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Pitavastatin Enhances Doxorubicin-induced Apoptosis in MCF7 Breast Cancer Cells

Saeb H. Aliwaini*, Tarek A. El-Bashiti and Khalid M. Mortaja

Department of Biology and Biotechnology, Faculty of Science, Islamic University of Gaza, Palestine

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Abstract

Breast cancer is the most common malignancy in women worldwide. While doxorubicin is part of the standard therapy for metastatic breast cancer, it has limited success. Pitavastatin has been shown to enhance the anti-cancer activity of certain therapeutics. The current study, therefore, explored the anti-cancer activity of the combined treatment of doxorubicin and pitavastatin in MCF7 breast cancer cells. Cell proliferation and viability assays demonstrated that combined doxorubicin and pitavastatin treatment resulted in synergistic cytotoxicity and cell death. Western blotting analysis showed that pitavastatin treatment resulted in increasing levels of p53 and the cell cycle regulator p21 in both doxorubicin treated and untreated cells. Furthermore, we demonstrated that apoptosis induced by the combined treatment occurs through the intrinsic pathway as evident from the activation of caspase 9, caspase 7 and the reduction of BCL-2 level. This study provides novel evidence to suggest that combined treatment of doxorubicin and pitavastatin may be effectively combined to treat breast cancer with the potential to minimize the side effects associated with high doses of doxorubicin.

Keywords: Apoptosis; Chemotherapy; Doxorubicin, MCF7; Pitavastatin, Synergism

1. Introduction

Breast cancer is the most common type of cancer worldwide, and it is estimated that one out of eight women will develop breast cancer in their lifetime (Cheng *et al*., 1998). In spite of the fact that most of the patients are diagnosed in early and curable stages, metastatic breast cancer occurs in one third of patients affecting bone, liver and lung, and ultimately leading to death (Malki *et al*., 2009). The natural anthracycline antibiotic doxorubicin is considered the most active single therapy available for metastatic breast cancer. Doxorubicin induces death of cancer cells by different mechanisms such as topoisomerase II-α (TOP2A) inhibition (Barni & Mandala, 2005). Although breast cancer is one of the chemo-sensitive tumours, its response to doxorubicin treatment ranges from 28% to 43% only (Taylor *et al*., 1991; AbuHammad & Zihlif, 2013). Doxorubicin resistance leads to an unsuccessful outcome in nearly 50% of treated patients, making resistance a major cause of treatment failure.

Statins are widely prescribed drugs used for the inhibition of the mevalonate pathway which is responsible for cholesterol synthesis in human cells (Gopalan *et al*., 2013; Warita *et al*., 2014). In addition, statins have a wide range of anticancer activities in different cancers (Al-Qatati and Aliwaini, 2017; Gopalan *et al*., 2013; Lee *et al*., 2012; Tu *et al*., 2011). For example, simvastatin was shown to induce cell cycle arrest through activation of Chk1 kinase and inhibition of Cdc25A, cyclin A and CDK2 in multiple myeloma cells (Tu *et al*., 2011). Simvastatin induced cell cycle arrest was accompanied by intrinsic apoptosis as demonstrated by diminished Bcl-2 protein levels, increased cytosolic cytochrome c and active caspase 9 and caspase 3 levels. Recent studies have shown that erivastatin, pitavastatin and fluvastatin are potent anti-proliferative drugs in glioblastoma cells (Lee *et al*., 2012; McFarland *et al*., 2014). Other studies showed the ability of fluvastatin to reduce tumour growth in high-grade, stage 0/1 breast cancer patients (Garwood *et al*., 2010). Pitavastatin has been shown to exert potent cytotoxic effects on glioblastoma growth in vivo (Lee *et al*., 2012). The mechanism of action of pitavastatin includes up regulation of the cell cycle regulator p21 and inhibition of NF-κB, which resulted in cell cycle arrest and apoptosis (Fujino *et al*., 2006; Wang J, e*t al*., 2006). Interestingly, autophagic cell death was also shown to be induced by pitavastatin (Tsuboi *et al*., 2009). Finally, our recent findings showed that pitavastatin and dacarbazine synergistically inhibit melanoma cell survival by inducing cell cycle arrest, apoptosis and autophagy (Al-Qatati and Aliwaini, 2017). Whether pitavastatin has the ability to enhance doxorubicin anticancer activity is mostly unknown.

