RESEARCH ARTICLE

Editorial Process: Submission:12/28/2025 Acceptance:07/18/2026 Published:07/28/2026

The Synergistic Effects of Metformin with Pitavstatin Against MDA-MB-468 Breast Cancer Cells

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Abstract

Background: Breast cancer is the most common malignancy among women and a leading cause of cancer-related mortality worldwide. Current therapeutic strategies are associated with several limitations, including cytotoxic side effects and multidrug resistance. As a result, a shift toward drugs with fewer adverse effects and greater efficacy must occur. Recent studies have demonstrated the anticancer potential of both metformin and pitavastatin. This study aimed to investigate the synergistic effects of their combination on MDA-MB-468 triple-negative breast cancer cells. **Materials and Methods:** The MTT assay was performed to assess the cytotoxicity of metformin and pitavastatin on MDA-MB-468 breast cancer cells. The anti-proliferative effects of the combined treatment were assessed using the trypan blue assay and the clonogenic assay. Additionally, a scratch assay was used to evaluate the migration ability of MDA-MB-468 cells after treatment with metformin and pitavastatin treatments. **Results:** Both metformin and pitavastatin significantly reduced the viability of MDA-MB-468 cells in a time- and dose-dependent manner. Metformin exhibited an IC_{50} value of 0.92mM, while pitavastatin showed an IC_{50} of 1.72µM. Notably, the combined treatment produced a stronger antiproliferative effect compared to either drug alone. Trypan blue data revealed a higher rate of cell death following combination therapy. Furthermore, colony formation was completely inhibited (100%) by the combined treatment compared with to the control group. The scratch assay demonstrated that the combination significantly suppressed cell migration, indicating a synergistic anti-migratory effect. **Conclusions:** These findings indicate that the combination of metformin and pitavastatin exerts a synergistic anticancer effect against MDA-MB-468 breast cancer cells and may represent a promising therapeutic strategy for triple-negative breast cancer.

Keywords: Breast cancer- apoptosis- MDA-MB-468- metformin- pitavastatin

Asian Pac J Cancer Prev, **27 (7)**, 2705-2712

Introduction

Cancer is one of the deadliest diseases and accounts for 13% of total deaths worldwide [1]. Poor diagnosis, a lack of effective treatment options, and high death rates are the main factors that have made cancer a serious issue. Cancer is described as an aggressive disease that results from the abnormal growth of cells that divide uncontrollably and can penetrate and destroy tissues [2]. Breast cancer is the most common cancer that occurs in women and a major cause of cancer-related deaths among females globally [3]. The incidence rate of female breast cancer worldwide was 11.7% of all cancer cases, with a projected 2.3 million new cases, according to data from 2020. Furthermore, with 685,000 fatalities worldwide, breast cancer is the fifth leading cause of cancer mortality [4]. There are several strategies to treat cancer depending on the type of cancer, timely diagnosis, and the patient's response to each treatment. These treatment methods include surgery, chemotherapy, hormone therapy, radiation therapy, immunotherapy, and targeted therapy [5]. Unfortunately, the unfavorable side effects and medication resistance brought on by the aforementioned treatments have restricted the success of the massive efforts made to treat cancer. This explains why there is a greater demand for novel treatments [1]. There is increasing interest in drug repurposing as a result of several tactics for more effective anti-cancer medications, such as employing medications like Metformin, Aspirin, and Pitavastatin that are already licensed for other indications to treat cancer. Pre-clinical, clinical, and observational investigations have therefore shown that molecules from a variety of therapeutic classes other than cancer have anti-tumor activity [6]. Metformin (1,1-dimethyl biguanide hydrochloride) is a relatively safe drug that is widely used to treat type II diabetes, obesity, and polycystic ovarian syndrome

