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Synergistic anticancer effect of combination treatment of vitamin D and pitavastatin on the HCC1937 breast cancer cells

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KEYWORDS

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Pitavastatin

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Apoptosis

Cell cycle arrest

ABSTRACT

Vitamin D (Vit D) has anticancer properties including activating cell senescence inhibiting cancer cell proliferation, inducing apoptotic cell death, and decreasing cancer cell migration. On the other hand, statins showed favorable anticancer activities including anti-survival, anti-proliferation, and anti-migration effects. The current study aimed to investigate the synergistic anticancer effect of Vit D and statins against HCC1937 triple-negative breast cancer cells. The antiproliferative effect was tested by MTT assay after 48 hours of the treatments. Trypan blue test and clonogenic assay were used to test the anti-survival activities of the treatments. The ability of the treatments to inhibit the migration ability was tested by scratch assay. Levels of the cell cycle and apoptotic markers were determined by western blotting. Results of the study revealed that all the tested compounds including Vit D, atorvastatin (Ator), simvastatin (Simv), and pitavastatin (Pita) inhibited HCC1937 breast cancer cell growth with different IC_{50} values ranging from 4.49-12.95 µM. Combined application of Pita and Vit D showed potent synergistic antiproliferative activities against HCC1937 breast cancer cells. The combined therapy of (1µM Vit D and 2 µM Pita) inhibited HCC1937 cell proliferation by cell cycle arrest and apoptosis as evidenced by increasing p21, p53, and cleaved PARP. Finally, the combined treatment decreased the p-STAT3 level in HCC1937 breast cancer cells. The results of the study can be concluded that the combined treatment of Pita and Vit D has a synergistic anticancer effect against HCC1937 breast cancer cells.

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1 Introduction

Nowadays, breast cancer is the most common invasive cancer in worldwide women. Based on WHO 2021 statistics, cancer is the second leading cause of death globally, and among the most commonly reported cancers, female breast cancer (**12**%) is the most frequently diagnosed cancer in the world (Sung et al. 2021). In Palestine, Breast cancer was the third largest cause of cancer mortality in 2018 at 12% and a more than 135% rise in breast cancer cases is expected by 2040 (AlWaheidi 2019). Immunohistochemical analysis revealed that breast cancers that express the estrogen receptor, progesterone receptor or both respond well to hormone therapy(Aliwaini et al. 2019; Porras et al. 2021). Another important progress in breast cancer therapy was identifying and targeting the Her2 subtype of epidermal growth factor receptors (EGFR) which improved the outcome of Her2-positive patients (Aliwaini et al. 2021). Triple-negative breast cancers (TNBCs) are called such because they lack receptors for estrogen, progesterone, and Her2. TNBCs are highly aggressive and resistant to conventional chemotherapy and are more common in individuals of African descent (Seachrist et al. 2021). It is important to note that more than 70% of TNBCs overexpress genes implicated in metastasis and invasion as well as genes involved in proliferation and resistance to apoptosis including AKT, PI3K, RAS, and NF-Kb (Porras et al. 2021). More importantly, mutations in p53 is reported to be one of the most common features of TNBCs and several studies indicate that these mutant p53 proteins enhance tumorigenesis and treatment resistance (O'Grady et al. 2020). Nowadays many researchers studied several anticancer agents and evaluated both monotherapy and combination therapy with currently used therapies to treat cancers and reduce the development of cancer cells in many organs (Aliwaini 2020).

Vitamin D (calcitriol) can be either endogenously synthesized in the skin from 7-dehydrocholesterol, upon exposure to the sun's ultraviolet light, or, in a small portion, can be absorbed from the diet (Young et al. 2021). There are two types of Vit D; the main form 25-hydroxycholecalciferol (25 (OH) D) and hormonal form 1, 25-dihydroxycholecalciferol (1, 25 $(OH)_2D$) in the liver and kidneys (Young et al. 2021). Several diseases have been found in animal studies to respond positively to Vit D and 1, 25 $(OH)_2D$ or their analogs, there have also been promising observations regarding adequate Vit D nutrition and cancer prevention that affects the development of cancer (Liu et al. 2021).

