Journal of Applied Pharmaceutical Science Vol. 15(06), pp 205-215, June, 2025
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2025.228633
ISSN 2231-3354

Persistent synergistic antiproliferative and apoptosis induction effects of PGV-1 and tamoxifen or 4-hydroxytamoxifen on ER-positive breast cancer cells

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ARTICLE HISTORY

Received on: 14/11/2024
Accepted on: 26/03/2025
Available Online: 05/05/2025

Key words:
PGV-1 (pentagamavunon-1), tamoxifen, 4-hydroxytamoxifen, co-chemotherapy, MCF-7 and T47D luminal breast cancer cell.

ABSTRACT

Tamoxifen (TAM) and its active metabolite, 4-hydroxytamoxifen (4-OHT), are major components of estrogen receptor (ER)-positive breast cancer treatment, but their efficacy is often limited by resistance and side effects. Pentagamavunone-1 (PGV-1), a curcumin analog, has demonstrated promising anticancer properties, suggesting its potential as a co-chemotherapy agent. This study evaluated the synergistic anticancer effects of PGV-1 in combination with TAM or 4-OHT on T47D and MCF-7 luminal breast cancer cells to enhance treatment efficacy and address resistance issues. The experimental design included cytotoxicity assays, washout persistent cytotoxicity assays, colony-formation tests, and cell cycle analysis using flow cytometry. Data were analyzed via ANOVA and post hoc Tukey HSD tests ($\alpha = .05$).-PGV-1, TAM, and 4-OHT individually showed potent cytotoxic effects on T47D and MCF-7 cells (IC_{50} < 10 µM) with minimal toxicity to normal cells. Combination treatments demonstrated significant synergy (combination index < 0.9) and persistent cytotoxicity, particularly for PGV-1 combined with 4-OHT, which strongly inhibited cancer cell colony formation. Cell cycle analysis revealed enhanced apoptosis, indicated by increased accumulation of cells in the sub-G1 phase ($p < 0.001$). Overall, combining PGV-1 with TAM or 4-OHT exhibited robust and sustained anticancer effects, presenting a promising luminal A breast cancer therapy strategy.

INTRODUCTION

Breast cancer remains a substantial health concern globally, with multiple subtypes requiring tailored specific treatment approaches. Luminal A breast cancer, which is characterized by hormone receptors such as estrogen receptor (ER) and progesterone receptor (PR) [1], accounts for approximately 40% of all breast cancer cases [2]. This subtype usually exhibits excessive ER and PR expression, promoting its pathogenesis and growth [3]. Tamoxifen (TAM) and its active metabolite, 4-hydroxytamoxifen (4-OHT), are crucial in treating hormone receptor-positive breast cancer, which makes them important therapeutic options [4]. TAM is a prodrug that undergoes a metabolic process to generate pharmacologically active metabolites, such as 4-OHT or 4-hydroxy-N-desmethyltamoxifen (endoxifen). This process is facilitated by cytochrome P450 [5]. These TAM metabolites, which have undergone hydroxylation (4-OHT and endoxifen), exhibit a high binding affinity for ER and demonstrate both antiestrogenic and anticancer effects [6]. TAMs function as selective ER modulators by attaching to the ER and inhibiting estrogen-induced signals [7]. 4-OHT is a metabolite of TAM that acts as a more powerful antagonist of ER than TAM and other metabolites, exhibiting a higher affinity for the ER [5]. This higher affinity results in a more efficient inhibition of the estrogen signaling pathways [6]. Furthermore, this mechanism is essential in treating luminal breast cancer,

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which is generally ER-positive. TAM and 4-OHT reduce the proliferation of cancer cells that depend on estrogen signaling for growth and development by inhibiting ERs [8]. These agents are widely used as the first-line treatment for ER-positive breast cancer, and they are particularly effective in postmenopausal women [9]. However, despite its efficacy, resistance and side effects challenge its clinical application.

Resistance mechanisms to TAM and 4-OHT involve several pathways, including loss of ER-alpha (ERα) expression and function, silencing of ER-β expression, ligand-independent growth factor signaling, altered availability of active TAM metabolites (such as 4-OHT), and regulation of autophagy and apoptosis [7–9]. This resistance substantially hinders the efficacy of TAM and 4-OHT in treating luminal breast cancer [9,10]. A combination therapy that includes TAM or 4-OHT with agents with different action mechanisms is required to overcome this resistance. This approach aims to target multiple pathways simultaneously, thereby enhancing therapeutic efficacy and reducing the likelihood of resistance development. For example, combining TAM with growth factor signaling pathway inhibitors or autophagy regulators could synergistically improve patient outcomes.

Pentagamavunon-1 (PGV-1), a promising anticancer agent synthesized by the Cancer Chemoprevention Research Center (CCRC) at Universitas Gadjah Mada (UGM), has shown potential cytotoxic effects in various cancer models, including breast cancer. Combining PGV-1 with conventional therapies such as TAM and 4-OHT presents a promising strategy to improve treatment outcomes in luminal breast cancer. Moreover, PGV-1 has been reported to inhibit proliferation in various breast, colon, liver, and leukemia cell lines and migration in metastatic breast cancer cells [11–13]. The mechanism of action of PGV-1 includes inhibiting cell cycle progression at prometaphase in K-562 leukemia cells [11] as well as inducing mitotic catastrophe in 4T1 and T47D cells [12] and inducing apoptosis in breast cancer cells (MCF-7, 4T1, and T47D) and liver cancer cells [14]. PGV-1 has also been extensively studied for its potential when combined with existing chemotherapy agents, such as doxorubicin in breast cancer cells and cisplatin in ovarian cancer cells. These findings demonstrate the potential for PGV-1 to be combined with TAM and 4-OHT to overcome resistance and increase therapeutic efficacy.

This study aimed to evaluate the potential of combining PGV-1 with TAM or 4-OHT by assessing their cytotoxicity, anti-proliferative effects, and induction of apoptosis (sub-G1 phase accumulation). The goal is to determine whether this combination produces a synergistic effect that could help overcome TAM resistance. By exploring the interaction between PGV-1 and TAM or its metabolites in luminal breast cancer cells (T47D and MCF-7), this research may offer new insights for treatment strategies. Further analysis will identify which cell type shows stronger responses, guiding more targeted therapies.

