

## RESEARCH ARTICLE

# Pentagamavunon-1 Enhances the Anticancer Effects of Doxorubicin on Triple-Negative Breast Cancer Cells in Monolayers and 3D Cancer Spheroid Models

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## Abstract

**BACKGROUND:** The 4T1 cells, a triple-negative breast cancer (TNBC) cell line, exhibit high malignancy and metastatic potential. As a primary treatment for TNBC, doxorubicin has limitations, including drug resistance mechanisms and severe side effects such as cardiotoxicity. Pentagamavunone-1 (PGV-1) exhibits antiproliferative and antimetastatic effects, induces prometaphase arrest, triggers cell senescence, and enhances reactive oxygen species (ROS) modulation, which may help overcome doxorubicin resistance. The selective cytotoxicity of PGV-1 against cancer cells suggests that it has a role in reducing systemic toxicity. Therefore, in this study, the anticancer effects of doxorubicin combined with PGV-1 was investigated.

**METHODS:** Monolayer/2D and spheroid/3D models of 4T1 cells were used to assess the effects of PGV-1, doxorubicin, and their combination. MTT assay was used to evaluate the cytotoxicity, colony formation assay was used to measure persistent antiproliferative effects, and spheroid volume analysis was performed to assessed tumor growth inhibition. Senescence-associated beta-galactosidase (SA- $\beta$ -gal) assay determined cellular senescence.

**RESULTS:** The combination of PGV-1 and doxorubicin significantly enhanced cytotoxicity, with IC<sub>50</sub> values of 0.57  $\mu$ M and 4.88  $\mu$ M, respectively ( $p=0.000$ ). A strong synergistic effect was observed, leading to persistent suppression of cancer cell proliferation and an 80% reduction in colony formation ( $p=0.007$ ). In the 3D spheroid model, combination treatment significantly reduced spheroid volume ( $p=0.002$ ) more effectively than monotherapy, indicating superior growth inhibition and cytotoxicity. It also increased SA- $\beta$ -gal, the senescence marker ( $p=0.010$ ).

**CONCLUSION:** The combination of PGV-1 and doxorubicin demonstrated potent anticancer effects in 4T1 monolayers and spheroid models by enhancing cytotoxicity and inducing cellular senescence. This combination confirmed its potential as a more effective therapeutic strategy.

**KEYWORDS:** 3D spheroid, 4T1 TNBC cell, doxorubicin, pentagamavunon-1, PGV-1, senescence

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## Introduction

Triple-negative breast cancer (TNBC) is a highly aggressive subtype of breast cancer that lacks estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth

factor receptor 2 (HER2) amplification.(1,2) This unique molecular profile limits the use of targeted hormonal and HER2-directed therapies, leading to poor prognosis and limited treatment options.(1) TNBC is characterized by high genetic heterogeneity, rapid disease progression, and an increased likelihood of metastasis compared to other

breast cancer subtypes.(3) Due to the absence of specific molecular targets, chemotherapy remains the mainstay treatment for TNBC, highlighting the urgent need for more effective therapeutic strategies. TNBC accounts for approximately 15-20% of all breast cancer cases worldwide and is associated with a higher mortality rate compared to other breast cancer subtypes.(4,5) In Indonesia, TNBC contributes significantly to breast cancer-related deaths, with limited access to effective treatment options further exacerbating the clinical burden. Given its aggressive nature and poor prognosis, developing novel therapeutic approaches for TNBC is crucial to improving patient survival rates.(6) The current standard treatment for TNBC primarily relies on conventional chemotherapy, including anthracyclines such as doxorubicin, and taxanes such as paclitaxel.(7,8) While these agents demonstrate initial efficacy, TNBC cells often develop resistance, leading to disease recurrence and treatment failure.(7,8) Additionally, chemotherapy is associated with severe adverse effects, including myelosuppression, neuropathy, and cardiotoxicity, which significantly impact patient quality of life. Therefore, strategies that enhance the efficacy of TNBC treatment while minimizing toxicity are highly needed.(9-12)

Doxorubicin remains a cornerstone in TNBC chemotherapy due to its potent cytotoxic effects. It exerts anticancer activity through the induction of reactive oxygen species (ROS), leading to DNA damage and apoptosis. (13,14) However, TNBC cells frequently develop resistance to doxorubicin-enhanced DNA repair and activation of survival signaling pathways.(15-17) Additionally, epigenetic modifications, including histone modifications and DNA methylation changes, have been implicated in doxorubicin resistance, altering the expression of key regulatory genes involved in cell survival and drug response. (18,19) Moreover, the tumor microenvironment, particularly hypoxia and stromal interactions, contributes to doxorubicin resistance by promoting epithelial-mesenchymal transition (EMT) and stemness properties in TNBC cells.(20) These limitations highlighted the need for combination therapies to enhance the effectiveness of doxorubicin while mitigating its adverse effects.

Pentagamavunon-1 (PGV-1), a synthetic curcumin analog, has emerged as a promising anticancer agent with superior chemical stability and enhanced antiproliferative activity compared to curcumin.(21-23) Unlike doxorubicin, which primarily induces apoptosis, PGV-1 exerts its cytotoxic effects by inducing prometaphase arrest, disrupting mitotic spindle formation, and promoting mitotic catastrophe, leading to cellular senescence or non-

apoptotic cell death.(21,23,24) PGV-1 has been reported to possess anti-metastatic properties by downregulating matrix metalloproteinases (MMPs), which are crucial for tumor metastasis.(25) Additionally, PGV-1 exhibits selective toxicity towards cancer cells while sparing normal cells, thereby reducing the likelihood of systemic toxicity. Preclinical studies have also shown that PGV-1 enhances ROS generation and inhibits nuclear factor kappaB (NF- $\kappa$ B) activation.(26) These properties suggest that PGV-1 has the potential as an adjuvant therapy to enhance chemotherapy efficacy while minimizing adverse effects.

