



Article

Synergistic Anti-Cancer Effects of ERB-041 and Genistein through Estrogen Receptor Suppression-Mediated PI3K/AKT Pathway Downregulation in Canine Mammary Gland Tumor Cells

Min-Jae Yoo, Ye-Ji Jang, Sang-Youel Park, Ja-Wun Choi *  and Jae-Won Seol * 

College of Veterinary Medicine, Jeonbuk National University, Iksan 54596, Jeollabuk-do, Republic of Korea; ymin105@naver.com (M.-J.Y.); yejiown25@gmail.com (Y.-J.J.); syipark@chonbuk.ac.kr (S.-Y.P.)

* Correspondence: jwchoi@jbnu.ac.kr (J.-W.C.); jwsseol@jbnu.ac.kr (J.-W.S.)

Abstract: Canine-mammary-gland tumors (CMTs) are prevalent in female dogs, with approximately 50% of them being malignant and often presenting as inoperable owing to their size or metastasis. Owing to poor outcomes, effective alternatives to conventional chemotherapy for humans are necessary. Two estrogen receptors, estrogen receptor alpha (ER α) and estrogen receptor beta (ER β), which act in opposition to each other, are involved, and CMT growth involves ER α through the phosphoinositide 3-kinases (PI3K)/AKT pathway. In this study, we aimed to identify the synergistic anti-cancer effects of ERB-041, an ER β agonist, and genistein, an isoflavonoid from soybeans known to have ER β -specific pseudo-estrogenic actions, on CMT-U27 and CF41.Mg CMT cell lines. ERB-041 and genistein synergistically inhibited cell proliferation and increased the number of annexin V-positive cells in both cell lines. Furthermore, we observed a synergistic increase in the Bax/Bcl-2 ratio and cleaved caspase-3 expression. Additionally, cell-cycle arrest occurred through the synergistic regulation of cyclin D1 and cyclin-dependent kinase 4 (CDK4). We also found a synergistic decrease in the expression of ER α , and the expression of proteins involved in the PI3K/AKT pathway, including p-PI3K, phosphatase and tensin homolog (PTEN), AKT, and mechanistic target of rapamycin (mTOR). In conclusion, ERB-041 and genistein exhibited a synergistic anticancer effect on CMTs, suggesting that cotreatment with ERB-041 and genistein is a promising treatment for CMTs.

Keywords: canine-mammary-gland tumors; estrogen receptor; ERB-041; genistein; PI3K/AKT pathway



Citation: Yoo, M.-J.; Jang, Y.-J.; Park, S.-Y.; Choi, J.-W.; Seol, J.-W.

Synergistic Anti-Cancer Effects of ERB-041 and Genistein through Estrogen Receptor Suppression-Mediated PI3K/AKT Pathway Downregulation in Canine Mammary Gland Tumor Cells. *Int. J. Mol. Sci.* **2024**, *25*, 2466. <https://doi.org/10.3390/ijms25052466>

Academic Editors: Barbara De Filippis, Marialuigia Fantacuzzi and Alessandra Ammazalorso

Received: 26 January 2024
Revised: 15 February 2024
Accepted: 16 February 2024
Published: 20 February 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Canine-mammary-gland tumors (CMTs) are the most common tumors in female dogs and approximately 50% of these tumors are malignant [1]. Approximately 50% of these tumors are inoperable because they are excessively large or metastasized; therefore, chemotherapy is administered [2]. The chemotherapeutic agents used in these cases are designed for humans and do not have a significant therapeutic effect in the treatment of CMTs [3]. In recent years, several methods have been used to treat CMTs, including surgery, radiotherapy, chemotherapy, and hormonal therapy. Chemotherapy is frequently used for CMTs that have metastasized or have a high probability of recurrence; however, the effectiveness of chemotherapeutic agents has not been proven, leading to high recurrence rates and poor prognosis [4,5]. Therefore, it is necessary to identify effective chemotherapeutic agents for the treatment of CMTs.

Estrogen is a steroid hormone that acts as a primary female sex hormone and affects many aspects of the body, including growth and differentiation. There are three main forms of estrogen: estrone (E1), estradiol (E2), and estriol (E3) [6]. These molecules primarily interact with two types of estrogen receptors, estrogen receptor alpha (ER α) and estrogen

receptor beta (ER β), to perform their roles. ER, a ligand-dependent transcription factor, regulates gene transcription through estrogen response elements, thereby promoting the normal biological functions of estrogen [7]. In numerous cases of breast cancer, the increased proliferation is commonly attributed to the activation of ER α by estrogen, which is counterbalanced by the existence of ER β , which imparts an inhibitory effect on cell growth [8]. In addition, previous studies have reported that high ER β levels reduce ER α expression [9]. In contrast to the way normal mammary glands express both ER α and ER β positively, CMTs have been reported to exhibit a predominantly ER β -positive status and a weaker intensity of ER α [10]. Despite these ER β -dominant features, CMTs are fatal with low survival rates [11].

The phosphoinositide 3-kinase (PI3K)/AKT pathway, which consists of PI3K, a phosphatase and tensin homolog (PTEN), AKT (also known as protein kinase B), and a mechanistic target of rapamycin (mTOR), is one of the most important pathways involved in cell death, metabolism, proliferation, and cell-cycle regulation [12]. In addition, PI3K, a key enzyme in this pathway, is considered an important therapeutic target because its gene *PI3KCA* is highly mutated in many tumors, including human breast cancer and CMTs, increasing the expression of downstream signals and promoting tumor growth [13,14]. Furthermore, in breast cancers, estrogen activates the PI3K/AKT pathway through ER α to increase downstream signaling, leading to tumor growth, invasion, and metastasis [15]. Therefore, it is essential to confirm whether the ER α inhibition effect occurs when treated with an ER β agonist. If this inhibitory effect leads to the inhibition of the PI3K/AKT pathway, it may contribute to the treatment of CMTs.

ERB-041 is a potent and selective ER β agonist and has been reported to exhibit anti-inflammatory effects in Phase II clinical trials for the treatment of rheumatoid arthritis [16–18]. ERB-041 inhibits triple-negative breast-cancer-cell invasion [19]. Furthermore, ERB-041 decreased cell migration, invasion, and proliferation and induced cell-cycle arrest by modulating the PI3K/AKT pathway in ovarian cancer cells [20]. Genistein is a phytoestrogen that belongs to the flavonoid family and is derived from soybeans and soy-derived foods [21]. Genistein has been reported to have anti-cancer effects in breast cancers, demonstrating its potential as a therapeutic agent [22]. Additionally, it inhibits the PI3K/AKT pathway in triple-negative breast cancer cells [23]. The most important characteristic of genistein is that as a phytoestrogen, it binds to and acts more specifically on ER β than ER α [24]. Both ERB-041 and genistein act as ER β agonists, showing selectivity with differences in the ligands that bind to ER β [25,26]. Therefore, the aim of the present study was to determine whether two agonists can have a synergistic anticancer effect on CMTs.

2. Results

2.1. ERB-041 and Genistein Synergistically Inhibit Proliferation of CMT Cells

To confirm the effective concentrations of ERB-041 and genistein against two CMT cell lines, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed on the CMT-U27 and CF41.Mg cell lines according to each determined concentration. At 10 μ M, ERB-041 induced significant proliferation inhibition in both cell lines, whereas genistein induced significant proliferation inhibition at 20 μ M in CMT-U27 and 80 μ M in CF41.Mg cells (Figure 1A,B). It is important to consider the fact that CMT-U27 has the characteristics of an epithelial cell and CF41.Mg has the characteristics of a mesenchymal cell, which means that the effective concentration of genistein for each is different. Therefore, we examined the synergistic effect of 8 μ M ERB-041 and 10 μ M genistein in CMT-U27 and 8 μ M ERB-041 and 20 μ M genistein in CF41.Mg cells, as concentrations that did not significantly reduce cell viability. We observed the morphology of CMT-U27 and CF41.Mg cells and performed an MTS assay. Morphological imaging of both cell lines showed that the combination of drugs synergistically reduced the number of cells and altered their morphology (Figure 1C,D). In the MTS assay, the treatment of CMT-U27 cells with ERB-041 and genistein inhibited cell proliferation by approximately 1.4% and 6.3%, respectively, compared to the control group, showing no

significant difference. However, upon cotreatment, a significant synergistic proliferation-inhibition effect of approximately 25.5% compared with the control group was observed (Figure 1E). Similarly, compared to the control group, the proliferation of CF41.Mg cells did not significantly differ with ERB-041 and genistein treatment. However, with cotreatment, a significant inhibitory effect of approximately 48.9% compared with that in the control group was observed (Figure 1F). These results indicate that ERB-041 and genistein synergistically inhibited cell proliferation in both CMT-U27 and CF41.Mg cell lines.

