Research Article

Concanavalin-A Shows Synergistic Cytotoxicity with Tamoxifen via Inducing Apoptosis in Estrogen Receptor-Positive Breast Cancer: *In Vitro* and Molecular Docking Studies

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Article Info

Abstract

Background: Tamoxifen (TAM) is the main treatment of estrogen receptor (ER)-positive breast cancer, however; its adverse effects and development of resistance hinder its use. Concanavalin A (Con A) is a mannose/glucose-binding lectin that has been reported to induce apoptosis in a variety of cell lines.

Methods: The effects of Con A on TAM-induced cell death in ERα positive cell line (MCF-7) were elucidated to identify the potential underlying molecular mechanisms using in silico (molecular docking) and in vitro (cytotoxicity assay, cell cycle analysis, annexin V-FITC apoptosis assay, and reverse transcription and quantitative real time-PCR) techniques as well.

Results: The results demonstrated that combined treatment with Con A and TAM reduced the expression of ERα, which showed clear synergistic effects on inhibiting the cell viability of MCF-7 cells. Interestingly, the combined treatment induces G1 phase arrest and reduces cyclin D1 activity while increasing apoptosis and autophagy as indicated by decreasing the expression level of anti-apoptosis gene BCl-2 and increased apoptosis/autophagic gene *BNIP3*. Molecular docking was conducted to evaluate the binding affinity of Con A towards ERα, and it revealed its potential activity as an ERα antagonist. Our data further indicated that Con A administration increased the drug reduction index of TAM.

Conclusion: Overall, our findings suggested that Con A could be used as an adjuvant agent with TAM to improve its effectiveness as an anticancer agent.

Introduction

Breast cancer (BC) is one of the most common malignant diseases and the leading cause of cancer-related death for women (2.09 million cases) worldwide.[1] The disease occurs mostly in women, but men may get it as well.[2] BC can invade the surrounding tissues or spread (metastasize) through the body to distant areas. BC is a heterogeneous disease comprised of several molecular subtypes, among which estrogen receptor-positive (ER[+], i.e., expressing estrogen receptors endogenously) is the most prominent type (about 75% of all patients). ERα is a transcription factor that regulates gene expression critical genes including cyclin D1, Bcl-2, and VEGF (Vascular endothelial growth factor), which play a significant role in the cell cycle, cell survival, and angiogenesis.[3]

Four major classes of pharmacological agents, referred to as endocrine therapy for ER[+] breast cancer, are now available in the clinic. These include selective estrogen receptor modulators (SERMs), selective estrogen receptor down regulators (SERDs), aromatase inhibitors (AIs), and luteinizing hormone-releasing hormone analogs (LHRH analogs).[4]

Tamoxifen (TAM) is a selective estrogen receptor modulator that is currently considered the first-line treatment for ER[+] BC in both pre-and post-menopausal women.[5,6] TAM has also been introduced to be efficient in the prevention and treatment of ER+ breast tumors. It works as a selective estrogen receptor modulator (SERM) which combines with estrogen receptors in BC cells and stops their growth and multiplication by depriving them of the estrogen hormone.[7] Besides, the toxicities of TAM, such as thromboembolic events and endometrial cancer, constitute a clinically significant issue, especially for their prevention. Moreover, nearly half of ER[+] BC patients do not respond to TAM. However, the positive response is usually shortened as most patients develop TAM resistance

within 2-5 years.[8] Therefore, new strategies are needed to enhance the efficacy of TAM in the prevention and/or treatment of ER⁺ BC. One such strategy is to examine the efficacy of TAM in lower doses, along with another apoptotic compound that their combination is required to be related to lower toxicity.[9]

Lectins from animal and plant origin are a family of proteins found in almost all foods, especially legumes and grains that induce apoptosis and autophagy of cancer cells and therefore possess the potential for the development of selective anticancer drugs.[10] Concanavalin A (Con A) is a legume lectin that was long-studied and reported to have anticancer effects against diverse human cancers through targeting programmed cell death (PCD).[11,12]

In continuation to our previous interesting work,[13-20] we thought to investigate the potential of Con A to enhance the antitumor efficacy and reduce the adverse effects of TAM, and to deduce its molecular mechanism of action by *in vitro* and molecular docking studies.