Combining PGV-1 with doxorubicin presents a novel therapeutic strategy to improve anticancer efficacy and overcome the limitations of single-agent therapy. Despite the promising potential of PGV-1, PGV-1 as a monotherapy may have limitations, including suboptimal tumor penetration and potential dose-dependent cytotoxicity. Several studies have explored PGV-1 as a monotherapy; however, its combination with standard chemotherapeutics, such as doxorubicin, remains underexplored. Given the necessity of doxorubicin in TNBC treatment, combinatorial approaches that exploit PGV-1's unique mechanism while minimizing doxorubicin's side effects are crucial.

Most preclinical studies evaluating the efficacy of TNBC therapies have relied on two-dimensional (2D) monolayer cell cultures. However, these models fail to accurately replicate the tumor microenvironment, particularly in terms of drug penetration and cell-to-cell interactions. In contrast, three-dimensional (3D) spheroid models provide a more physiologically relevant system that better mimics *in vivo* tumor architecture and drug resistance mechanisms.(27) Compared to 2D monolayer cultures, 3D spheroids better represent tumor heterogeneity, hypoxia gradients, and drug diffusion barriers, making them a more predictive model for therapeutic responses. (28-30) In TNBC research, the 4T1 murine breast cancer cell line serves as a well-established model due to its EMT characteristics and aggressive metastatic potential.(3,25) This study was conducted to investigate the potential of PGV-1 in combination with doxorubicin for TNBC treatment using a 3D spheroid model. Specifically, the cytotoxic and synergistic effects of PGV-1 and doxorubicin on TNBC cells in both monolayer and spheroid models were evaluated, their impact on spheroid volume reduction as an indicator of tumor growth inhibition and the induction of cellular senescence through senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal) staining were assessed. Furthermore, in this study, the efficacy of the combination therapy to single-agent treatments to determine its therapeutic advantage was

also compared. The findings of this study are expected to provide new insights into the therapeutic potential of PGV-1 as an adjuvant for doxorubicin in TNBC, supporting the development of more effective and less toxic treatment strategies.

## Methods

### Cultivation of Monolayer (2D) Cell Model

The 4T1 breast cancer cells (ATCC No. CRL-2539TM; ATCC, Manassas, VA, USA), Vero normal kidney cells (ATCC No. CCL-81TM; ATCC), and NIH-3T3 fibroblast cell (ATCC No. CRL-1658TM; ATCC) were procured from the American Type Culture Collection (ATCC) and cultured in accordance with ATCC's recommended medium. Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. 12800-01710X1L; Gibco, Grand Island, NY, USA) was used as the culture medium for 4T1, Vero, and NIH-3T3 cells. The media were enriched with glucose and supplemented with 10% (v/v) fetal bovine serum (FBS) (Cat. No. 26140079; Thermo Fisher Scientific, Waltham, MA, USA), HEPES buffer (Gibco), and sodium bicarbonate (Cat. No. S5761-500G; Sigma-Aldrich, St. Louis, MO, USA). The cells were maintained in a humidified incubator at 37°C with a 5% CO<sub>2</sub> atmosphere.

### Treatment and Study Design

The combination treatment was performed using sub-doses of PGV-1 and doxorubicin at 1/8, 1/4, and 1/2 of their respective IC<sub>50</sub> values, which were 0.626, 1.25, and 2.5 μM, and 70, 140, and 280 nM to evaluate their synergistic effects. The 4T1 cells were planted at 3×10<sup>3</sup> cells/well and normal cells (Vero and NIH-3T3) at 4×10<sup>3</sup> cells/well on 96-well plates and left overnight. Treatment was administered using doxorubicin and PGV-1 at concentrations of 1–10 μM, with an untreated control group serving as a baseline reference. In the colony formation test, 1×10<sup>3</sup> cells/well of 4T1 cells were cultured in 6-well plates and subjected to sub-IC<sub>50</sub> concentrations of 2.5 μM PGV-1, 280 nM Dox, and their combination for 24 hours.

### Cytotoxic Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Cat. No. M2128-100MG; Sigma-Aldrich) test was conducted to evaluate the cytotoxicity profile of doxorubicin and PGV-1 compounds. PGV-1, which was obtained from the Cancer Chemoprevention Research Center (CCRC) at the Faculty of Pharmacy, Universitas

Gadjah Mada, and Doxorubicin Hydrochloride (Cat. No. 040-21521; FUJIFILM Wako Pure Chemical, Osaka, Japan) were used as a therapeutic agent in this study. The test was conducted singly to determine the IC<sub>50</sub> value and selectivity index (SI) on cancer and normal cells. Furthermore, a combination test was conducted to assess the synergistic effect by obtaining the combination index (CI) value after 24 hours of incubation. The drug medium was discarded and replaced with a medium containing MTT reagent, then incubated for 4 hours until formazan crystals were formed. (31) The reaction was stopped with an SDS-stopper solution, and absorbance was measured at a wavelength of 595 nm using a microplate reader (BioRad, Hercules, CA, USA). The IC<sub>50</sub> [1], SI [2], and CI [3] calculations were performed according to standard procedures, as follow:

$$[1] IC_{50} = \frac{\text{cell absorbance with treatment} - \text{media control absorbance}}{\text{cell control absorbance} - \text{media control absorbance}}$$

$$[2] SI = \frac{IC_{50} (\text{non-cancerous cell})}{IC_{50} (\text{cancerous cell})}$$

$$[3] CI = \frac{D_1}{D_{x1}} + \frac{D_2}{D_{x2}}$$

### Colony Formation Assay

Colony formation models were employed to verify the permanent effects of PGV-1, doxorubicin, and their combination on inhibiting cancer cell growth. After the 4T1 cells were cultured and subjected to various combination of treatments, the chemicals were removed and replaced with a new, drug-free medium. The cells were then cultured for up to 14 days. The established cell colonies were stained with 0.01% (w/v) crystal violet in dH<sub>2</sub>O for 45 minutes, following fixation with a 4% paraformaldehyde solution for 30 minutes. The colonies were ultimately measured utilizing ImageJ software (National Institutes of Health, Bethesda, MA, USA).