The present study therefore aimed to explore the effect of combined pitavastatin and doxorubicin treatment in breast cancer cells. The presented data shows that pitavastatin enhances doxorubicin induced cytotoxicity in MCF7 cells and further demonstrates that this occurs through induction of intrinsic apoptosis.

* Corresponding author e-mail: Saib.iwini@gmail.com; siwini@iugaza.edu.ps.

2. Materials and Methods

2.1. Cell culture and treatments

The human breast cancer cells MCF7 were obtained from Prof. Rana Abu-Dahab, The University of Jordan. They were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% CO_2 balanced air incubator at 37°C. Pitavastatin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and doxorubicin (Sigma-Aldrich, St Louis, MO, USA) were dissolved in dimethyl sulfoxide (DMSO) and water, respectively, to give stock concentration of 5 mM which were stored for no more than 5 days. Control cells were treated with equivalent concentrations of DMSO (vehicle).

2.2. Cytotoxicity assays

Breast cancer cells were seeded in 96-well plates at 8×10^3 cells per well and allowed to settle for 48 hours. Cells were treated individually with 0-5.0µM of pitavastatin, or 0.0- 0.5µM doxorubicin or vehicle for 48 hours (Al-Qatati and Aliwaini, 2017). Cytotoxicity was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Aliwaini *et al*., 2013) as per manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Briefly, 10 µL of MTT solution were added to each well and cells were incubated at 37°C for 4 hours, followed by addition of 100 µL solubilization buffer (10% sodium dodecyl sulfate /SDS) in 0.01M hydrochloric acid (HCl) and incubation for 16 hours at 37°C. Absorbance at 585 nm was determined for each well and the mean cell viability was calculated as a percentage of the mean cell survival of the vehicle control.

2.3. Viability assay

The trypan blue exclusion method was used to determine the total cell number and the proportion of live and dead cells. Briefly, MCF-7 cells were grown on 6-well plates at a density of 6×10^4 cells/well. After 24 hours, the cells were treated with different concentrations either of doxorubicin or pitavastatin for 48 hours. To determine whether the combined treatment of pitavastatin and doxorubicin may have a greater anti- cancer effect on MCF7 cells than doxorubicin alone, breast cancer cells were treated with 1 µM pitavastatin for 1 hour followed by treatment with increasing concentrations (0.05-0.5µM) of doxorubicin for 48 hours. After the 48 hours, cells were trypsinized, centrifuged, resuspended in 0.5 ml of the medium, mixed thoroughly with 50 µl of 0.4% Trypan blue (w/v), at room temperature for 5 min. The cells were then counted using a hemocytometer. Each experiment was repeated three times with triplicate samples in each. Statistically, the percentage of cell death was calculated by counting at least 300 cells per sample in several randomly selected fields using the formula of percentage of cell death = number of dead cells/number of total cells × 100.

2.4. Western blotting

To test whether pitavastatin induces cell cycle arrest and apoptosis, breast cancer cells were treated with vehicle, pitavastatin (1.0µM), doxorubicin (0.3µM) or pitavastatin- doxorubicin (1.0 and 0.3 µM, respectively) and western blotting with an antibody to p53 and p21, PARP cleavage, caspase 7, caspase 9, BCL-2 was performed. Cells were harvested and protein prepared as described previously (Aliwaini *et al*., 2013). Primary antibodies used were: anti-PARP1/2 (sc-7150), anti-p53 (sc-126), anti-p21 (sc- 756), anti-caspase 7 (sc-56063) and anti-caspase 9 sc-56076 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-actin (Sigma, St. Louis, MO, USA), anti-BCL-2 (sc-509). After primary antibody incubation, membranes were incubated with HRP-conjugated secondary antibodies at appropriate ratio (1:5000) (BioRad) and antibody-reactive proteins were visualized using the electrochemiluminescence reaction (ECL) detection system (Thermo Scientific, Hudson, NH, USA).

2.5. Statistical analysis

Results presented are the means ± SEM (the standard error of the means) of three independent experiments. Statistical analysis of data was performed using the 2-sample *t*-test (Excel, Microsoft, Redmond, WA, USA) or two-way ANOVA (Graph Pad Prism, La Jolla, CA, USA) and $p<0.05$ was considered statistically significant.

3. Results

3.1. Pitavastatin enhances doxorubicin induced death of breast cancer cells

The anti-proliferative effect of pitavastatin on breast cancer cell line was determined by MTT assay. MCF7 cells were treated using different concentrations (0.0µM–5.0µM) of pitavastatin. The results showed that pitavastatin exerts a pronounced anti-proliferative effect on MCF7 cells with an IC50 value of (3.38µM) (Figure 1a). While low concentrations of pitavastatin (0.5µM) and (1.0µM) show little cytotoxic effect on MCF7 cells, 5µM induces a very strong cytotoxic effect.