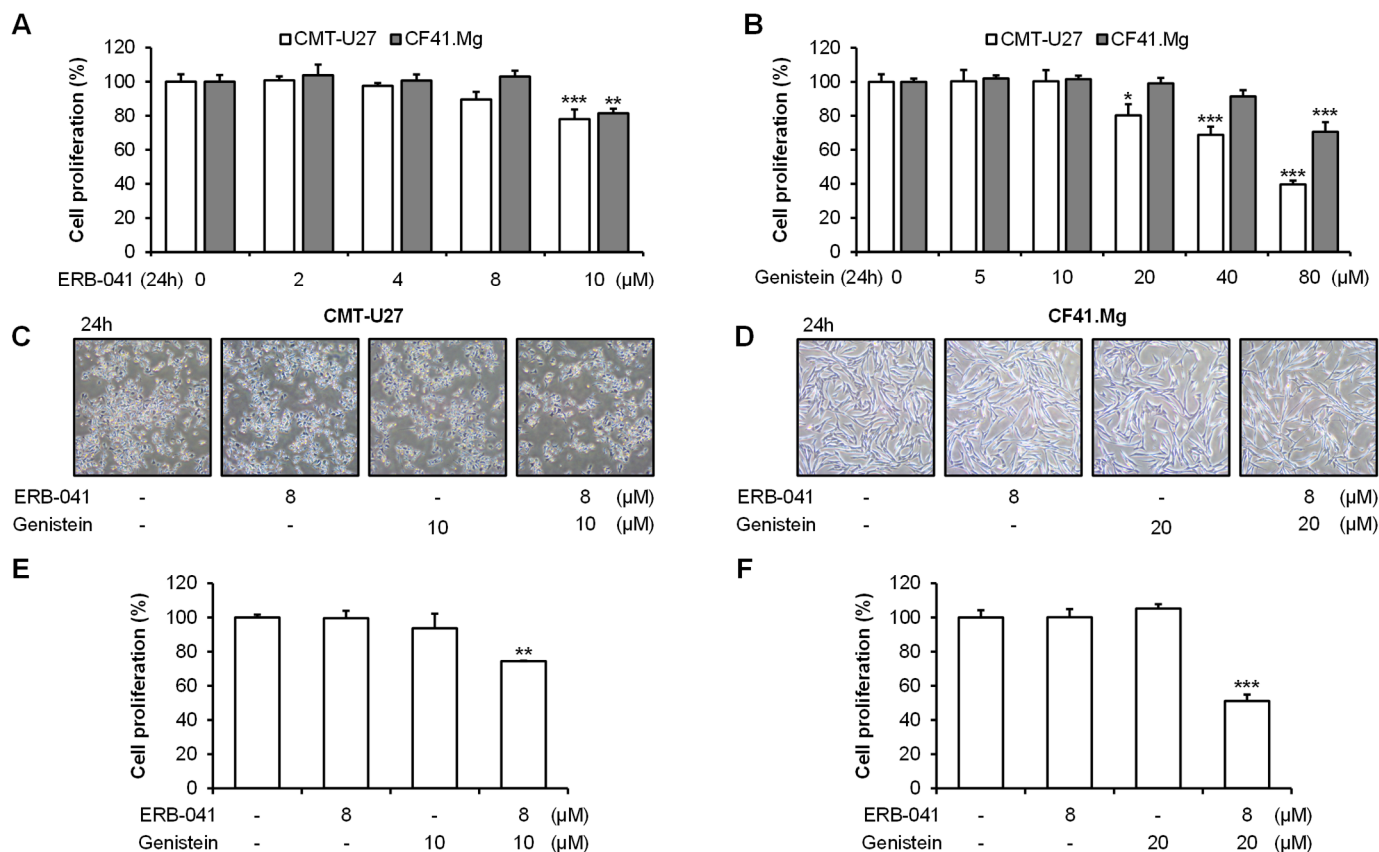


Figure 1. ERB-041 and genistein synergistically suppress the proliferation of CMT cells. (A,B) Cell viability at indicated concentrations of ERB-041 and genistein in CMT-U27 (A) and CF41.Mg (B); (C,D) morphological images of CMT-U27 (C) and CF41.Mg (D); (E,F) synergistic cell viability reduction in CMT-U27 (E) and CF41.Mg (F) following treatment with ERB-041 and genistein at the indicated concentrations. Magnification, 100 $\times$. Values are mean $\pm$ SD. * p < 0.05; ** p < 0.01; *** p < 0.001 versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

2.2. ERB-041 and Genistein Synergistically Induce Apoptosis in CMT Cells by Regulating the Expression of Bcl-2/Bax/Caspase-3

To determine the effects of ERB-041 and genistein on CMT cell death, we performed an Annexin-V/Propidium Iodide (PI) assay. The results showed that in CMT-U27 cells, ERB-041 treatment increased cell death by approximately 5-fold compared to that in the control group, and genistein treatment increased cell death by approximately 2-fold. In addition, the combination treatment induced approximately 6.5-fold more cell death than the control (Figure 2A). Similarly, in CF41.Mg cells, ERB-041 and genistein increased cell death by approximately 1.6- and 1.8-fold, respectively, compared to that in the control group, and by approximately 2.4-fold when combined compared to that in the control group (Figure 2B). To confirm apoptosis induction, Western blotting was performed to determine the expression of Bcl-2, Bax, caspase-3, and cleaved caspase-3. The Bax/Bcl-2 ratio in CMT-U27 cells was not significant when treated with ERB-041 alone but increased significantly

to approximately 25% compared to that in the control group when treated with genistein alone and to approximately 48% when treated with both agonists (Figures 3A and S1). Caspase-3 expression did not differ significantly between the treatment and control groups. In contrast, cleaved caspase-3 expression showed no difference when treated with genistein alone, but showed a significant increase of approximately 98% when treated with ERB-041 and an increase of approximately 120% when cotreated compared to that in the control group (Figures 3B and S1). Protein expression in the CF41.Mg cells was similar to that in the Western blot-treated CMT-U27 cells. In CF41.Mg, a significant difference was absent in the Bax/Bcl-2 ratio when treated with ERB-041 alone. However, when treated with genistein alone or cotreated, the Bax/Bcl-2 ratio increased by approximately 30% and 143%, respectively, compared to that in the control group, which was highly significant (Figures 3C and S2). Caspase-3 expression was reduced by approximately 31%, 11%, and 10% compared with that in the control group when treated with ERB-041, genistein, or both, respectively, and the expression of cleaved caspase-3 was reduced by approximately 1%, 10%, and 12%, respectively, compared with that in the control group (Figures 3D and S2). Activated caspase-3 was identified using immunocytochemistry. A significant difference was absent in the expression of activated caspase-3 between the ERB-041 and genistein alone groups. However, with cotreatment, CMT-U27 and CF41.Mg showed significant increases of approximately 245% and 34%, respectively, compared to that in the control group (Figure 3E–H). These results indicate that ERB-041 and genistein synergistically induced apoptosis in both CMT-U27 and CF41.Mg cell lines.

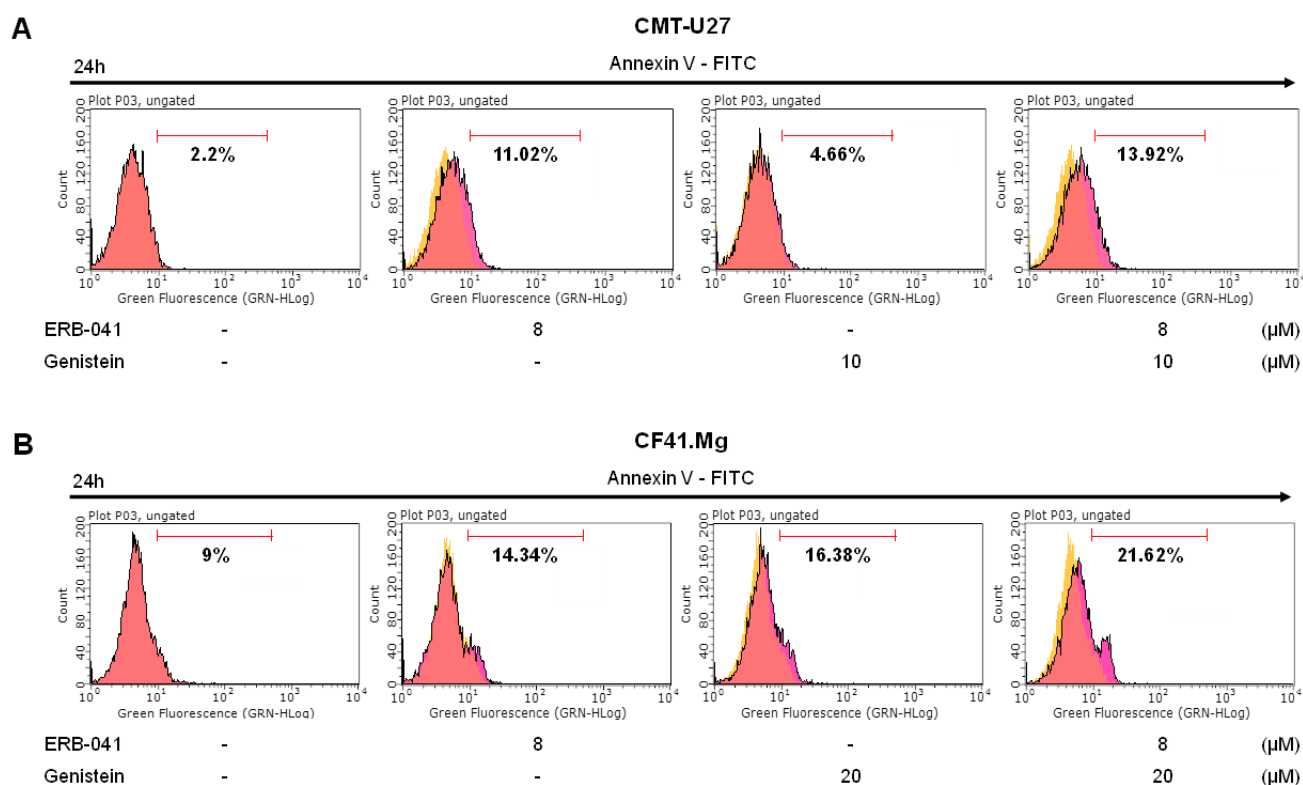


Figure 2. ERB-041 and genistein synergistically induced apoptosis in CMT cells. (A,B) Images of Annexin-V/PI staining results of CMT-U27 (A) and CF41.Mg (B) cells treated with ERB-041 and genistein at the indicated concentrations.

