

Phospholipase A2 increases the sensitivity of doxorubicin induced cell death in 3D breast cancer cell models

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SUMMARY

Doxorubicin is a common drug used for various cancer treatments including breast, ovarian, bladder, and lung cancer. However, inefficient penetration of cancer drugs into the interior of the three-dimensional (3D) tumor tissue limits the drug delivery. Increasing the dosage of drugs is an unacceptable solution, as patients may have a toxic reaction. Therefore, efficient drug delivery is crucial. We hypothesized that the addition of phospholipase A2 (PLA2), an enzyme found in bee venom that hydrolyzes the bond connecting fatty acid and glycerol, would increase the permeability of the drug for efficient drug penetration. We designed a 3D spheroid tumor model because it mimics an actual tumor environment better than the monolayer two-dimensional (2D) cell culture. We performed dye permeability assay using 5-chloromethylfluorescein diacetate (CMFDA), a green fluorescent probe, on 3D spheroid cells with concentration-dependent PLA2 treatment. We found that 1 mM PLA2 had the highest percentage of green fluorescence cells, indicating the highest permeability. Subsequently, we hypothesized that co-treatment of PLA2 and doxorubicin may increase drug sensitivity of doxorubicin. After analyzing the half-maximal inhibitory concentration, IC_{50} (639.8 nM) of doxorubicin on the MDA-MB-231 3D spheroid, the co-treatment of PLA2 and doxorubicin exhibited lower 3D spheroid cell viability compared to doxorubicin treatment alone. This indicates that PLA2 may be a useful drug to assist in more effective doxorubicin treatment for breast cancer. Increased efficiency in drug delivery would allow lower concentrations of drugs to be used, minimizing damage to normal cells.

INTRODUCTION

Cancer is a disease characterized by the uncontrollable growth and division of cells (1). Breast cancer is the most diagnosed cancer worldwide and the second most common in the U.S. (2). Breast cancer mortality has significantly increased worldwide over the past 25 years. Also, breast cancer is one of the most frequent causes of cancer death for

women worldwide (3).

Although conventional chemotherapy has helped many breast cancer patients, one of the main drawbacks of chemotherapy is the low penetration of drugs into three-dimensional (3D) tumor tissues (4). Therefore, high efficiency of drug penetration through the interior of the tumor tissue is required to avoid high-dose requirements and adverse side effects (5). In this study, we focused on PLA2, an enzyme found in bee venom for enhancing chemotherapy drug delivery (6). PLA2 disrupts the cell membrane and enhances the penetration of bee venom inside the host cells. It cleaves the fatty acid section of phospholipids through hydrolysis of the bond connecting fatty acid and glycerol (6). Since the outer layer of cells is composed of a phospholipid bilayer, PLA2 can effectively make the membrane more permeable through cleaving sections of the fatty acid (7).

To test whether PLA2 enhanced the drug delivery on breast cancer cells, we used MDA-MB-231 cell line, which is a type of epithelial cell found in breast tissues (3). This breast cancer cell line is frequently used for medical research properties. MDA-MB-231 is triple-negative, which means that it does not have estrogen receptors (ERs), progesterone receptors (PRs), or human epidermal growth factor receptor 2 (HER2) amplification (3). Also, this cell line exhibits aggressive metastasis in xenograft models (3).

To better mimic the breast cancer tumor tissue, we used a 3D cell culture model in this research. The limitation to the normal method of adding media on a flat surface is that cells usually grow in a two-dimensional (2D) layer (8). However, a 3D model shows a stronger resemblance to the actual structure of tumor tissues in the human body (8). To set up a 3D culture model, we used the hanging drop method in this study (9).

Doxorubicin is an anti-cancer chemotherapy drug used against breast, uterine, ovarian, lung, and cervical cancer. It is an anthracycline antibiotic, which is known to bind nucleic acids presumably by intercalation with the DNA double helix. When it is intercalated into DNA, it inhibits topoisomerase II and disrupts DNA repair and replication (10). DNA binding of doxorubicin causes cell cycle arrest and increases oxygen radicals that induce apoptosis (10). However, the use of this drug is limited by its dose-dependent toxicity. A recent

study indicated that overuse of doxorubicin due to poor drug penetration may cause multidrug resistance in tumors as well as serious side effects such as congestive heart failure (11).