To confirm the inhibitory action of doxorubicin on MCF7 cells, the cells were treated with the drug at concentrations of 0.0µM–5.0µM and the proliferative activity was determined by MTT assay. The results show that both high and low concentrations of doxorubicin have cytotoxic effect on MCF7 cells with IC50 of (0.29µM). Low concentration of doxorubicin (0.25µM) killed 30 % of MCF7 cells, while high concentration (0.5µM) killed 60 % of MCF7 cells (Figure 1b). Figure 1b demonstrates that the combined treatment resulted in enhanced cytotoxic activity compared with doxorubicin treatment alone. While doxorubicin treatment induced 50% inhibition at 0.29µM, pre-treatment of cells with 1µM pitavastatin for 1 hour resulted in ~80% cell death at 0.3µM doxorubicin concentration.

To test the ability of pitavastatin to increase the percentage of cell death induced by doxorubicin, breast cancer cells were treated with 1µM pitavastatin for 1 hour followed by treatment with doxorubicin of for 48 hours. Figure 1c demonstrates that while doxorubicin treatment induced about 40% cell death, combined treatment resulted in about 70% cell death. These results demonstrate that combined pitavastatin and doxorubicin treatment cause a synergistic cytotoxic effect in breast cancer cells.

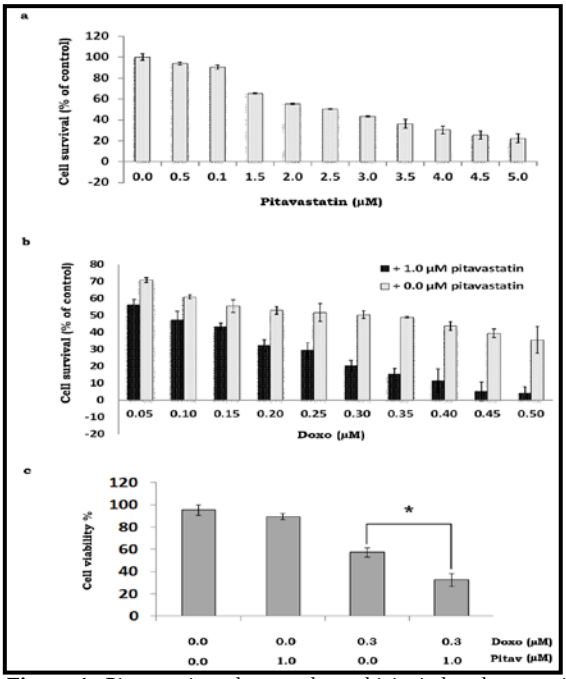

Figure 1. Pitavastatin enhances doxorubicin induced cytotoxic effect in breast cancer cells**.** Cell survival (MTT assay) of breast cancer cells (MCF7 cells) treated with increasing concentrations of pitavastatin (0.0μM - 5.0μM) or vehicle for 48h (a). MCF7 cells were treated with 1.0 μM pitavastatin for 1 hour and increasing concentrations (0.05-0.5μM) of doxorubicin (b). MCF7 cells treated with single treatment (vehicle, pitavastatin or doxorubicin) or combined treatment of 1.0 μM pitavastatin and 0.3μM doxorubicin (c). Results are presented as the mean percentage ± standard error of the mean of untreated cells and represent the pooled results of at least three experiments performed in quadruplicate.

3.2. Pitavastatin activates cell cycle regulators p53 and p21 levels in MCF7:

Figure 2 shows that p53 and its downstream target p21 increased in response to pitavastatin treatment. More importantly, while doxorubicin treatment also induced slight increase in both proteins, the combined treatment significantly increased both p53 and p21 levels.