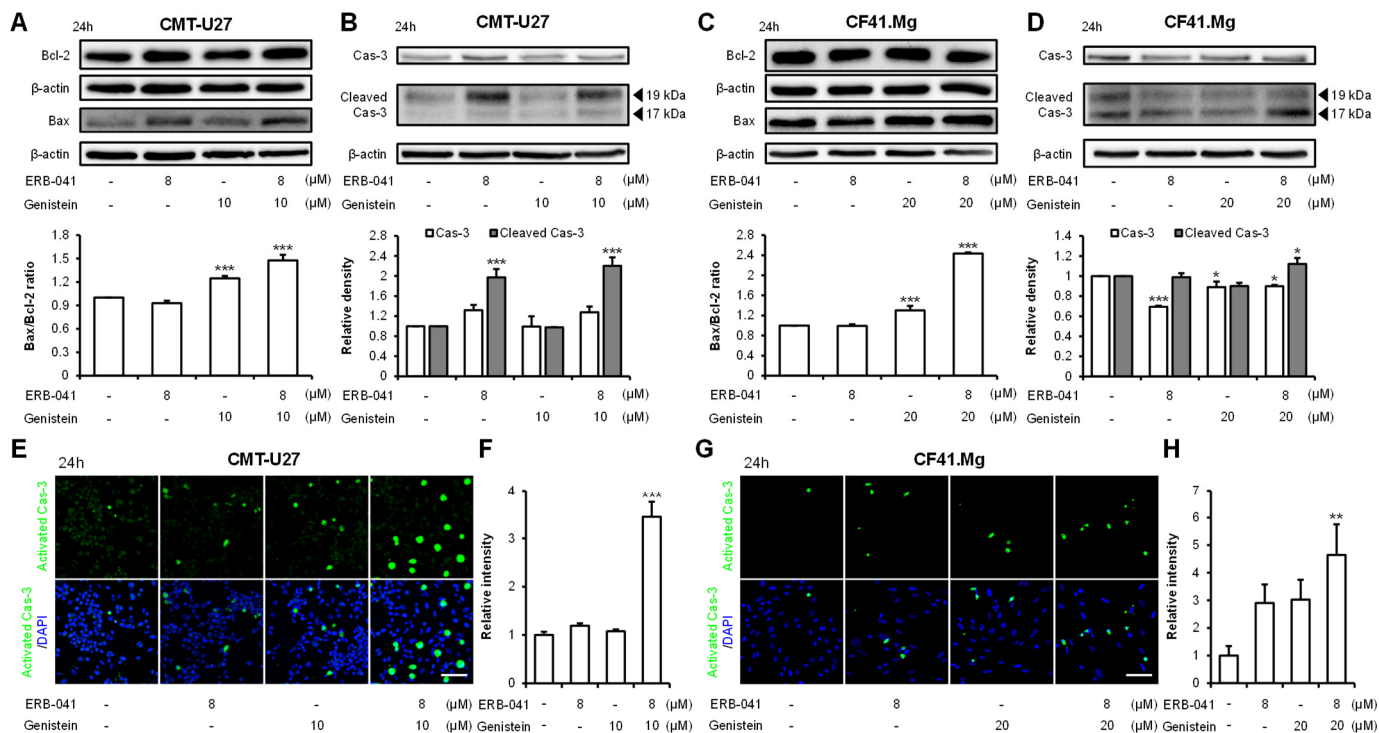


Figure 3. ERB-041 and genistein are involved in the regulation of Bcl-2, Bax, and caspase-3. (A,B) Western blot images and quantification of the expression of Bcl-2, Bax (A), caspase-3, and cleaved caspase-3 (B) in CMT-U27 upon ERB-041 and genistein treatment at the indicated concentrations and the expression of Bcl-2, Bax (C), caspase-3, and cleaved caspase-3 (D) in CF41.Mg; (E–H) immunocytochemistry images and quantitative analysis of activated caspase-3 in CMT-U27 (E,F) and CF41.Mg (G,H). Cas-3, caspase-3. Scale bar, 50 μm. Values are mean ± SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

2.3. ERB-041 and Genistein Synergistically Induce Cell-Cycle Arrest by Regulating Cell-Cycle-Related Proteins in CMT Cells

To investigate the effects of ERB-041 and genistein on the cell cycle of CMT-U27 and CF41.Mg, we performed Western blotting with anti-CDK4 and -cyclin D1 antibodies and a cell-cycle arrest assay (Figures 4A–E and S3). When CMT-U27 cells were treated with ERB-041, genistein, and both, the expression of CDK4 increased by approximately 8%, 5%, and 19%, respectively, compared to that of the control group. In contrast to the expression of CDK4, cyclin D1 expression was reduced by approximately 13%, 13%, and 28% in the three groups, respectively, compared to that of the control group. Subsequently, a cell-cycle arrest assay revealed a G0/G1 phase arrest. The number of cells in the G0/G1 phase increased synergistically from approximately 48% in the control to 61% with ERB-041 treatment alone, 60% with genistein treatment alone, and 64% with cotreatment. A Western blotting and cell-cycle arrest assay in CF41.Mg cells showed contrasting effects to that in CMT-U27 cells (Figures 4F–J and S3). The expression of CDK4 in the CF41.Mg cells decreased by approximately 19%, 23%, and 35% compared to that in the control group when treated with ERB-041, genistein, or both, respectively. Conversely, the expression of cyclin D1 increased by approximately 24%, 48%, and 66%, respectively, in each group compared to that in the control group. In the cell-cycle arrest assay, a significant difference in cell number was observed only in the cotreated group with ERB-041 and genistein. The number of cells in the G0/G1 phase decreased by approximately 26% in the cotreatment group compared to the control group, and the number of cells in the G2/M phase increased by approximately 11% compared to that in the control group, indicating G2/M phase arrest.

These results indicate that ERB-041 and genistein synergistically regulate the expression of cell-cycle-related proteins to induce cell-cycle arrest in CMT cells.

