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Original Article

Cancer as an infectious disease: A different treatment alternative using a combination of tigecycline and pyrvinium pamoate — An example of breast cancer

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KEYWORDS
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Abstract *Background:* Tigecycline is an antibiotic that well tolerated for treating complicated infections. It has received attention as an anti-cancer agent and expected to solve two major obstacles, sides effects that accompany chemotherapy and drug resistance, in

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Pyrvinium pamoate;
Tigecycline

the breast cancer treatment. However, previous studies reported that the levels in the blood are typically low of tigecycline, so higher doses are needed to treat cancer, that may increase the risk of side effects. To achieve better anti-cancer effects for tigecycline, we need to find a novel adjunct agent.
Methods: In this study, we used different concentration of pyrvinium pamoate combined with tigecycline to treat cell. And assess the effect of two drugs in inhibit cell proliferation, induce cell autophagy, or increase cell apoptosis to evaluate the consequent of combined therapy.
Results: We observed that after the combined therapy, the cell cycle arrest at G1/s phase, the level of p21 increased, but decreased the levels of CDK2. Others, two drugs via different mechanisms to inhibit cancer cell proliferation and with selective cytotoxic to different cell lines. That could enhance the effect of breast cancer treatment.
Conclusion: Combining low dose of tigecycline use with pyrvinium pamoate is a novel approach for breast cancer treatment. Appropriate combined therapy in breast cancer is recommended to improve outcomes. Other problems like drug resistance occur in patients or the microbes surrounding breast tissues would confer susceptibility to cancers then influence the effectiveness of treatment, which could be improved through combined therapy.

Introduction

Breast cancer is one of the most common causes of death in females in Europe and the United States.[1] In Taiwan, it is the most frequently diagnosed type of cancer and its mortality rate is the fourth highest. With changes in lifestyle and the westernization of eating habits, the prevalence of breast cancer is increasing in Taiwan. Clinically, surgery, chemotherapy, or both are used to treat breast cancer. Unfortunately, even with these treatments, the death rate remains unacceptably high, possibly owing to two major factors. First, there are many sides effects accompany chemotherapy, e.g., immunosuppression, inflammation of surrounding tissues, and infections.[2] Second, patients develop resistance to chemotherapeutic drugs. Commonly, drug resistance inevitably occurs in many patients in the late stage of breast cancers.[3,4] Thus, developing new strategies and agents to treat breast cancer is urgently needed.

Recently, bacterial communities within the breast tissue have been considered to be a factor that can cause breast cancer. Other evidence suggests that microbes surrounding human tissues can confer susceptibility to cancer and alter treatment efficacy.[5] Based on the idea that these bacteria can interfere with therapy, there has been a growing interest over the past decade in using antibiotic drugs for breast cancer treatment.

Among such antibiotics, tigecycline is safe and well tolerated for treating complicated infections and is an effective anti-cancer treatment.[6] Moreover, tigecycline may be used as an adjunct to treatment in cancers such as gastric cancer, chronic myeloid leukemia, prostate cancer, or breast cancer.[7] However, following dosing at moderate levels, tigecycline levels in the blood are typically low, meaning that higher doses are needed to treat cancer, meanwhile increasing side effect risks,[8] which is a major drawback of tigecycline. In 2013, the FDA issued a warning about an increased risk of death with tigecycline. In addition, as tigecycline is a bacteriostatic antibiotic, it relies on the host's own immune system to eradicate bacteria. To achieve better anti-cancer effects and to reduce the drug dose to avoid side effects, we need another novel adjunct agent.

Another FDA-approved anthelminthic drug, pyrvinium pamoate (PP), has recently been reported to have novel effect in tumor treatment. PP can inhibit cancer cell proliferation and stimulate apoptosis in cancer cells.[9] Numerous studies have shown that PP is effective in treating colon cancer, prostate cancer, and breast cancer.[10] Under conditions of glucose deprivation, PP exerts preferential cytotoxicity against various cancer cell lines. Even under normoglycemic conditions, PP can inhibit cancer cell proliferation by blocking the mitochondrial electron-transport chain.[11] Thus, in this study, we aimed to investigate the combination effects of tigecycline and PP through different pathways on breast cancer.

Methods

Cell culture and spheroid formation

Two human breast cancer cell lines, MDA-MB-231 and MCF-7, and one primary human aortic endothelial cells (HAECs) were obtained from American Type Cell Culture (ATCC, Bethesda, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum by the microfluid-based hanging-drop culture system.[12] Cells were seeded at a density of 5×10^4 per well and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Under these conditions, the cells aggregated to form spheroids in three days.