Materials and Methods
Tamoxifen citrate salt, Con A, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), Dimethyl sulfoxide (DMSO), Annexin-V Staining Kit, RNAs-A, Propidium Iodide (PI), and triton x-100 were purchased from Sigma (United States). Dulbecco's Modified Eagle's Medium (DMEM), streptomycin, and Fetal Bovine Serum (FBS) was from GIBCO (Invitrogen Co., CA, USA)

In vitro studies
Cell culture and treatment
The *in vitro* cytotoxic activity of Con A and TAM against ER⁺ MCF-7 breast cancer cell line was measured using MTT assay as described in a previous study,[21] with some modifications. Briefly, MCF-7 cells were cultured at a concentration of 4×10^4 cells/cm² in DMEM medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2, 95% air, and complete humidity. Seeding density was determined manually by a hemocytometer using 0.4% trypan blue. At 40-50% confluency (48 hours post-seeding), the cultivated cells were treated with either TAM or Con A alone at different concentrations (from 0.39 μM to 100μM)- which is a commonly used concentration range used to study the efficacy of the tested compound at different concentrations- for 24 h treated, and cells were left to grow for another 24 hours.

Cytotoxicity assay
Once cultured MCF-7 cells reached ~90% confluency, 50 μl of MTT (1mg/ml in PBS) was added to the culture medium, and cells were incubated at 37°C with 5% CO_2 for 3h. Next, the cells were washed and reincubated for an additional 5 min with 200 μl of DMSO. The optical density (OD) of the wells was determined using a plate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm. The percentage of cell viability was calculated by (OD treated well – OD blank)/ (OD untreated control – OD Blank) x 100. The percent of cytotoxicity equals 100 – cell viability %. The MTT assays were performed at least three times for each concentration to determine the half-maximal inhibitory concentration (IC_{50}) values of TAM and Con A.

Combination index analysis
The efficacy of the drug combination was tested according to the fixed ratio or ray design of the two drugs.[22] In this study, efficacy was measured by percent inhibition of cancer cell proliferation for the two drugs individually, and in combination for a series of different concentrations (from 0.39 μM to 100μM) for 24 h. In the combination treatment, the concentration ratio of the two drugs is fixed. Dose-response data are the input data used to calculate the combination index (CI) by CompuSyn software version 1.0 (Ting Chao Chou and Nick Martin, Paramus, NJ, USA), which is based on the Chou–Talalay method to determine the nature of the interaction between the two-/three-agents.[23] Based on CI values, the extent of synergism/antagonism may be determined.[24] Whereas, CI < 1 refers to synergism; CI = 1 refers to an additive effect, and CI > 1 refers to antagonism. Besides, the drug reduction index (DRI) values above 1 indicate a preferred reduction in the dose of the drug combination compared to monotherapy.

Cell cycle analysis
Flow cytometry was used to detect both cell cycle phases and apoptosis in untreated or treated MCF-7 cell cutlers as previously discussed.[25] Simply, MCF-7 BC cells were seeded at 8×10^4, supplied with 5% CO_2, and incubated at 37°C overnight. The IC_{50} of the three treatments (TAM, ConA, and combination) were applied to treat MCF-7 cells to record their effect on the cell population compared to the media (control). 48 h later, centrifugation of cell pellets for 5 min at 300g was done. After, cell pellets were fixed in 70% ethanol on ice for 15 min to be used for cell cycle analysis. The aforementioned pellets were treated with the staining solution of propidium iodide (PI) for 1 h at room temperature. Finally, the stained cells were preserved at 4°C in dark till their analysis through flow cytometry.

Annexin V-FITC apoptosis assay
FITC Annexin-V/PI kit was used to detect apoptosis according to the manufacture's protocol. Briefly, treated cells were washed using PBS, then a binding buffer (200 mL) was added containing Annexin V-FITC (5 mL) and PI solution (10 mL) for staining. After keeping at 25 ℃ for 15 min., the flow cytometry analysis was applied to detect the apoptotic cells. This procedure was repeated triplicate and fluorescence-activated cell sorting (FACS) was used to analyze the samples.[21]

Reverse transcription and quantitative PCR
The combining effects of Con-A on TAM mRNA expression for the target genes (*ERα, Cyclin-D1, Bcl-2,* and *BNIP3*)

Table 1. Primers sequences of the target genes (ER$_\alpha$, Cyclin-D1, Bcl-2, and BNIB3) and the housekeeping gene (GAPDH).