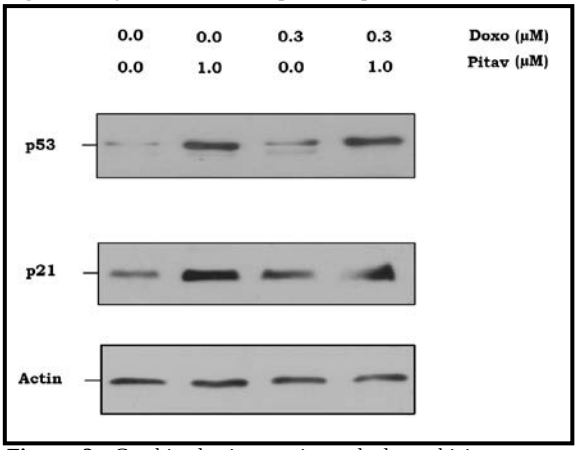

Figure 2. Combined pitavastatin and doxorubicin treatment activates cell cycle regulators. Breast cancer cells were treated with 1.0 μM pitavastatin (Pitav), 0.3μM doxorubicin (Doxo), pitavastatin- doxorubicin (1.0 and 0.3μM, respectively) or vehicle. Protein extracts were analyzed by SDS-PAGE (8-15%) and western blotting was performed by using antibodies against p53 and p21. Actin was detected as a loading control.

3.3. Pitavastatin increases apoptosis induced in MCF7 by Doxorubicin:

Total protein was extracted and resolved by SDS-PAGE and analyzed by western blot for cleaved PARP1. Figure 3 shows that combined treatment of pitavastatin-doxorubicin induced high level of PARP cleavage. However, single treatment of doxorubicin (0.3μM) or pitavastatin (1.0μM) induced very low levels of PARP cleavage. Figure 3 further shows that pitavastatin, doxorubicin and the combined (pitavastatin-doxorubicin) treatments decreased the anti-apoptotic protein BCL-2 and increased the apoptotic proteins (active caspase 9 and active caspase 7).

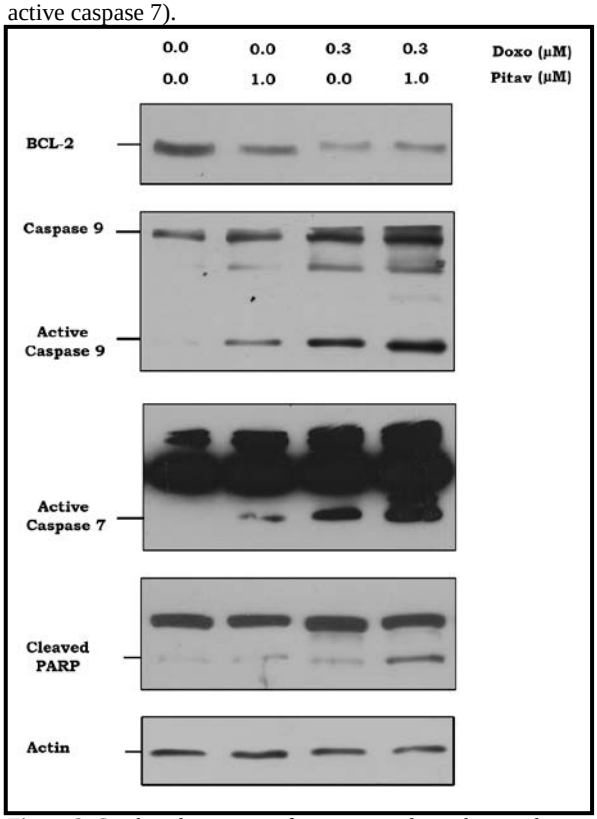

Figure 3. Combined treatment of pitavastatin-doxorubicin induces intrinsic apoptosis in breast cancer cells. MCF7 cells were treated with either vehicle, 1.0μM pitavastatin, 0.3μM doxorubicin or pitavastatin- doxorubicin (1.0 and 0.3μM, respectively). Protein extracts were analyzed by SDS-PAGE (8 and 15%) and western blotting using antibody to BCL-2, caspase 9, caspase 7 and cleaved PARP. Actin was detected as a loading control.

4. Discussion

Breast cancer continues to be the leading cause of cancer deaths among women, and its treatment is constantly evolving as new technologies, drugs, and strategies are discovered (Westbrook and Stearns, 2013). The choice of therapy depends on the molecular profile and the stage of breast cancer (Massarweh and Schiff, 2006). Doxorubicin is one of the most active therapeutics for metastatic breast cancer which inhibits tumor growth by inducing cell cycle arrest and apoptosis (Barni and Mandala, 2005). In spite of its strong anti-cancer activity, doxorubicin can treat less than 43% of metastatic breast cancer patients (Taylor *et al.*, 1991; AbuHammad and Zihlif, 2013;). In addition to that, doxorubicin resistance

leads to an unsuccessful outcome in nearly 50% of treated patients. Therefore, there is a need for new strategies to empower the anticancer effect of doxorubicin and to decrease its side effect.