O'Brien et al.(2022) stated that statins are routinely used to treat hyperlipidemia, but may also have antineoplastic effects. It can also be applied to the treatment of other diseases, particularly cancer, in which progression depends on increased migration, survival, and ultimately proliferation (Göbel et al. 2019). Statins competitively inhibit the rate-limiting enzyme of the mevalonate pathway, 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) leading to low levels of isoprenoids geranylgeranyl pyrophosphate (GGPP) and farnesyl pyrophosphate (FPP) (Guerra et al. 2021; Razali et al. 2018). The current study tested the possible synergistic cytotoxic effect between statins (simvastatin, atorvastatin, and pitavastatin) and Vit D. The most potent combination of Vit D and pitavastatin was further investigated.

2 Materials and methods

2.1 Cell culture

Breast cancer cells HCC1937 were maintained in RPMI1640 supplemented with 10% FBS, 2 mM l-glutamine, 100 IU/Ml penicillin, and 100 µg/mL streptomycin. Cells were cultured at 37°C in a humidified CO_2 incubator and subcultured every 3-5 days.

2.2 Cytotoxicity assay

Cell viability was determined by measuring the capacity of reducing enzymes present in viable cells to convert MTT to formazan crystals as described by Aliwaini et al. **(**2021). Briefly, cells were incubated with increasing concentrations of the following combinations Vit D + simvastatin, Vit D + atorvastatin, or Vit D + pitavastatin and incubated for 48 hrs, then 10 µL MTT was added to each well. After 4 hrs of incubation at 37°C, 100 µL of the solubilizing solution was added to each well, with shaking for 1 hr to dissolve the formazan crystals. The color intensity of the blue formazan solution formed in each well was measured at 570/690 nm using a BIO-TEK Instruments EL 312e microplate reader (Bio-Tek Instruments, Winooski, VT). The percentage of cell viability was calculated relative to vehicle-treated control designated as 100% viable cells. Data were fitted to a sigmoidal dose-response model (GraphPad Prism®, GraphPad Software Inc).

2.3 Clonogenic survival assay

The clonogenic survival assay was performed to determine the long-term survival of HCC1937 cells after different treatments. Cells were seeded and treated with statins, Vit D, or the combination of Vit D and Pita Twenty-four hours after treatment, cells were trypsinized, re-suspended in fresh medium, and re-plated at a low density of 1000 cells per well in 6-well plates. Cells were allowed to grow and monitored for colony formation for 14 days. Media were routinely changed every 2 to 3-day intervals. Live cells were washed 3 times with 1 X PBS, fixed for 15 minutes in methanol: acetic acid (3:1), and excess fixative 3 times washed off with 1 X PBS. Thereafter cells were stained for 15 minutes with 0.5% crystal violet (Sigma-Aldrich, USA) in 100% methanol (Aliwaini et al. 2015). Colonies were imaged and both size and number of colonies were quantified using Image J together with Origin Pro 2021 software. The plating efficiency was calculated and presented ±SEM.

2.4 Growth curve assay

Breast cancer cells were seeded for 24 hours to settle and then treated with Vit D, Pita, the combination (Vit D and Pita), or vehicle. The number of cells was assessed after 24, 48, and 72 hours of the treatment.

2.5 Scratch motility assay

Cells were grown to confluence and a linear scratch was made through the monolayer using a sterile 200 µl pipette tip. To remove cell debris, the growth medium was replaced and several markings were made along the edges of the scratch line which were used as reference points and the wound widths were measured at the time of the scratching (0 hours) and after the indicated treatments. Pictures were taken using an inverted light microscope (Olympus 1X71, USA) and camera (Zeiss AxioCam, Germany) respectively. Migration distances were measured using Axiovert software (Zeiss, Germany).

