

The effect of Vorinostat (SAHA) and ERL on the expression profile of P21 and PARP in MCF-7 breast cancer cell line

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ABSTRACT

The fight to defeat cancer is viewed as the greatest challenge of modernistic medicine. Despite many forms of cancers, breast cancer still the most frequently diagnosed one among females worldwide, numerous chemotherapeutic drugs have been developed to overcome this type of cancer. At present, much clinical interest is focused on combination with HDAC inhibitors, those combination therapy has the greatest biological rationale. In the current study, the antiproliferative effect of a combination of Vorinostat (SAHA) and Erlotinib in breast cancer cell line (MCF7) were evaluated. Cytotoxicity effect of the drugs single and in combination was measured by MTT assay. As well, the expression levels of *P21* and *PARP* genes was measured by qRT-PCR after 24 h of treatment. MTT assay exposed that combination of erlotinib and vorinostat drugs synergistically induce cytotoxic effect in breast cancer cell lines. qPCR results demonstrated synergistic upregulation in the mRNA levels of *P21* and *PARP* at the used concentrations. Our data provide indication that synergistic antiproliferative effects of Erlotinib and Vorinostat are related to the up-regulation of *P21* and *PARP* genes and propose that the combination therapy may have therapeutic value in breast cancer treatment.

Keywords

Breast cancer (BC); PARP; P21; ERL; SAHA

Introduction

Cancer is a complicated disease that expresses itself in a lot of forms, all manifested by the same uncontrolled proliferation of cells (Allen and Chen, 2013; Hajiloo et al., 2013). Currently, cancer consider one of the most typical threatening diseases in the world (Rasouli&Zarghami, 2018). Cancer continues to be the major cause of death in developed countries as well as second reason of death in developing countries. The burden of cancer is getting rise in the developing countries because of aging of population as well as, increasingly, an acceptance of lifestyle choices related to cancer including being overweight or having obesity, smoking, and westernized diets. (Jemal et al., 2011).

Several types of cancer affect human health, but (BC) is considered the most common cause of cancer death in females as it is affecting around one in nine women worldwide (Jemal et al., 2010). Yearly, around 1.38 million new cases were diagnosed with breast cancer of which about 35% mortality rate (Halimi et al., 2013).

The rate of (BC) in Egypt is 49.6 per 100.000 individuals which represent 18.9% of all cancer cases in the country

(El-Hadidy et al., 2012; Enders et al., 2013). In developing countries about half the (BC) cases and 60% of the deaths are estimated to happen (Mackay et al., 2006). However, there is a huge difference in breast cancer incidence among women where Caucasian having the highest incidence while Asian women having the lowest risk (He and Chen, 2013).

(BC) risk factors could be allotted to one of these four groups that including genetic\ family history, hormonal\ reproductive, proliferative benign (BC), and density of mammography. And now these four factors have been carefully studied and a precise quantitative estimates for the risk are available for many of them (Cuzick, 2012).

There is a genetic susceptibility, reaching up to 30% of the hereditary of (BC) (Heyn et al., 2013). Drug resistance increasing limits the efficiency of targeted drugs. Alternative approaches now are using different combinations of drugs to inhibit several pathways that could be more effective strategy for cancer treatment (Jeannot et al., 2016)

In clinical settings, combination of different chemotherapeutic drugs to exhibit additive or synergistic effects, is commonly used. This combination approach should not only improve therapeutic efficacy but also will treat metastatic breast cancer by using mechanistically different approaches, in consequence enhancing therapeutic effectiveness (Zhuang et al., 2021)

Also, HDACs have been reported to modify the function of numerous genes, especially those involved in the differentiation, cell cycle, and apoptosis. Moreover, HDACs able to up regulate the expression level of several genes that involved in the process of tumor invasion, neo-vascularization, and metastasis (Wu et al., 2020).