METHODS

Chemical compounds

We obtained PGV-1 (2,5-bis(4-hydroxy-3,5-dimethylbenzylidene)cyclopentanone) from the CCRC, Faculty of Pharmacy, UGM. We purchased TAM and 4-hydroxytamoxifen (4-OHT) from FUJIFILM (Wako, Japan).

Cultivation of luminal a breast cancer cells; Vero and NIH-3T3 normal cells

Breast cancer cell lines MCF-7 (ATCC-HTB-22™) and T47D (ATCC-HTB-133™) as well as normal fibroblast cell line NIH-3T3 (ATCC-CRL-1658™) and normal kidney cell line Vero (ATCC-CCL-81™) were acquired from Prof. Masashi Kawaichi at the Nara Institute of Science and Technology (NAIST, Japan). T47D cells were grown in Roswell Park Memorial Institute media, and 4T1, Vero, and NIH-3T3 cells were cultivated in Dulbecco's modified Eagle's medium. HEPES, sodium bicarbonate, and 10% (v/v) fetal bovine serum were added to these culture mediums, which have a high glucose content. Cells were incubated at 37°C in a 5% CO_2 atmosphere in a humid environment [14,15]. This study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, in collaboration with Dr. Sardjito General Hospital. The ethical approval was granted under the reference number KE/FK/0058/EC/2022 in January 2022.

Cytotoxic MTT assay

Cytotoxic effects of PGV-1, alone or in combination, were assessed using the MTT assay/3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MCF-7 (3.5×10^3 cells/well), T47D (5×10^3 cells/well), Vero, and NIH-3T3 cells (7×10^3 cells/well) were plated in 96-well plates and cultivated for 24 hours until 80% confluence. Cells were treated with varying PGV-1 doses (0.1–10 µM) for 24 hours [12,16]. After treatment, the MTT solution was added for 4 hours to form formazan crystals, which were dissolved with 10% sodium dodecyl sulfate in 0.01 N hydrochloric acid and left overnight. Absorbance was measured at 595 nm using a microplate reader, and IC_{50} values were calculated from triplicate data. The selectivity index (SI) was determined using the IC_{50} of PGV-1, TAM, or 4-OHT in normal and cancer cells, with SI > 3 indicating strong selectivity [15]. The combination effect was analyzed using three concentrations (⅛, ¼, and ½ IC_{50}) and the Chou–Talalay method, with CI values < 1 indicating synergism, CI = 1 indicating additive effects, and CI > 1 indicating antagonism [17,18].

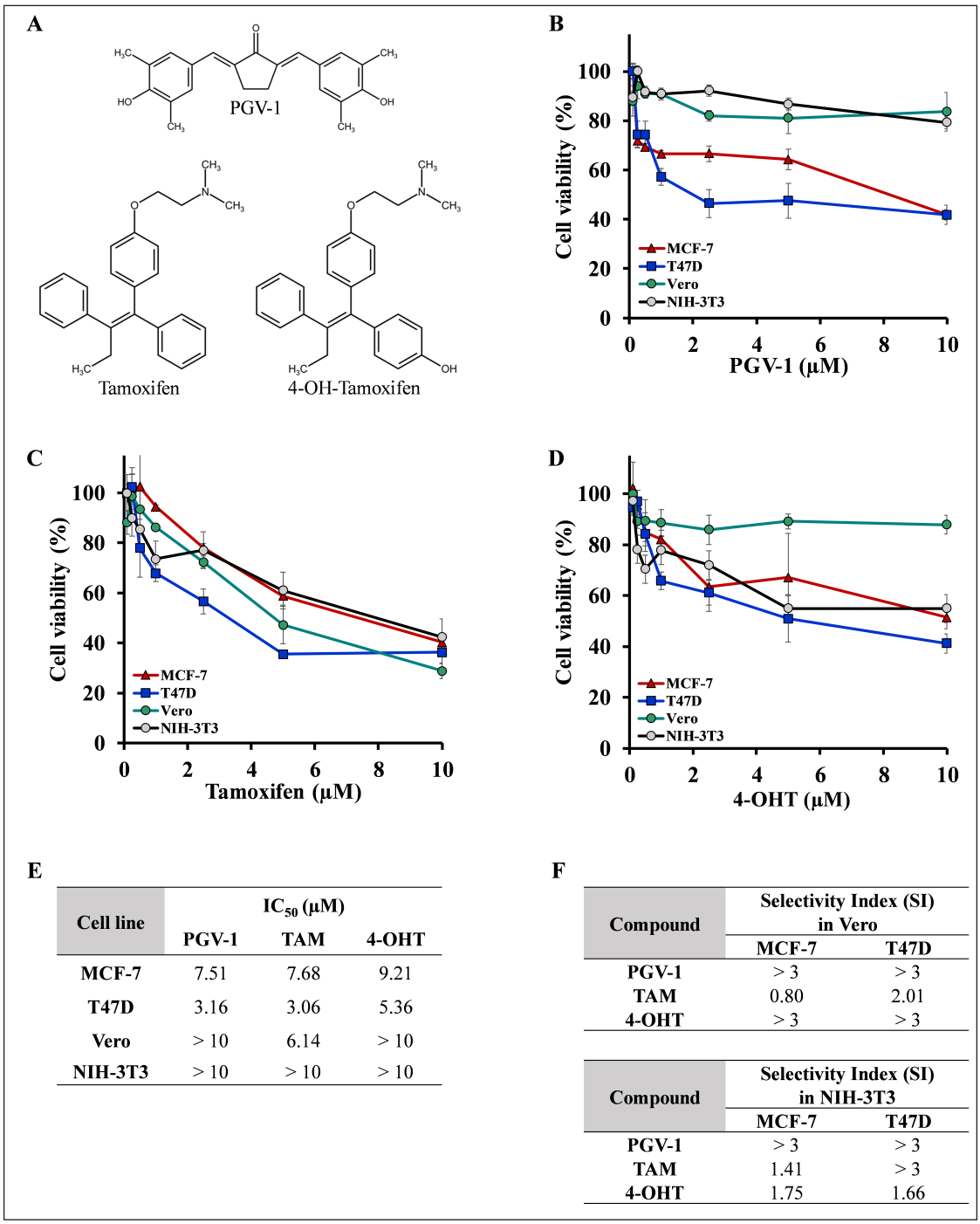
Washout proliferation assay

To ascertain the irreversible impact of PGV-1, TAM, 4-OHT, and the combination on impeding cancer cell proliferation after eliminating the compounds from the medium, cells were treated with PGV-1, TAM, 4-OHT, and the combination for 24 hours. Following this treatment, the compounds were withdrawn by substituting the medium with a fresh medium devoid of the compounds for the ensuing 5 days. Viable cells were then quantified through MTT assay.