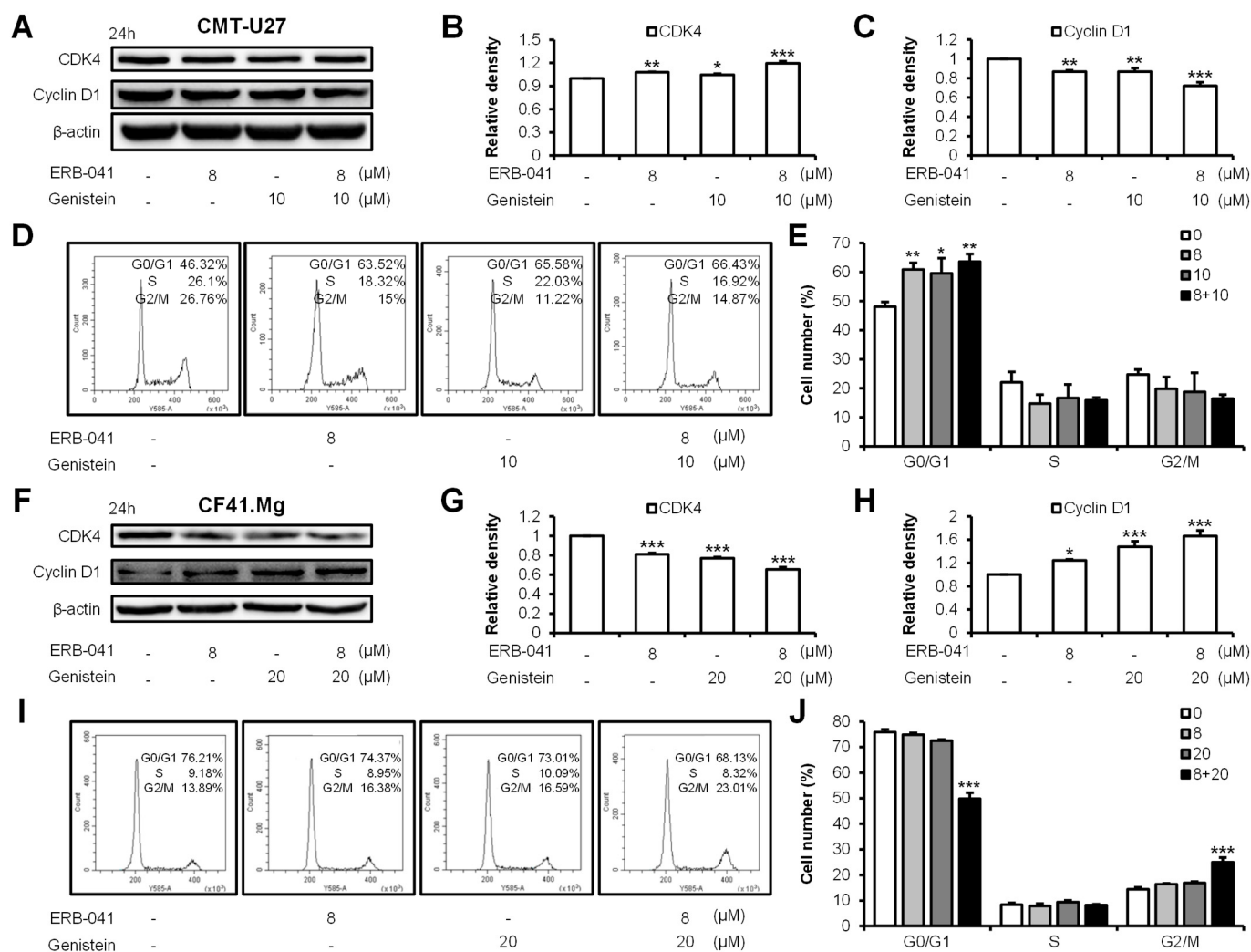


Figure 4. ERB-041 and genistein induce cell-cycle arrest in CMT cells through synergistic regulation of CDK4 and cyclin D1. (A–C) Western blotting images and quantification of CDK4 (A,B) and cyclin D1 (A,C) following treatment with indicated concentrations of ERB-041 and genistein in CMT-U27; (D,E) cell-cycle-arrest assay data by flow cytometry (D) and quantitative analysis (E) in CMT-U27; (F–H) Western blotting images and quantification of CDK4 (F,G) and cyclin D1 (F,H) after treatment with ERB-041 and genistein at the concentrations indicated in CF41.Mg; (I,J) cell-cycle-arrest assay data by flow cytometry (I) and quantitative analysis (J) in CF41.Mg. CDK4, cyclin-dependent kinase 4. Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

2.4. ERB-041 and Genistein Synergistically Inhibit the Expression of ER α

Western blot analysis was conducted to assess the alterations in the expression of ER α and ER β upon treatment with ERB-041 and genistein, which both function as ER β agonists. Upon treatment with ERB-041, genistein, and their combination, in CMT-U27 cells, the expression of ER β exhibited significant reductions by approximately 18%, 36%, and 54%, respectively, compared to that in the control group. Similarly, the expression of ER α showed substantial decreases of approximately 11%, 19%, and 29% compared to that in the control group upon treatment with ERB-041, genistein, and cotreatment, respectively (Figures 5A and S4). CF41.Mg cells showed similar results, with ERB-041 alone, genistein alone, and combined treatment reducing ER β expression by approximately 4%, 13%, and

24%, respectively, compared to that of the control group. The expression of ER α was not significantly different from that of the control group in the ERB-041 treated group, whereas it was significantly reduced by approximately 32% and 37% in the genistein and cotreated groups, respectively, compared to that in the control group (Figures 5B and S4). Immunocytochemistry was then performed to confirm the Western blotting results for ER β and ER α . The expression of ER β in CMT-U27 was reduced by approximately 8%, 20%, and 22% when treated with ERB-041 alone, genistein alone, and both treatments together, respectively, compared to that in the control group. ER α expression was significantly different in the three groups, showing a decrease of approximately 29%, 35%, and 31%, respectively, compared to that in the control group (Figure 5C). CF41.Mg cells showed the same results, with ER β expression significantly declining by approximately 36%, 47%, and 51% compared to that in the control group when treated with ERB-041 alone, genistein alone, or together, respectively. The expression of ER α was greatly reduced in the three groups to approximately 26%, 55%, and 40%, respectively, compared to that in the control group. The most significant decrease occurred in the group treated with genistein alone. However, all three groups showed substantial differences (Figure 5D). These results suggest that ER α is repressed by the ER β agonists ERB-041 and genistein in both CMT cell lines.

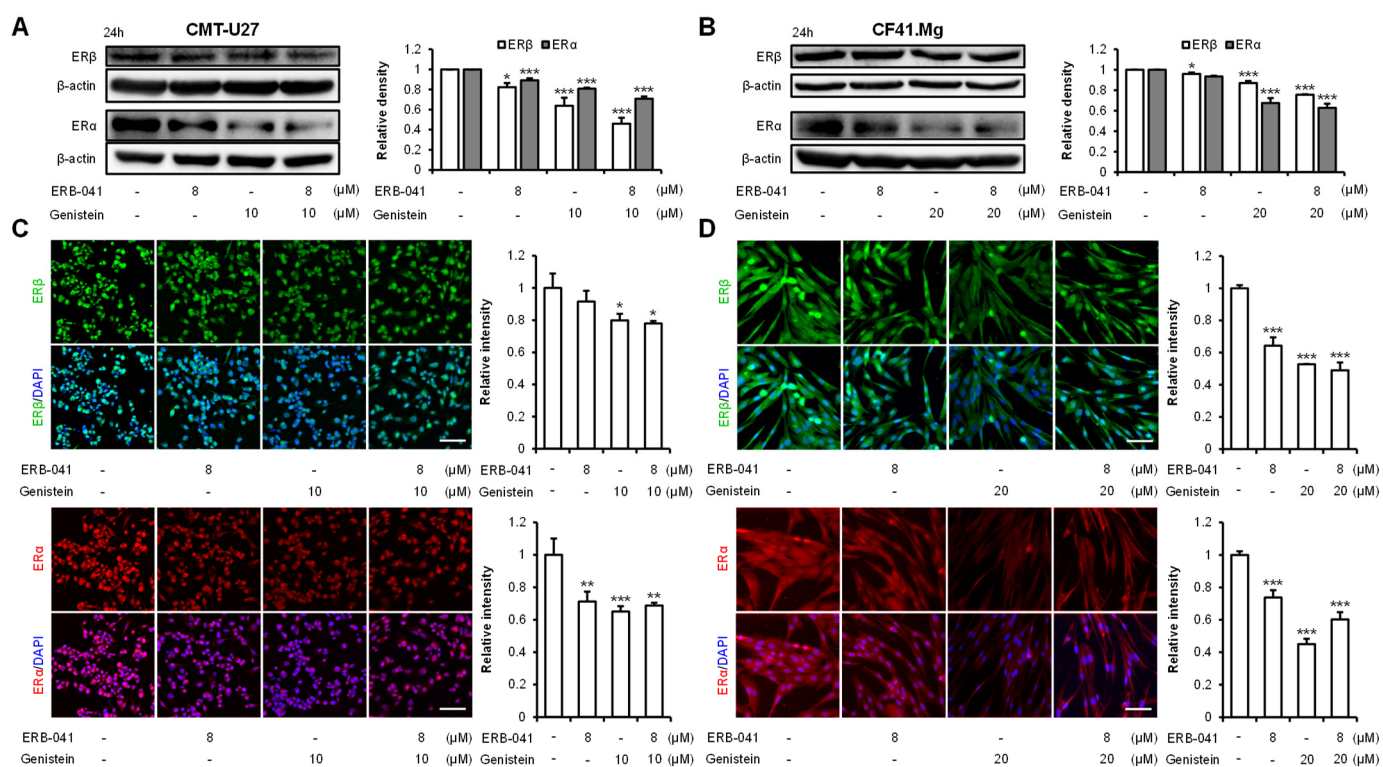


Figure 5. Effects of ERB-041 and genistein treatment on the expression of ER β and ER α . (A,B) Western blot images and quantitative analysis of ER β and ER α upon treatment with ERB-041 and genistein at the concentrations indicated in CMT-U27 (A) and CF41.Mg (B); (C,D) immunocytochemistry images and quantification of ER β and ER α in CMT-U27 (C) and CF41.Mg (D). Scale bar, 50 μ m. ER β , estrogen receptor beta; ER α , estrogen receptor alpha. Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

2.5. ERB-041 and Genistein Synergistically Inhibit the Expression of the PI3K/AKT Pathway-Related Proteins

To confirm that the previously identified inhibition of ER α expression in CMT cells by ERB-041 and genistein leads to the inhibition of the PI3K/AKT pathway, Western blotting was performed. In CMT-U27, Western blot analysis showed that the protein expression levels of p-PI3K, PTEN, p-AKT, and p-mTOR were reduced. The p-PI3K level was reduced