Materials and Methods

Cell line culture and maintenance

Breast cancer (MCF-7) cell line was purchased from the Holding Company for Vaccines and Biological Products (VACSERA), Cairo, Egypt. The cells were kept in (RPMI)1640 Roswell Park Memorial Institute media (Gibco, USA) using a bicarbonate buffering system and variable amounts of amino acids and vitamins complemented by 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin mix (Invitrogen Life Technologies). Cells were seeded in 12-well U-bottom microplates (Nunc, Denmark) and incubated at 37 °C for 24 h in a fully humidified atmosphere of 5% CO₂.

Chemotherapeutic drugs

Two chemotherapeutic drugs: Vorinostat (SAHA) and Erlotinib (TKI) were purchased from Santa Cruz Biotechnology (USA).

Drug preparation and doses

A stock of (16 µM) of Erlotinib and (10.5 µM) of Vorinostat drugs and a drug combination of both with a concentration (8 µM + 5.25 µM, respectively) was prepared and used for the treatment of the MCF-7. 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 will be mixed separately and added to the wells containing the MCF-7.

Cell viability test

Before exposing the cells to any treatment, MTT test will be performed to evaluate the number of viable cells. The cytotoxic effects of Erlotinib and Vorinostat in vitro on breast cancer (MCF-7) cell line was tested with a rapid colorimetric assay using MTT assay and compared with the untreated controls where Viable cells with active metabolism change MTT into formazan and dead cells lose this ability and consequently show no signal.

DNA extraction

Genomic deoxyribonucleic acid was extracted from both treated and non-treated cells for downstream analysis and stored in -20 °C until being used. Extraction was performed using Cell Biolab DNA extraction kit (USA) following the kit's instructions.

Real-Time PCR (qPCR)

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

RNA extraction

Total RNA was extracted using QiaGen column RNAeasy kit. RNA will be measured and then prepared to further analysis. Further, cDNA was synthesized using RT reverse transcriptase II kit. We followed the kit's instructions.

PCR array

PCR array kit targeting breast cancer will be purchased from QiaGen (UK). All tests will be performed in the supplied plate that is pre-loaded with 24 target gene. We followed the kit's instructions.

Cell cycle analysis using flow cytometry

Concisely, cells (2.5×10^5 cells/mL) were seeded in culture dish and treated with Vorinostat (SAHA) and Erlotinib alone and in combination for 24 h. After 24 h incubation, cells were harvested and kept in 70% cold ethanol at 4°C for 1 h. After fixation, cells were washed with PBS and incubated with 0.5 mg/mL RNase A (Sigma) at 37°C for 1 h. Nuclear DNA was stained using propidium iodide (PI) (50 μ g/mL) under subdued light for 30 min at room temperature. The DNA histograms, reflecting cell cycle distribution, were assessed by using BD FACSanto II Calibur Flow Cytometer (Becton-Dickinson, San Jose, CA, USA).

Apoptosis assay

The induction of apoptosis was analyzed using a FITC-Annexin V apoptosis detection kit according to the manufacturer's instructions. Briefly, cells (2.5×10^5 cells/mL) were seeded in culture dish and treated with Vorinostat (SAHA) and Erlotinib alone and in combination for 24 h. Cells were harvested, washed with cold PBS and resuspended in the Annexin V-binding buffer. Then cells were incubated with Annexin V-FITC and PI at room temperature for 15 min, and then analyzed by flow cytometry by using a BD FACSanto II Calibur Flow Cytometer (Becton-Dickinson). The flow cytometry results were compared with conventional cell count and morphology under a fluorescence microscope.