Gene	Primer sequence
Erα	F 5'- GCTTACTGACCAACCTGGCAGA -3' R 5'- GCTTACTGACCAACCTGGCAGA -3'
Bcl-2	F 5'- GACTTCGCCGAGATGTCCAG -3' R 5'- CAGGTGCCGGTTCAGGTACT -3'
Cyclin D1	F 5'-AGACCTGCGCGCCCTCGGTG-3', R 5'-GTAGTAGGACAGGAAGTTGTTC-3'.
BNIP3	F 5'-CCACAA AAA GCAGATGCT CA-3' R 5'-AAGAGG CGCTTT TTCACAAT-3'
GAPDH	F 5'- GGCAAATTCAACGGCACAGT -3' R 5'- AGATGGTGATGGGCTTCCC -3'

were recorded using quantitative PCR. The housekeeping gene (*GAPDH*) in MCF-7 cells was done. Moreover, the primer sequences for the genes described in our study are depicted in Table 1. Herein, RNA from MCF-7 BC cells was obtained by many treatments for 48 h. The procedure was run at 95 ºC/30s, then 40 cycles of 95 ºC/5s, and 60 ºC/30s. 2$^{-\Delta\Delta Ct}$ method[26] was used to analyze the obtained data as the average from the triplicate measurements.

Docking studies
Protein-protein docking for Con A and ERα was performed, and ERα was downloaded and prepared with its co-crystallized inhibitor, 4-Hydroxytamoxifen (4-OHT) molecules. Docking studies using MOE 2019 drug design suite[27] were done to evaluate the binding affinity of lectin towards ERα and confirm its inhibiting activity in combination with the co-crystallized OHT molecules.

Preparation of target proteins
The X-ray structure of the lectin (Con A) was obtained from the Protein Data Bank in Europe (https://www.ebi.ac.uk/pdbe/, PDBe code 1jbc) while that of ERα was extracted from (https://www.rcsb.org/, PDB code 2JF9) which was found to be composed of three subunits (namely A, B, and C) and each subunit containing a molecule of its co-crystallized inhibitor (4-OHT).[28] The two proteins were prepared for docking studies using Quickprep where automatic correction was applied to check for any errors in the connections of the atoms and the type, hydrogen atoms with their standard 3D geometry were added, and all atoms were made free during minimization.

Protein-protein docking
The protocol of docking of the aforementioned proteins was applied, where the prepared ERα protein was identified as the receptor, the lectin Con A protein was identified as the ligand, and the docking was started as a protein-protein docking process using hydrophobic patch potential. The preplacement poses were (10000), the placement poses were (1000), and the refinement poses were (100). At the end of the docking process, the resulted 100 poses were carefully studied, and the best one with the best protein-protein interactions was selected and further studied for energy calculations.

Statistical analysis
Mean ± standard error of the mean (SEM) was applied to express the experimental results. Moreover, one-way ANOVA was used to analyze the obtained data, and a significant difference was considered by a p-value < 0.05.

Results and Discussion
In vitro studies
TAM is the first-line treatment of ER⁺ breast tumors that inhibit cancer cell proliferation by antagonizing the transcription factor estrogen receptor. Unfortunately; 30–50 % of females with ER⁺ BC show primary or secondary resistance to TAM. *De novo* or intrinsic resistance to TAM appears in many cases resulting in tumor recurrence, progression, and metastasis.[29] To overcome this critical problem, we tested the possibility of enhancing the efficacy of low doses of TAM by combining it with Con A, which has been demonstrated to have antiapoptotic and anticancer activities.