Doxorubicin alone or in combination with other drugs is a common method used for many different types of cancers including breast, ovarian, bladder, and lung (12). However, a high dosage of drug usage may be harmful to the patients, as the drugs also harm normal cells (13). In addition, this drug may not penetrate to the core of tumors. Therefore, the efficiency of drug delivery is highly important (14). We hypothesize that the addition of PLA2 will increase the permeability of the cells for efficient drug delivery. This increased permeability would allow lower concentrations of drugs to be used, which would minimize damage to normal cells.

To test our hypothesis, we used a 3D spheroid cell culture to mimic the 3D structure of breast cancer tumors. In addition, a dye permeability assay was performed on concentration dependent PLA2 treatment to find the optimal PLA2 concentration. Lastly, the co-treatment of PLA2 and doxorubicin showed greater drug penetration, leading to a higher rate of cell death.

RESULTS

To find the optimized cell number for 3D cell culture, we tested different initial cell concentrations and analyzed the morphology of the cells. 2D cell culture of MDA-MB-231 showed a monolayer growth (Figure 1A). 3D culture of MDA-

MB-231 cells aggregated and formed 3D spheroids (Figure 1B–E). One visible pattern was that as the concentration of cells increased, the size of the cell cluster also increased (Figure 1). We concluded that 3×10^4 cells/ml would be the optimal concentration for 3D cell formation. With the hanging droplets of 1×10^4 cells/ml and 2×10^4 cells/ml, the microscope could not focus on the cell formation, so it was difficult to interpret the results. For concentrations of 4×10^4 cells/ml and above, the hanging droplet was unstable and difficult to control. Therefore, we used 3×10^4 cells/ml as the fixed concentration for downstream experiments.

To test whether PLA2 can be applied to increase the permeability of the 3D breast cancer cell membrane, 5-chloromethylfluorescein diacetate (CMFDA) dye, a green fluorescence dye that stains live cells, was used for the dye-permeability assay. As a result, even though $1 \mu\text{M}$ CMTFDA dye can stain almost 100% of the breast cancer cells in 2D culture conditions, 3D culturing conditions showed a relatively low level of green fluorescent cells (Figure 2). The negative control (3D) was a spheroid sample without treatment of $1 \mu\text{M}$ CMTFDA dye and showed that no cells were stained (Figure 2). Without PLA2, only about 60% of the cells were stained (Figure 2). The remaining 40% of the 3D spheroid cells, especially those that are in the interior, may not be stained well because of the limitation of CMTFDA in penetrating the 3D structure of the breast cancer cells. We further analyzed three different treatment concentrations of PLA2: 1 mM, 2