by approximately 39% and 52% with ERB-041 treatment alone (84 and 54 kDa), 32% and 47% with genistein treatment alone, and 50% and 51% with their cotreatment, respectively, compared to that in the control group (Figures 6A and S5). The expression of PTEN was reduced by approximately 27% compared to that in the control group when treated with ERB-041 alone, 43% when treated with genistein alone, and 45% when treated together; the expression of p-PTEN was decreased by 2%, 24%, and 18%, respectively, compared to that in the control group (Figures 6B and S5). AKT expression was only significant in the cotreatment group, increasing by approximately 23% compared with that in the control group. In contrast, the expression of p-AKT decreased by approximately 10% compared to that in the control group with ERB-041 alone, by approximately 19% with genistein alone, and by approximately 28% with cotreatment, with the decrease being significant in all groups (Figures 6C and S5). Furthermore, the expression of mTOR was reduced by approximately 5%, 3%, and 3% compared to that in the control group when cells were treated with ERB-041 alone, genistein alone, and both, respectively, and p-mTOR expression was decreased by approximately 26%, 25%, and 53%, respectively, compared to that in the control group (Figures 6D and S6). In CF41.Mg cells, a similar pattern of protein expression to that observed in CMT-U27 was observed with Western blotting. The expression of p-PI3K was significantly reduced by 27% and 5% when treated with ERB-041 alone (84 and 54 kDa) and by 55% and 32% when treated with genistein alone and together, respectively, compared to that in the control (Figures 6E and S6). The expression of PTEN increased by approximately 3% compared to the control group when treated with ERB-041 alone, decreased by approximately 14% when treated with genistein alone, and by approximately 27% when treated with both, compared to that in the control group. The expression of p-PTEN declined by approximately 22% compared to that in the control group control when cotreated, which was the only significant finding (Figures 6F and S6). The expression of AKT decreased by approximately 21% compared to that in the control group when treated with genistein alone and by approximately 24% when cotreated with ERB-041 and genistein, whereas the expression of p-AKT was significantly reduced by approximately 12%, 14%, and 29% compared to that in the control group when treated with ERB-041 alone, genistein alone, and cotreated, respectively (Figures 6G and S7). The expression of mTOR increased by approximately 4%, 28%, and 19% when treated with ERB-041 alone, genistein alone, and their combination, respectively, compared to that in the control group. Furthermore, p-mTOR expression was reduced by approximately 16% in the cotreatment group compared to that in the control group, which was highly significant (Figures 6H and S7). We then performed immunocytochemistry to confirm the synergistic effect of ERB-041 and genistein on the inhibition of the PI3K/AKT pathway, as observed through Western blotting. The results showed that the expression of p-PI3K in CMT-U27 cells showed a significant synergistic decrease, with reductions of approximately 26%, 29%, and 36% compared to that in the control group upon treatment with ERB-041 alone, genistein alone, and their cotreatment, respectively (Figure 7A). The expression of PTEN also exhibited a synergistic effect, showing reductions of approximately 28%, 25%, and 40%, respectively, in the three treatment groups compared to that in the control group (Figure 7C). The expression of p-PI3K in CF41.Mg cells was reduced by approximately 24% compared to that in the control group when treated with ERB-041 alone, 23% when treated with genistein alone, and approximately 50% when treated with cotreatment, with significance only in the cotreatment group (Figure 7B). In addition, PTEN expression was reduced by approximately 21%, 4%, and 68% in the three treatment groups compared to that in the control group, with significance only in the cotreatment group (Figure 7D). These results indicated that ERB-041 and genistein synergistically inhibited the expression of PI3K/AKT pathway-related proteins in both CMT cell lines.

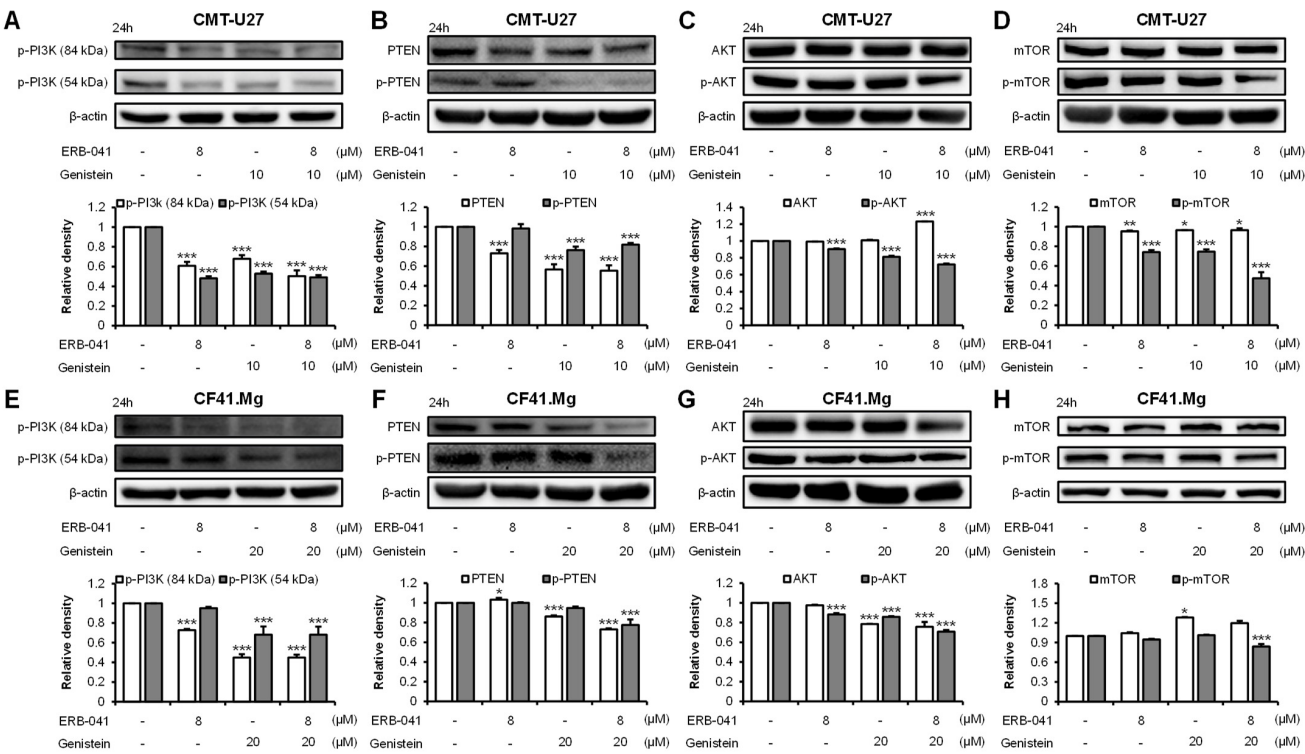


Figure 6. Western blotting results demonstrate that ERB-041 and genistein synergistically regulate the expression of the PI3K/AKT pathway. (A–D) Western blot images and quantification of p-PI3K (A), PTEN, p-PTEN (B), AKT, p-AKT (C), mTOR, and p-mTOR (D) expression in CMT-U27 following ERB-041 and genistein treatment; (E–H) Western blot images and quantification of the expression of PI3K/AKT-pathway-related proteins in CF41.Mg of ERB-041 and genistein at the indicated concentrations. PI3K, phosphoinositide 3-kinases; PTEN, phosphatase and tensin homolog; mTOR, mechanistic target of rapamycin. Values are mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

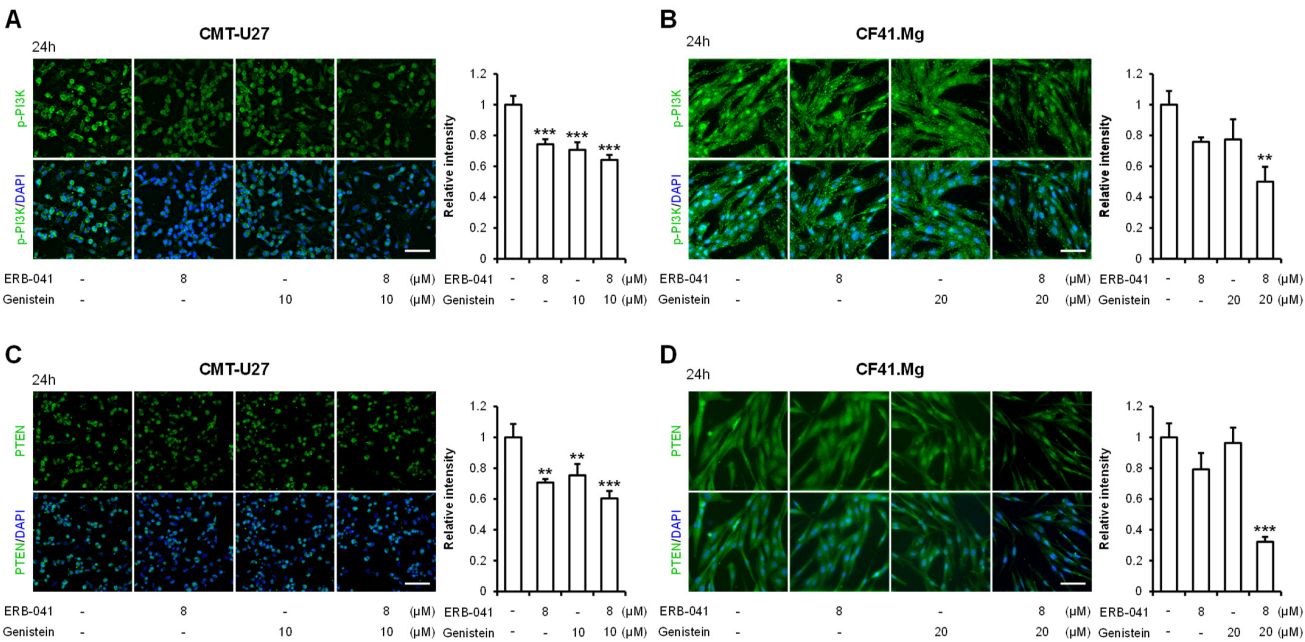


Figure 7. Immunocytochemistry results demonstrate that ERB-041 and genistein synergistically

reduce the expression of the PI3K and PTEN. (A–D) Immunocytochemistry images and quantitative analysis of the p-PI3K expression in CMT-U27 (A) and CF41.Mg (B) when treated with ERB-041 and genistein at the indicated concentrations and PTEN expression in CMT-U27 (C) and CF41.Mg (D). PI3K, phosphoinositide 3-kinases; PTEN, phosphatase and tensin homolog. Scale bar, 50 μ m. Values are mean $\pm$ SD. ** $p < 0.01$; *** $p < 0.001$ versus untreated cells by one-way ANOVA followed by Bonferroni post hoc test.