Half maximal inhibitory concentration (IC50) of PP in the presence of a fixed concentration of tigecycline in three cell lines

As shown in our previous study, we developed a microfluidic-based hanging-drop culture system to form cell

spheroids. After seeding the cells for 72 h, the MDA-MB-231, MCF-7, and HAEC spheroids were washed with phosphate buffered saline (PBS) and then cultured in fresh media for 72 h containing drugs namely tigecycline (Sigma—Aldrich, St. Louis, MO, USA), PP (Sigma—Aldrich, St. Louis, MO, USA) or a combination of the two. Tigecycline was used at a fixed concentration of 10 μM in the presence of increasing concentration of PP (0 nM—4000 nM) to evaluate the effect of the combination of these two drugs on three different cell lines, namely MDA-MB-231, MCF-7, and HAEC. After 3 days of treatment, the spheroids were harvested from the microfluidic device. Cell morphology and average spheroid diameters were assessed by light microscopy (CiS, Nikon, Tokyo, Japan). Following this, the spheroids were treated with trypsin to separate them into single cells. Isolated cells were seeded in 96-well plates then incubated at 37 °C for 24 h. Cell viability was assessed using Cell Counting Kit-8 (Dojingdo, Kumamoto, Japan) according to the manufacturer's protocol, and a TECAN 200/200 Pro multimode microplate reader (TECAN Trading AG, Männedorf, Switzerland).

Combination therapy

After the cells were seeded for 72 h, drugs were added to the culture system. In this study, three drug conditions were assessed, namely tigecycline, PP, and a combination of each drug at half of a single-dose to evaluate the effect of drugs on cancer cells.

Cell cycle analysis

After treatment with tigecycline and/or PP for 72 h, the cell cycles in the three cell lines were assessed by flow cytometry. A Cycletest Plus DNA Reagent Kit was used to evaluate the effect of both drugs to induce cell cycle arrest.[13] The methods were conducted in accordance with manufacturer guidelines, and the stained cells were then analyzed by flow cytometry (LSRFortessa X-20, BD Biosciences, Franklin Lakes, NJ, USA) at an excitation of 488 nm.

Western blotting

Cells were treated with drugs for 72 h, harvested and lysed in RIPA buffer (Sigma—Aldrich, St. Louis, MO, USA) for western blot analysis. Western blotting was performed using antibodies to the following cell cycle marker proteins, CDK2 (#2546 S), Cyclin D1 (#2978 S), p21 (#2947 S) and the autophagy marker proteins, SQSTM1 (#PA5-78268), LC3I/LC3II (#2775) and β-Actin (#3700 S) was used as a loading control. All antibodies were from Cell Signaling Technology (Cell Signaling Technology, Inc., MA, USA). Protein bands were detected using a western lightning plus-ECL, enhanced chemiluminescence substrate (PerkinElmer Inc., MA, USA).

Autophagy assay

An autophagy assay kit (Merck KGaA, Darmstadt, Germany) was used to measure cell autophagy based on a fluorescent autophagosome marker ($\lambda ex = 360/\lambda em = 520$ nm). The cells were seeded in a 96 well black plate at a density of 2×10^4 per well and the methods were conducted in accordance with manufacturer guidelines. Following the induction of autophagy, the fluorescence intensity measured using a microplate reader.

Apoptosis analysis using annexin V staining

Before analysis, the spheroids were disaggregated into a single-cell suspension using trypsin dissociation. The cells were the washed twice in PBS and centrifuged at $1200 \times g$ for 5 min. The cells were then resuspended in 400 μL PBS. Phosphatidylserine externalization was assessed by quantifying surface Annexin V-FITC and PI (Becton Dickinson, USA) using a flow cytometer. Cells that were both PI negative and annexin V negative were considered healthy cells, PI negative and annexin V positive cells were considered apoptotic, and cells that were both PI positive and annexin V positive were considered necrotic.[14]

Measurement of reactive oxygen species (ROS) for DNA damage

ROS was measured using the CellROX® Green Reagent (Thermo Fisher Scientific Inc., MA, USA). The cell-permeant dye exhibited bright green photostable fluorescence upon oxidation by ROS and subsequent binding to DNA and were analyzed by flow cytometry with absorption/emission maxima of ~485/520 nm. The method was conducted in accordance with manufacturer guidelines.