2.6 Western blotting

Cells were harvested and protein was prepared as described previously (Aliwaini et al. 2021). Primary antibodies used for western blotting are anti-p-Stat3 (sc-8059), anti-PARP1/2 (sc-7150), anti-caspase-9 (sc-56076), anti-p53 (sc-126), anti-p21 (sc-756) and anti-α-Tubulin (sc-8035) (Santa Cruz, California, USA).

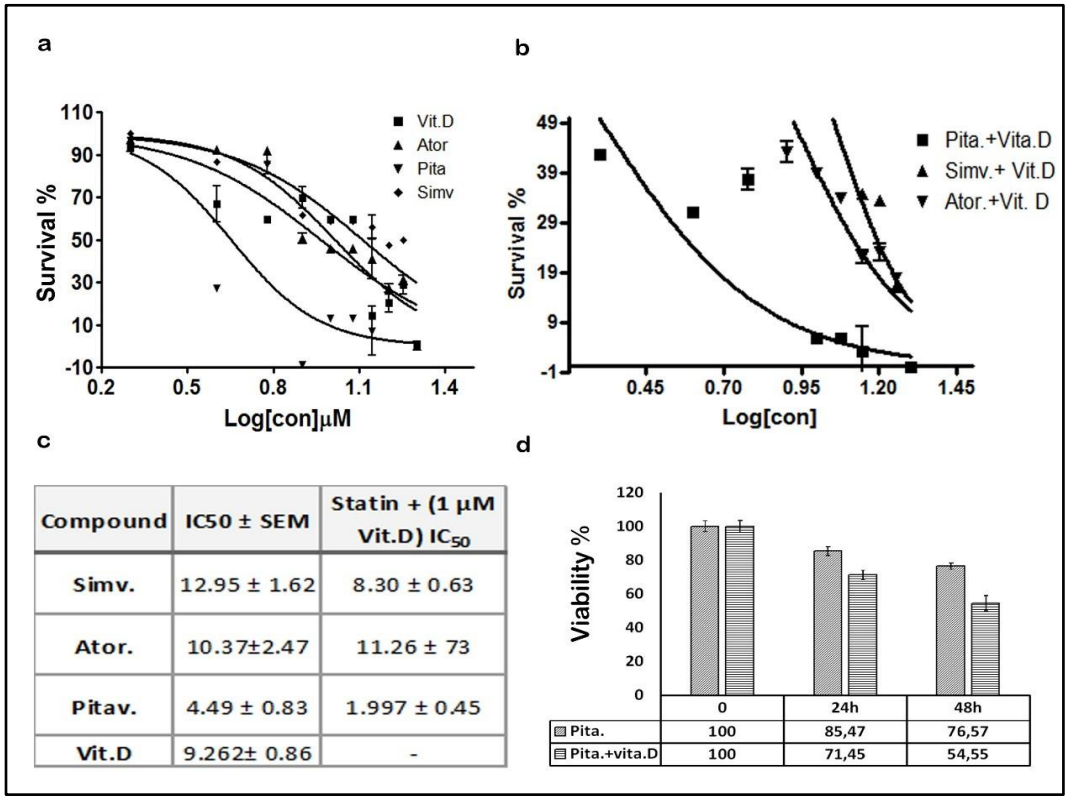
2.7 Statistical analysis

Data presented are mean ± SEM (Standard error of the means) of appropriate replicates. Statistical significance was assessed between the groups using the Student‘s t-test. A value of $P< 0.05$ was accepted as statistically significant.