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(PCOS) [7]. According to laboratory studies, Metformin is associated with a significant reduction in the incidence of cancer. Its ability to activate AMP-kinase, which in turn suppresses the mammalian target of rapamycin (mTOR), may further contribute to its possible anti-tumorigenic impact [8]. Metformin causes cell cycle arrest in the G0-G1 phase and increases p53, p21, and Cyclin D1 when cancer cell lines are treated [9]. Pitavastatin is a synthetic drug that significantly lowers serum total cholesterol, LDL cholesterol, and triglycerides and has several pharmacodynamic and pharmacokinetic properties that are significantly different from other statins [10]. It targets and inhibits 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR), the rate-limiting enzyme in cholesterol biosynthesis [11, 12]. Numerous investigations have demonstrated that Pitavastatin treatment reduces cancer by causing apoptosis and cell cycle arrest in a number of cancer cell lines [13, 14]. Pitavastatin, when treated with different cancer cell lines caused cell cycle arrest and apoptosis by arise in the level of Cyclin E1, Cyclin D1, p53, and its downstream target p21 proteins were associated with cell cycle arrest [13]. According to some research, the combination of the two treatments (Metformin and Pitavastatin) activates autophagy and apoptosis in pancreatic cancer cells by inhibiting the phosphatidylinositol 3-kinase (PI3K)/mTOR pathway [15]. Other studies confirmed a significant cytotoxic effect against HCC1937 and BT-20 breast cancer cells when the combination treatment was used of Pitavastatin and Metformin [16, 17]. This study aimed to evaluate the effect of a single dose of Pitavastatin and Metformin and their combination against MDA-MB-468 breast cancer cell lines in vitro.

Materials and Methods

Study design and site of experiment conducting

Triple-negative breast cancer cells, MDA-MB-468, were employed in an in vitro investigation. The studies were conducted in the biotechnology labs of the Islamic University of Gaza between January 10, 2020, and January 10, 2023.

Cell culture and treatments

The MDA-MB-468 cells were a gift from prof. Adrienne Lesley Edkins (Department of Biochemistry and Microbiology, Rhodes University). The human MDA-MB-468 breast cancer cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM). 10% fetal bovine serum (FBS), 100 µg per milliliter of streptomycin, and 100 U per milliliter of penicillin were added to this medium. Cells were then maintained at 37°C in an incubator with 5% carbon dioxide and 95% air humidity, changing the medium every two or three days [18].

Preparation of drugs

Metformin and Pitavastatin were both bought from the Middle East Pharmaceutical company. Metformin was dissolved in 1ml of sterile distilled water, then heated at 100°C by water bath for 5 minutes and put on a vortex for 2 minutes, and this process was repeated several times to obtain stock 0.5M of Metformin stored at -20°C. To obtain stock 2mM of Pitavastatin held at -20°C, the medication was dissolved in 100µl of DMSO (dimethyl sulfoxide), heated by a heat block for two minutes at 95°C, and vortexes for two minutes. The final concentration was then obtained by preparing dilutions in the proper media for the cell line. Control cells (vehicle) were treated with DMSO at the same concentrations [16].

Cytotoxicity assays (MTT)

MDA-MB-468 cells were seeded at a density of 2×10^5 cells/well in 96-well plates, and they were let to settle overnight. Then, for 48 hours, cells were treated with various concentrations of Metformin (0-5µM) and Pitavastatin (0-5mM) or vehicle. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test) test was used to determine cytotoxicity, according to the manufacturer's directions for use (Roche, USA). In summary, after one week of incubation of treated cells, 10 microliters of MTT solution were applied to each well, and the cells were cultured at 37°C for 4 hours before being exposed to 100 microliters of DMSO for 30 minutes at 37°C. Absorption was measured at 550 nm per well using the ELISA reader, and the average cell survival was computed as a percentage of control. Lastly, the IC_{50} values were calculated using a Microsoft linear equation by [19] and EC_{50} values were calculated which is the concentration of a drug that gives a half-maximal response or effect [20, 21].

Cell morphology

An inverted microscope was used to view the morphological changes in cells following seeding and treatment for each experiment.

Drug combination analysis

Prior to the cytotoxic test, cells were treated with varying doses of Metformin, either by itself or in conjunction with Pitavastatin.

Viability assay

The percentage of living and dead cells as well as the total number of cells were determined using the trypan blue exclusion method. Cells were cultured in a 6-well plate at a density of 1.5×10^4 cells/well and incubated for 24 hours at 37°C in a 5% CO2 and 95% air-humidified incubator. After one day, Metformin and Pitavastatin were given to the cells for 4 and 8 days at various concentrations. After that, the cells were trypsinized, centrifuged, resuspended in media (0.5mL), and well mixed with 25µl trypan blue at each time point, then left to stain for 5 minutes at room temperature. The cells were then counted using a hemocytometer. Three separate samples were used for each experiment, which was conducted three times. According to [22], the percentage of cell viability was computed statistically by counting the unstained (viable) and blue-stained (nonviable) cells per sample in several randomly selected fields and applying the following formula:

% Cell viability = total stained cells (nonviable) * 100 / total cells (stained + unstained)

Scratch motility assay

In the in vitro scratch motility experiment, MDA-MB-468 cells were cultured in 6 well tissue culture plates, and with a sterile microliters pipette tip, a linear wound was generated by scratching the monolayer. Cells were cultured at 95% confluency with 95 × 10^4 cells/well.To eliminate cell debris, the growth media was renewed. Then several reference points were generated around the scratch line's edges and wound width that were recorded at the time of scratching (0 hours) and at intervals of 24, 48, and 72 hours. Using an inverted light microscope and camera, images were obtained and migration expanses were estimated according to [23].

Clonogenic survival assay

MDA-MB-468 cells were pre-cultured at a density of 1.5 × 10^4 cells/well in a 6-well plate, treated with particular concentrations of Metformin and Pitavastatin for 24 hours, and then re-plated at low density (500 and 1000 cells) and incubated for two weeks to test the cells' capacity to produce clones. Beyond that period at room temperature, methanol fixation was made to surviving cells for 5 minutes, followed by staining with 10% Giemsa dye in deionized water (Sigma, USA) for 20 minutes. The stained colonies were then washed twice in 1ml Phosphate Buffer Saline (PBS). The number of colonies calculated in the sample is divided by the number of colonies estimated in the control multiplied by 100 percent to get the percentage variation in surviving colonies [18].

Statistical analysis

The average standard error of the means (SEM) of three different experiments was displayed. The data were statistically analyzed using the 2-sample t-test, and $p<0.05$ was deemed to be statistically significant. The acquired data were analyzed using Image J software, Microsoft Excel 2013, Combenefit program, and a phase-contrast microscope.

Results

Cytotoxic effect of Metformin on MDA-MB-468 breast cancer cell line

MTT assay demonstrated that Metformin significantly reduced the viability of MDA-MB-468 cells in a dose- and time-dependent manner. The IC_{50} value was 0.92mM (Figure 1), with 25% inhibition at 0.3mM and 80% inhibition at 5mM after one week. Metformin also showed significant cytotoxicity with an EC_{50} of 1.01mM (Figure 2). Metformin effectively inhibited cell proliferation in MDA-MB-468 cells in a dose-dependent manner.

Cytotoxic effect of Pitavastatin on MDA-MB-468 breast cancer cell line

Pitavastatin suppressed cell proliferation by 24.5% at 0.3μM and by 56.9% at 5μM, according to the results, which demonstrated that the drug has a strong dose-dependent anti-proliferative impact on MDA-MB-468 cells (IC_{50} value of 1.72μM) (Figure 3). Furthermore, Pitavastatin promotes an important cytotoxic effect on MDA-MB-468 cells with an EC_{50} of 0.416μM (Figure 4). Pitavastatin exhibited strong dose-dependent antiproliferative activity against MDA-MB-468 cells.

Metformin and Pitavastatin synergistically inhibit the proliferation of MDA-MB-468 breast cancer cells line

The combination treatment was more successful than the single treatment at preventing the proliferation of cells, as seen in Figure 5. While a single treatment of 0.3μM Pita or 0.6mM Met inhibited cells growth by 41% and 28%, respectively, the combined treatment (0.3μM Pita + 0.6mM Met) at 16 of the Loewe program inhibited cells proliferation by more than 76% Table 1. Additionally, morphological alterations were observed in the treated