Statistical analysis

A student's *t*-test was used to compare the means of two independent sample groups. A p-value of less than 0.05 was considered to indicate statistical significance. All experiments were conducted in at least triplicate for statistical analysis, and the mean ± standard deviation was determined.

Results

IC50 of PP in the presence of a fixed concentration of tigecycline

The IC_{50} for tigecycline has been found to be in the range of 10—50 μM in the MCF-7 and MDA-MB-231 cell lines. In combination with 10 μM tigecycline, the IC_{50} for PP was 750 nM in MDA-MB-231 cells, 300 nM in MCF-7 cells, and 300 nM in HAEC (Fig. 1). In subsequent experiments, tigecycline was used at 10 μM and PP at 300 nM for solitary therapy and half does of two drugs to evaluate the effects of combination therapy.

Spheroid growth and cell viability after combination treatment

Treatment with tigecycline alone caused a disruption of the cell spheroids formed by all three cell lines (Fig. 2a).

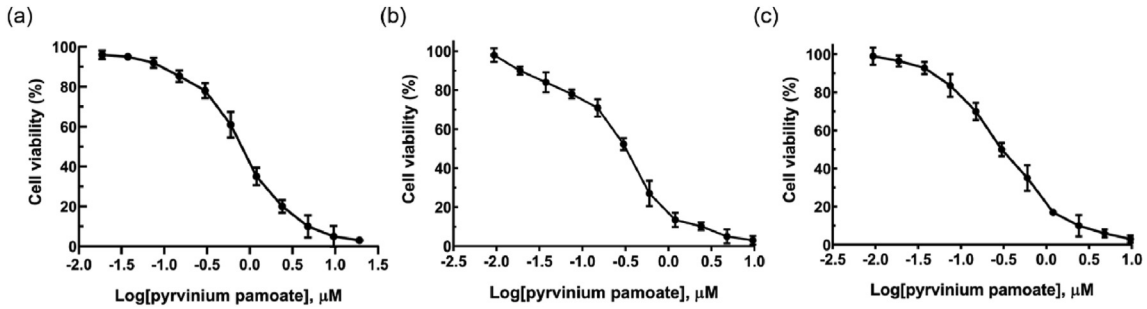

Figure 1. The IC_{50} for pyrvinium pamoate in combination therapy in the three cell lines: (a) MDA-MB-231, (b) MCF-7, and (c) HAECs.

Similar data was seen for PP treatment alone (Fig. 2b). The combination of two drugs caused a significant disruption of the spheroids formed by MDA-MB-231 and MCF-7 cells as early as day 3. In contrast, combination treatment did not disrupt HAEC spheroids but in fact increased their diameter over time (Fig. 2c).

The effect of these drugs on cell viability was determined. After 3 days there were no significant differences in the viability of cells treated with tigecycline. In contrast to the two other cell lines, PP treatment alone increased the viability of MDA-MB-231 cells to 107.11 ± 31.71%. Combination therapy, the viability of the two cancer cell lines significantly decreased to 30% but had no effect on normal cells. At day 7, the viability of the two cancer cell lines treated with tigecycline and/or PP was 40% but the viability of HAEC treated with combined therapy was considerably increased 1.5-fold (Fig. 3).

Cell cycle analysis

Fig. 4 shows the results of a flow cytometric analyses of the cell cycle in these three cell lines after 72 h of drug treatment. In MDA-MB-231, the percentage of cells in G1/S increased, and decreased in G2 after tigecycline treatment. After PP treated, the percentage of cells in G1/S considerably increased and the proportion in G2 decreased significantly. However, compared with no treatment, combination therapy didn't have a significant effect on the percentage of cells in G1/S. In contrast, in MCF-7 cells combination therapy resulted in a clear accumulation of cells in G1/S and a similar result was seen with PP treatment. There were no significant effects of these three drug treatment regimes in normal HAEC. In the three cell lines, combination therapy markedly increased the levels of p21 and decreased the levels of CDK2 but didn't significantly affect Cyclin D1 levels. In contrast, treatment of the three cell lines with tigecycline,