3 Results

3.1 Effect of Statins and Vit D on HCC1937 breast cancer cell line proliferation

We initially tested the antiproliferative effect of three widely used statins including atorvastatin (Ator), simvastatin (Simv), and Pita (Pita) and Vit D (Vit D) on the viability of HCC1937 breast cancer cells. Results presented in figure 1 (a) revealed that low concentrations (less than 20 µM) of all tested statins and Vit D (less than 10 µM) induced significant anti-proliferative effects in a dose-dependent manner. In



Compound	IC50 ± SEM	Statin + (1 µM Vit.D) IC_{50}
Simv.	12.95 ± 1.62	8.30 ± 0.63
Ator.	10.37±2.47	11.26 ± 73
Pitav.	4.49 ± 0.83	1.997 ± 0.45
Vit.D	9.262± 0.86	-

Figure 1 Cytotoxicity of Vit D and statins against breast cancer cells (1 a and b); Cell viability was tested using the MTT assay and data were expressed as a percent of vehicle-treated control ± SEM ; (1 c) table summarizes the IC_{50} values of different treatments; (1 d) Cell viability was assessed by trypan blue assay after 48 hours of the indicated treatments. The data represents pooled results of at least three independent experiments performed in quadruplicate.

comparison to other statins, Pita showed the most potent antiproliferative effect with an IC_{50} of 4.49 µM. To test the possible synergistic effect of different statins with Vit D, cells were pretreated with a low dose of Vit D (1µM) and then treated with Simv, Atoror, and Pita, and were tested by MTT assay. Figure 1 (b, c) shows that both Pita and Simv have more potent cytotoxic effects in the presence of Vit D than the single treatment of each of them. However, the most synergistic effect was observed by combining Pita and Vit D treatments. The IC_{50} value of Pita in the presence of Vit D was 1.997 µM. While Pita treatment induced a significant cell death of about 24 % after 48 hours of the treatment, the combined treatment (Pita and Vit D) killed more than 45 % of breast cancer cells (Figure 1d). Altogether, these data indicate that the combined treatment of Pita and Vit D has a significant cytotoxic effect against HCC1937 breast cancer cells.

3.2 Effect of combined treatment of Pita and Vit D on growth and colony formation ability of breast cancer cells

Both Vit D and Pita were found to effectively reduce the clonogenic formation of cancer cells in vitro (Figure 2 a, b). However, this reduction was augmented by combining Pita and Vit D. The percentage of plating efficiency decreased from 37% for control cells to about 10% for the combination (Vit D + Pita) treated cells. To examine the synergistic effect of Vita D and Pita on the growth of human breast cancer cells, a growth curve assay was performed. Results of this study revealed that both Vit D and Pita treatments inhibited the growth rate after four days of the treatment. However, the combined treatment inhibited cancer cells more significantly (Figure 2 c). Taken together, these data show that the combined treatment of Vit D and Pita inhibits the growth and colony formation ability of HCC1937 breast cancer cells.