Colony-formation assay

MCF-7 and T47D cells, with a concentration of 1×10^3 cells/well, were placed in 6-well plates. On the subsequent day,

they were administered different doses of experimental medication for 24 hours. Subsequently, the medium was substituted and cells were grown for a further 12 days in a medium devoid of drugs. Cells were then immobilized using a 4% paraformaldehyde solution and treated with a staining solution comprising 0.1% gentian violet in 20% methanol. After staining, the cells were washed with 1× phosphate-buffered saline to ensure complete release of gentian violet. Colonies were then photographed with a camera. Colonies formed in the dish were counted and measured regardless of their size, including small, medium, and large colonies.



E

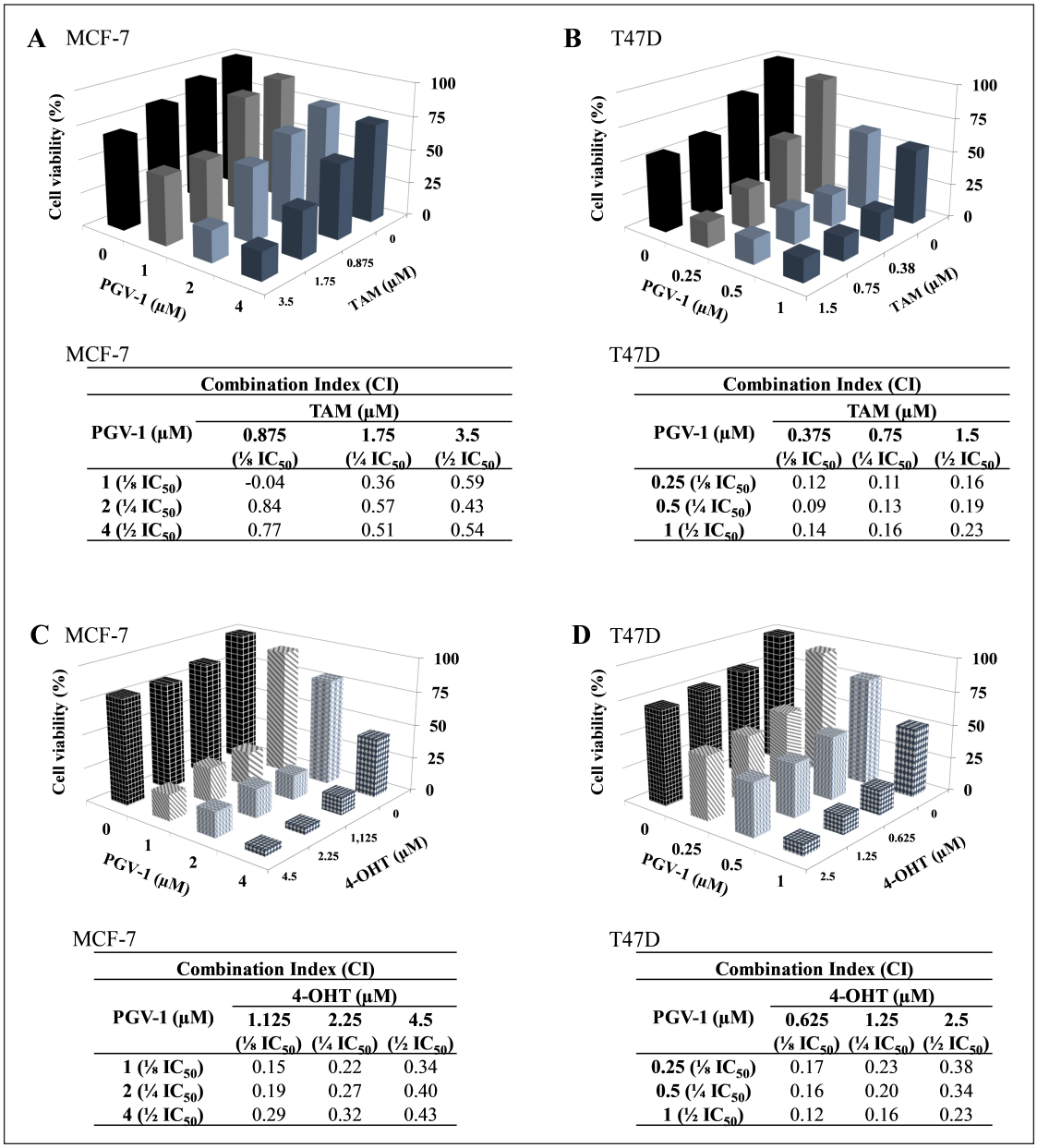
Cell line	IC_{50} (μM)		
	PGV-1	TAM	4-OHT
MCF-7	7.51	7.68	9.21
T47D	3.16	3.06	5.36
Vero	> 10	6.14	> 10
NIH-3T3	> 10	> 10	> 10

F

Compound	Selectivity Index (SI) in Vero	
	MCF-7	T47D
PGV-1	> 3	> 3
TAM	0.80	2.01
4-OHT	> 3	> 3

Compound	Selectivity Index (SI) in NIH-3T3	
	MCF-7	T47D
PGV-1	> 3	> 3
TAM	1.41	> 3
4-OHT	1.75	1.66

Figure 1. Chemical structure and cytotoxic effects of PGV-1, tamoxifen (TAM), and 4-hydroxy tamoxifen (4-OHT) on MCF-7, T47D, Vero, and NIH-3T3 cells. Cell viability was assessed using an MTT assay after incubating cells with the compounds for 24 hours. (A) Cell viability profiles following treatment with PGV-1, Panel C shows the profiles after treatment with TAM, and Panel E shows the profiles after treatment with 4-OHT. (B) PGV-1, TAM, and 4-OHT. (D) IC_{50} values of PGV-1, TAM, and 4-OHT for each cell line. (F) Selectivity index (SI) of PGV-1, TAM, and 4-OHT on MCF-7 and T47D cells compared with Vero and NIH-3T3 cells.