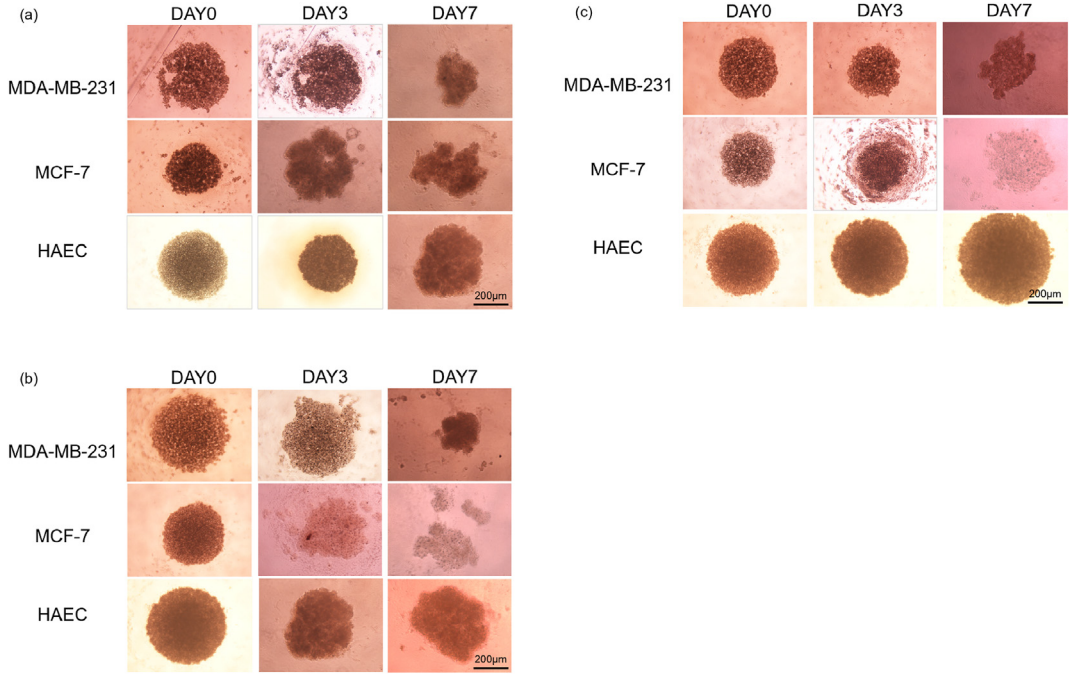

Figure 2. Changes in spheroid appearance after treatment with (a) tigecycline only, (b) pyrvinium pamoate only, and (c) combination therapy.

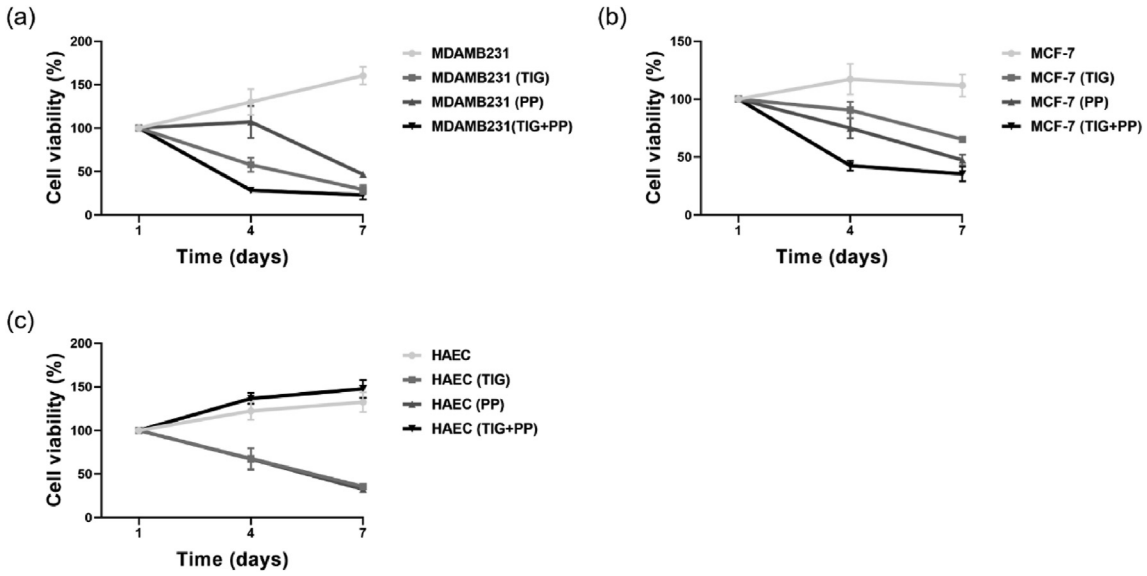

Figure 3. Cell viability in the three cell lines after treatment with tigecycline only, pyrvinium pamoate only, and a combination of tigecycline and pyrvinium.

considerably increased the levels of Cyclin D1, CDK2, and p21. A similar result appeared for PP.