Con A enhances the cell proliferation inhibitory effects of TAM
MTT assay was performed to describe the antiproliferative effect of the combination of Con A and TAM, cell viability assays were performed in the ER+ MCF-7 BC cell line. Our data revealed that MCF-7 cells when incubated with different concentrations of Con A and TAM for 24 h, resulted in a more potent cellular proliferation inhibitory activity than TAM alone as indicated by the MTT reduction assay (Figure 1A). As the calculated IC$_{50}$ of Con A was 7.55 µM, and of TAM was 2.75 µM, while the combined Con A and TAM drug showed IC$_{50}$ of 0.9 µM, which indicates that it is more cytotoxic on cells than Con A or TAM alone (Figure 1B). These results are similar partly to Shi *et al.*[12] study, who reported that Con A-induced dose- and time-dependent cell death in MCF-7. Those authors reported that the 15 µg/mL of Con A-induced inhibitory rate of MCF-7 cells reached nearly 50% (IC$_{50}$). The current data similarly indicated that Con A has a great cytotoxic effect on MCF-7 cells that may potentiate the antiproliferative effects of TAM.

To further describe and quantify the combination effects for two-drug either synergistic, antagonistic or additive; MCF-7 cells were exposed to Con A, TAM, or in combination (at a fixed ratio). The combination index-affected fraction (CI-Fa) curve clarified that the values of the CI were < 1 at low and moderate fa values (IC$_{10}$–IC$_{70}$) which confirms the synergistic effect of Con A with TAM on MCF-7 cells (Figure 1C). The dose reduction index (DRI) represents a multiple of the dose reduction of the tested toxin combinations compared to each toxin at the same inhibition rate. The DRI curve showed that both Con A and TAM had a DRI value > 1 indicating an inhibitory effect. So, their combined treatment was better than each drug alone, suggesting that Con A could be advantageous to decrease the side effects of TAM in the combination therapy. Herein, the DRI for Con A is superior to that of

TAM suggesting that its therapeutic combination with the latter could result in a TAM dose reduction and consequently reducing its side effects as well (Figure 1D).

Con A synergizes with TAM in inducing G1 growth arrest

The mainstream anticancer treatment induces cytotoxic effects and DNA damage leading to cell cycle arrest at G1, S, G2, and consequently preventing the replication of the damaged DNA which if not repaired, may cause tumorigenesis or apoptosis.[30] Therefore, to investigate whether the aforementioned cytotoxic effects of the combined therapy might affect the cell cycle, MCF-7 cells treated with different concentrations of Con A and or TAM were analyzed using flow cytometry. Flow cytometry is a technique that measures many physical and biological characteristics that include a particle's size, internal complexity, or relative granularity and DNA content using the fluorescence intensity of certain DNA-intercalating fluorescent dyes such as Propidium Iodide.[31] In the present study, the cell cycle assay of MCF-7 cells following different treatments showed altered cell cycle patterns compared to untreated controls (Figure 2).

The percentages of cells in G0/G1 were increased in all treatments, especially in the combined treatment of Con A/TAM, which showed a significant (P<0.05) increase compared with untreated MCF-7 cells. Treatment of cells with Con A and with TAM decreased the percent of cells in the S phase but not significantly compared to that of the control group (p > 0.05). Cells treated with Con A and with TAM showed a significant (p < 0.05) decrease in the G2/M phase (3.53% and 4.12% respectively) in comparison to

that of the control group (12.79%). Besides, the combined treatment, Con A/TAM showed significantly (p < 0.05) decreased G2/M compared with control untreated MCF-7 cells (Figures 2A, B). These results indicate that Con A synergistically with TAM could inhibit the growth of ER+ MCF-7 cancer cells by arresting the cell cycle at G1.

Con A enhances the apoptotic effects of TAM

Besides cell cycle blockage at the G0/G1 phase of cell progression, another mechanism called apoptosis may be implicated in the cytotoxic effects of TAM and Con A on MCF-7 cell lines. Previous studies indicated that apoptosis is related to cell cycle arrest, whereas the compounds which can induce cell cycle arrest and apoptosis are considered to have anticancer potential.[32,33] The rates of cell apoptosis were evaluated in the present study by two different methods, the pre-G1 phase of the DNA-cell cycle and by Annexin-V/PI dual staining assays using flow cytometry. The percentages of apoptotic cells in pre-G1 were found to increase significantly from 1.17 ± 0.39 % for the control group to 29.91 ± 1.89 %, 33.4 ± 1.89 %, 44.9 ± 1.89 %, following exposure to Con A, TAM, and their combination, respectively (Figure 2C).