### Cultivation of Cancer Cells in 3D Spheroids Models

Triple-negative 4T1 breast cancer cells were cultivated in a three-dimensional configuration utilizing a 1% agarose-based matrix embedding technique in 96-well plates. Initially, 50 μL of DMEM complete culture media with 1% agarose (Cat. No. A9539-100G; Sigma) was introduced into each well and let to harden, creating a gel matrix. Subsequently, 50 μL of cell-free growth media was applied to the solidified gel. A 2×10<sup>3</sup> cells/well of 4T1 cells was inoculated in 20 μL of culture media, ensuring meticulous placement into the liquid medium above the gel matrix for sufficient liquid coverage. The wells were subsequently cultured under

circumstances that promoted the development of a singular 4T1 cell spheroid per well. The cells were cultured for seven days to facilitate the creation and compaction of spheroids. After the incubation, each well housed a single spheroid prepared for treatment with the test chemical.

### Spheroid 3D Antiproliferation Assay

The 4T1 spheroid cells were allowed to aggregate and form a single spheroid per well in 96-well plates over a seven-day development period. Afterward, the growth medium was replaced with a treatment medium containing PGV-1, doxorubicin, and their combination for 3 days. After the 3-day treatment, the press was replaced with a compound-free medium, and the spheroids were cultured for an additional 10 days. The dimensions of the spheroids were monitored daily using microscopy, and the diameter was measured with ImageJ software. These measurements were used to calculate the volume of the 4T1 spheroids for each treatment condition.

### SA- $\beta$ -gal Assay in 3D Spheroids Cells

All treated cells were incubated in a 4% formaldehyde solution for 10 minutes, washed with phosphate buffered saline (PBS), stained with a 0.2% X-gal solution (FUJIFILM Wako Pure Chemical), and then incubated for 72 hours prior to microscopic observation in phase contrast. The characterization of spheroid cells was conducted by interpreting qualitative observations of color intensity, wherein the presence of green staining in the peripheral region of the spheroid signified cellular senescence.

### Statistical Analysis

The data were statistically analyzed using SPSS software version 25 (IBM Corporation, Armonk, NY, USA), with results presented as mean $\pm$ SD. All experiments were conducted in triplicate (n=3) to ensure the reliability and reproducibility of the data. The *p*-value indicated the significance of the observed differences. A one-way analysis of variance (ANOVA) was performed on the experimental data, followed by Bonferroni post-hoc tests. Statistical significance was denoted by asterisks (\**p*< 0.05) in figures.

## Results

### Anti-proliferative Effects of PGV-1 and Doxorubicin on TNBC and Normal Cells

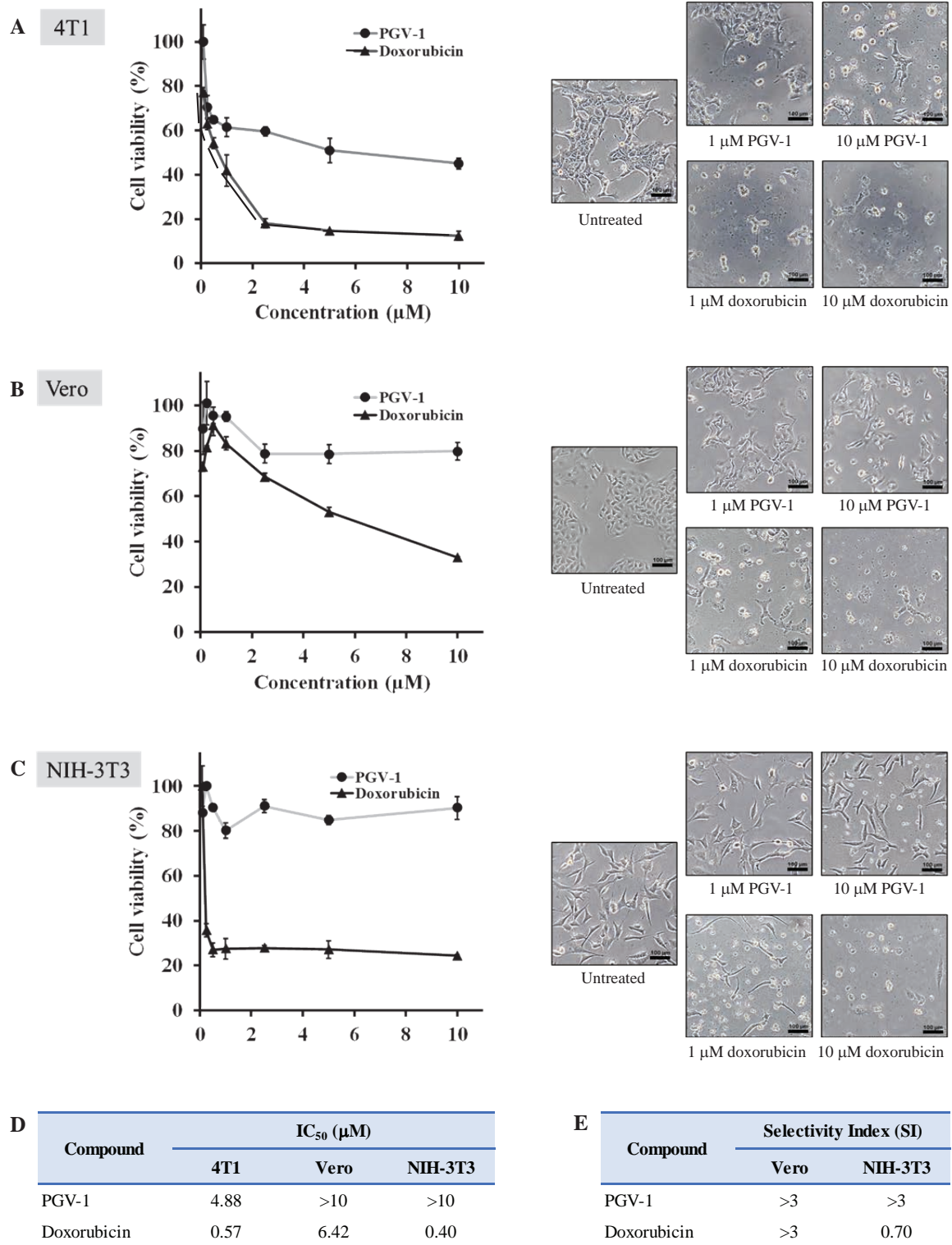
The results showed that PGV-1 exerts a cytotoxic effect on murine 4T1 breast cancer cells but through a mechanism