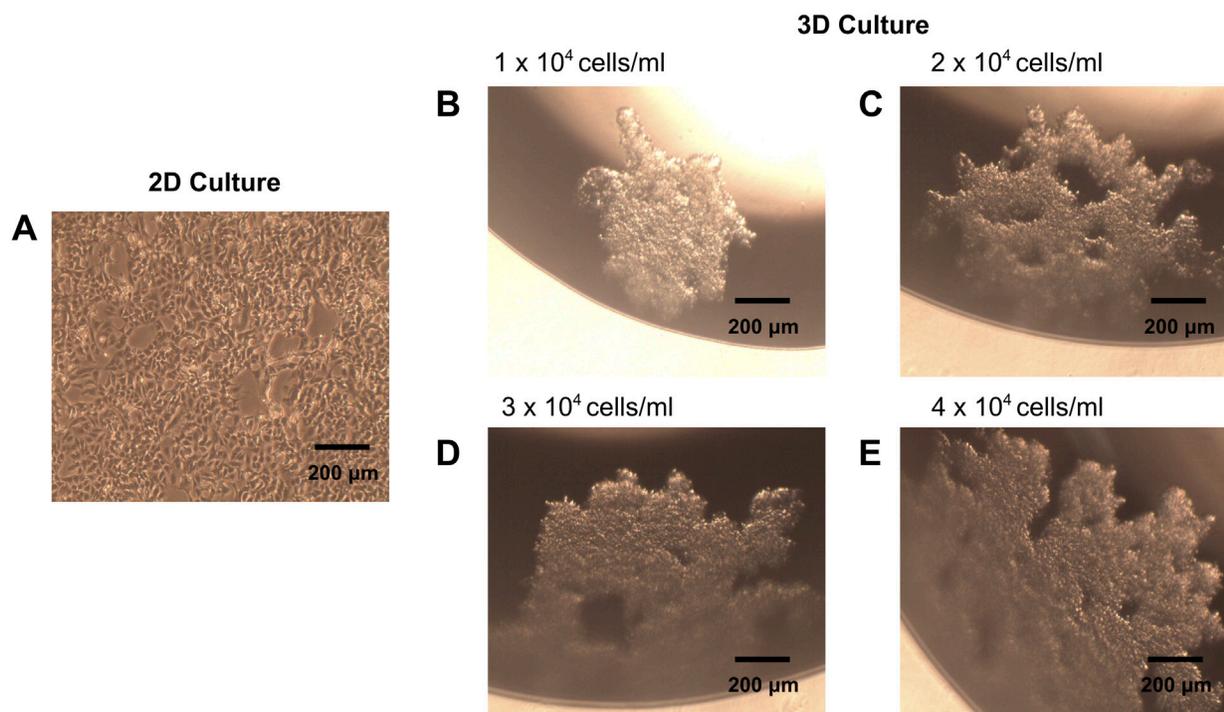


Figure 1. The image of MDA-MB-231 cell morphology after 2D and 3D culture. Different initial cell concentrations were used, and the morphology of the cells were photographed. Images show MDA-MB-231 cells grown as (A) a flat monolayer in 2D culture conditions or a 3D spheroid culture with (B) 1×10^4 cells/ml, (C) 2×10^4 cells/ml, (D) 3×10^4 cells/ml, or (E) 4×10^4 cells/ml. Images were taken with a brightfield inverted-microscope.

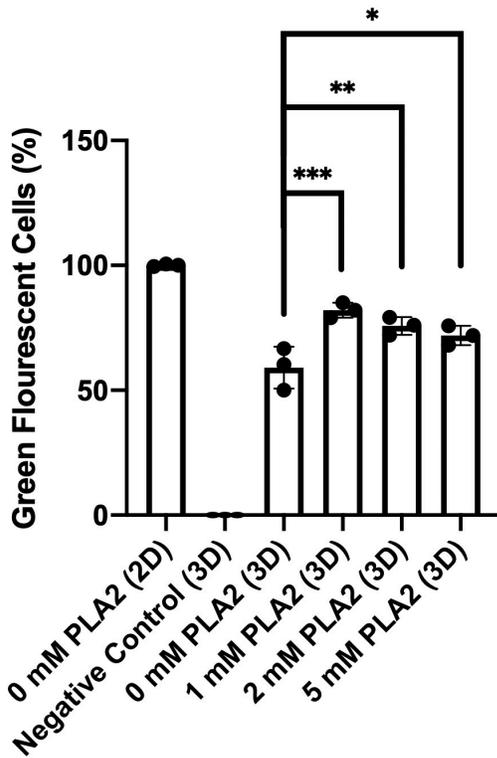


Figure 2. Dye permeability assay on concentration-dependent PLA2 treatment on 2D and 3D MDA-MB-231 cells. 1 μ M CMFDA and fluorescent probes were used to check cell membrane permeability for 10 min. After the cells were washed with the PBS to remove the remaining CMFDA from the cell media cell strainer was used to remove the cell clump. The cells were then dissociated into single cells with Trypsin-EDTA buffer and green fluorescent positive cells were quantified using an automated cell counter. The mean and the standard deviation are presented in the bar graph. Two-way ANOVA with Tukey's multiple comparisons test, p -value < 0.05 (*), p -value < 0.01 (**), p -value < 0.001 (***), N = 3.

mM, 5 mM on spheroids. Even though 1 mM PLA2 showed the highest percentage of green fluorescent cells compared to control ($p = 0.0003$), 2 mM and 5mM PLA2 did not further improve CMFDA penetration (Figure 2). Overall, this result indicates that PLA2 may disrupt the cell membrane thus increasing the cell permeability of CMFDA dye.