Also, Annexin V apoptosis assay revealed that, while most cells in the control group were negative to both Annexin V-FITC and PI stains, cells treated with Con-A, TAM or their combination showed significant increases in the ratios of both early and late apoptosis cells, while the percentage of the viable cells was decreased (Figure 3A). Meanwhile, the necrotic cell population was also slightly increased (Figure 3B). Our study has shown that treatment
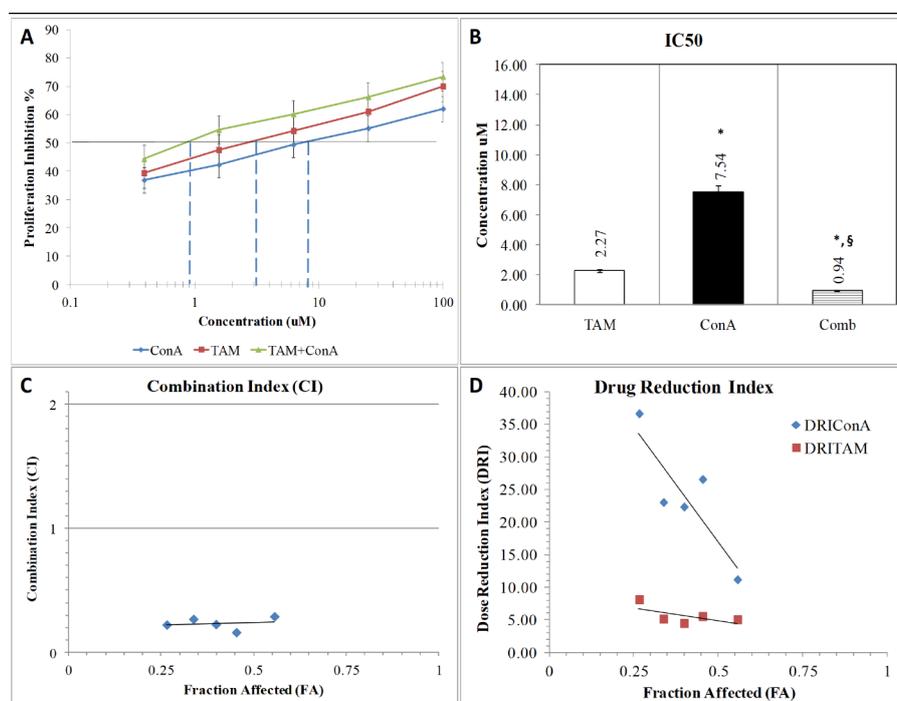