distinct from that of doxorubicin. The cell viability demonstrated that doxorubicin significantly reduced the viability of 4T1 cells compared to PGV-1, with IC<sub>50</sub> values of 0.57  $\mu$ M and 4.88  $\mu$ M, respectively (Figure 1A). This suggests that doxorubicin was more effective in eliminating cancer cells but also exhibited more significant toxicity towards normal cells. After treatment, observations of the morphology of 4T1 cells further support these findings, showing that doxorubicin induced a more pronounced cytotoxic effect than PGV-1.

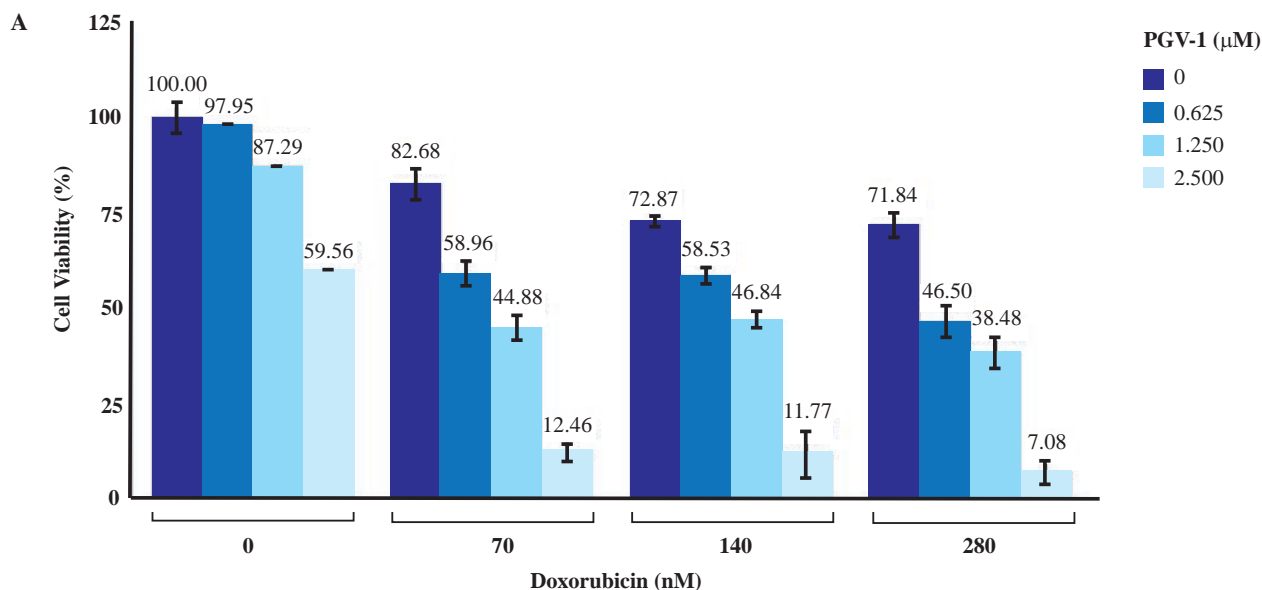
Furthermore, evaluations of non-cancerous Vero and NIH-3T3 cells were carried out to test the selectivity of the two compounds. The viability test results showed that PGV-1 had lower toxicity to normal cells than doxorubicin. The IC<sub>50</sub> value of PGV-1 on Vero and NIH-3T3 cells was more than 10  $\mu$ M (Figure 1B and 1C), while doxorubicin had an IC<sub>50</sub> of 6.42  $\mu$ M on Vero cells and 0.40  $\mu$ M on NIH-3T3 cells (Figure 1B and 1C). The microscopic observations also support this finding, showing that Vero and NIH-3T3 cells treated with doxorubicin experienced significant morphological changes, including shrinkage and detachment from the substrate. In contrast, cells treated with PGV-1 maintained their normal morphological appearance. From this data, SI was calculated to assess the degree of selectivity of the compound for cancer cells compared to normal cells. PGV-1 has an SI of more than 3 for Vero and NIH-3T3 cells, indicating better selectivity for cancer cells. In contrast, doxorubicin exhibited a low SI for NIH-3T3 cells (0.70) (Figure 1E), indicating significant toxicity to normal cells (*p*=0.005).