Autophagy

As shown in Fig. 4, tigecycline treatment caused an increase in both the levels of SQSTM1/p62 and LC3 II in the three cell lines. A similar effect was seen following PP treatment. After combination therapy, the levels of SQSTM1/p62 decreased and LC3 II considerably increased in the two cancer cell lines but interestingly there was no significant effect in HAEC.

Apoptosis

As shown in Fig. 5, after 72 h of treatment, tigecycline had no significant effect on apoptosis in any of the three cell lines. In contrast, PP induced a significant degree of apoptosis in all three cell lines. Treatment of MDA-MB-231 and MCF-7 cells with the combination of tigecycline and PP increased the number of cells in both early and late-stage apoptosis. Gratifyingly, combination therapy didn't induce apoptosis in normal HAEC. Thus, we concluded the combination of low doses of tigecycline and PP is selectively cytotoxic toward breast cancer cells.

ROS

We measured intracellular ROS in the three cell lines exposed to tigecycline, PP, or the combination (Fig. 6). As a result, tigecycline didn't significantly increase either ROS level in the three cell lines treated with tigecycline whereas pyrvinium treatment increased ROS in the two cancer cell lines. Interestingly, combination therapy considerably increased the levels of ROS in both cancer cell lines but had no effect in normal cells.

Discussion

In this study, we found the combined effect of the antibiotic tigecycline (TIG) and the anthelminthic drug, pyrvinium pamoate (PP) had anti-cancer activity from in vitro experiments. Here, we used a microfluidic-based hanging-drop culture system to form cell spheroids and used this for drug delivery. Because of the heterogeneity of breast cancer, two cancer cell lines, MDA-MB-231, and MCF-7 were used in this study. There are very different tissues surrounding breast tumors such as adipocytes, inflammatory cells, and endothelial cells.[15] We chose HAECs as a model for surrounding normal tissue, to compare the effect of drugs. Numerous evidences have suggested that breast cancer can induce resistance via different mechanisms including increased ALDH activity, enhanced DNA repair mechanisms, enhanced ROS scavenging, and induction of autophagy, as well as others.[16] We assessed several of these processes to assess whether combination therapy, acting through one of these mechanisms, can achieve a better treatment effect. Our results showed that the synergistic effect of TIG with PP for breast cancer cells was cell-specific. Compared with solitary TIG or solitary PP therapy, three cell lines, including MDA-MB-231, MCF-7, and HAEC, cell activity were inhibited and apoptosis got induced. Conversely, low dose TIG combined with PP could effectively inhibit tumor cancer activity, induced autophagy, and increased the level of ROS to induce tumor apoptosis but showed no effect on normal cells.

To evaluate the effect of combined therapy, we first evaluated the half-maximal inhibitory concentration of two drugs in three cell spheroids. In previous studies, the solitary dose of TIG inhibits spheroids formation with an IC-50 between 10 and 50 μM,[17] and the solitary dose of PP is 500 nM—2000 nM.[10] The mechanism of tigecycline is target mitochondria dysfunction to modulate the metabolism of cancer cell. While, PP was reported that can modulate mitochondria respiratory activity to reduce oxidative