Figure 1. Effect of TAM and/or Con A treatment on the growth of MCF-7 cells. **A)** Inhibitory effect on MCF-7 proliferation rate. **B)** IC$_{50}$ of Con A and/or TAM. **C)** Synergistic effect indicated by CI < 1. **D)** DRI for Con A and TAM per Fa that represents the fraction of cell proliferation inhibition (where 0% inhibition Fa = 0% and 100% inhibition Fa = 1). Three independent experimental data were summarized as mean ± SD. *, § Significantly different from TAM-treated or Con A treated cells at p-value < 0.05.
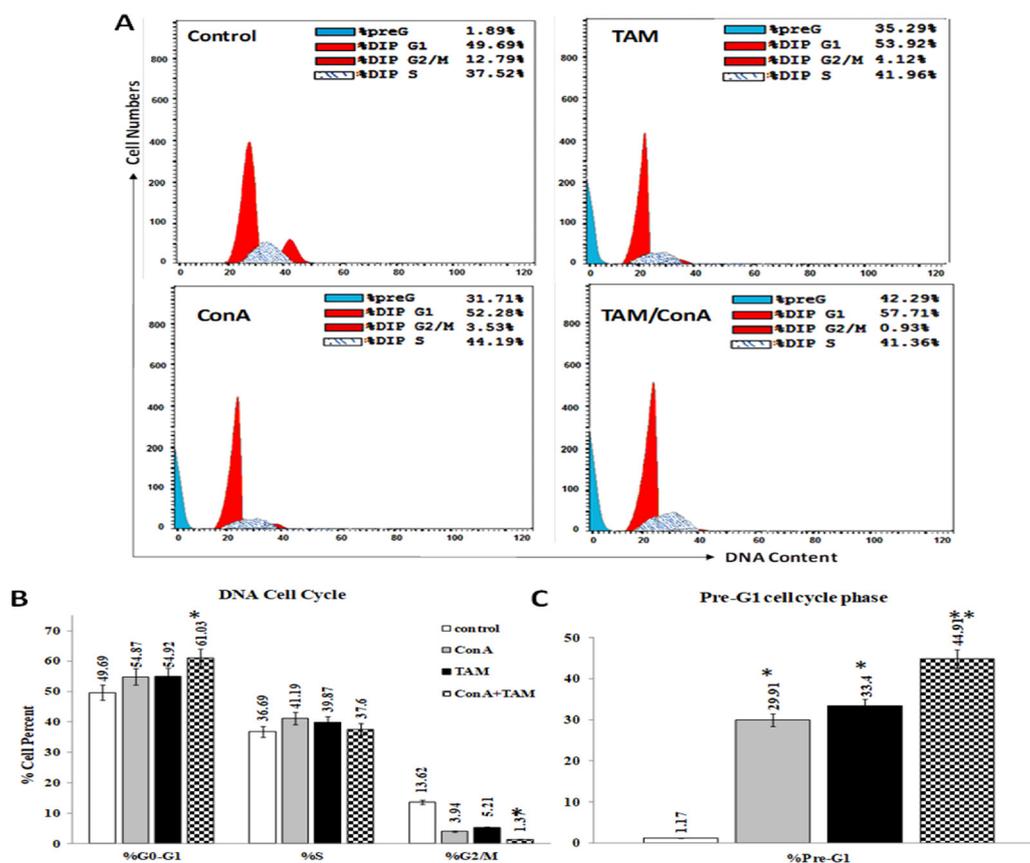
Figure 2. Impact of Con A and/or TAM on the cell cycle phases of MCF-7 cells. **A)** Representative DNA-cytograms of the different treatments as determined by flow cytometry. **B)** The cell cycle phases. **C)** Apoptotic cells as determined by the pre-G1 phase of the cell cycle. The values indicate the mean±SD of the three experiments. *, ** Significantly different compared to the untreated control cells at p-value < 0.05, and < 0.01 respectively.

of ER⁺ MCF-7 cells with Con-A induced inhibition of cell proliferation by increased cell apoptosis, and augmented the antiproliferative properties of TAM, suggesting that Con-A could be a promising drug for cancer treatment. These data are in line with previous studies that reported the ability of Con-A to induce apoptosis in certain types of tumors.[10,11]

Molecular mechanism of the G1 arrest and apoptosis induced by Con A and TAM

ERα is a key transcription factor in breast cancer that participates in a variety of different signaling pathways. It promotes the expression of the oncogenic protein cyclin D1 that regulates cell proliferation through its regulation of G1-S cell cycle progression.[34] Also, it was reported that downregulation of ERα accompanied by retardation of the S-phase, and reducing the expression of cyclin D1, consequently leading to G1 arrest.[35] In agreement with the abovementioned data, we found that the inhibition of MCF-7 cell growth was accompanied by the downregulation of ERα mRNA that was treated with Con A (0.73- fold), TAM (0.55-fold), and a combination of Con A and TAM (0.37-fold) (Figure 4A).