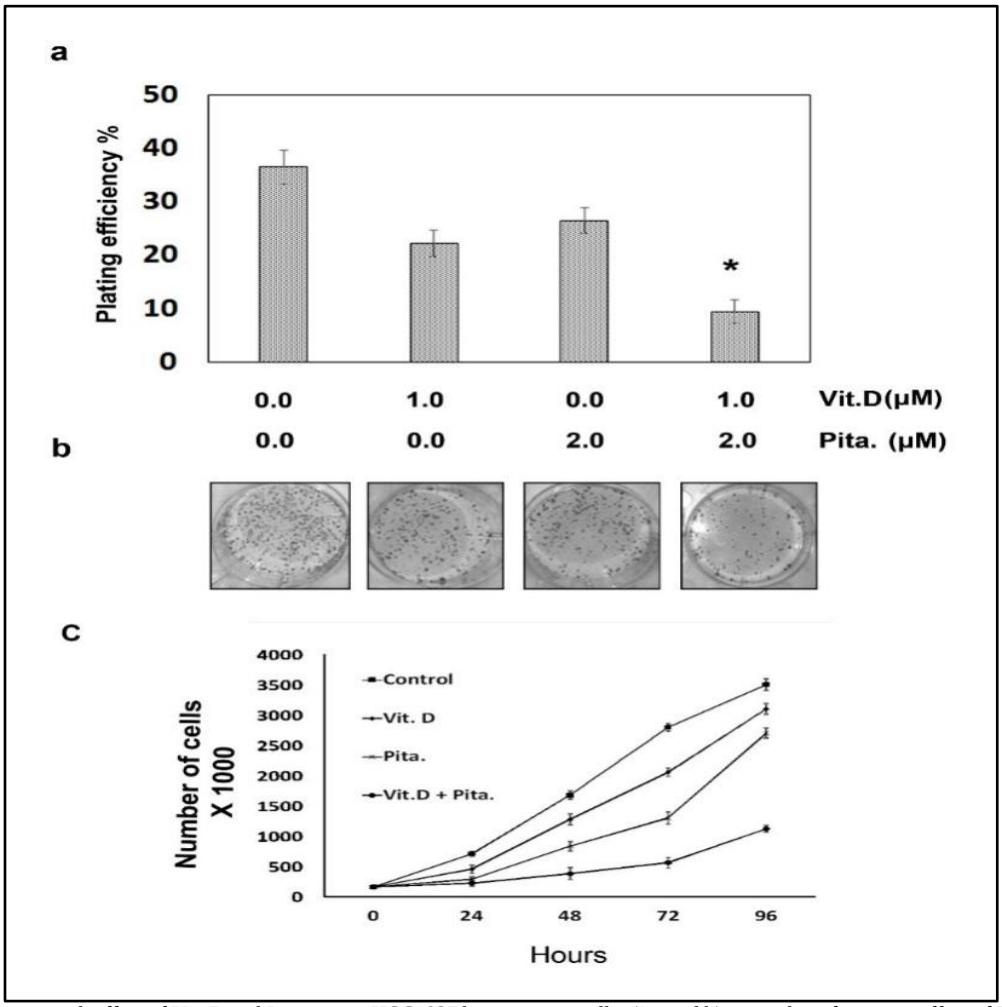


Figure 2 Anti-growth effect of Vit. D and Pita against HCC1937 breast cancer cells; (2 a and b) anti-colony formation effect of Vit D and Pita against HCC1937 cells (2a) indicated the effect of the treatment on the plating efficiency of the cell lines, (2b) Representative images of clonogenic results for HCC1937 cells treated as indicated on the figure, (2c) The curves of HCC1937 breast cancer cell line treated as indicated in the figure. The difference was significant after the sixth day ($P<0.05$).

3.3 Combined application of Pita and Vit D on the migration ability of breast cancer cells

To further explore the anti-migration activity of the combined treatment (Vit D and Pita), a wound-healing assay was performed. HCC1937 cells were exposed to Pita, Vita D, or the combination of Vit D and Pita for 48 hours. Results of the study revealed a significant reduction in cell motility in all tested treatments; however, the combination of Vit D and Pita showed the most potent anti-migratory effect (Figure 3 a, b). Results of the study also suggested that the combination of these two agents inhibits the migration ability of HCC1937 cells by more than 50% after 48 hours of the treatment.

3.4 Effect of combined treatment of Vit D and Pita on cell cycle arrest in HCC1937 cells

Application of Vit D and Pita as a single treatment or in combination can also induce cell cycle arrest. Levels of the cell cycle markers were determined by western blotting for a protein harvested from HCC1937 breast cancer cells treated with single or combined treatments of (1µM Vit D or 2µM Pita). Results presented in Figure 4 show that Vit D as a treatment or combination with Pita inhibits the phosphorylation of p-STAT3 protein in HCC1937 cells. Importantly, combined treatment of Vit D (1µM) and Pita (2µM) induced lower levels of p-STAT3 than the single treatment. Inhibition of p-STAT3 led us to test the possible effect of single or combined treatments on the expression of P53 protein which plays an important role in cell cycle regulation by stimulating the p21 gene to express P21 protein. P21 protein inhibits Cdk and induces G1 cell cycle arrest. The results presented in Figure 4 revealed that the combined treatment induces high levels of P53 and P21 proteins in the HCC1937 cell. Altogether, these findings clearly show that combined treatment of Vit D and Pita induces G1 cell cycle arrest by inhibiting the STAT3 pathway and inducing P53 and P21 expression in HCC1937 breast cancer cells.