RESULTS

Measuring cell cytotoxicity

MTT assay was used to determine cell viability, cytotoxicity after incubation for 24 h. In every treatment (vorinostat, erlotinib and their combination) was showed that MCF-7 cell toxicity was increased by using a concentration of 10.5 μ L of vorinostat (SAHA) (D1) which increase from 0.6465 OD to 0.7265 OD comparing to control cells, while at concentration of 16 μ L of Erlotinib (D2) we noticed a significant increase in cell cytotoxicity that raised from 0.6465 OD to 0.866 OD compared to control cells. Even more, a combination of both drugs under a concentration of (5.25 μ L of SAHA + 8 μ L of Erlotinib) (D3) showed synergistic effect on cell toxicity raised from 0.6465 OD to 0.844 OD in control cells. As shown in Figure 1A, While in Figure 1B the effect of chemotherapeutic drugs on normal WISH cells demonstrated a significant increase in cell cytotoxicity in all treatments (D1, D2 and D3).

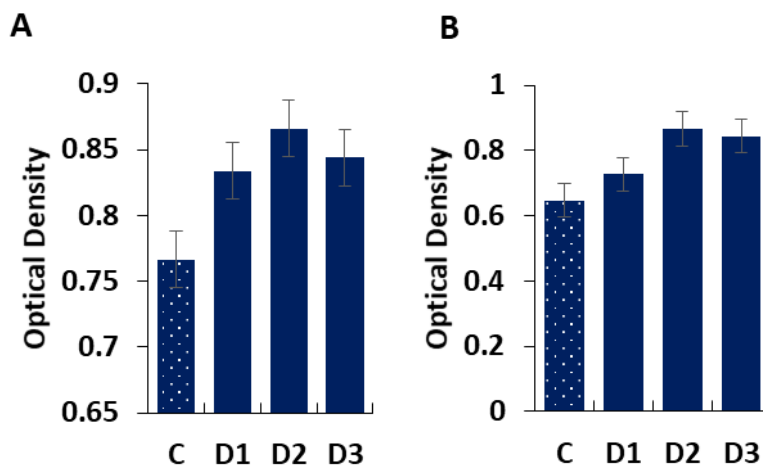


Figure 1: Mitochondrial reductase enzyme activity as measured after treating the normal and malignant cells with different types of chemotherapeutic drugs D1 (SAHA), D2 (erlotinib) and D3 (combination) for 24 h. A: breast cancer MCF-7 cell line, B: WISH (normal) cells.

***P21* and *PARP* gene expression level in MCF-7 Breast Cancer cell line**

The present data in Figure 2 showed that *P21* gene expression was up regulated in MCF-7 cells treated with dual drugs (SAHA and Erlotinib) than each drug alone compared to control cells.

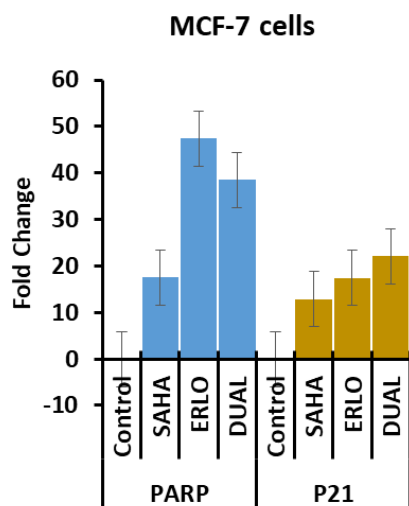


Figure 2: effect of SAHA (vorinostat), erlotinib and combination of both chemotherapeutic drugs on expression level of *P21* and *PARP* genes in MCF-7 breast cancer cell line.

On the other side we noticed that *PARP* gene in MCF-7 cells was up regulated in cells treated with Erlotinib more than that treated with dual drugs and SAHA alone comparing these results with control.

Drugs effect on cell-cycle progression

Flow cytometry was used to study the cell cycle distribution in MCF-7 cells exposed to HDAC inhibitors (SAHA), Erlotinib and combination of both chemotherapeutic drugs. Treatment with SAHA lead to cell cycle arrest at G2/M phase that rose from 10.36% in MCF7 alone to 33.69% in treated cells, slightly decrease in S phase and significant reduction in G0/G1 phase as shown in Figure 3.