MCF-7

PGV-1 (μM)	Combination Index (CI) TAM (μM) 0.875 (⅛ IC_{50})	1.75 (¼ IC_{50})	3.5 (½ IC_{50})
1 (⅛ IC_{50})	-0.04	0.36	0.59
2 (¼ IC_{50})	0.84	0.57	0.43
4 (½ IC_{50})	0.77	0.51	0.54

T47D

PGV-1 (μM)	Combination Index (CI) TAM (μM) 0.375 (⅛ IC_{50})	0.75 (¼ IC_{50})	1.5 (½ IC_{50})
0.25 (⅛ IC_{50})	0.12	0.11	0.16
0.5 (¼ IC_{50})	0.09	0.13	0.19
1 (½ IC_{50})	0.14	0.16	0.23

MCF-7

PGV-1 (μM)	Combination Index (CI) 4-OHT (μM) 1.125 (⅛ IC_{50})	2.25 (¼ IC_{50})	4.5 (½ IC_{50})
1 (⅛ IC_{50})	0.15	0.22	0.34
2 (¼ IC_{50})	0.19	0.27	0.40
4 (½ IC_{50})	0.29	0.32	0.43

T47D

PGV-1 (μM)	Combination Index (CI) 4-OHT (μM) 0.625 (⅛ IC_{50})	1.25 (¼ IC_{50})	2.5 (½ IC_{50})
0.25 (⅛ IC_{50})	0.17	0.23	0.38
0.5 (¼ IC_{50})	0.16	0.20	0.34
1 (½ IC_{50})	0.12	0.16	0.23

Figure 2. Combined cytotoxic effects of PGV-1, TAM, and 4-OHT on MCF-7 and T47D cells. The combination index (CI) for both cell lines was calculated as described in the method. (A) MCF-7 cell profile after exposure to PGV-1 and TAM combination. (B) Viability profile of T47D cells under the same treatment conditions. (C, D) Combination treatment of PGV-1 and 4-OHT, respectively.

Cell cycle analysis

MCF-7 cells (2.5×10^5 cells/well) and T47D cells (3×10^5 cells/well) were separately treated with PGV-1 at ¼ and ½ of their respective IC_{50} concentrations, or in combination, for a period of 24 hours. Next, the cells were separated by trypsinization, washed with PBS at a concentration of 1×, and spun at a speed of 2,500 rpm for 5 minutes. After centrifugation, the cells were treated with 70% ethanol to fix them. They were then stained using a solution called propidium iodide (PI)/RNase staining buffer, following the instructions provided by the manufacturer. In addition, 0.1% Triton X-100 was added to the staining process. The DNA-PI complex within the cells was analyzed for fluorescence using a BD-FACS Canto™ II Flow Cytometer (USA).

Statistical analysis

The data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical analysis was performed using SPSS software (version 25, SPSS Inc., Chicago, USA) and reported as mean ± SD. Differences were considered significant based on *p*-values, with statistical comparisons conducted using one-way analysis of variance (ANOVA), followed by Tukey's HSD and Bonferroni post hoc tests. The

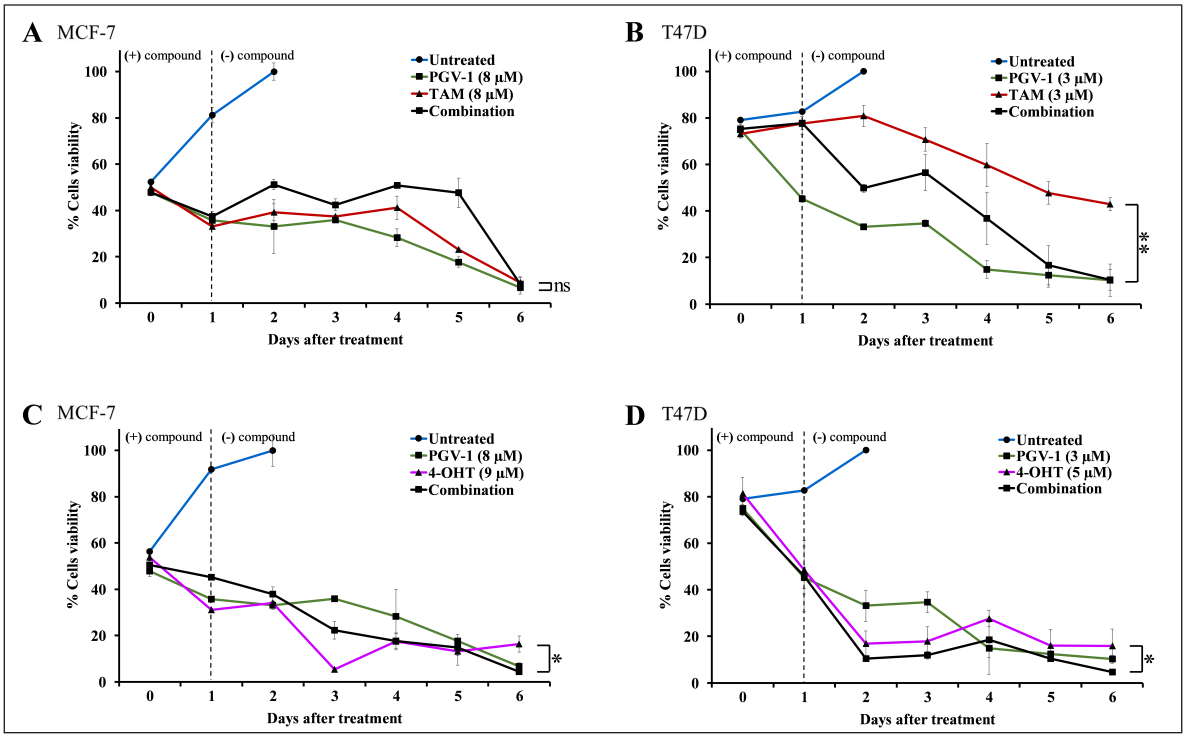


Figure 3. The persistence cytotoxicity of PGV-1, TAM, or 4-OHT. MCF-7 (3.5 × 10^3 cells/well) and T47D (5 × 10^3 cells/well) were grown for 24 hours and then treated with samples at IC_{50} for 24 hours. These cells were then replaced with fresh medium and continuously incubated for up to 6 days. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns: not significant.

levels of significance are indicated by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****p < 0.0001 for each figure.