3. Discussion

In numerous cases of human breast cancer, growth is commonly promoted by estrogen-activated ER α , which is counterbalanced by the cell growth-inhibitory effects of ER β [27]. Similarly, in veterinary research, ER α activated by E2 has been demonstrated to play an important role in the growth of CMTs [28]. In addition, the development of CMTs is estradiol-dependent, wherein many of these patients express high tissue levels of ER α or elevated concentrations of serum E2 [28,29]. However, the selective estrogen receptor modulators such as tamoxifen and raloxifene, which act as antagonists to ER α , did not show a significant effect on CMTs, probably owing to a low affinity for the receptor [10]. Furthermore, the selective activation of ER α or ER β can be influenced not only by the affinity for selective receptor binding but also by the selective activation of each receptor subtype [30]. In this study, we aimed to determine whether treatment with ERB-041 and genistein, which act as ER β agonists, can exert anti-cancer effects on CMTs by reducing the expression of ER α . Our results demonstrate that ERB-041 and genistein synergistically decreased ER α expression, resulting in PI3K/AKT pathway inhibition and the induction of cell apoptosis and cell-cycle arrest in both CMT-U27 and CF41.Mg cell lines.

Numerous studies suggest that the induction of apoptosis and cell-cycle arrest are major strategies in anti-cancer therapy [31,32]. Previous studies have shown that ERB-041 and genistein increase the Bax/Bcl-2 ratio and expression of cleaved caspase-3, a marker of apoptosis induction in various human cancers [33,34]. Our findings indicate that ERB-041 and genistein synergistically inhibited the proliferation of CMT-U27 and CF41.Mg cells. In addition, Annexin-V/PI staining demonstrated a synergistic increase in apoptosis when both cell lines were treated with ERB-041 and genistein. Moreover, synergistic effects were identified through the ratio of Bax/Bcl-2 and the expression of cleaved caspase-3, which are proteins involved in apoptosis. The Bax/Bcl-2 ratio and cleaved caspase-3 expression in both cell lines significantly increased in the cotreatment group. In addition, immunocytochemistry results confirm that the expression of activated cas-3 was significantly increased in both cell lines in the cotreatment group. Regarding cell-cycle arrest, previous studies have reported that ERB-041 induced G1-phase arrest in human cancers by decreasing cyclin D1 expression, which regulates the cell-cycle transition from the G1 phase to S phase through PI3K/AKT pathway inhibition [34]. In contrast, genistein induces G2/M phase arrest through PI3K/AKT pathway inhibition in several human tumors, and cyclin D1 is upregulated [35,36]. Our cell-cycle-arrest assay results for CMT-U27 and CF41.Mg cells were very different. In CMT-U27 cells, there was an increase in CDK4 expression and a decrease in cyclin D1 expression, which synergistically induced G0/G1 phase arrest. In contrast, in CF41.Mg, the expression of CDK4 decreased and that of cyclin D1 increased, synergistically inducing G2/M phase arrest. We assumed that these contrasting results were due to differences in the characteristics of the two cell lines. In a previous study, it was reported that when human liver cancer cell lines HepG2 and Hep3B were treated with doxorubicin, the two cell lines responded differently to the drug, with HepG2 showing a G1 arrest and Hep3B showing a G2/M arrest [37]. These results are consistent with those of previous studies, and this study confirmed the same effect in both CMT cell lines. Based on these results, we conclude that ERB-041 and genistein synergistically exert anticancer effects by inducing apoptosis and cell-cycle arrest in CMT cells.

Next, we determined whether these anti-cancer effects of ERB-041 and genistein were mediated through reduced ER α expression and through the PI3K/AKT pathway. ERB-041

and genistein have been reported to reduce the expression of ER α in human tumors, and ER α has been reported to regulate apoptosis in breast cancer [38–40]. Furthermore, the expression of the PI3K/AKT pathway in breast cancer is ER α -dependent. In detail, the underlying mechanism involves ER α directly binding to PI3K, leading to an increase in PI3K expression and a decrease in the expression of PTEN, which functions in opposition to PI3K [41,42]. Our results showed that the expression of ER β was synergistically reduced by ERB-041 and genistein in CMT-U27 and CF41.Mg cell lines despite treatment with ER β agonists. Noteworthy, our experiments showed a significant decrease in the expression of ER α regardless of the decreased expression of ER β , contrary to what is commonly known about ER β inhibiting ER α [9]. In addition, Western blotting demonstrated that the expression of p-PI3K, p-AKT, and p-mTOR was synergistically reduced in both cell lines. The expression of PTEN, a negative regulator of PI3K, was reduced, despite the decreased expression of p-PI3K [43,44]. We focused on PTEN to understand the paradoxical expression of these proteins. The decreased expression of proteins involved in the PI3K/AKT pathway is speculated to be attributable to the reduced expression of ER α , leading to a diminished direct binding to PI3K. The expression of PTEN is decreased when PI3K expression is inhibited [45]. Furthermore, knockdown of PTEN induces a decrease in ER β expression [46]. Based on the results reported in previous studies, we speculated that the direct effects of ERB-041 and genistein reduced the expression of ER α and PI3K, which in turn reduced the expression of PTEN, and that this would have led to a reduction in the expression of ER β . Therefore, we determined that ERB-041 and genistein exert their anti-cancer effects by directly inhibiting the expression of ER α , which in turn downregulates the expression of the PI3K/AKT pathway in both CMT cells.

In conclusion, our results showed that treatment of two different CMT cells with ER β agonist ERB-041 and genistein synergistically decreased the PI3K/AKT pathway expression by decreasing the expression of ER α . Thus, we suggest that cotreatment with ERB-041 and genistein has the potential to be effectively used in the treatment of CMTs.

4. Materials and Methods

4.1. Cell Culture and Reagents

CMT-U27 and CF41.Mg cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 medium (CMT-U27 cells; HyClone, Logan, UT, USA) or Dulbecco's modified Eagle's medium (CF41.Mg cells; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO, USA), 100 unit/mL of penicillin (100 unit/mL) and 100 μ g of streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All cells were incubated at 37 °C in 5% CO₂. ERB-041 and genistein were purchased from Sigma-Aldrich (ERB-041; PZ0183, Genistein; G6649).

4.2. MTS Assay

To assess the synergistic effect of palmatine on cell viability, we conducted a CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (Promega Corporation, Madison, WI, USA) based on the MTS assay. CMT-U27 cells were plated at 1×10^4 cells and CF41.Mg cells were plated at 5×10^3 cells in 96-well plates with 100 μ L of medium and incubated at 37 °C for 24 h. Then, the cells were treated with ERB-041 and genistein for 24 h at each concentration. Following treatment, 20 μ L of CellTiter 96[®] AQueous One Solution Reagent was added to each well and incubated for 2 h at 37 °C. The absorbance was measured at 490 nm using a microplate reader (Spectramax M2; Molecular Devices, San Jose, CA, USA).

4.3. Annexin-V/PI Staining

Cell death in CMT-U27 and CF41.Mg cells was evaluated by flow cytometry using an Annexin-V assay (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) according to the manufacturer's protocol. Annexin-V content was estimated by measuring the fluorescence at

488 nm (excitation) and 525 nm (emission) using the Guava easyCyte HT system (Millipore, Billerica, MA, USA).

4.4. Western Blotting

CMT-U27 and CF41.Mg cells were lysed in cold lysis buffer supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Protein extraction was followed by separation using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent transfer to nitrocellulose membranes. To inhibit non-specific binding, the membranes were treated with 5% skim milk and then incubated overnight at 4 °C with the following primary antibodies in a blocking buffer: rabbit polyclonal anti-ER β (ab3576; 1:1000; Abcam, Cambridge, MA, USA), mouse monoclonal anti-ER α (MA1-12692; 1:1000; Invitrogen, Carlsbad, CA, USA), rabbit polyclonal anti-p-PI3K (AF3242; 1:1000; Affinity biosciences, Cincinnati, OH, USA), rabbit polyclonal anti-AKT (9272; 1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA), rabbit polyclonal anti-p-AKT (9271; 1:1000; Cell Signaling Technology), rabbit monoclonal anti-mTOR (2983; 1:1000; Cell Signaling Technology), rabbit monoclonal anti-p-mTOR (5536; 1:1000; Cell Signaling Technology), rabbit polyclonal anti-PTEN (bs0686-R; 1:1000; Bioss, Inc., Beijing, China), mouse monoclonal anti-p-PTEN (sc-377573; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-Bcl-2 (Sc-7382; 1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-Bax (2772; 1:1000; Cell Signaling Technology), rabbit polyclonal anti-caspase-3 (9662; 1:1000; Cell Signaling Technology), mouse monoclonal anti-Cyclin D1 (AHF0082; 1:1000; Invitrogen), rabbit polyclonal anti-CDK4 (11026-1-AP; 1:1000; Proteintech, Chicago, IL, USA) and mouse monoclonal anti- β -actin (A5441; 1:1000; Sigma-Aldrich). Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Chemiluminescent signals were amplified using WESTSAVE Gold (LF-QC0103; Abfrontier, Seoul, Republic of Korea) or WESTSAVE Star (LF-QC0106; Abfrontier) and subsequently captured using a Fusion FX7 acquisition system (Vilbert Lourmat, Eberhardzell, Germany). Band densities were quantified using Quantity One (version 4.6.6) and normalized to that of β -actin. The intensity was then presented relative to that of the control.