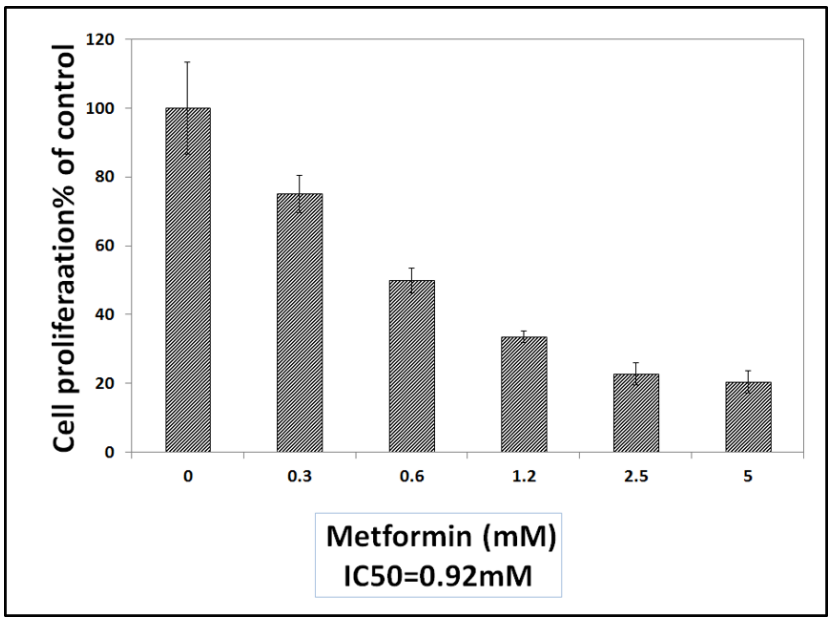

Figure 1. Metformin Induced Cytotoxicity on MDA-MB-468 Breast Cancer Cell Line. Using the MTT assay, figure (1) illustrates the cytotoxicity of Metformin on MDA-MB-468 breast cancer cells following a week of treatment. The findings are the average % ± SEM of a minimum of three quadruplicate studies.

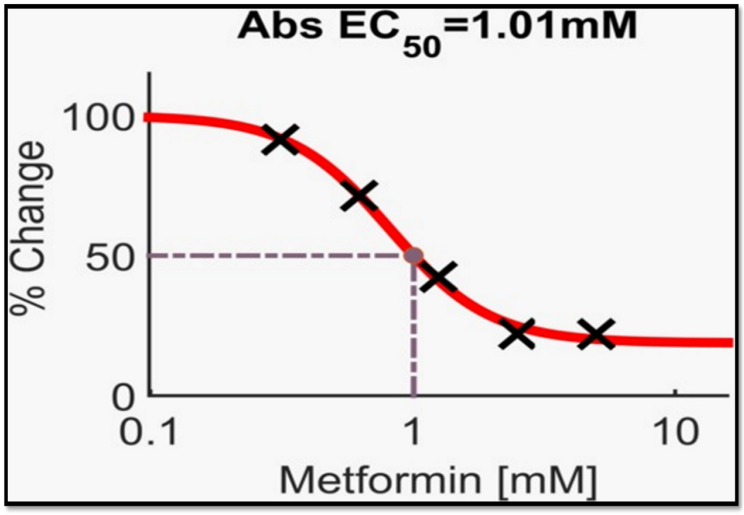

Figure 2. Dose-Dependent Curve of Metformin Cytotoxicity on MDA-MB-468 Breast Cancer Cells. The outcomes show how Metformin affects MDA-MB-468 cells in a dose-dependent manner in at least three quadruplicate MTT assays. The Combenefit program is then used to process and evaluate the data.

Table 1. Comparative Analysis of Drug Concentrations and % Inhibition

Drug	Concentration	IC_{50}	EC_{50}	inhibition
Metformin	0.3mM	0.92 mM	1.01mM	28%
Pitavastatin	0.6µM	1.72µM	0.416µM	41%
Mix	(0.3µM Pit + 0.6µM Met)	-	-	76%

cells when viewed under an inverted microscope. These changes included a decrease in cell confluency and a loss of attachment, were more pronounced with combination treatment. These results demonstrated the synergistic cytotoxic effect of using Metformin and Pitavastatin together to treat MDA-MB-468 cells.

Metformin and Pitavastatin synergistically induce cell death of MDA-MB-468 breast cancer cell line

Trypan blue exclusion assay showed that single treatments with Pitavastatin treatment induced 9.5% and 11.6% cell death at 4 and 8 days, respectively. Cell death from a single Metformin dose was 7.16% after 4 days and 14.34% after 8 days. Remarkably, The combination treatment increased cell death to 14.1% at 4 days and 37.67% at 8 days (Supplementary Figure 1). Combination therapy synergistically enhanced cell death compared with individual treatments.