3-Hydroxy-3-methylgutarylCoA(HMG-CoA) reductase inhibitors, commonly referred to as the statins have therapeutic and preventative effects in different diseases (Chan *et al*., 2003). Interestingly, statins exert anti-proliferative and anti-cancer effect against a range of types of tumour (Tsuboi *et al*., 2009; Tu *et al*., 2011; Yang and Chen, 2011). The present study, therefore, explored the *in vitro* efficacy of a combined treatment of doxorubicin with pitavastatin in human breast cancer cells. The results of the present study provide several lines of evidence to suggest that pitavastatin may synergistically improve the anti-cancer activities of doxorubicin. The inhibitory concentration 50% (IC_{50}) of doxorubicin in MCF7 ranges between 0.3 to 0.6 µM as reported by previous studies (Osman *et al*., 2012; Buranrat *et al*., 2017). This is close to the results of the current study where doxorubicin inhibited MCF7 growth with an IC50 of 0.29µM. On the other hand, previous studies showed that pitavastatin has a potent cytotoxic effect against MCF7 cells with IC50 around 10 µM (Wang and Kitajima, 2007). The results of our study showed that pitavastatin inhibits MCF7 cells with IC50 of 3.38µM. The difference between the two IC50s might be ascribed to the different techniques used in the two studies. The present study further demonstrated that cancer cells pre-treated with pitavastatin (1µM) are sensitised to doxorubicin resulting in ~50% cell death at doxorubicin dose less than 0.1µM.

Furthermore, the present study revealed that the mechanism of action by which pitavastatin-doxorubicin combined treatment inhibits cancer growth involves cell cycle arrest and intrinsic apoptosis. P53 is major anticancer transcription factor which induces growth arrest and or apoptosis in cells exposed to chemotherapies. Previous studies provided evidence that doxorubicin mediated cytotoxicity depends at least partially on its ability to activate of p53 protein (Kotamraju *et al*., 2000; Wang *et al*., 2004). Similarly, other studies have indicated that doxorubicin treatment results in cell cycle arrest at different phases, and this was accompanied with increasing levels of p53 and p21 proteins (Vali *et al*., 2015; Buranrat *et al*., 2017). The current study showed similar results, as doxorubicin treatment induced slightly increasing level of p53 and p21. The increase in the cycle regulator p21 is usually considered a marker of cell cycle arrest at G1 phase (Aliwaini *et al*., 2015). These data confirm previous data that doxorubicin treatment stops breast cancer cell cycle at G1 stage. Furthermore, doxorubicin has been shown to induce a high level of active caspase 3, an established marker of apoptosis (Buranrat *et al*., 2017). The same study showed that doxorubicin induces intrinsic apoptosis mainly as evident by the release of cytochrome c from mitochondria of breast cancer cells. Similar data was also recorded by our study, as doxorubicin induced high level of active caspase 7 and 9 which are documented markers of intrinsic apoptosis (Aliwaini *et al*., 2015).

Notably, pitavastatin was also demonstrated to inhibit proliferation of breast cancer cells in a dose-dependent manner and to inhibit NF-κB pathway (Wang and Kitajima, 2007). However, there is no adequate data in literature whether pitavastatin induces cell cycle arrest and/or apoptosis and what the exact mechanism behind its inhibitory effect is. The current study provides evidence that pitavastatin treatment mainly increases p53 and p21 proteins and induces cell cycle arrest. This effect is significantly increased when breast cancer cells are treated with both pitavastatin and doxorubicin.

Furthermore, the present study demonstrated that the cytotoxic effect of pitavastatin-doxorubicin includes the induction of intrinsic apoptosis. Notably, whilst doxorubicin treatment induced low level apoptosis, as evident by PARP cleavage and caspases level, combined pitavastatin-doxorubicin treatment resulted in significant levels of apoptosis as evidenced by the increased level of apoptosis markers.

5. Conclusions

The presented results suggest that the combined treatment of pitavastatin-doxorubicin provides a synergistic anti-cancer effect through cell cycle arrest and apoptosis. While the study was performed in vitro, there is a need to determine if pitavastatin would protect against Dox-induced chronic cytotoxicity, and enhance Dox anticancer activities against breast cancer in vivo. The strategy of combined treatment may potentially be applied in future clinical trials and, if successful, could help to improve chemotherapy selection for the benefit of breast cancer patients.

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