Next, we identified the IC_{50} of doxorubicin, which is the dosage that results in 50% cell death. IC_{50} is most often used as a measurement of antagonist, or inhibitory drug potency, as well as a quantification of the toxicological effects of inhibitory compounds. We treated the 3D spheroid with increasing concentrations of doxorubicin and measured cell viability. As the concentration of doxorubicin increased, the percentage of cell death also increased. The doxorubicin IC_{50} of a 3D spheroid MDA-MB-231 was found to be 639.8 nM (Figure 3).

We then hypothesized that co-treatment of doxorubicin with PLA2 on 3D spheroid MDA-MB-231 would decrease the cancer cell viability, leading to efficient drug penetration. Four

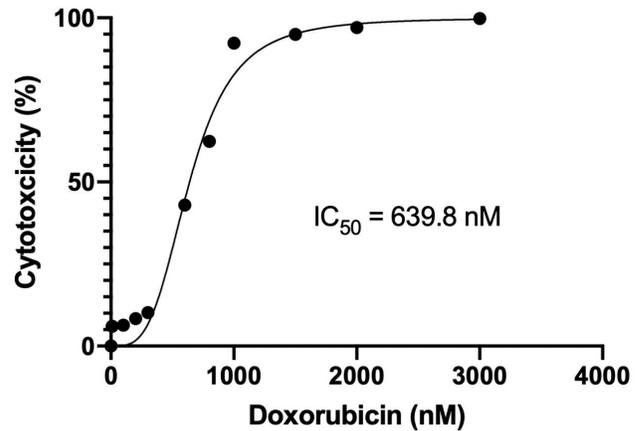


Figure 3. Cytotoxicity of doxorubicin in 3D MDA-MB-231 cells. The cell cytotoxicity was measured by the Prestobleue assay to identify IC_{50} of doxorubicin. The following doxorubicin concentrations were tested: 0, 10, 100, 200, 300, 600, 800, 1000, 1500, 2000, and 3000 nM. The IC_{50} value for doxorubicin was 639.8 nM, N = 1.