### Synergistic Effect of PGV-1 and Doxorubicin on 4T1 Cells

The combination of PGV-1 and doxorubicin exhibited a synergistic cytotoxic effect on 4T1 breast cancer cells. The combination of various concentrations of PGV-1 (0.625, 1.25, and 2.5  $\mu$ M) with doxorubicin (70, 140, and 280 nM) resulted in a more significant reduction (*p*=0.026) in cell viability compared to the use of each compound individually alone (Figure 2A). Increasing the concentration of PGV-1 combined with doxorubicin significantly reduced cell viability, indicating an increase in cytotoxic effects when the two agents were co-administered. In addition, the CI value in Figure 2B confirmed the synergistic interaction between PGV-1 and doxorubicin. A CI smaller than 1 indicated a synergistic effect, where the lower the CI value, the stronger the synergy. At PGV-1 concentrations of 1.25 and 2.5  $\mu$ M in combination with all tested doxorubicin concentrations, the CI values were consistently below 0.5, indicating a strong



**Figure 1.** Cytotoxic effects of doxorubicin and PGV-1 on 4T1, Vero, and NIH-3T3 cells. A: Cell viability profiles of 4T1 cells after 24-hour treatment with varying concentrations of doxorubicin (0–10 µM) and PGV-1 (0–10 µM). B: Cell viability profiles of NIH-3T3 cells after 24-hour treatment with varying concentrations of doxorubicin (0–10 µM) and PGV-1 (0–10 µM). C: Cell viability profiles of Vero cells after 24-hour treatment with varying concentrations of doxorubicin (0–10 µM) and PGV-1 (0–10 µM). Representative microscopic images of cell morphology after treatment are displayed on the right side of each graph, showing untreated cells, PGV-1-treated cells at 1 µM and 10 µM, and doxorubicin-treated cells at 1 µM and 10 µM. Black bar: 100 µm. D: IC<sub>50</sub> values of doxorubicin and PGV-1 in the tested cell lines were determined using non-linear regression analysis. E: SI values of doxorubicin and PGV-1, calculated as the ratio of IC<sub>50</sub> in normal cells (Vero and NIH-3T3) to IC<sub>50</sub> in 4T1 cells. SI value greater than 3 indicates selective cytotoxicity toward cancer cells.



**B**

PGV-1 (μM)	Doxorubicin (nM)		
	70	140	280
0.625	0.00	-0.20	0.26
1.250	0.19	0.26	0.22
2.500	0.15	0.16	0.17

**Figure 2. Synergistic cytotoxic effects of PGV-1 and doxorubicin on 4T1 cells.** A: Cell viability graph illustrating the combination of doxorubicin and PGV-1 at concentrations of 1/8, 1/4, and 1/2 of IC<sub>50</sub> for each compound. B: Combination index table for each treatment. CI values are categorized as follows: CI < 1 (synergism), CI = 1 (additive effect), and CI > 1 (antagonism).

synergistic effect. Even at the lowest PGV-1 concentration (0.625 μM), the combination with doxorubicin showed CI values approaching or below zero at higher doxorubicin concentrations, further strengthening the indication of synergy.

**Persistent Breast Cancer Cell Growth Suppression**

The colony formation assay results showed that the combination of PGV-1 and doxorubicin significantly inhibited ( $p=0.007$ ) the proliferation of 4T1 breast cancer cells compared to single treatments. In Figure 3A, the control treatment without compounds produced many evenly distributed colonies. Treatment with 2.5 μM PGV-1 alone significantly reduced the number and size of colonies, demonstrating a potent antiproliferative effect. Meanwhile, treatment with 280 nM doxorubicin also decreased the number of colonies, but to a greater extent than PGV-1. The combination of PGV-1 and doxorubicin exhibited a more pronounced inhibitory effect than doxorubicin alone, significantly reducing the number of colonies ( $p=0.000$ ).

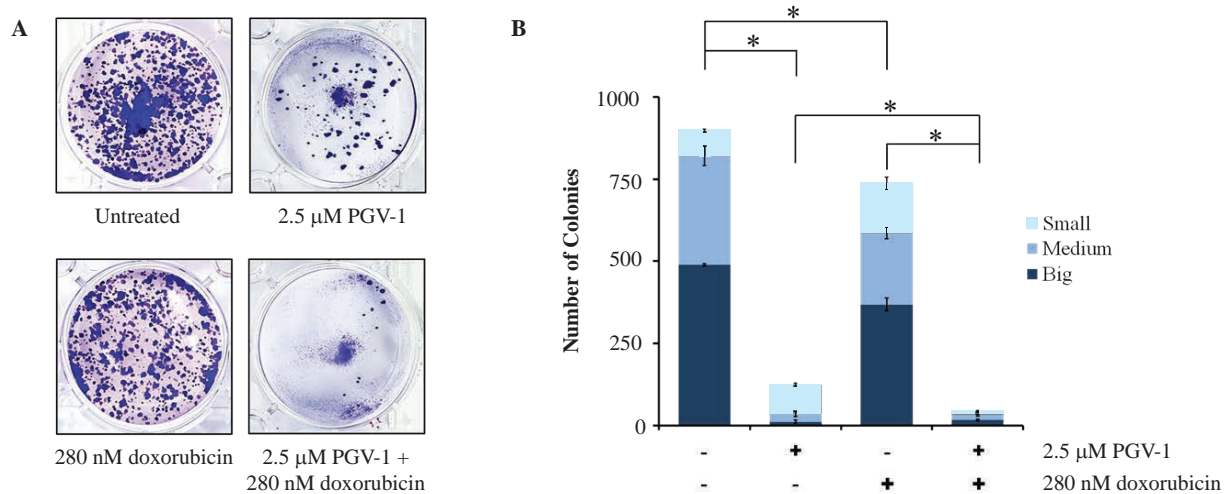
In Figure 3B, the number of colonies according to size (small, medium, and large) was quantified. In the control treatment, the number of colonies approached nearly 1,000, with a prevalence of medium and large colonies. The single PGV-1 treatment significantly reduced the number

of colonies, with most remaining colonies being small. Doxorubicin treatment also reduced the number of colonies but remained higher than that of PGV-1. The combination of PGV-1 and doxorubicin produced the most significant inhibitory effect ( $p=0.007$ ), resulting in a significantly lower number of colonies compared to a single treatment, indicating a synergistic effect in inhibiting the formation of cancer cell colonies.