Besides, the levels of Cyclin D1 mRNA were downregulated significantly posttreatment with combined Con A and TAM more than either treatment alone (Figure 4B). These data confirm the previous finding that blocking ERα induces cell cycle arrest at G1 through downregulating cyclin D1.[36] Moreover, Katary *et al.*[37] have reported that mechanisms linking the reduction of the oncogenic protein cyclin D1 correlate with a reduction in the cellular component of anti-apoptotic molecules NF-κB, Bcl-2 proteins leading to induction of apoptosis. Thereby, emphasizing the role of Bcl-2 in inducing apoptosis in ER⁺ MCF-7 cells. Bcl-2 is an anti-apoptotic protein that inhibits apoptosis either by sequestering the apoptosome assembly of caspases or by preventing the release of cytochrome c and AIF (apoptosis-inducing factor).[38] In the present study, it was found that *Bcl-2* gene expression was decreased (0.3-fold) compared to the control following treatment of MCF-7 BC cells with Con A (Figure 4C). Furthermore, the cells treated with combined Con A/TAM showed an enhanced reduction in Bcl-2 expression than either monotherapy alone. Data from these experiments suggest that Con A may act as an apoptosis inducer that works synergically with TAM in decreasing the expression of Bcl-2 in ER⁺ BC cells with Con A (Figure 4C). Furthermore, the cells
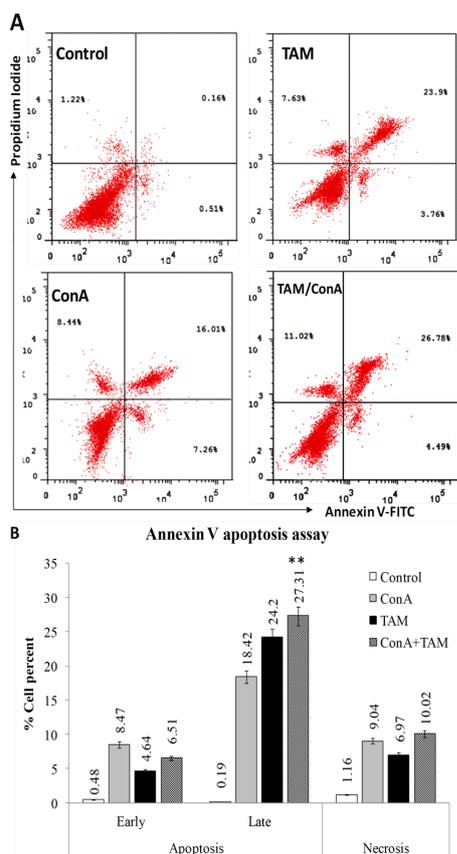
Figure 3. The effects of Con A, TAM, or combination Con A/TAM on the rate of apoptosis in MCF-7 cells. **(A)** Representative cytogram of cells stained with AnnexinV-FITC/PI. **(B)** Distribution of apoptotic cells after the different treatments. The values represent the mean ± SD (n = 2). * and **, significantly different compared to the untreated control cells at p-value < 0.05, and < 0.01, respectively.

treated with combined Con A/TAM showed an enhanced reduction in Bcl-2 expression than either monotherapy alone. Data from these experiments suggest that Con A may act as an apoptosis inducer that works synergically with TAM in decreasing the expression of Bcl-2 in ER⁺ human BC.

Con A enhances the autophagic effects of TAM

On the other hand, BNIP3 (BCl-2/adenovirus E1B 19 kDa interacting protein) is a pro-apoptosis protein regulated by the methylation status of its promoter, which has been implicated in inducing necrosis, autophagy, and/ or apoptosis.[39] It has been shown that BNIP3 expression is increased in hypoxic regions of breast tumors.[40] Autophagy refers to an evolutionary conversed process for maintaining homeostasis and eliminating harmful cells.[32] It was reported that Con A can inhibit cancer cell growth through binding to mannose glycoproteins and is proposed to make an autophagic pathway in hepatoma ML-1 ce37. This is indicated by the formation of LC3-II which is an autophagy marker, and induction of BNIP3 which is a protein associated with autophagy; suggesting Con A can induce mitochondrial apoptosis and BNIP3-mediated mitochondrial autophagy, and therefore causing cancer cell death.[10] In the present work (Figure 4D), treatment with Con A and TAM drugs stimulated the overexpression of BNIP3 protein (3.48-fold), TAM (4.32-fold), and combination with both (7.53-fold) compared to the control cells (1-fold), suggesting that combined treatment of MCF-7 cells with Con A and TAM drug stimulate BNIP3-induced autophagic cell death.