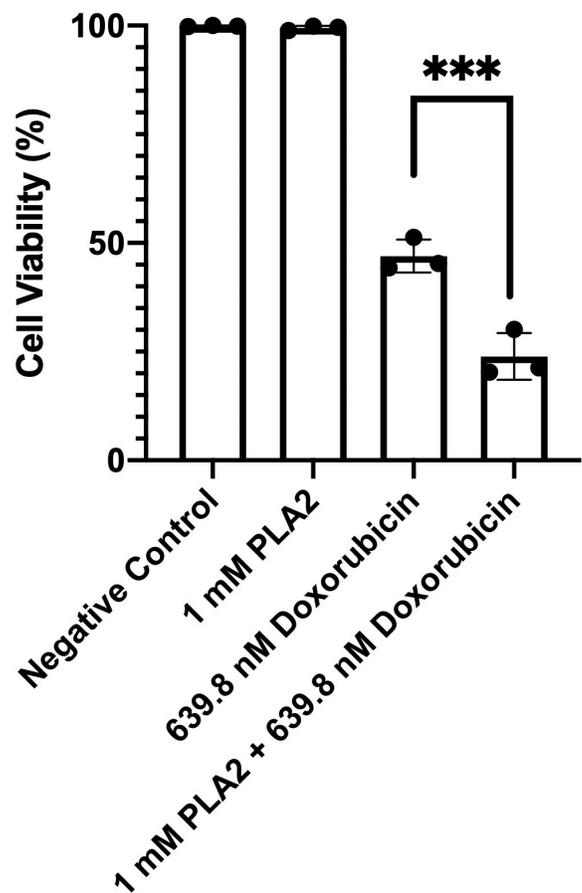


Figure 4. Effect of PLA2 and doxorubicin treatment on cell viability of MDA-MB-231 3D spheroid cells. Negative control (no PLA2 or doxorubicin treatment), 1mM PLA2, 639.8 nM doxorubicin, and 1 mM PLA2 with 639.8 nM doxorubicin treatment was tested. The cell viability was quantified after 24 hr treatment. The mean and the standard deviation are presented in the bar graph. Two-way ANOVA with Tukey's multiple comparisons test, p -value < 0.001 (***), N = 3.

different conditions of treatment were tested: Negative control (no PLA2 or doxorubicin treatment), 1 mM PLA2, 639.8 nM doxorubicin, and 1 mM PLA2 with 639.8 nM doxorubicin (**Figure 4**). The treatment of 1 mM PLA2 showed near 100% cell viability, indicating that membrane disruption by PLA2 did not affect cell viability (**Figure 4**). The 639.8 nM (IC₅₀) doxorubicin treatment decreased the cell viability to around 45%, which was consistent with our previous experiment ($p = 0.00009$) (**Figure 4**). During the co-treatment of PLA2 and doxorubicin, cell viability significantly decreased compared to treatment of doxorubicin alone ($p = 0.0002$) (**Figure 4**). In conclusion, the co-treatment of PLA2 and doxorubicin was more effective in increasing drug sensitivity than the individual treatment of PLA2 and doxorubicin.

DISCUSSION

We concluded that PLA2 may be a useful drug to assist in more effective doxorubicin treatment for breast cancer. Also, we designed this experiment to include a 3D cell culture because it mimics an actual tumor condition better than the monolayer cell morphology of 2D cell cultures. A 3D cell culture can model interactions between cellular and extracellular models. It also more accurately depicts cell morphology, polarity, and division (15).

Conventional chemotherapy for cancer treatment in clinical practice has many limitations (16). One of the critical problems is the lack of targeting the delivery of anticancer compounds selectively into solid tumor tissue to execute anticancer activities (17). The lack of permeability of chemotherapy drugs requires a high concentration of drug treatment, which often induces serious side effects (18). Our result agrees with the previous study that doxorubicin alone did not effectively induce cell death in 3D spheroid cancer cells than 2D cultured cells. Also, we discovered that PLA2 can increase the doxorubicin permeability in 3D cancer cell model. PLA2 may disrupt the cancer cell membrane and allow the drugs to enter the tumor tissue easily. Therefore, many different combination treatments of chemotherapy drugs with PLA2 should be tested in the future.

A recent study targeted membrane integrity of cancer cells to increase the efficacy of anticancer drugs (19). A previous study also found that the presence of 200 kHz tumor-treating fields (TTFields), which are high-frequency electric fields, increased cellular membrane permeability in glioblastoma (20). Also, treatment of TTFields has been shown to improve the median survival of glioblastoma patients with temozolomide treatment (21). These results indicate that targeting the cancer cell membrane can be an excellent strategy to increase drug efficacy.

This study found that PLA2 co-treatment with doxorubicin can be used for greater drug penetration, leading to a higher rate of cell death due to targeted drug delivery. We used the enzymatic activity of PLA2 disrupting the integrity of the cancer cell membrane to increase the drug penetration. However, since PLA2 can also disrupt the normal cell's membrane,

the controlled-release capsule method may be best for this PLA2 co-treatment with doxorubicin. PLA2 co-treatment with doxorubicin must be specifically released near solid tumors; otherwise, it may disrupt the membrane of normal cells and eventually induce cell death.