**Effect of PGV-1 and Doxorubicin in Reducing 3D Spheroid 4T1 Cell Size**

According to the objective study, observations of 4T1 breast cancer cell spheroids after treatment with PGV-1, doxorubicin, and their combination revealed a significant growth inhibitory effect (Figure 4A and Figure 4B). In Figure 4A, a single treatment with 20 μM PGV-1 or 20 μM doxorubicin resulted in a decrease in spheroid size compared to the untreated control, as observed on day-12. Spheroids in the control group retained their size, while the single treatment led to gradual shrinkage.

In the combined treatment of 10 μM PGV-1 and 20 μM doxorubicin, a more pronounced decrease in spheroid volume was observed compared to single treatment, indicating a synergistic effect of the two compounds in inhibiting spheroid cell growth (Figure 4B). This sustained



**Figure 3. Inhibition profile of 4T1 cell colony formation after administration of doxorubicin and PGV-1, both alone and in combination.** A: Morphology of 4T1 cell colonies after crystal violet staining following post-treatment with doxorubicin and PGV-1 at a concentration of  $1/2 IC_{50}$ , alone and in combination. B: Graph depicting the number of 4T1 cell colonies after treatment.

suppression suggests that the combination treatment could be more effective in limiting tumor progression. Daily spheroid volume quantification data showed that the combination treatment produced a more consistent and significant inhibitory effect than the single treatment. These findings highlight the potential of combining PGV-1 with doxorubicin for *in vivo* tumor treatment, where tumor heterogeneity and microenvironmental factors may influence therapeutic outcomes.

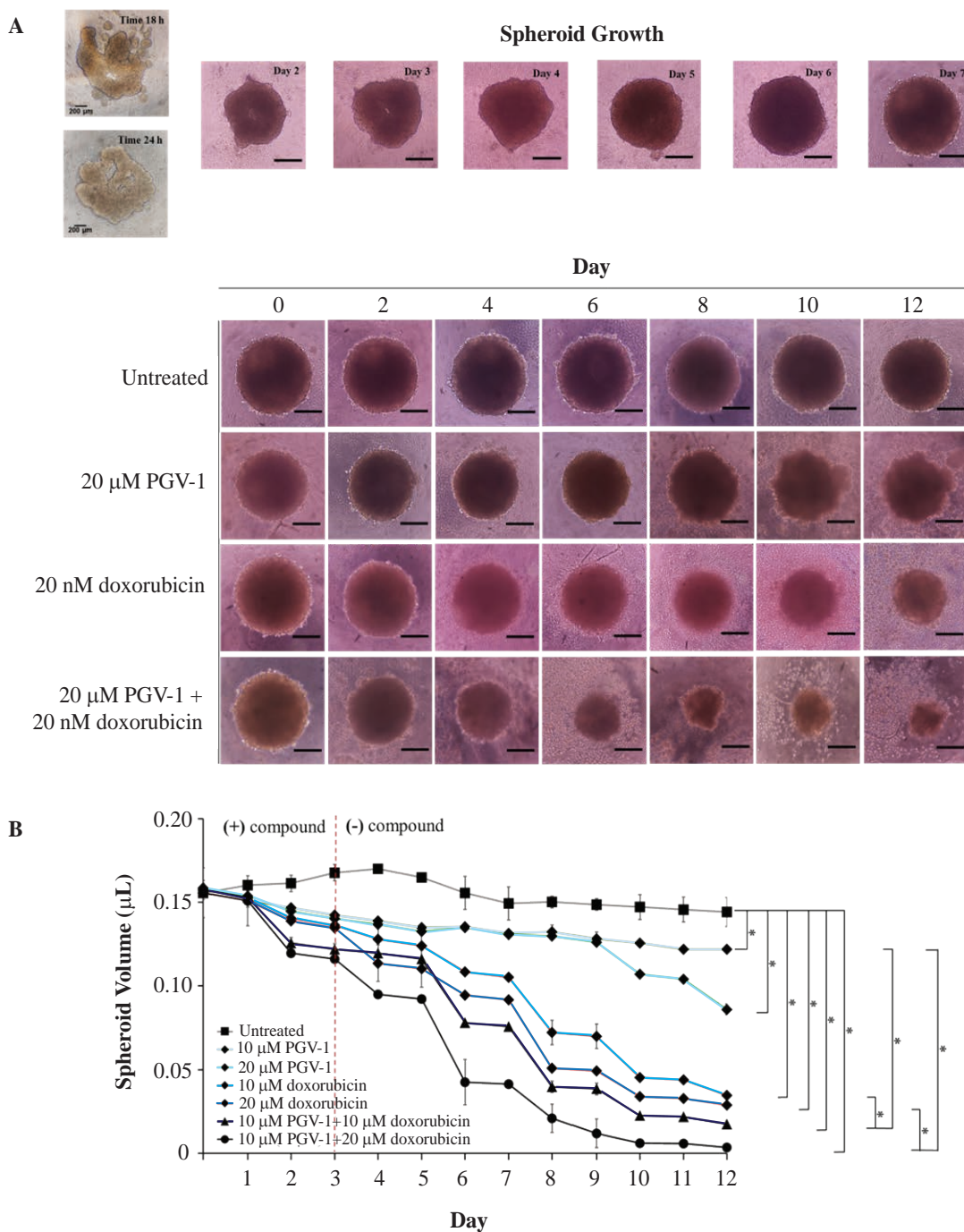
### Senescence Induction of Doxorubicin and PGV-1 in 4T1 3D Spheroid Cells