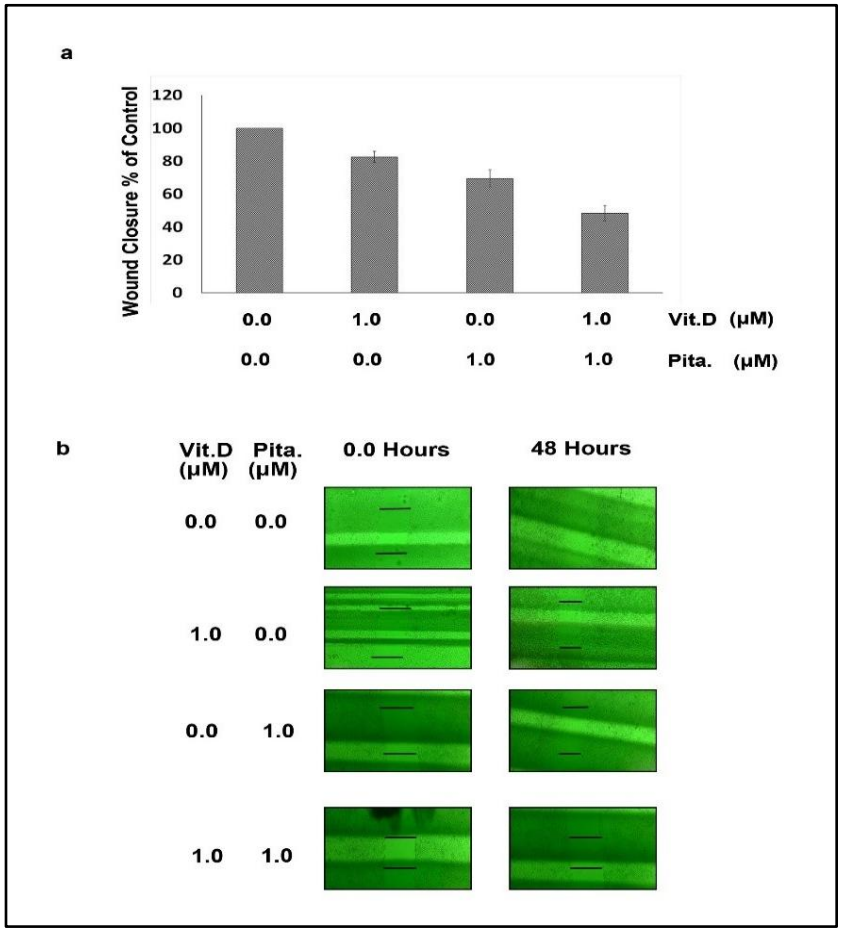


Figure 3 Vit D and Pita inhibited the migration of breast cancer cells; (3a) The graph shows the ability of the treatments to inhibit the migratory ability of HCC1937 breast cancer cells (3b) At specified time points (0 and 96) hours cells were photographed using (10x; Olympus 1X71). Assays were done in duplicate and two independent experiments were performed.

