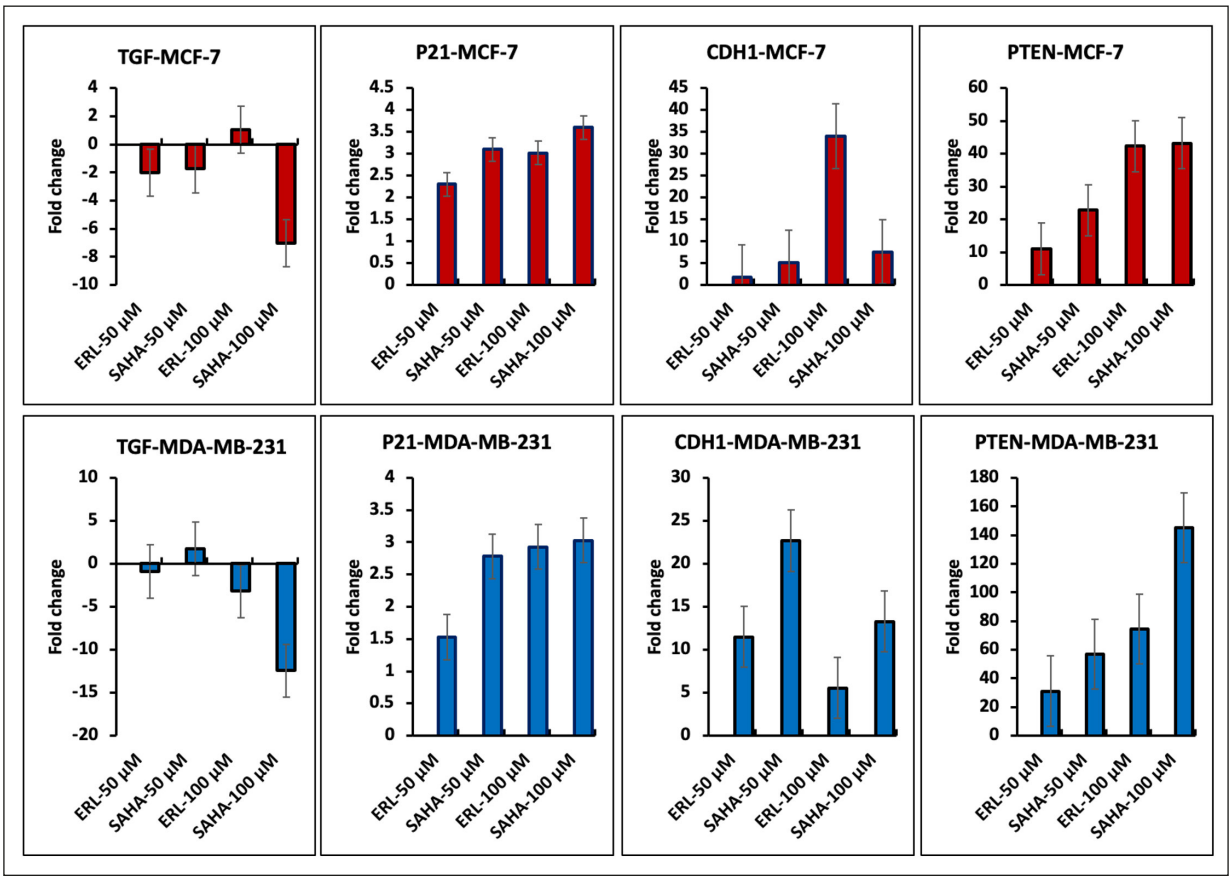

Figure 6. The gene expression profiling of *PTEN*, *P21*, *TGF*, and *CDH1*.

SAHA also triggered cell cycle arrest by upregulating CDKN1A and downregulating CCND1, which encodes the proteins p21WAF1/CIP1 and cyclin D1. However, in the present study, this effect was executed *via* the overall activation of *PTEN, P21, TGF*, and *CDH1,* although some concentration had a downregulating effect on these genes; the 100 µM of both ERL and SAHA on *P21, TGF,* and *PTEN.* However, when loaded on TiO_2 NPs, ERL and SAHA could arrest breast cancer cells at the G2/M phase, which indicates the cytotoxic effect of these treatment[29]. Though they are capable of inducing apoptosis in BC cells, ERL and SAHA could also increase the level of apoptosis in lung cancer cells.

Limitations

Although these data can be helpful to open the gate for BC treatment, further studies are needed to address more deep questions.

Conclusions

Breast cancer is a global health problem that affects women in different ages. Investigating different types of agents to treat the disease became mandatory, especially with the fact that every individual has a unique disease. The present study investigated the role of ERL and SAHA in inducing apoptosis and upregulating *CDH1* and other related genes. This approach might help designing new modalities to address the need for reliable chemotherapeutic drugs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

Not applicable.

Authors' Contributions

Authors contributed equally and made a significant contribution to the work reported. Authors contributed to the study design, execution, methodology, acquisition of data, software, validation, data analysis, and interpretation, took part in writing, original draft preparation, reviewing, and editing.

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Informed Consent

Not applicable in this study.

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