Observations of SA- $\beta$ -gal staining indicated variations in the level of senescence among 4T1 breast cancer cell spheroids treated with PGV-1, doxorubicin, and their combination. In untreated spheroids, SA- $\beta$ -gal staining was minimal at the edge and the center, indicating that the cells within the spheroid demonstrated a low level of senescence (Figure 5). In spheroids treated with 5  $\mu$ M doxorubicin, there was an increase in SA- $\beta$ -gal staining, particularly at the edge of the spheroid, indicating that cells in this area experienced more significant senescence than those in the center. The darker color at the edge signifies an accumulation of aging cells in that region. Treatment with 10  $\mu$ M PGV-1 resulted in a more uniform increase in senescence compared to doxorubicin, as indicated by the distribution of SA- $\beta$ -gal staining across the spheroid. The combination of 10  $\mu$ M PGV-1 and 5  $\mu$ M doxorubicin resulted in the most intense and widespread staining, synergistically enhancing senescence induction in 4T1 cancer cell spheroids.

## Discussion

The results of this study indicate that PGV-1 has the potential to be a more selective chemotherapy agent than doxorubicin. The cytotoxic effect of PGV-1 on cancer cells is more significant than on normal cells, as shown by the higher  $IC_{50}$  value and improved selectivity index. This suggests that PGV-1 could be a safer therapeutic candidate with a reduced risk of side effects compared to doxorubicin. Regarding molecular mechanisms, the antiproliferative effect of PGV-1 may be linked to the inhibition of the G2/M phase, examined explicitly in previous studies, including the arrest at the prometaphase phase in the cell cycle.(21,23) PGV-1 is known to inhibit cancer cells in the G2/M phase, preventing them from progressing to the mitotic stage.(20,26) In contrast to doxorubicin, which induces DNA damage by inhibiting topoisomerase II, PGV-1 significantly stabilizes the senescence state and inhibits proliferation without causing extensive apoptosis.(14-16)

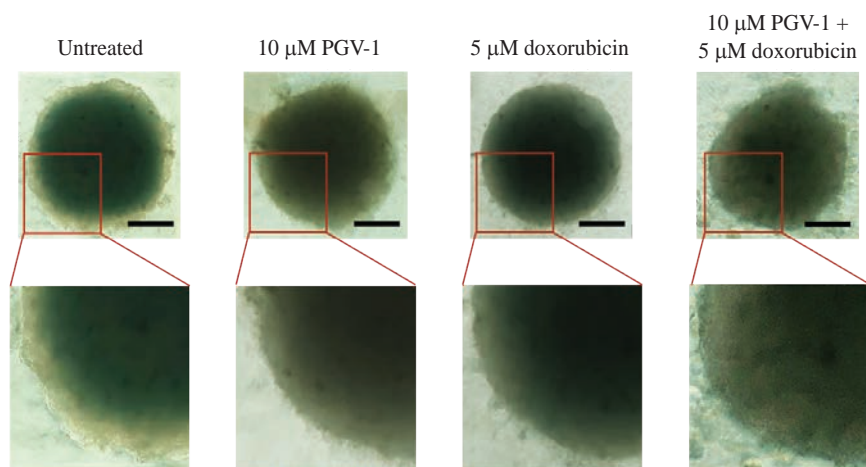
Observation of cell morphology indicated that PGV-1 did not cause drastic changes in normal cells, while doxorubicin significantly induced severe damage to cell morphology, suggesting death. This finding is supported by previous studies indicating that PGV-1 can inhibit the activation of NF- $\kappa$ B.(32) NF- $\kappa$ B is known to play a role in the expression of various genes that support proliferation, including cyclin D1, MMPs, and vascular endothelial growth factor (VEGF), while also inducing tumor necrosis factor (TNF)- $\alpha$ , which supports inflammation and survival.(33)



**Figure 4. The effect of PGV-1 and doxorubicin on tumor spheroid growth inhibition, both individually and in combination.** A: The morphology of 4T1 spheroid cells after treatment with doxorubicin and PGV-1 on days 0, 2, 4, 6, 8, 10, and 12 reveals post-treatment changes. Observations continued after the drug medium was removed on day 3. Black bar: 200 μm. B: A graph representing the observed cellular morphological changes illustrates daily variations and differences in effects between the treatments.

In contrast, p53 counteracts NF-κB by driving apoptosis, balancing cell fate decisions.(25,32,34) By suppressing the NF-κB pathway, PGV-1 can reduce the angiogenesis and invasion of cancer cells (32), resulting in a more persistent antiproliferative effect can be achieved compared to doxorubicin.

The combination of PGV-1 and doxorubicin exhibited a significant synergistic effect in reducing 4T1 cell viability, as indicated by a low CI value. This synergy may be driven by increased oxidative stress, with PGV-1 enhancing ROS production, thereby amplifying doxorubicin-induced DNA damage.(23) However, excessive ROS accumulation could



**Figure 5. The effect of doxorubicin and PGV-1 on senescence induction in 4T1 spheroid cells, both individually and in combination.** SA- $\beta$ -gal staining of 4T1 cell spheroid tissue. The morphological representation of 4T1 cell spheroids was examined using a microscope to observe the condition from the edge layer to the center of the spheroid cells. The green color indicates cells undergoing cellular senescence. Representative images for cell senescence staining are displayed on the red box. Black bar: 250  $\mu$ m.

also harm normal cells, underscoring the need for dose optimization to balance efficacy and toxicity. Further studies are needed to assess the toxicity of ROS in normal tissues and explore potential protective strategies.