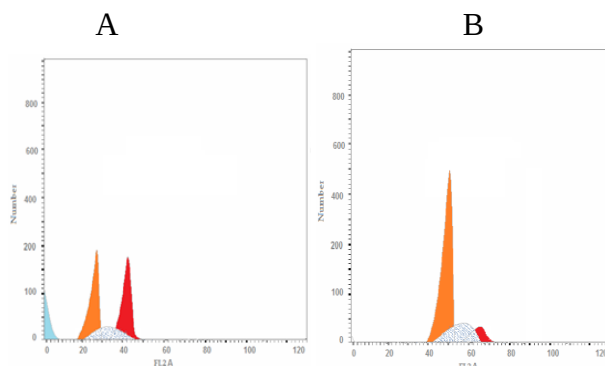


Figure 3: A: effect of SAHA (vorinostat) on cell cycle progression of MCF-7 Breast Cancer cell line. B: control sample of MCF-7 cell line.

While treatment with erlotinib showed that MCF7 cell line underwent a significant increased cell cycle arrest in G2/M phase raised from 10.36% in MCF7 alone to 45.05% in treated cells with a slightly decrease in S phase and notably decrease in G0/G1 phase as shown in Figure 3 (C).

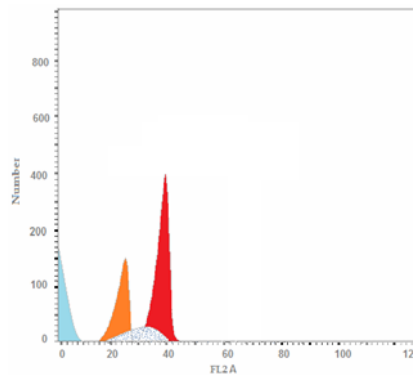


Figure 3 C: effect of erlotinib on cell cycle progression of MCF-7 Breast Cancer cell line.

On the other hand, Combination treatment with HDAC (SAHA) and EGFR (erlotinib) synergistically increases cell cycle arrest at G2/M phase with significant decrease in S and G0/G1 phase respectively Figure 3 (D).

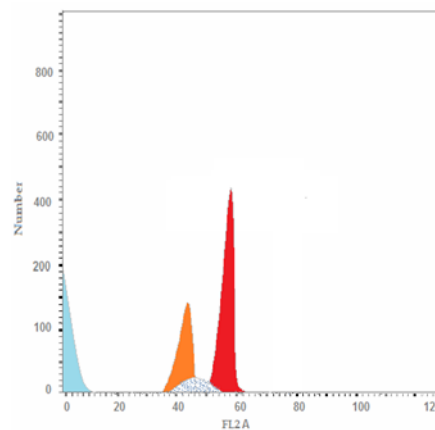


Figure 3 D: The effect of dual chemotherapeutic drugs on cell cycle progression of MCF-7 Breast Cancer cell line.

We therefore investigated that treatment with a combination of SAHA (vorinostat) and Erlotinib have an increased cell cycle arrest at G2/M phase on Breast Cancer cell line (MCF7) than each drug alone.

The apoptotic effect on MCF7 breast cancer cells

Apoptosis induction was studied by annexin V and PI biomarkers. Erlotinib promote apoptosis (late apoptosis) in MCF7 breast cancer cell line increasing while SAHA when subjected to MCF-7 showed early apoptosis with 11.16% and 5.5% late apoptosis. Figure 4 A, B, C and D.