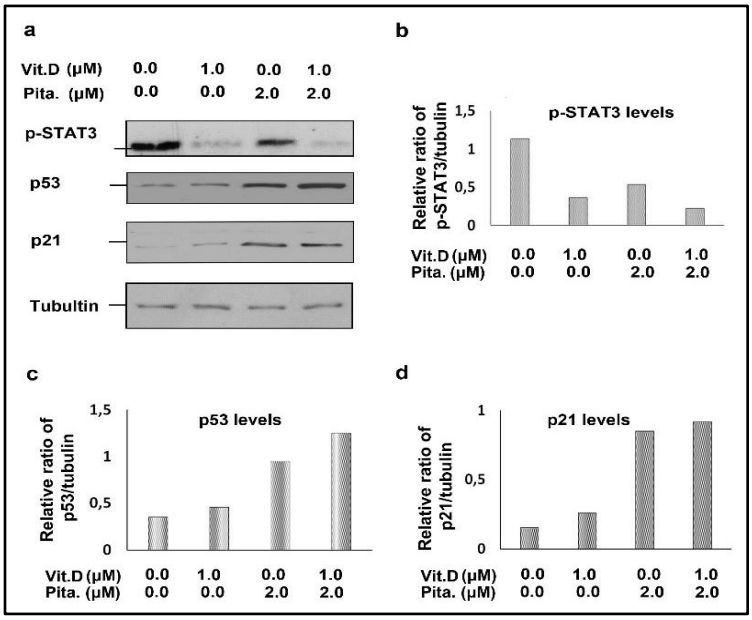


Figure 4 Vit D and Pita treatments induced cell cycle arrest in HCC1937 breast cancer cells. (4a) Western blotting of proteins from the cancer cells treated with indicated treatments for 48 hours and analyzed with antibodies to p STAT-3, p53, and p21. Tubulin was used as a loading control; (4 b,c,d) Densiometric readings of the specific proteins relative to tubulin

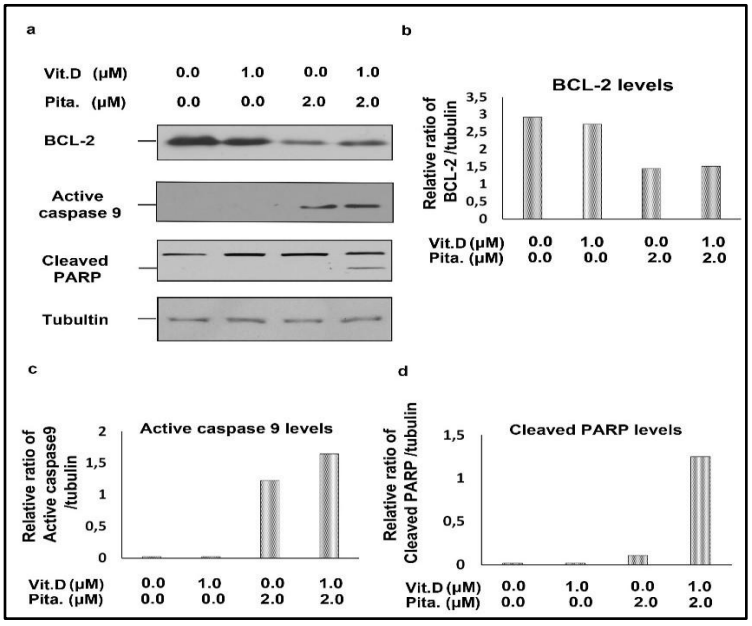


Figure 5 Vit D and Pita treatments induced apoptosis in HCC1937 breast cancer cells, (5a) Western blotting of proteins from the cancer cells treated with indicated treatments for 48 hours and analyzed with antibodies to BCL-2, caspase 9 and cleaved PARP. Tubulin was used as a loading control; (5 b,c,d) Densiometric readings of the specific proteins relative to tubulin

3.5 Combined treatment of Vit D and Pita induces apoptosis in HCC1937 cells

To test whether Vit D, Pita, or the combination (Vit D and Pita) also induces apoptosis in cancer cells, in this study, western blotting was performed and apoptotic markers were detected. Results presented in Figure 5 showed that the combined application of Vit D and Pita downregulated BCL-2, the anti-apoptotic protein, and induced both active caspase 9 and cleaved PARP after 48 hrs of the treatment. These findings indicate that combined treatment of Pita and Vit D induces intrinsic apoptosis in HCC1937 cells.