4.5. Cell-Cycle-Arrest Assay

CMT-U27 and CF41.Mg cells were seeded in 6-well plates at 5×10^5 and 25×10^4 cells, respectively. After treatment, cells were fixed overnight in ice-cold ethanol/PBS (7:3). The cells were then washed twice with PBS and incubated with FxCycle™ PI/RNase Staining Solution (F10797; Invitrogen) for 30 min at room temperature. The cells were analyzed using a CytoFLEX S V4-B2-Y0-R3 Flow Cytometer (C02948; Beckman Coulter, Brea, CA, USA) and the data were analyzed using CytExpert software (version 2.5.0.77).

4.6. Immunocytochemistry

CMT-U27 and CF41.Mg cells were cultured on gelatin-coated coverslips. Cells were fixed with 2% paraformaldehyde for 20 min at 4 °C and permeabilization using 0.5% Triton X-100 in PBS for 10 min. Blocking was performed using 5% animal serum (donkey or goat) in 2% bovine serum albumin in PBS for 1 h at room temperature. Subsequently, the cells were treated with primary antibodies against anti-ER β (1:200; Abcam), anti-ER α (1:200; Invitrogen), anti-p-PI3K (1:200; Affinity Biosciences), and anti-PTEN (1:200; Bioss) in a blocking solution at 4 °C overnight. The cells were incubated with Alexa Fluor™ 594 conjugated goat anti-mouse IgG (A-11005; 1:1000; Invitrogen) or Alexa Fluor® 488 conjugated goat anti-rabbit IgG (ab150077; 1:1000; Abcam). The cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). The cells were then mounted using mounting medium (Dako, Carpinteria, CA, USA) and images were captured using a THUNDER Imager 3D Live Cell & 3D Cell Culture System (Leika Microsystems, Wetzlar, Germany). The mean fluorescence intensity for each channel in the three distinct regions was quantified using ImageJ software (version 1.52a). The fluorescence intensity was expressed relative to that of the control.

4.7. Statistical Analysis

All data are presented as mean $\pm$ standard deviation (SD). Statistical significance between groups was determined using an unpaired Student's *t*-test. One-way analysis of variance (ANOVA), followed by Bonferroni post hoc tests, was conducted to determine significant differences among multiple groups. Statistical analyses were performed using GraphPad Prism software (version 5.0). Statistical significance was set at $p < 0.05$.

Supplementary Materials: The supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms25052466/s1>.

Author Contributions: Conceptualization: M.-J.Y., J.-W.C. and J.-W.S.; data analysis and curation: M.-J.Y., J.-W.C. and J.-W.S.; visualization: M.-J.Y.; investigation: M.-J.Y. and Y.-J.J.; methodology: M.-J.Y., J.-W.C. and J.-W.S.; project administration and supervision: S.-Y.P., J.-W.S. and J.-W.C.; funding acquisition: J.-W.S.; writing—original draft: M.-J.Y.; writing—review and editing: M.-J.Y., J.-W.C. and J.-W.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education (NRF-2020R111A3068208), and was supported by ‘regional innovation mega project’ program through the Korea Innovation Foundation funded by Ministry of Science and ICT (Project Number: 2023-DD-UP-0031).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Acknowledgments: We thank Hanna Kang, Center for University-wide Research Facilities (CURF) at Jeonbuk National University, for use of their super-resolution confocal-laser-scanning microscope.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Valdivia, G.; Alonso-Diez, Á.; Pérez-Alenza, D.; Peña, L. From conventional to precision therapy in canine mammary cancer: A comprehensive review. *Front. Vet. Sci.* **2021**, *8*, 623800. [\[CrossRef\]](#)
- Cassali, G.D.; Jark, P.C.; Gamba, C.; Damasceno, K.A.; Lima, A.E.; Nardi, A.B.d.; Ferreira, E.; Horta, R.S.; Firmo, B.F.; Sueiro, F.A. Consensus Regarding the Diagnosis, Prognosis and Treatment of Canine and Feline Mammary Tumors-2019. *Braz. J. Vet. Pathol.* **2020**, *13*, 555–574. [\[CrossRef\]](#)
- Marconato, L.; Lorenzo, R.M.; Abramo, F.; Ratto, A.; Zini, E. Adjuvant gemcitabine after surgical removal of aggressive malignant mammary tumours in dogs. *Vet. Comp. Oncol.* **2008**, *6*, 90–101. [\[CrossRef\]](#)
- Sorenmo, K.U.; Worley, D.R.; Goldschmidt, M.H. Tumors of the mammary gland. In *Withrow and MacEwen's Small Animal Clinical Oncology*; Elsevier: Amsterdam, The Netherlands, 2013; pp. 538–556.
- Simon, D.; Schoenrock, D.; Baumgärtner, W.; Nolte, I. Postoperative adjuvant treatment of invasive malignant mammary gland tumors in dogs with doxorubicin and docetaxel. *J. Vet. Intern. Med.* **2006**, *20*, 1184–1190. [\[CrossRef\]](#)
- Ceccarelli, I.; Bioletti, L.; Peparini, S.; Solomita, E.; Ricci, C.; Casini, I.; Miceli, E.; Aloisi, A.M. Estrogens and phytoestrogens in body functions. *Neurosci. Biobehav. Rev.* **2022**, *132*, 648–663. [\[CrossRef\]](#)
- Faltas, C.L.; LeBron, K.A.; Holz, M.K. Unconventional Estrogen Signaling in Health and Disease. *Endocrinology* **2020**, *161*, bqaa030. [\[CrossRef\]](#)
- Dall, G.V.; Hawthorne, S.; Seyed-Razavi, Y.; Vieusseux, J.; Wu, W.; Gustafsson, J.-A.; Byrne, D.; Murphy, L.; Risbridger, G.P.; Britt, K.L. Estrogen receptor subtypes dictate the proliferative nature of the mammary gland. *J. Endocrinol.* **2018**, *237*, 323–336. [\[CrossRef\]](#) [\[PubMed\]](#)
- Dyson, M.T.; Bulun, S.E. Cutting SRC-1 down to size in endometriosis. *Nat. Med.* **2012**, *18*, 1016–1018. [\[CrossRef\]](#)
- Torres, C.G.; Iturriaga, M.P.; Cruz, P. Hormonal carcinogenesis in canine mammary cancer: Molecular mechanisms of estradiol involved in malignant progression. *Animals* **2021**, *11*, 608. [\[CrossRef\]](#) [\[PubMed\]](#)
- Tewari, D.; Patni, P.; Bishayee, A.; Sah, A.N.; Bishayee, A. Natural products targeting the PI3K-Akt-mTOR signaling pathway in cancer: A novel therapeutic strategy. In *Seminars in Cancer Biology*; Academic Press: Cambridge, MA, USA, 2022; pp. 1–17.
- Engelman, J.A.; Luo, J.; Cantley, L.C. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. *Nat. Rev. Genet.* **2006**, *7*, 606–619. [\[CrossRef\]](#)
- Castel, P.; Toska, E.; Engelman, J.A.; Scaltriti, M. The present and future of PI3K inhibitors for cancer therapy. *Nat. Cancer* **2021**, *2*, 587–597. [\[CrossRef\]](#)