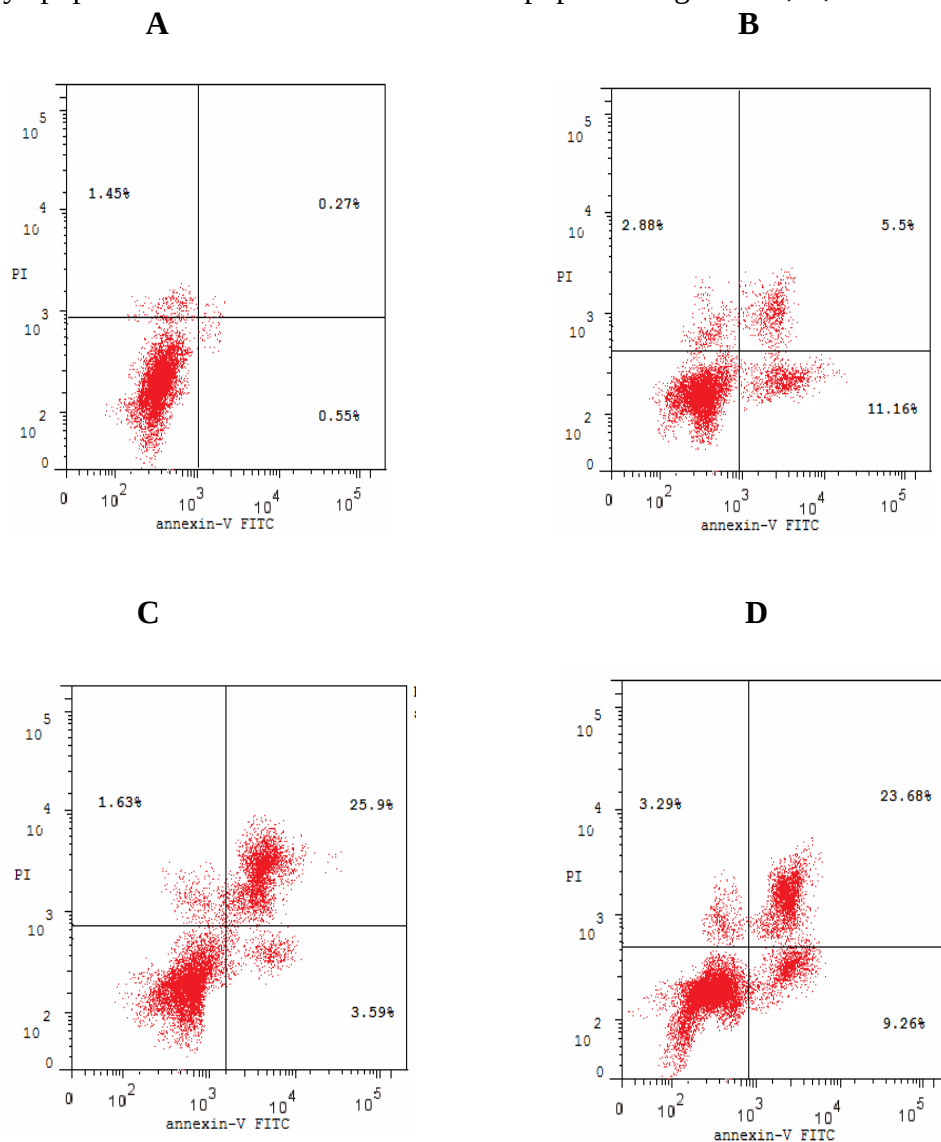


Figure 4: A: Illustrates apoptosis dot plot of control sample (MCF-7), B: apoptosis dot plot of (MCF-7 with SAHA), C: apoptosis dot plot of (MCF-7 with ERL) and D: apoptosis dot plot of (MCF-7 with DUAL drugs).

On the other hand, the combination of both drugs Erlotinib and (vorinostat) SAHA induced synergistic cell death. Apoptotic cells were markedly increased in MCF7 with 23.68% late apoptosis compared to normal cells of 9.03%.

These results revealed that both Erlotinib, SAHA and a combination of both induced apoptosis in MCF-7 cell line.

Discussion

Breast cancer is a crucial health problem in developing countries where its incidence and death rate has increased in past decades (Rivera-Franco and Leon-Rodriguez, 2018).