4 Discussion

Several previous studies showed that statins have effective anticancer properties on several types of cancer cells. In 2021, Rezano et al. (2021) showed that simvastatin has a cytotoxic effect on MDA-MB-231 and MCF7 with an IC_{50} of 4.5μM and 8.9μM respectively (Rezano et al. 2021). Similarly, O'Grady et al. (2020) found that simvastatin has an IC_{50}value of 40.8 μM while it was reported 49.1μM for atorvastatin on triple-negative breast cancer cell lines compared to non-triple negative breast cancer cell lines (O'Grady et al. 2020; Rezano et al.2021). Tilija Pun et al. (2022) showed that Pita induced apoptosis in oral (SCC4 and SCC15) and colon (HT29, HCT116, and SW480) cancer cell lines when treated with 0.25–0.5 μM of Pita In 2020, in vitro study by Aliwaini et al. (2021) showed that combined treatment between Pita and doxorubicin has a synergistic cytotoxic antiproliferative effect against MCF7 breast cancer cells with an inhibitory concentration of 1 μM.

Based on the results of this study, it can be suggested that Vit D has strong effects on the cell cycle where it plays a critical role in regulating proteins related to the cell cycle checkpoints such as P53, P21, P27, and Cdk. Many researchers and reviewers had highlighted that there was an association between Vit D and cell cycle arrest. Li et al. (2019) reported that Vit D3 could increase gene expression of Vit D receptor (VDR) and p21 in pancreatic cancer cells (PC). Similarly, Bao et al. (2014) also investigated the antiproliferative properties of Vit D and cisplatin with single or combined treatment on gastric cancer cells. The same study showed that Vit D had a synergistic effect on gastric cancer cells (GC) by inducing cell cycle proteins such as p21 and p27 and there was a larger number of cells in the G0/G1 in the combined treatment than the single treatment (Bao et al. 2014)**.** Ya-li zhang et al.(2021) have reported that Vit D had a synergistic effect on the K562 cell line and its effect was significantly increased after being treated in a combination with arsenic trioxide. Vit.D treatment induced G_0/G1 cell cycle arrest and elevated the mRNA levels of VDR, p21, and p27 significantly (Charoenngam and Holick 2020; Morris 2005). Along with the same line, other researchers such as Zheng et al. (2019) evaluated the anticancer activity of different concentrations of Vit D on MCF7 and MDA-MB-453 breast cancer cell lines after 48 hours. They observed that Vit D treatment downregulated the gene expression of Ras and ERK and induced cell cycle arrest in both breast cancer cell lines (Zheng et al. 2019).

Several publications like Lee et al. (2020) evaluated the anticancer activity of Pita on oral squamous cell carcinoma cell lines (OSCC15 and OSCC4) they concluded that Pita treatment upregulated FOXO3a by modulating the AMPK and Akt pathways. Similarly, Wang et al. (2016) found that simvastatin arrested the cell cycle of bladder cancer cell lines (BCa) in G_0/G1(Wang et al. 2016). In agreement with these findings, Al-Qatati and Aliwaini (2017) showed that Pita induced cell cycle arrest on human melanoma cell line A375 and WM115. Western blotting assay of the treated cell lines with Pita showed a significant increase in the expression of p53 and p21. Furthermore, the combined treatment of dacarbazine and Pita synergistically increased p53 and p21. Another study by Wang et al.(2020) examined different concentrations of atorvastatin treatment on hepatocellular carcinoma (HCC) and demonstrated that there was a clear increase in the expression of the inhibitory proteins p21 and p53 in a dose-dependent manner which played a vital role in the suppression of cell growth (Wang et al. 2020). Our present study shows that the combination of Vit D and Pita inhibited the p-STAT3 and increased p53 and p21 proteins in HCC1937. The present study also showed that a significant reduction in cell migration (30%) was observed for HCC1937 cells exposed to a combination of 1μM Vit D and 1μM Pita after 48 hours.

Conclusion

Taken together these observations indicate the potent synergistic anticancer effect of Vit D and Pita against the HCC1937 triple negative breast cancer cells. This combined treatment showed a significant anti-proliferative effect by inducing cell cycle arrest and apoptosis in breast cancer cells.

Conflict of Interest Statement

There are no conflicts of interest.

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