14. Arendt, M.L.; Sakthikumar, S.; Melin, M.; Elvers, I.; Rivera, P.; Larsen, M.; Saellström, S.; Lingaas, F.; Rönnerberg, H.; Lindblad-Toh, K. PIK3CA is recurrently mutated in canine mammary tumors, similarly to in human mammary neoplasia. *Sci. Rep.* **2023**, *13*, 632. [\[CrossRef\]](#)
15. Han, R.; Gu, S.; Zhang, Y.; Luo, A.; Jing, X.; Zhao, L.; Zhao, X.; Zhang, L. Estrogen promotes progression of hormone-dependent breast cancer through CCL2-CCR2 axis by upregulation of Twist via PI3K/AKT/NF- κ B signaling. *Sci. Rep.* **2018**, *8*, 9575. [\[CrossRef\]](#)
16. Harris, H.A. Preclinical characterization of selective estrogen receptor beta agonists: New insights into their therapeutic potential. In *Tissue-Specific Estrogen Action*; Ernst Schering Foundation Symposium Proceedings; Springer: Berlin/Heidelberg, Germany, 2006; pp. 149–161. [\[CrossRef\]](#)
17. Jiang, Q.; Li, W.; Zhu, X.; Yu, L.; Lu, Z.; Liu, Y.; Ma, B.; Cheng, L. Estrogen receptor β alleviates inflammatory lesions in a rat model of inflammatory bowel disease via down-regulating P2X7R expression in macrophages. *Int. J. Biochem. Cell Biol.* **2021**, *139*, 106068. [\[CrossRef\]](#)
18. Wang, X.L.; Cheng, W.J.; Dai, H.H.; Han, S.P.; Fu, S.L. ERB-041, a selective ER beta agonist, inhibits iNOS production in LPS-activated peritoneal macrophages of endometriosis via suppression of NF-kappaB activation. *Mol. Immunol.* **2009**, *46*, 2413–2418. [\[CrossRef\]](#)
19. Hinsche, O.; Girgert, R.; Emons, G.; Gründker, C. Estrogen receptor β selective agonists reduce invasiveness of triple-negative breast cancer cells. *Int. J. Oncol.* **2015**, *46*, 878–884. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Chan, K.K.L.; Siu, M.K.Y.; Jiang, Y.X.; Wang, J.J.; Leung, T.H.Y.; Ngan, H.Y.S. Estrogen receptor modulators genistein, daidzein and ERB-041 inhibit cell migration, invasion, proliferation and sphere formation via modulation of FAK and PI3K/AKT signaling in ovarian cancer. *Cancer Cell Int.* **2018**, *18*, 65. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Křížová, L.; Dadáková, K.; Kašparovská, J.; Kašparovský, T. Isoflavones. *Molecules* **2019**, *24*, 1076. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Jiang, H.; Fan, J.; Cheng, L.; Hu, P.; Liu, R. The anticancer activity of genistein is increased in estrogen receptor beta 1-positive breast cancer cells. *OncoTargets Ther.* **2018**, *11*, 8153–8163. [\[CrossRef\]](#) [\[PubMed\]](#)
23. Sharifi-Rad, J.; Qaispe, C.; Imran, M.; Rauf, A.; Nadeem, M.; Gondal, T.A.; Ahmad, B.; Atif, M.; Mubarak, M.S.; Sytar, O. Genistein: An integrative overview of its mode of action, pharmacological properties, and health benefits. *Oxid. Med. Cell. Longev.* **2021**, *2021*, 3268136. [\[CrossRef\]](#) [\[PubMed\]](#)
24. Barkhem, T.; Carlsson, B.; Nilsson, Y.; Enmark, E.; Gustafsson, J.-Å.; Nilsson, S. Differential response of estrogen receptor α and estrogen receptor β to partial estrogen agonists/antagonists. *Mol. Pharmacol.* **1998**, *54*, 105–112. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Farkas, S.; Szabó, A.; Hegyi, A.E.; Török, B.; Fazekas, C.L.; Ernsts, D.; Kovács, T.; Zelena, D. Estradiol and estrogen-like alternative therapies in use: The importance of the selective and non-classical actions. *Biomedicines* **2022**, *10*, 861. [\[CrossRef\]](#) [\[PubMed\]](#)
26. Pike, A.C.W.; Brzozowski, A.M.; Hubbard, R.E.; Bonn, T.; Thorsell, A.G.; Engström, O.; Ljunggren, J.; Gustafsson, J.-Å.; Carlquist, M. Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **1999**, *18*, 4608–4618. [\[CrossRef\]](#) [\[PubMed\]](#)
27. Chang, E.C.; Frasier, J.; Komm, B.; Katzenellenbogen, B.S. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology* **2006**, *147*, 4831–4842. [\[CrossRef\]](#) [\[PubMed\]](#)
28. Sorenmo, K.U.; Durham, A.C.; Radaelli, E.; Kristiansen, V.; Peña, L.; Goldschmidt, M.H.; Stefanovski, D. The estrogen effect; clinical and histopathological evidence of dichotomous influences in dogs with spontaneous mammary carcinomas. *PLoS ONE* **2019**, *14*, e0224504. [\[CrossRef\]](#)
29. Abdelmegeed, S.M.; Mohammed, S. Canine mammary tumors as a model for human disease. *Oncol. Lett.* **2018**, *15*, 8195–8205. [\[CrossRef\]](#)
30. Leitman, D.C.; Paruthiyil, S.; Vivar, O.I.; Saunier, E.F.; Herber, C.B.; Cohen, I.; Tagliaferri, M.; Speed, T.P. Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. *Curr. Opin. Pharmacol.* **2010**, *10*, 629–636. [\[CrossRef\]](#)
31. Fatemizadeh, M.; Tafvizi, F.; Shamsi, F.; Amiri, S.; Farajzadeh, A.; Akbarzadeh, I. Apoptosis Induction, Cell Cycle Arrest and Anti-Cancer Potential of Tamoxifen-Curcumin Loaded Niosomes Against MCF-7 Cancer Cells. *Iran. J. Pathol.* **2022**, *17*, 183–190. [\[CrossRef\]](#)
32. Kouroshnia, A.; Zeinali, S.; Irani, S.; Sadeghi, A. Induction of apoptosis and cell cycle arrest in colorectal cancer cells by novel anticancer metabolites of *Streptomyces* sp. 801. *Cancer Cell Int.* **2022**, *22*, 235. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Tophkhane, C.; Yang, S.; Bales, W.; Archer, L.; Osunkoya, A.; Thor, A.D.; Yang, X. Bcl-2 overexpression sensitizes MCF-7 cells to genistein by multiple mechanisms. *Int. J. Oncol.* **2007**, *31*, 867–874. [\[CrossRef\]](#)
34. Chaudhary, S.C.; Singh, T.; Talwelkar, S.S.; Srivastava, R.K.; Arumugam, A.; Weng, Z.; Elmets, C.A.; Afaq, F.; Kopelovich, L.; Athar, M. Erb-041, an estrogen receptor- β agonist, inhibits skin photocarcinogenesis in SKH-1 hairless mice by downregulating the WNT signaling pathway. *Cancer Prev. Res.* **2014**, *7*, 186–198. [\[CrossRef\]](#)
35. Park, C.; Cha, H.-J.; Lee, H.; Hwang-Bo, H.; Ji, S.Y.; Kim, M.Y.; Hong, S.H.; Jeong, J.-W.; Han, M.H.; Choi, S.H.; et al. Induction of G2/M Cell Cycle Arrest and Apoptosis by Genistein in Human Bladder Cancer T24 Cells through Inhibition of the ROS-Dependent PI3k/Akt Signal Transduction Pathway. *Antioxidants* **2019**, *8*, 327. [\[CrossRef\]](#)
36. Aprilliantina, Y.S.; Novita, H.D.; Sadono, E.G.; Aldina, R. Protective Effect of Genistein on Cyclin D1 Expression in Malignant Ocular Melanoma Cells. *Med. Arch.* **2021**, *75*, 180–183. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Qiu, G.H.; Xie, X.; Xu, F.; Shi, X.; Wang, Y.; Deng, L. Distinctive pharmacological differences between liver cancer cell lines HepG2 and Hep3B. *Cytotechnology* **2015**, *67*, 1–12. [\[CrossRef\]](#) [\[PubMed\]](#)

38. Hwang, K.-A.; Park, M.-A.; Kang, N.-H.; Yi, B.-R.; Hyun, S.-H.; Jeung, E.-B.; Choi, K.-C. Anticancer effect of genistein on BG-1 ovarian cancer growth induced by 17 β -estradiol or bisphenol A via the suppression of the crosstalk between estrogen receptor alpha and insulin-like growth factor-1 receptor signaling pathways. *Toxicol. Appl. Pharmacol.* **2013**, *272*, 637–646. [[CrossRef](#)] [[PubMed](#)]
39. Chimento, A.; De Luca, A.; Avena, P.; De Amicis, F.; Casaburi, I.; Sirianni, R.; Pezzi, V. Estrogen receptors-mediated apoptosis in hormone-dependent cancers. *Int. J. Mol. Sci.* **2022**, *23*, 1242. [[CrossRef](#)] [[PubMed](#)]
40. Lu, W.; Katzenellenbogen, B.S. Estrogen Receptor- β modulation of the ER α -p53 loop regulating gene expression, proliferation, and apoptosis in breast cancer. *Horm. Cancer* **2017**, *8*, 230–242. [[CrossRef](#)]
41. Lee, Y.R.; Park, J.; Yu, H.N.; Kim, J.S.; Youn, H.J.; Jung, S.H. Up-regulation of PI3K/Akt signaling by 17 β -estradiol through activation of estrogen receptor-alpha, but not estrogen receptor-beta, and stimulates cell growth in breast cancer cells. *Biochem. Biophys. Res. Commun.* **2005**, *336*, 1221–1226. [[CrossRef](#)]
42. Noh, E.-M.; Lee, Y.-R.; Chay, K.-O.; Chung, E.-Y.; Jung, S.H.; Kim, J.-S.; Youn, H.J. Estrogen receptor α induces down-regulation of PTEN through PI3-kinase activation in breast cancer cells. *Mol. Med. Rep.* **2011**, *4*, 215–219. [[CrossRef](#)] [[PubMed](#)]
43. Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S.I.; Puc, J.; Miliarensis, C.; Rodgers, L.; McCombie, R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **1997**, *275*, 1943–1947. [[CrossRef](#)]
44. Maehama, T.; Dixon, J.E. The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3, 4, 5-trisphosphate. *J. Biol. Chem.* **1998**, *273*, 13375–13378. [[CrossRef](#)] [[PubMed](#)]
45. Mukherjee, R.; Vanaja, K.G.; Boyer, J.A.; Gadal, S.; Solomon, H.; Chandarlapaty, S.; Levchenko, A.; Rosen, N. Regulation of PTEN translation by PI3K signaling maintains pathway homeostasis. *Mol. Cell* **2021**, *81*, 708–723.e705. [[CrossRef](#)] [[PubMed](#)]
46. Treeck, O.; Schüler-Toprak, S.; Skrzypczak, M.; Weber, F.; Ortmann, O. Knockdown of PTEN decreases expression of estrogen receptor β and tamoxifen sensitivity of human breast cancer cells. *Steroids* **2020**, *153*, 108521. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.