Effect of SAHA, Erlotinib and combination on MCF-7 Breast Cancer cells

Significant increase in cell toxicity in MCF-7 cell line treated with erlotinib was found. The control was lower than the ERL-treated MCF-7 breast cancer cells. These results were in consistent with other studies such as (Blumenberg, 2014) who explained that therapies which inhibit EGFR became a model for targeted treatment of cancers in human and use inhibitors of (EGFR) kinase, Erlotinib and Gefitinib.

While cytotoxic activity of SAHA on MCF-7 was investigated and found an increase in effect of the drug which rise from 0.6465 in control sample to 0.7265 in treated MCF-7 and 0.844 OD in MCF-7 treated with combination drugs, which mean that treatment with erlotinib and the combination therapy was much more effective than using SAHA alone (Chen, et al., 2013) as numerous studies supported the use of HDAC inhibitors in combination with EGFR-TKIs in NSCL cancer cells to iterate EGFR-TKI sensitivity (Jeannot et al., 2016).

These findings came in line with previous studies which declare that SAHA as an HDAC inhibitors can destroy cancer cells through the process of activation of tumor-suppressor genes and apoptotic pathways. However, progressive outcomes and low therapeutic efficacy are often reported in cancer therapies. To improve the HDACi therapy against tumors and deadly metastasis combination treatment (Lin et al., 2012) with erlotinib was used to increase efficacy of the drug. Moreover, non-toxic treatments using a combination of HDACis and TKIs have been established (Gerber et al., 2015; Gray et al., 2014).

Effect of chemotherapeutic drugs on gene expression

SAHA was indicated to induce growth inhibition, apoptosis and cell cycle arrest, Breast Cancer cells, probably by modulating cell cycle and apoptosis regulatory proteins, such as CDK inhibitors *p21* and *p27*, *pRb*, and other differentiation and/or growth inhibition-associated genes (Huang & Pardee, 2000; Harper et al., 1993). These finding are in line with our results that showed upregulation in *p21* gene expression in MCF-7 breast cancer cell line treated with SAHA. Previous Preclinical in vitro and in vivo studies showed that treatment with SAHA induces *p21* (Bali et al., 2005), concurrently HDIs are able to induce apoptosis or inhibit cell cycle by regulating expression of several genes (Wawruszak et al., 2015).

Effect of Erlotinib on *p21* gene expression in MCF-7 breast cancer cell lines

In our study, the expression of *p21* gene has been up regulated after treatment of MCF7 cells with erlotinib and these results come in consistent with previous studies which demonstrated that erlotinib can induce the expression level of *p21* and *p27* cell cycle regulatory proteins, and also induce of apoptosis regulatory protein Bim, in a *p53*-dependent manner (Amin et al., 2009; Ling et al., 2007).

A growing body of indication suggests that erlotinib can induce the expression of *p21* and *p27* (Amin et al., 2009).

We can conclude that erlotinib can induce cell-cycle and apoptosis via *p53*- dependent induction of *p21* (Amin et al., 2009).

Effect of combination of both chemotherapeutic drugs on *p21* gene expression

Our data indicated that *p21* gene expression has been upregulated after treatment with combination therapy at a synergistic way more than either agent alone (Lin et al., 2012).

Furthermore, (Hou et al., 2018) demonstrated that combination of drug therapy that target different oncogenic cell signaling pathways can decrease the side-effects and complications in patients and increase the efficacy of treatment and hence decrease morbidity in comparison with conventional chemotherapy.

Finally, a combination of EGFR-TKI with other treatments could be a useful approach in Breast Cancer therapy (Su et al., 2016).

Effect of combination of both chemotherapeutic drugs on *PARP* gene expression

It is recognized that chemotherapeutic therapy can induce deoxyribonucleic acid damage, and repair of DNA damage could affect the outcome of therapy (Casorelli et al., 2012). (Song et al., 2018) showed that DNA damage, if not repaired correctly, can lead to genetic instability, that may lead to the development of cancer. So we can conclude with (Rojo et al., 2012) that DNA damage is an obligatory trigger for the activation of the abundant nuclear enzyme *PARP*. Moreover a number of investigators have determined that the activation of *PARP* can lead to rapid depletion of NAD and ATP with subsequent cell death (Filipovic et al., 1999).