RESULTS

Cytotoxic effects of PGV-1, TAM, and 4-OHT on luminal breast cancer cells

To elucidate the potential of the antitumor activity and selectivity of PGV-1, TAM, and 4-OHT, we performed *in vitro*-based cytotoxicity assays on breast cancer cells (MCF-7 and T47D) and normal cells (Vero and NIH-3T3). We monitored the cytotoxic effects for 24 hours. This study revealed that these three compounds were potent for breast cancer cells with IC_{50} of < 10 µM (Fig. 1B–D). However, T47D cells appeared more sensitive to these compounds than MCF-7 cells, as indicated by 50%–60% lower IC_{50} values than those in MCF-7 cells (Fig. 1E). Moreover, in normal cells, both compounds showed IC_{50} values of > 10 µM, except for TAM in Vero cells (6.14 µM). Based on the SI calculation, we determined that PGV-1 was selective toward cancer cells, whereas TAM and 4-OHT were not as selective as PGV-1 (Fig. 1F).

The cytotoxicity assay revealed increased cell death and proliferation inhibition when PGV-1 was combined with TAM compared to individual treatments. Persistent cytotoxic effects were confirmed in the washout assay. The colony-formation assay showed synergistic inhibition of colony formation and cancer cell proliferation. Cell cycle analysis indicated enhanced apoptosis, demonstrated by sub-G1 phase accumulation in the combination treatment.

PGV-1 in combination with TAM and 4-OHT synergistically suppressed the growth of breast cancer cells

A combination cytotoxic test was conducted to determine whether PGV-1 synergized with TAM or 4-OHT. A synergistic parameter was obtained by calculating the CI value, which was < 1 when comparing the combination therapy to the solo treatment at a dosage below the IC_{50} threshold. Fig. 2A and 2B graphically depicts the cytotoxic effects of the combination of PGV-1 and TAM on MCF-7 and T47D cells, respectively. Results showed that the combination of TAM and PGV-1 has a synergistic effect on both cell types, as evidenced by a CI value of < 1. Furthermore, co-administration of 4-OHT and PGV-1 on MCF-7 and T47D cells demonstrated a synergistic impact, as illustrated in Fig. 2C and 2D. The T47D cells exhibited a more prominent reaction, consistently displaying lower CI values. In summary, a combination of PGV-1 with TAM and 4-OHT exhibited a synergistic impact on inhibiting the proliferation of luminal breast cancer cells.

The results showed that combining TAM or 4-OHT with PGV-1 produced a synergistic cytotoxic effect on breast cancer cells (MCF-7 and T47D). While TAM alone exhibited cytotoxic effects on both cell lines, the combination with PGV-1 enhanced this effect (Fig. 2A, B). Similarly, 4-OHT, a weaker metabolite of TAM, showed less cytotoxicity alone but displayed synergy when combined with PGV-1 (Fig. 2C and D). T47D cells were more sensitive to both single and combination treatments compared to MCF-7 cells, responding more strongly to TAM, 4-OHT, and PGV-1 (Fig. 1B–E).