There are several limitations to this study. First, the concentration of PLA2 used in this study does not represent the reality for actual *in vivo* use. The tumor's microenvironment *in vivo* contains complex interaction between different cells such as endothelial cells from blood vessels, immune cells, and normal fibroblast cells near the cancer cells (22). Since we only tested *in vitro* conditions, in which only cancer cells were present in the samples, a higher concentration of PLA2 may be needed for actual *in vivo* use. Using a higher concentration of PLA2 may also cause severe side effects disrupting the membrane of normal cells around the tumor tissue. Therefore, optimized dosage of PLA2 for *in vivo* use must be identified.

Also, we only tested one type of breast cancer cell line *in vitro*. Therefore, an *in vivo* experiment would be necessary to assess the actual anti-cancer effect. Most importantly, we did not analyze the effect of PLA2 on normal cells. PLA2 might also destroy the membranes of normal cells, leading to cell death. In the future, we plan to extend our research by using the co-treatment of PLA2 with various cancer drugs. We also plan to evaluate the effect PLA2 has on normal cells. Ultimately, we are looking for a novel drug treatment method that will specifically target cancer cells.

MATERIALS AND METHODS

Cell culture maintenance

MDA-MB-231 cells were purchased from Korea Cell Line Bank (KCLB). Cells were maintained in RPMI 1640 media (Gibco) supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin and streptomycin antibiotics (Gibco). The cells were stored inside a 37°C, 5% CO₂ incubator (Eppendorf).

Hanging drop 3D spheroid culture

After the cells were detached from the cell culture plate surface with 0.05% Trypsin-EDTA buffer (Gibco), the complete medium was added to stop trypsinization. The cells were counted with the Luna Fluorescence Cell Counter (Logos), the cells were seeded on the cover lid of 100 mm tissue culture dish by adding a 30 μ L drop. Then, 10 mL of cell medium was placed in the bottom of the dish to act as a hydration chamber. Then the lid was inverted onto the medium-filled bottom chamber and incubated in the 37°C, 5% CO₂ cell incubator. The 3D spheroid cultures were maintained for five days before the downstream experiments.

Cell imaging

After the lid containing the 3D spheroid cells was placed on the inverted microscope (Nikon), the brightfield image of the cells was captured by imaging software provided by Nikon. The scale bar was added on the image based on the

magnification of the microscope.

Dye permeability assay

The CMFDA (5-chloromethylfluorescein diacetate) fluorescent probe was used to check cell membrane permeability. After the spheroids were collected from the hanging drop, the cells were incubated with 1 μM CMFDA for 10 min, the cells were washed with the PBS to remove the remaining CMFDA from the cell media. The cells were then dissociated into single cells with Trypsin-EDTA buffer. A 40 μm cell strainer (SPL) was further used to remove the clumps from the prepared samples. All experiments were performed with three replicates for each experimental group. Dye permeability assay was conducted at 37°C.

Fluorescent cell quantification

Luna-FL (Logos Bio), an automated fluorescence cell counter, was used to quantify the green fluorescent cells from each sample. After the cells were injected on the sample loading slide (Logos Bio), the integrated analysis software provided the percentage of green fluorescent positive cells.

Cell viability assay

Prestoblue (Invitrogen) was used to quantify cell cytotoxicity (dead cells) and cell viability (live cells). The final concentrations of doxorubicin (Sigma) (0, 10, 100, 200, 300, 600, 639.8, 800, 1000, 1500, 2000, and 3000 nM) were used to treat on the hanging droplets for 24 hr. For PLA2 co-treatment with doxorubicin, 1mM PLA2 and 639.8 nM doxorubicin was treated for 24 hr. Then, the cells were placed into 96 well plates for the cell viability assay. After measuring 570 nm absorbance using a microplate reader (Biotek), the cell cytotoxicity was calculated. IC_{50} value was calculated by the Prism 8 program. All experiments were performed with three replicates for each experimental group. Cell viability assay was conducted at 37°C.

Statistical test

All statistical tests were performed using the GraphPad Prism 8 program. Two-way ANOVA and Tukey's multiple comparison post hoc test were used. A p value less than 0.05 was considered statistically significant.

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