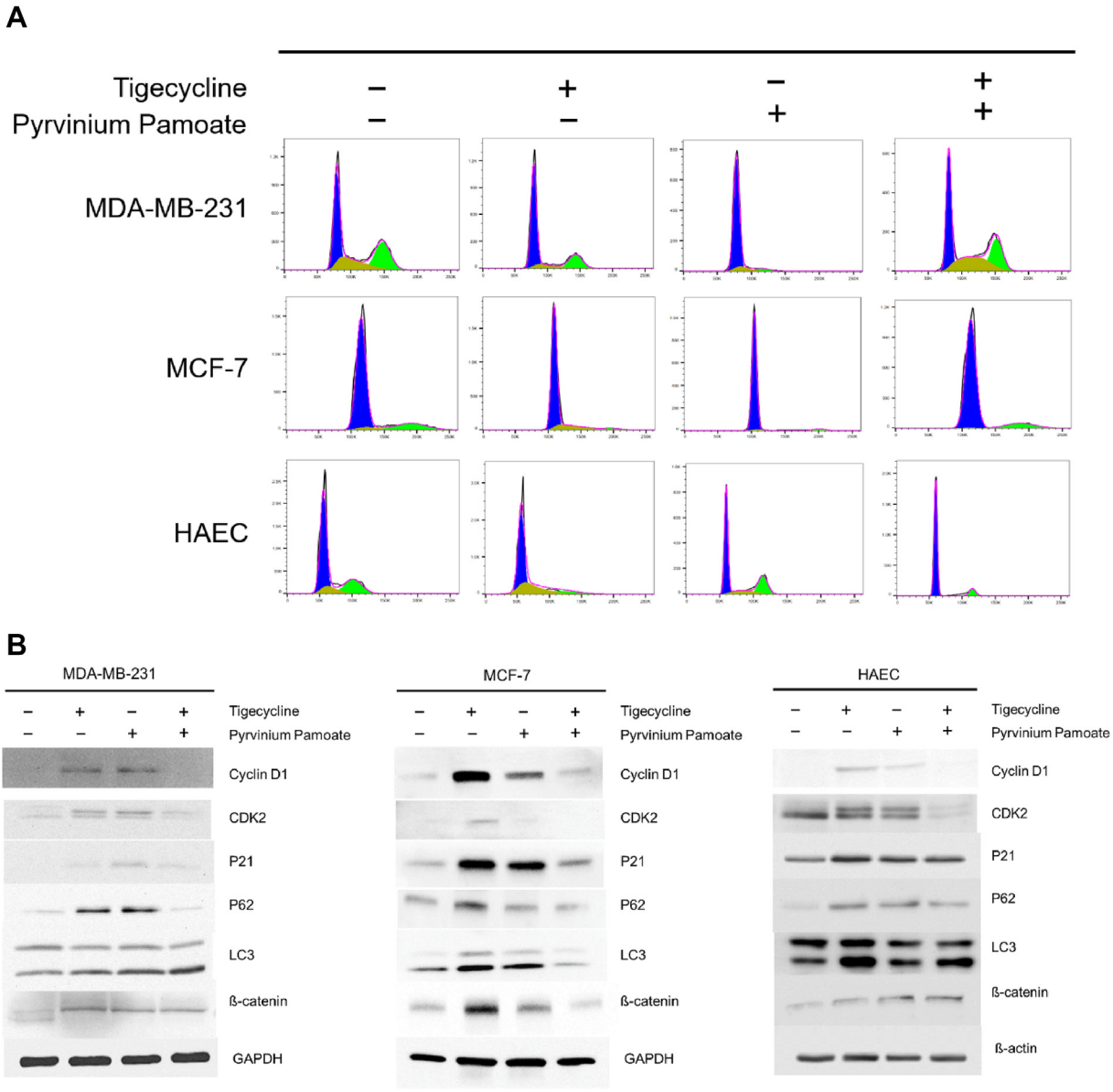

Figure 4. Effect of tigecycline only, pyrvinium pamoate only, and their combination on cell cycle and autophagy in the three cell lines. (A) FACS analysis of the cell cycle. (B) Western blotting of cell cycle and autophagy associated proteins.

phosphorylation and energy production. The common characteristics in these two drugs is target mitochondria to effect cell viability. But there were just minor mitochondria dependent in normal cell. The result of this study showed the same tendency. Compared with solitary TIG or PP,[18] a quantitatively similar result showed that the combined therapy with a lower dose of TIG and PP, also has the capacity to inhibit tumor-sphere growth, and without significant damage on a normal cell. We found that all cell types were damaged by high dose of tigecycline and PP. However, in the combined therapy group, MDA-MB-231 and MCF-7 spheroids started to exhibit hollow structure at the third day of treatment, and spheroid structure collapsed by day 7. And the structure of HAEC spheroids remained unchanged; on the contrary, their diameter increased with increasing culture period. Similar results were noted for cell viability. Thus, low concentrations of tigecycline and PP are selectively toxic to different cells.

Additionally, according to previous studies, TIG would induce cell cycle arrest at G1 phase.[18,19] In our study, flow cytometric analyses revealed that all cell lines responded to cell accumulation in the G1/S phase. For MDA-MB-231 cells, the percentage of cells in G1/S phase increased and in G2 phase decreased after both tigecycline treatment as well as PP treatment. However, in MDA-MB-231 cells, combined therapy didn't significantly alter the G1/S phase. In addition, MCF-7 cell accumulation was clearly observed in the G1/S phase. Moreover, HAECs weren't significantly damaged drug treatment compared with those after no treatment. We also accessed the key markers Cyclin D1,[20] p21,[21] and CDK2[22] that controlled the G1/S transition alter between three cell lines via western blot assay. We