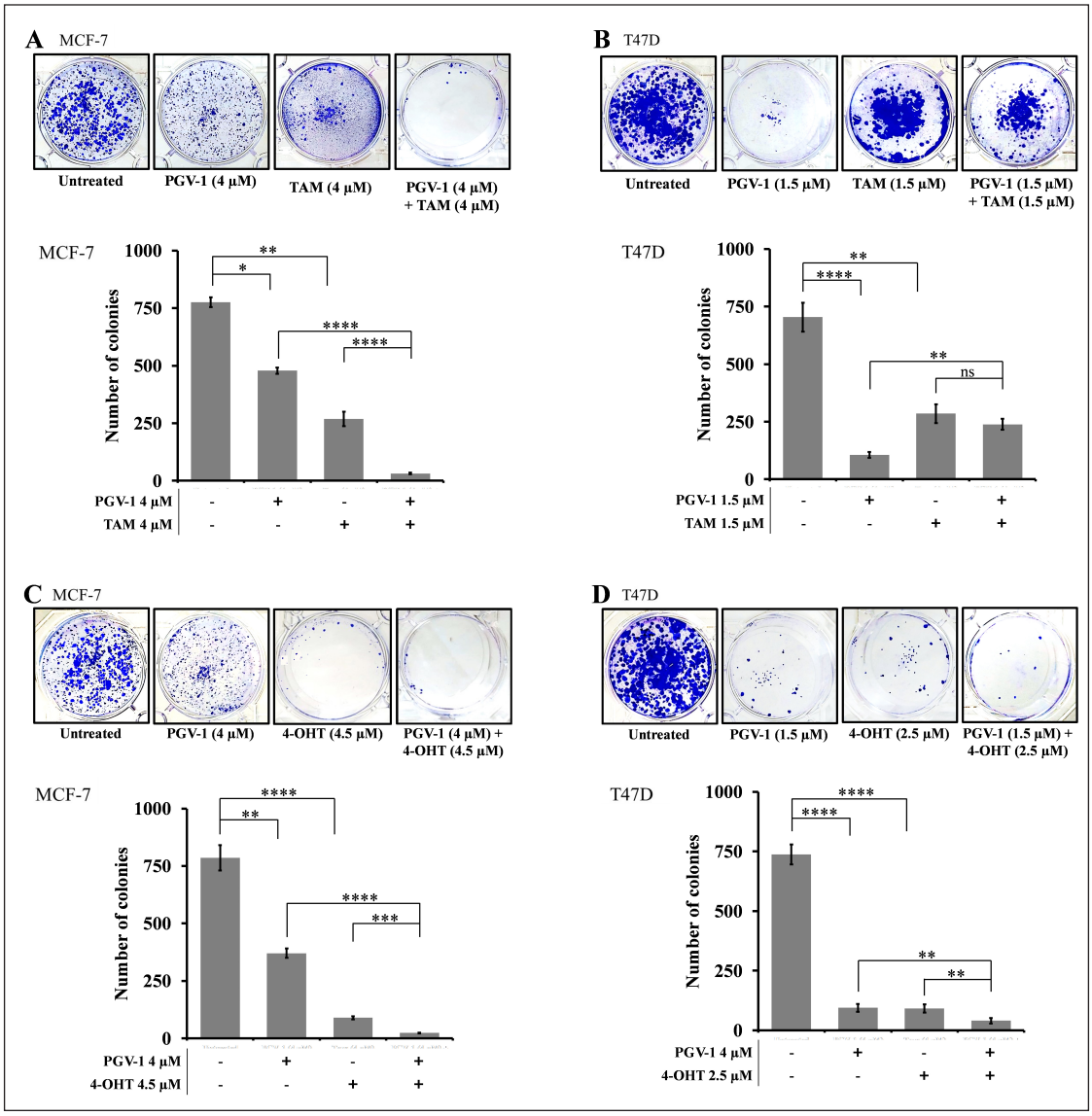


Figure 4. Colony-formation assay of PGV-1 and 4-OHT. The experimental cells were grown and then incubated with ½ IC_{50} samples for 1 day, replaced with fresh media, and then continuously incubated for up to 2 weeks. Colony-formation assays were then performed as described in the method. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns: not significant.

Persistent breast cancer cell growth suppression of combination of PGV-1 and TAM or 4-OHT

To assess recurrence after treatment discontinuation, we conducted washout experiments (Fig. 3) and colony-formation assays (Fig. 4). Results showed that PGV-1, TAM, 4-OHT, and their combinations sustained suppression of cancer cell growth. Even without further treatment, cell viability continued decreasing until day 6 across all groups (Fig. 3). Among the compounds, 4-OHT had the most persistent inhibitory effect, followed by PGV-1 and TAM (Fig. 4A–D). Overall, T47D cells were more responsive to treatment than MCF-7 cells.

In the T47D colony-formation assay, both combinations inhibited colony formation, but the PGV-1 and TAM combination showed no significant improvement over TAM alone and less inhibition compared to PGV-1 alone (Fig. 4B). This suggests that PGV-1's dominant growth-suppressive effect was reduced by TAM. However, the combination increased the inhibitory effect on MCF-7 cells (Fig. 4A). Single treatments with PGV-1 and 4-OHT significantly inhibited colony formation in both cell types, and combining 4-OHT with PGV-1 further enhanced this effect in MCF-7 ($p < 0.001$) and T47D cells ($p < 0.01$) (Fig. 4C and D).

Cell cycle profiles

We investigated whether the cytotoxic and antiproliferative effects of TAM or 4-OHT, alone or combined with PGV-1, were linked to cell cycle modulation. Cells were treated with ¼ and ½ IC_{50} concentrations (Fig. 5). TAM and 4-OHT caused significant sub-G1 accumulation, while PGV-1

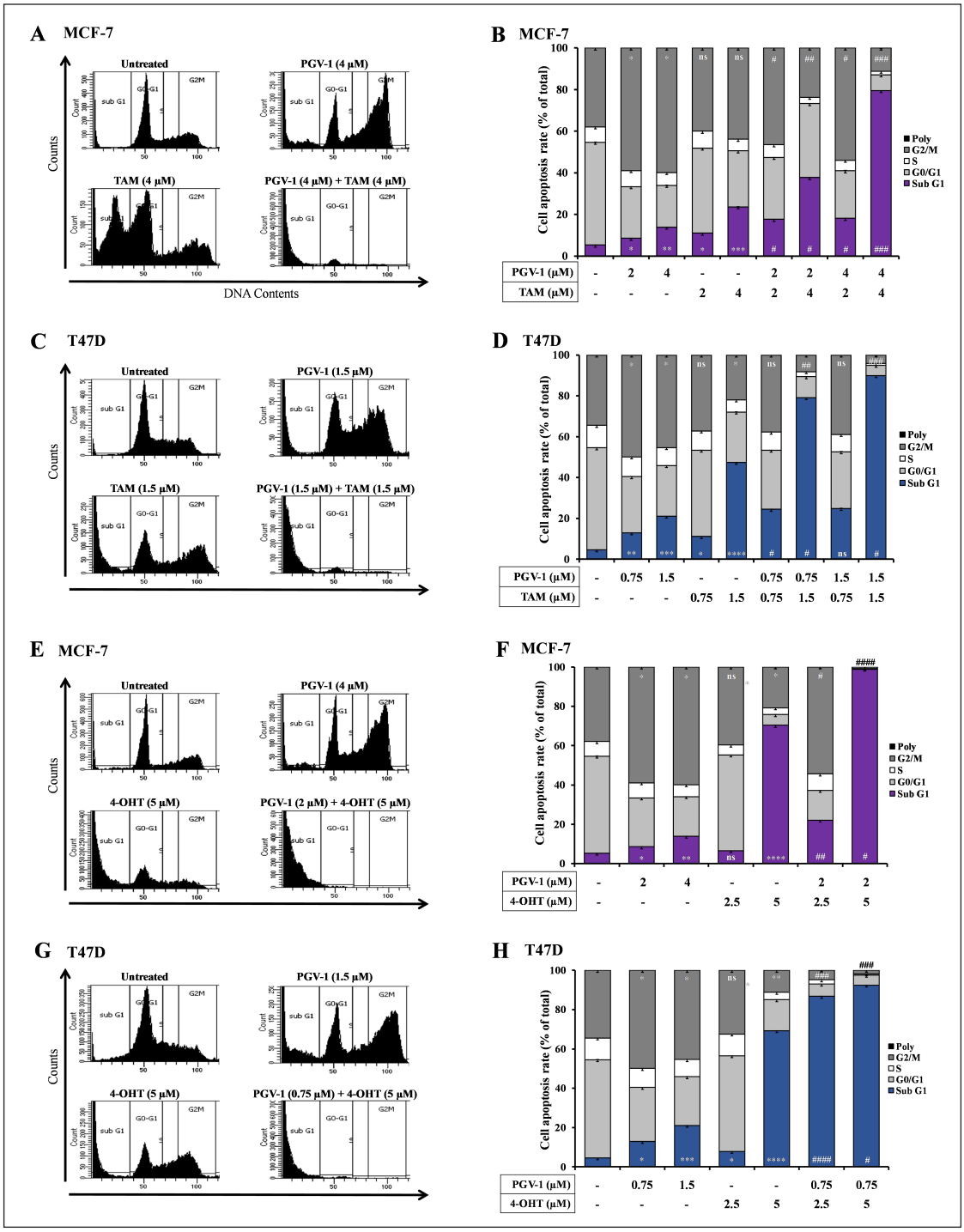


Figure 5. Cell cycle profiles of MCF-7 and T47D after 24-hour treatment with the concentration of ¼ and ½ IC_{50} of PGV-1, TAM, and 4-OHT in both the single and combination treatments. A, C, E, and G: Flow cytograms of each treatment. B, D, F, and H: Graphs of the percent cell population of each phase. (*) single treatment compared with untreated (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns: not significant). (#) combination treatment compared with the single treatment of TAM or 4-OHT (#$p < 0.05$; ##$p < 0.01$; ###$p < 0.001$; ####$p < 0.0001$; ns: not significant).