Metformin and Pitavastatin synergistically impeded the proliferation of the MDA-MB-468 breast cancer cell line

Growth curve analysis demonstrated that the

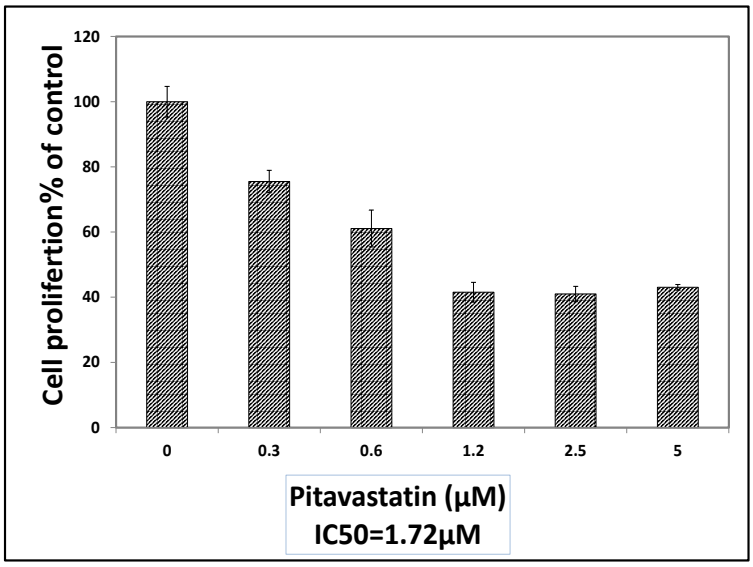

Figure 3. Pitavastatin Induced Cytotoxicity on MDA-MB-468 Breast Cancer Cells Line. The figure (3) shows the cytotoxicity of Pitavastatin on the MDA-MB-468 breast cancer cells using the MTT assay after one week of the treatment. Results represent the mean percentage ± SEM of at least three experiments performed in quadruplicate.

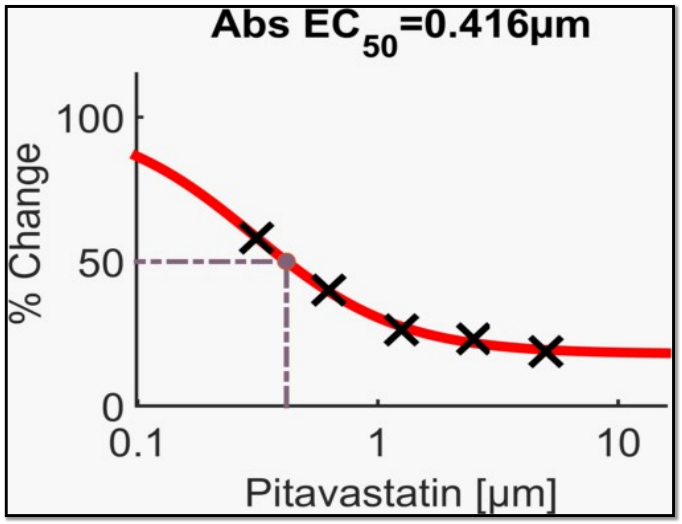

Figure 4. Dose-Dependent Curve of Pitavastatin Cytotoxicity on MDA-MB-468 Breast Cancer Cells Line. Pitavastatin's dose-dependent action on MDA-MB-468 cells is demonstrated by the results of at least three quadruplicate MTT assays. Following that, the Combenefit program is used to process and evaluate the data.

Table 2. Effect of Metformin, Pitavastatin, and Combination on MDA-MB-468 Migration (Wound Healing Assays)

Time	Control	Pit	Met	Drugs (mix)
24hr	21.40%	15.90%	10.10%	5.30%
48hr	36.20%	28.90%	14.20%	10.90%
72hr	44.40%	34.60%	19.70%	14.40%

combination treatment markedly suppressed MDA-MB-468 cell proliferation over an eight-day period. Notably, after two days of treatment, Supplementary Figure 2 shows that 0.6mM Metformin and 0.3µM Pitavastatin acted synergistically to reduce the rate of cell proliferation. Treated cells exhibited a pronounced decline in growth rate after day 6, whereas control cells continued to proliferate until the end of the experiment. Overall, the combined treatment with Metformin and Pitavastatin significantly inhibited long-term cell proliferation.