To evaluate the synergism of PGV-1 and doxorubicin in a physiologically relevant system, a 3D spheroid model more closely resembling the *in vivo* tumor microenvironment was utilized.(35,36) Spheroids are formed through increased cell-to-cell adhesion instead of adhesion to the substrate, featuring an outer layer of proliferating cells and an inner core that undergoes necrosis due to nutrient and oxygen limitations.(37) TNBC 4T1 cell line effectively demonstrated spheroid formation as a relevant model for assessing the combined effects of these two compounds.

PGV-1 alone induced spheroid disaggregation, though less effectively than doxorubicin. However, their combination significantly disrupted spheroids, indicating impaired cell adhesion and suppressed proliferation. This effect likely stems from the disruption of key adhesion molecules, particularly E-cadherin and integrins, which regulate spheroid stability. Similar effects have been observed with compounds like  $\alpha$ -mangostin, which destabilize spheroids by disrupting cell-to-cell and cell-to-extracellular matrix (ECM) interactions, leading to growth suppression.(38) E-cadherin regulates cellular adhesion, while integrins mediate cell-to-ECM interactions, both of which are critical for maintaining spheroid integrity. These findings suggest that targeting adhesion molecules may represent a promising strategy to enhance the therapeutic efficacy of anticancer agents in 3D tumor models.(37) Spheroid formation occurs in three stages: initial integrin-ECM-mediated aggregation, a delay phase for E-cadherin accumulation, and final compaction due to homophilic E-cadherin interactions.(37) The significant disaggregation in this study indicates that PGV-1 and doxorubicin disrupt

one or more of these adhesion stages. Aggregation plays a critical role in maintaining the proliferative capacity of spheroids, as cell-to-cell and cell-to-ECM interactions provide essential signals for survival and growth.(39) Disruption of these interactions can lead to reduced proliferation due to increased apoptosis or cell cycle arrest. PGV-1 is known to suppress cyclooxygenase-2 (COX-2) and VEGF expression, contributing to reduced cell proliferation and tumor angiogenesis ability.(34) Thus, the observed disaggregation may suggest impaired adhesion and reduced proliferative potential of spheroid cells. This combination not only directly inhibits cancer cell growth but also disrupts essential factors that support tumor survival and expansion in a three-dimensional environment. Loss of adhesion significantly affects tumor progression. However, in cancer therapy, disrupting adhesion can enhance treatment efficacy by preventing the formation of protective clusters and improving drug penetration. The combination of PGV-1 and doxorubicin may be more effective in suppressing tumors by reducing cellular cohesion and increasing therapeutic effectiveness accessibility.

The induction of senescence through the combination of PGV-1 and doxorubicin in the spheroid model also offers new insights into the potential for long-term therapy. Doxorubicin is known to induce senescence primarily at the edges of the spheroid, likely due to the limited penetration of the drug into the spheroid's core. In contrast, PGV-1 demonstrated a more uniform effect in inducing senescence, potentially due to its capacity to suppress the NF- $\kappa$ B pathway and enhance the expression of senescence markers such as p21 and p16.(22,40) This induction of senescence could be a promising therapeutic strategy for preventing tumor recurrence after treatment.

This study demonstrates that PGV-1 is a more selective chemotherapeutic agent compared to doxorubicin, offering

sustained antiproliferative effects. When administered in combination, PGV-1 synergistically enhances the efficacy of doxorubicin while concurrently reducing its dosage and toxicity, thereby potentially minimizing cardiotoxicity and myelosuppression. This combination approach targets multiple tumorigenic pathways, resulting in significant inhibition of spheroid growth and colony formation, ultimately leading to persistent suppression and apoptosis. Mechanistically, PGV-1 facilitates spheroid disaggregation and promotes cellular senescence, as indicated by an increase in SA- $\beta$ -gal activity, while doxorubicin induces apoptotic cell death. These findings highlight the potential of PGV-1 to enhance the efficacy of doxorubicin while minimizing toxicity, thereby necessitating further preclinical and clinical evaluation. Additional research is necessary to explore molecular mechanisms underlying spheroid adhesion loss, including cadherin-integrin signaling, ECM remodeling, and EMT. A comprehensive understanding of these pathways could enhance the strategies employed in combination therapy for TNBC, with particular emphasis on *in vivo* validation and adhesion-targeting methodologies to improve treatment outcomes.

## Conclusion

This study demonstrates that PGV-1 exhibits robust antiproliferative effects on cancer cells, showing more excellent selectivity and potentially fewer adverse effects compared to doxorubicin. Thus, PGV-1 emerges as a promising candidate for a safer chemotherapeutic option. The synergistic combination of PGV-1 and doxorubicin substantially enhances cytotoxicity, reduces spheroid volume, and disrupts cell adhesion mechanisms that are crucial for maintaining tumor integrity. In monolayer cultures, the optimal concentrations identified were 2.5  $\mu$ M PGV-1 and 280 nM doxorubicin; conversely, in three-dimensional spheroid models, the most effective concentrations were 10  $\mu$ M PGV-1 and 20  $\mu$ M doxorubicin.

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## Authors Contribution

AN were involved in the conceptualization of the study. DRR was involved in the data investigation, data analysis, and writing the original manuscript draft. AN, RM, and RIJ were involved in the data validation, supervision, as well as reviewing and editing the manuscript. AN was performing the funding acquisition. All authors contributed to the critical editing of the paper.

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