induced G2/M arrest (Fig. 5A–H). Combining TAM with PGV-1 led to a marked decrease in the G2/M population and a sharp increase in sub-G1 accumulation in both MCF-7 and T47D cells, particularly at ½ IC_{50} TAM ($p < 0.001$ for MCF-7 and $p < 0.01$ for T47D) (Fig. 5A–D). Similarly, combining 4-OHT with PGV-1 significantly decreased the G2/M population and increased sub-G1 accumulation at ¼ IC_{50} 4-OHT ($p < 0.05$) (Fig. 5E–H).

DISCUSSION

This study investigated the effects of combining PGV-1 with TAM or 4-OHT in treating luminal breast cancer using T47D and MCF-7 cell lines. Results showed increased cytotoxicity with T47D cells exhibiting higher sensitivity than MCF-7 [19]. We found increased cytotoxicity of the combination treatment with T47D cells showing higher sensitivity than MCF-7, which is also consistent with other studies showing T47D to be more sensitive than MCF-7 [19]. Both are ER-positive luminal A breast cancer types [1,20], but they have different molecular status. One of the differences is that MCF-7 has functional wild-type *p53*, while T47D expresses nonfunctional mutant *p53* [21,22]. The study found synergistic anticancer effects in T47D cells with mutant *p53*, which impair cell cycle control and apoptosis, increasing resistance to DNA damage-induced cell death [22] and making them susceptible to treatments targeting alternative pathways. The significance of *p53* status is crucial for determining potential synergistic effects [23]. However, other variables may also influence this, requiring further research to explore the impact of *p53* and additional factors. T47D cells were more sensitive to PGV-1, TAM, and 4-OHT, possibly due to molecular traits like cytoplasmic nonfunctional *p53* [24–26], which diminishes their ability to regulate the cell cycle and apoptosis. In contrast, MCF-7's functional wild-type *p53* in the nucleus contributes to lower sensitivity to chemotherapeutics [24–26]. Overall, the combination of ER status and nonfunctional *p53* in T47D cells enhances sensitivity to these treatments compared to MCF-7. While *p53* status is essential, further research is needed to understand the mechanisms behind the cytotoxic response. Inactivation of *p53* has been shown to increase the sensitivity of cancer cells to various chemotherapeutic agents [26,27]. When *p53* is inactive or a nonfunctional mutant, as seen in T47D, cells lose their ability to repair DNA or arrest the cell cycle, which enhances their susceptibility to chemotherapy-induced damage. This finding supports the conclusion that nonfunctional *p53* in T47D cells may contribute to increased sensitivity to chemotherapeutic agents, as reflected in the results of our study.

In addition to differences in *p53* status, T47D and MCF-7 have 164 differentially expressed proteins that influence therapeutic response [28]. In T47D, the expression of proteins that facilitate cell death induction, such as BH3-interacting domain death agonist p11 and cytochrome c releasing factor [28], may increase its susceptibility to the combination of PGV- 1, TAM, and 4-OHT. Meanwhile, MCF-7 expresses proteins associated with the prevention of chemotherapy-induced protein damage and inhibition of apoptosis initiation as well as protective proteins from oxidative stress, such as Heat shock 70 kDa protein 1 (HSP70), Peroxiredoxin-4, Glutathione S-transferase Mu 2, and 17b-hydroxysteroid dehydrogenase (17β-HSD10 and 17β-HSD10), which are highly expressed in MCF7 [28]. MCF-7 cells are more responsive to estrogen, which promotes faster cell growth than T47D. Estrogen stimulates TGFα production in MCF-7 with a significant increase of up to 200-fold. TGFα, as a growth factor, promotes cell proliferation by activating mitogenic signaling pathways [29]. ETHE1, a mitochondrial protein, is highly expressed in MCF-7 cells compared to T47D. ETHE1 plays a critical role in suppressing p53-induced apoptosis and may help maintain cell viability even under stressful conditions where p53 activation occurs, potentially reducing its therapeutic response [28]. The different proteome profiles suggest that the sensitivity of T47D treatment is influenced by p53 status and its specific molecular properties, which requires additional investigation to optimize therapeutic synergy.

In the cytotoxic washout and colony-formation assays, combining PGV-1 with TAM or 4-OHT significantly inhibited cell growth, with MCF-7 cells showing higher sensitivity than T47D cells. The combination treatments had a synergistic effect on MCF-7 cells, strongly suppressing proliferation and colony formation compared to single treatments. However, in T47D cells, the PGV-1 and TAM combination did not significantly inhibit colony growth compared to TAM alone. In contrast, PGV-1 combined with 4-OHT showed stronger colony inhibition than 4-OHT alone, suggesting that 4-OHT is more effective in suppressing colony formation in both cell types.

Previous studies have shown that PGV-1 at sub-IC_{50} modulates cells in the G2/M phase arrest, specifically prometaphase arrest [11,12], whereas TAM and 4-OHT modulate cells in the G0/G1 phase arrest [30]. We confirmed these results in the present study. When we observed the cell cycle in the combination treatment of PGV-1 with TAM or 4-OHT, we noted a significant increase in the sub-G1 phase. TAM has been shown to induce apoptosis in various cancer cell lines, increasing the sub-G1 population through caspase activation and intrinsic apoptotic pathways, which is often mediated by mitochondrial dysfunction and cytochrome C release [5,31]. Additionally, TAM causes cell arrest in the G1 phase by reducing cyclin expression and increasing cyclin-dependent kinase inhibitor levels [30]. Our results confirmed these findings. Combining PGV-1 with TAM or 4-OHT resulted in significantly increased sub-G1 accumulation. PGV-1 induces mitotic arrest in the prometaphase (G2/M) phase [11,12], whereas TAM and 4-OHT increase apoptosis, leading to an increased synergistic effect on sub-G1 accumulation [32]. The increase in the sub-G1 phase indicates apoptosis, although additional apoptosis data are needed for confirmation. Thus, the combination of PGV-1 and TAM or 4-OHT shows the potential to increase the efficacy of cancer therapy through cell cycle modulation and apoptosis induction.