Metformin and Pitavastatin synergistically inhibit cell migration of MDA-MB-468 breast cancer cell line

Scratch wound-healing assays showed that untreated cells closed approximately 44.4% of the wound area after 72 hours. In contrast, cells treated with 0.6mM Metformin or 0.3µM Pitavastatin alone closed the wound area by about 19.7% and 34.6%, respectively, whereas cells

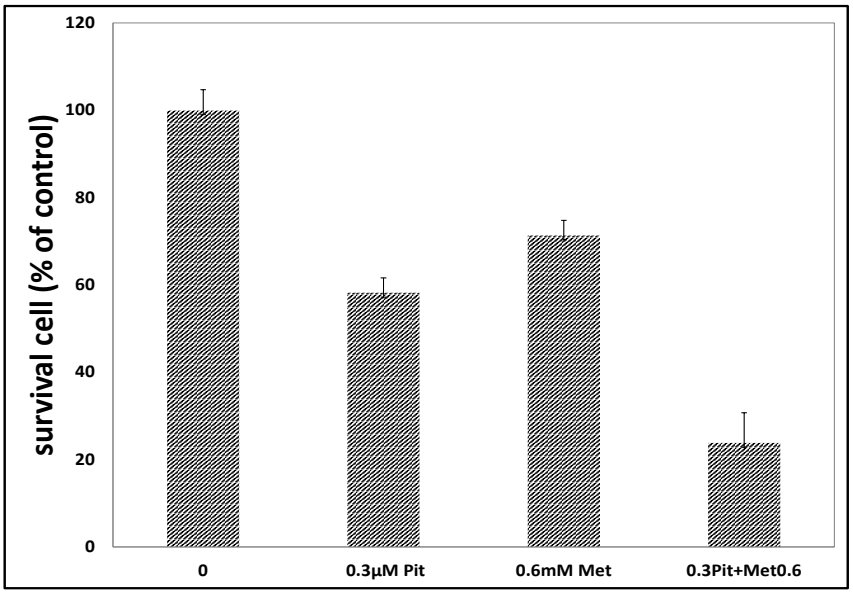

Figure 5. Metformin and Pitavastatin Synergistically Inhibit MDA-MB-468 Breast Cancer Cell Proliferation. Using the MTT assay, figure (5) illustrates the antiproliferative effects of Metformin and Pitavastatin combination treatment on MDA-MB-468 cells after one week.

treated with the combination closed around 14.4% of the wound area (Table 2, Supplementary Figure 3). The results revealed that the percentage of cell migration inhibition was significantly higher in the combination-treated cells than in cells treated with either drug alone.

Metformin and Pitavastatin synergistically inhibit colony formation ability of MDA-MB-468 breast cancer cell line

Clonogenic assays showed that single-agent treatments reduced colony formation, whereas the combination completely abolished colony growth (100%) at both seeding densities (500 and 1000 cells per well). Untreated cells formed 8 colonies at 500 cells and 95 colonies at 1000 cells. Pitavastatin reduced colony formation by 75% and 88.4%, respectively, while Metformin reduced it by 87.5% and 95.8%. The combination treatment resulted in complete inhibition of colony formation in both conditions (Supplementary Figure 4). These findings demonstrate that the combination significantly inhibits MDA-MB-468 cell colony formation.

Discussion

In the present study, the antiproliferative effects of Metformin and Pitavastatin, either alone or in combination, were evaluated against MDA-MB-468 breast cancer cells using the MTT assay. The findings demonstrated that Metformin and Pitavastatin together have a synergistic cytotoxic impact when used to treat breast cancer cells. The viability assay showed that the combined treatment was able to induce cell death more than using individual drugs. Metformin and Pitavastatin worked in concert to slow the rate of cell division, particularly after two days of treatment.

Metformin has been shown in numerous studies to have antiproliferative properties against various cancer cell types. According to a study by Lee et al. [24], the IC_{50} value for the toxicity of Metformin on HCC1937 breast cancer cells was 7.4mM after 48 hours , while another study found that the IC_{50} on the MCF-7 breast cancer cell line was 5.8mM after 72 hours [25]. Anyway, the breast cancer cell lines treated with Metformin have IC_{50} values ranging between 5 and 20mM [26]. Although our experiments demonstrated that Metformin treatment has lethal effects on MDA-MB-468 breast cancer cell lines, the IC_{50} values were 0.92mM. A previous study showed that Pitavastatin has a potent cytotoxic effect against MCF-7 cells with an IC_{50} around 10μM [14], and another study revealed the IC_{50} on the same cell line was 7.2mM after 48 hours [27]. After treatment of MDA-MB-468 breast cancer cells with Pitavastatin in our lab, we obtained an IC_{50} equal to 1.72μM in a dose-dependent manner. The difference in IC_{50} compared to the previous studies may be because of the difference in conditions, including incubation time and cancer cell lines.