In the present study qRT-PCR was performed to examine the association of *PARP* gene expression with therapeutic responses in MCF-7 breast cancer cell line treated with chemotherapy (SAHA, erlotinib and a combination of both drugs). Moreover our findings came in line with (Hou et al., 2018) which found that the expression levels of cleaved-caspase-3, cleaved-caspase-8, cleaved-*PARP* in the combination group were significantly increased compared with those in the control and the two individual groups.

Meanwhile, our results also showed upregulation of *PARP* gene expression in MCF-7 when treated with erlotinib in a higher manner than of those cells treated with combination therapy or SAHA.

At last Treatment with SAHA, erlotinib and a combination of both drugs synergistically induced activation *PARP* in both cell lines tested. These findings are indeed in line with previous literatures that elucidate the synergistic effect of combination treatment with multiple agents [Blumenberg, 2014; Siraj et al., 2018).

Effect of SAHA, Erlotinib and combination on apoptosis level

Apoptosis

Generally cancer cells that has been treated with a combination therapy demonstrated a significant enhancement in the antiproliferative and apoptotic effects of these treatments as compared to monotherapy alone (Bachawal et al., 2010).

In our study, the induction of apoptosis by SAHA, erlotinib, and their combination on MCF-7 breast cancer cell line was confirmed by annexin V/PI double staining using flow cytometry technique.

We found that Erlotinib promote apoptosis (late apoptosis) in MCF7 breast cancer cell line increasing from 0.27% to 25.9 % in treated cells while treatment with SAHA was found to induce late apoptosis but less significant comparing to MCF7 (from 0.27 % to 5.5% in treated cells and even more in combination therapy results showed also late apoptosis reached 23.68% and this could relay to that SAHA data (HDACis) can increase sensitivity to EGFR-TKIs and also can reverse resistance to EGFR-TKIs as reported in (Han et al., 2015).

Furthermore (Wu et al., 2017) confirmed that combined treatment of HDAC inhibitor vorinostat and epidermal growth factor receptor (EGFR) inhibitor synergistically induced apoptosis in MCF-7 cells.

Previous preclinical in vitro and in vivo studies have shown that treatment with SAHA induces cell cycle growth arrest, associated with differentiation and apoptosis of breast cancer cells (Bali et al., 2005).

Synergistic and additive tumor cell apoptosis has been observed when combining pan-HDAC inhibitors with cytotoxic therapies that induce DNA damage (Namdar et al., 2010).

Cell cycle arrest

our study also illustrated the effect of SAHA, erlotinib and a combination treatment on MCF7 cell line that underwent a significant increased cell cycle arrest in G2/M phase raised from 10.36% in MCF7 alone to 45.05% in MCF7 with erlotinib and 33.69% with SAHA and finally 51.06% with combination therapy.

These findings was supported by previous researchers who explained the efficacy of combination therapy in several settings (Zhang et al., 2008; Park et al., 2015; Librizzi et al., 2016).

Conclusion

In the present study we investigated the effects of the TKI (erlotinib) and HDAC inhibitors (vorinostat) as single and in combination on cell proliferation and survival by the MTT assay, respectively; apoptosis was also investigated by flow cytometry. Gene expression was evaluated by real-time polymerase chain reaction (RT-PCR), cell cycle distribution, apoptosis, and methylation pattern of both *P21* and *PARP* gene expression were measured.

Numerous lines of evidence have suggested that combination treatments including HDAC inhibitors with TKIs could have synergistic effects in cancer cells. These data provide evidence that synergistic antiproliferative effects of Erlotinib and Vorinostat are linked to the up regulation of *P21* and *PARP* genes and suggest that their combination may have therapeutic value in treatment of Breast Cancer.

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