Our findings revealed that combining PGV-1 with TAM or 4-OHT improved the therapeutic effectiveness when compared to employing single drugs. In the studies, the T47D cell line exhibited more sensitivity than MCF-7. Although both are luminal A breast cancer types, they differ in molecular properties such as ER subtype expression, *p53*, and caspase status. MCF-7 (ERα+/ERβ−) and T47D (ERα+/ERβ+) are recognized models for ERα+ luminal A carcinoma [20,32,33]. ERα controls genes related to cell proliferation, differentiation, and migration through endocrine and paracrine pathways [20,29,34,35]. ERβ promotes apoptosis, inhibits ERα proliferation, and prevents metastasis [36,37]. The ERβ expression is generally associated with a favorable outcome in ERα+ breast cancer [36,38]. Relevance of ERβ in ERα− breast cancer is still contested, with some research

associating it with poor prognosis and others claiming that ERβ deletion enhances cell-invasion ability [38,39]. MCF-7 cells have a high ERα/ERβ ratio, but T47D cells have a low ratio, which impacts their responsiveness to estrogen and cell proliferation [34]. These feature changes may explain the diversity in cell sensitivity, which needs more investigation to determine the process. Our study suggested that combining PGV-1 with TAM or 4-OHT enhanced therapeutic efficacy compared to single-drug treatments. In this study, the T47D cell line showed greater sensitivity than MCF-7 although not for all test results and both were equally classified as luminal A breast cancer subtype. These differences may be attributed to their distinct molecular characteristics, including ER subtype expression, *p53* status, and caspase activity. MCF-7 cells (ERα+/ERβ−) and T47D cells (ERα+/ERβ+) are established models for ERα+ luminal A carcinoma [20,33,34]. ERα regulates genes associated with cell proliferation, differentiation, and migration through endocrine and paracrine mechanisms [20,35,36]. Yu *et al.* [20] using T47D cells, a luminal A model of breast cancer, showed that ERα plays a role in regulating the specific effects of progesterone through hormonal pathways [20]. Ichikawa *et al.* [30] found that ERα induces cycle arrest in the G1 phase by expressing cyclin-dependent kinase inhibitors, an essential mechanism in controlling the proliferation and differentiation of cancer cells in the MCF-7 model [30]. A study on various breast cancer cells, including MCF-7, reported that activation of ERα by phytoestrogens such as genistein and apigenin increased the expression of genes related to proliferation, confirming the involvement of ERα in the endocrine mechanism of proliferation regulation [36]. In addition, Lu and Katzenellenbogen [37] showed that ERα interacts with *p53* in modulating the expression of genes controlling proliferation, differentiation, and apoptosis in MCF-7 and T47D cells, with ERα acting as a key regulator in the ERα-*p53* feedback loop [37]. These findings reinforce the role of ERα as a key regulator in luminal A breast cancer.

In contrast, ERβ is opposed to promoting apoptosis, inhibiting ERα-driven proliferation, and preventing metastasis [37,38]. ERβ expression is generally associated with good outcomes in ERα+ breast cancer [37,39]. However, its relevance in ERα− breast cancer is controversial, with some studies linking ERβ expression to poor prognosis, while others have shown that ERβ deletion increases cell invasiveness [39,40]. The different ERα/ERβ ratios between MCF-7 (high ERα/ERβ) and T47D (low ERα/ERβ) cells may explain the different responses to estrogen signaling and sensitivity to treatment. These findings indicate that ER subtype expression influences therapeutic response, although the molecular mechanism still requires further study. This study demonstrates the potential of PGV-1 to enhance the anticancer effects of TAM and 4-OHT *in vitro*. It also lays the groundwork for further *in vivo* studies to test its application in more physiologically relevant models. Tumor-bearing animal models are recommended to confirm the efficacy and safety of the PGV-1 combination. Further studies are also needed to explore the molecular mechanisms behind the different sensitivities between T47D and MCF-7 cells, particularly concerning the role of ER subtype expression. In addition, an analysis of apoptotic proteins, such as caspases, Bax, and Bcl-2, is proposed to elucidate further the apoptotic mechanisms underlying the effects of the PGV-1 combination.

CONCLUSION

In summary, our data suggest that PGV-1 improves TAM and 4-OHT activity toward luminal breast cancer cells: MCF-7 and T47D cells. The combination treatment demonstrated synergistic cytotoxicity, inhibition of colony-formation assay, and cell cycle arrest at the G0/G1 phase with increased population at sub-G1, indicating apoptosis. Further molecular investigations are warranted to completely determine the underlying mechanisms.

ACKNOWLEDGMENT

We gratefully acknowledge the support provided by the Rekognisi Tugas Akhir 2024 (RTA 2024) at Universitas Gadjah Mada under grant number 5286/UN1.P1/PT.01.03/2024. The data presented in this study were parts of Desty Restia Rahmawati's dissertation data which is under scholarship of the Education of Master Degree Leading to Doctoral Program for Excellent Graduates (PMDSU) from the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia which grant number 008/E5/PG.02.00/PL.PMDSU/2024 and 2060/UN1/DITLIT/Dit-Lit/PT.01.03/2024.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study protocol was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, in collaboration with Dr. Sardjito General Hospital, Indonesia (Approval No.: KE/FK/0058/EC/2022 in January 2022).

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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How to cite this article:
Rahmawati DR, Nurrochmad A, Meiyanto E, Jenie RI. Persistent synergistic antiproliferative and apoptosis induction effects of PGV-1 and tamoxifen or 4-hydroxytamoxifen on ER-positive breast cancer cells. J Appl Pharm Sci. 2025;15(06):205–215. DOI: 10.7324/JAPS.2025.228633