This study, showed that when used the combination of Metformin and Pitavastatin have a synergistic reduction in cell proliferation, more than when used alone. These findings align with previous studies reporting enhanced inhibitory effects of combined Metformin and Pitavastatin on BT-20 breast cancer cells, a single treatment dose of 0.3μM Pita or 0.6mM Met inhibited cell growth by 25.6% and 11.9%, respectively, the combined treatment (0.3μM Pita plus 0.6mM Met) inhibited cell proliferation by more than 42% [17], and based on [16] HCC1937 breast cancer cell lines were treated with Pitavastatin and Metformin alone or in combination for 48 h, the result of an inhibitory effect of 1μM Pita and 5mM Met separately on the cells was 3.8% and 19.6%, respectively, while the combination of both increased the percentage to more than 48.0%, and another study showed a significant synergistic reduction in cell viability on pancreatic cancer cells (ASPC-1 and PANC-1 cells) when used in combination of Metformin (30mM) and Pitavastatin (10μM) [15].

We discovered in our research that Metformin and Pitavastatin both inhibit cell migration in varying amounts, and that the combination of the two medications prevents migration in excellent proportions. Several studies indicate that Pitavastatin and Metformin separately or combined, leads to inhibited cell migration and invasion [28]. Numerous studies have demonstrated that Pitavastatin treatment dramatically reduces the migration of some breast cancer cells [29, 30]. On the other hand, Metformin exerts inhibitory effects on breast cancer cells such as MDA-MB-231 [31]. Furthermore, the HCC1937 breast cancer cell lines' growth and migration were more strongly inhibited by Pitavastatin and Metformin together, and the wound closed by approximately 1% in comparison to the control [16].

In the present investigation, the combination of 0.3μM Pita and 0.6mM Met was more effective than each monotherapy in decreasing the number of colonies by 100% in the 500 and 1000 cells. Our findings are consistent with recent research, which found that 5μM of Pitavastatin inhibited hepatocellular cancer [32] and that it decreased the number of MCF-7 and MDA-MB-231 colonies [29]. Furthermore, Metformin was observed to decrease colony formation in four cell lines (MCF-7/713, SKBR-3, MCF-7, and BT-474) in a dose-dependent manner, while treating the MCF-7 breast cancer cells with 2mM Met was effective in inhibiting 75% of the colonies [33]. Research indicates that, as compared to the control, a combination of Metformin (5mM) and Pitavastatin (1μM) clearly reduced the formation of HCC1937 cell colonies [16].

Overall, the present study demonstrates that the combination of Metformin and Pitavastatin exerts a potent synergistic anticancer effect in MDA-MB-468 cells by inhibiting cell viability, proliferation, migration, and clonogenic survival. These findings support the potential of this combination as a promising therapeutic strategy for breast cancer treatment.

In conclusions, the current research provides a wealth of evidence supporting the synergistic anticancer effect of Metformin and Pitavastatin. The current study offers multiple pieces of evidence that, when compared to each treatment alone, the combination of Metformin and Pitavastatin significantly reduces the value of IC_{50} and is cytotoxic to MDA-MB-468 breast cancer cells in a time and concentration-dependent manner. Additionally, the trypan blue dye exclusion assay demonstrated that, in contrast to each treatment alone, the combination of

Metformin and Pitavastatin also causes cell death and an anti-proliferative effect in the MDA-MB-468 cell line. Metformin and Pitavastatin work better synergistically to restrict MDA-MB-468 cell movement and stop colony formation. We advise that more research be done to look at the combined anticancer effects of Metformin and Pitavastatin on both normal cells and other cancer cell lines in light of these positive results. It has been necessary to do additional research to fully comprehend the potential in vivo effects of the combination of Metformin and Pitavastatin compounds.

Author Contribution Statement

All experimental assays were devised and carried out by Alnahhal, N., who also made a significant contribution to the writing and development of the report. Aliwaini, S., was the second major contributor to the manuscript's drafting and oversaw several procedures for the execution of experimental assays. Adris, M, was the main supervisor of this study, he reviewed the writing, and editing. Altaher, A. He was responsible for the chemical analysis of the compounds that were manufactured. All authors have approved the final version of the manuscript.

Acknowledgements

The authors would like to thank Islamic University of Gaza, Faculty of Sciences, Department of Biological Sciences and Biotechnology for providing the laboratory facilities to conduct this research.

Ethical approval

There was no need for institutional ethics committee approval because this was an experimental in vitro study.

Availability of data and materials

This published article contains all of the data that was gathered throughout this experimental investigation.

Conflict of interest

None.

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