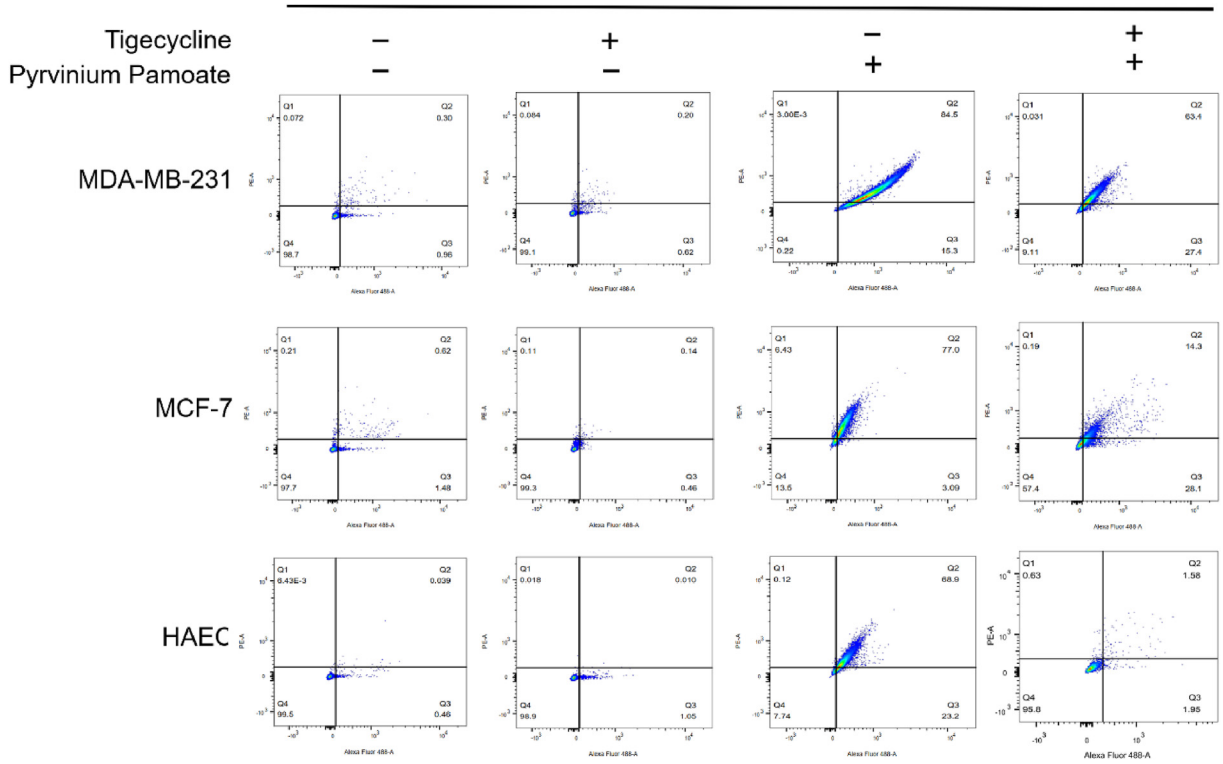

Figure 5. Effect of tigecycline only, pyrvinium pamoate only, and their combination on apoptosis.

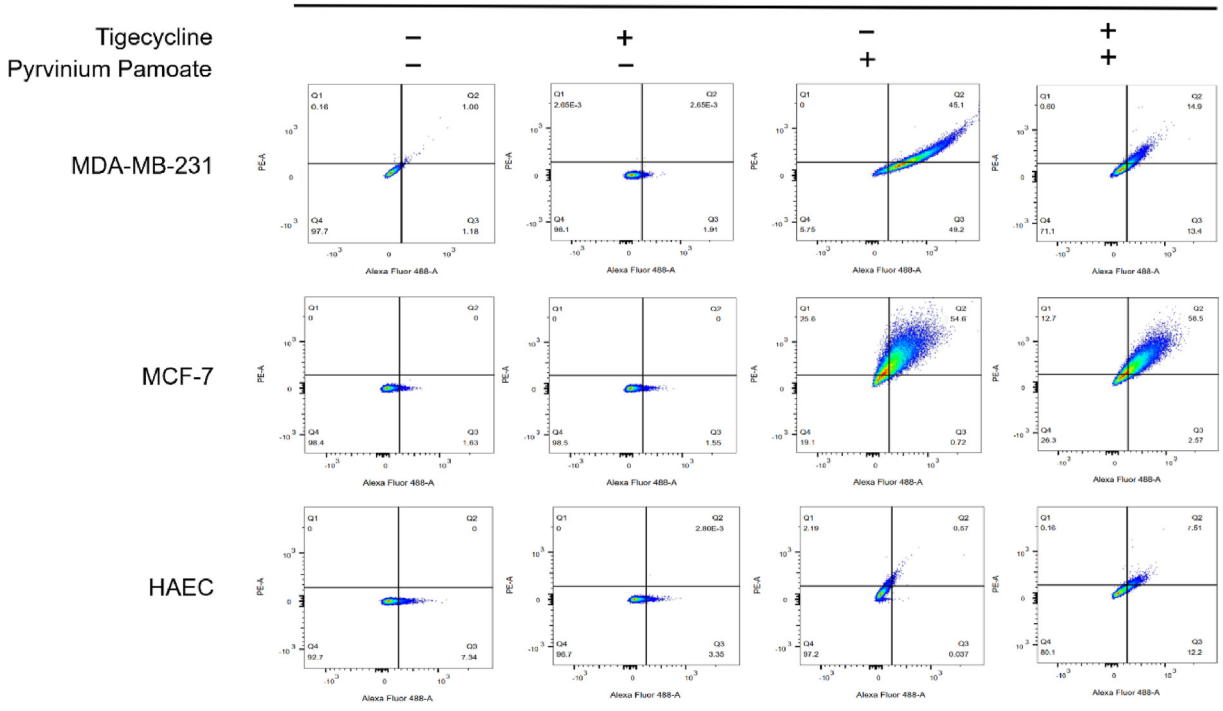

Figure 6. Effect of tigecycline only, pyrvinium pamoate only, and their combination on ROS levels.

found that in three cell lines, combined therapy markedly increased p21 levels and decreased CDK2 levels. However, no significant change in Cyclin D1 was noted in the three cell lines. In contrast, after tigecycline treatment was done in all three cell lines, the levels of Cyclin D1, CDK2, and p21 increased considerably. Similar results were noted with PP treatment. Thus, we inferred tigecycline combines with PP may have a synergistic effect on cancer, even at low doses.

We also found that two drugs, especially as combined therapy, were functionally involved in cell autophagy. Moreover, the levels of SQSTM1/p62 decreased in two breast cancer cell lines and those of LC3 II considerably increased; interestingly, no significant effect was noted in HAECs. Thus, the degradation of SQSTM1/p62 and accumulation of LC3-II-positive autophagosomes demonstrated efficient autophagic flux. These results are consistent with previous data,[23] that demonstrated cell cycle arrest and induce autophagy in cancer cells in the combination therapy group.

At last, in the apoptosis experiment, 72 h of treatment with tigecycline and PP in MDA-MB-231 cells increased the ratio of early-stage to late-stage apoptosis. In addition, the early-stage and late-stage apoptosis ratio in MCF-7 cells significantly increased. The combined therapy didn't induce apoptosis in the normal cells, HAEC. However, we didn't find a significant effect on apoptosis in the tigecycline-treated group. According to previous reports, TIG would not induce cell apoptosis, the data in our study is consistent with referential studies.[24] However, PP induces cell apoptosis then causes cell death.[25] Finally, we investigated whether cell apoptosis was induced by DNA damage by measuring intracellular ROS level in three cell lines. All ROS levels didn't significantly increase in three cell lines treated with tigecycline; however, PP treatment increased ROS levels in two cancer cell lines and the similar result showed in combined therapy. Interestingly, we didn't observe any significant effect in HAEC cells. From our results, we conclude that the combination of tigecycline and PP can have a selective cytotoxicity toward breast cancer cells.

In conclusion, the clinical significance of this study is that it introduces a novel method of using antibiotics to treat cancer which would solve some problems in later stage of cancer. TIG would act by binding to mitochondrial 28 S ribosomes inhibiting mitochondrial translation and cell proliferation[7]; then PP would change the product activity from mitochondrial electron-transport chain to prevent ATP production and stimulate apoptosis in the cancer cell.[10] Both two drugs have a novel effect on cancer therapy, but either of them needs to work solitary at high concentrations. In this study, we proved that two agents could treat breast cancer via different pathway and let cell cycle arrest, inhibit cell proliferation, induce autophagy, even induce apoptosis in lower dose. Other problems like drug resistance occur in patients[4] or there were higher relative abundances of Bacillus, Enterobacteriaceae, and Staphylococcus in patients would confer susceptibility to cancers then influence the effectiveness of treatment, also could be improved through combined therapy.[17] Future work may use other cancer cell lines, animal models and clinical trials to further evaluate the functionality of the protection liner and improve the design.

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