Molecular docking studies

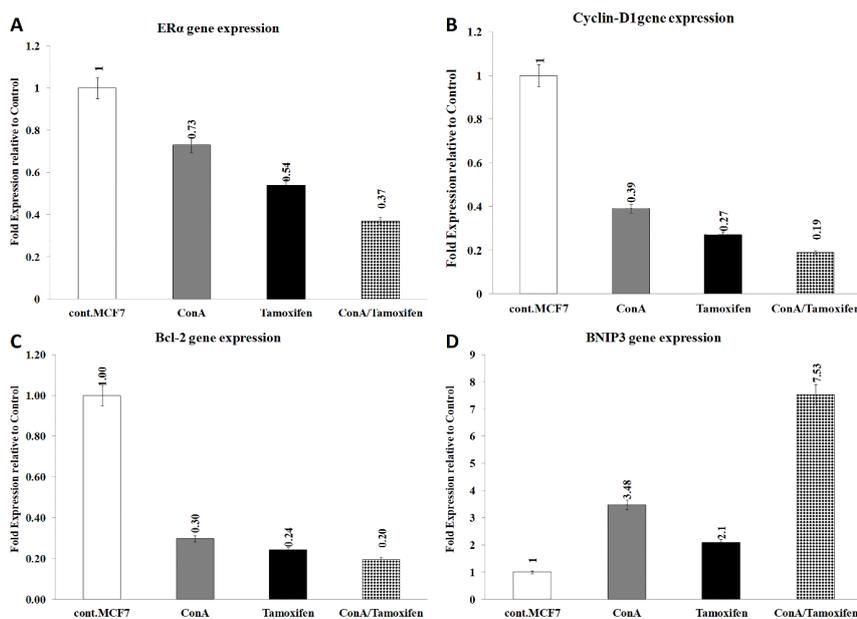Molecular docking is an approach used to model the

Figure 4. The effect of treating ER⁺ MCF-7 cell lines with Con A and/or TAM on the relative expression of *ERα* **(A)**, *Cyclin D1* **(B)**, *BCl-2* **(C)**, and *BNIP3* **(D)**. The values represent the mean ± SD (n = 2). *,** Significantly different from corresponding untreated control cells at p-value < 0.05 and < 0.01, respectively.
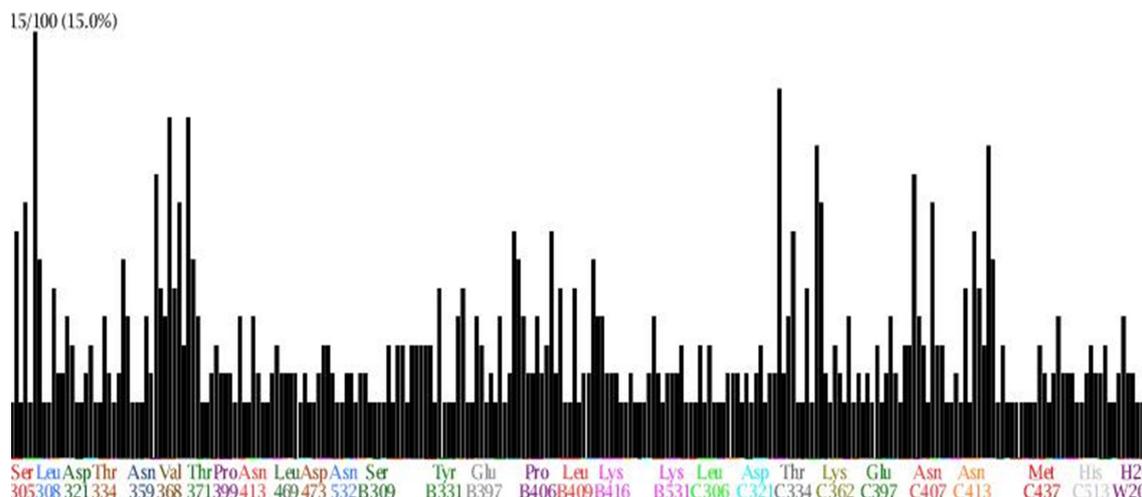
Figure 5. Protein-ligand interaction fingerprints (PLIF) for ERα-lectin interactions.

interaction between certain molecules and a protein at the cellular level that allows the researcher to study the interactions of small molecules inside the binding pocket of target receptors and to explain the predicted mechanisms of action as well.[41-44] Therefore, molecular docking analysis was conducted to investigate whether the induction of apoptosis, autophagy, and G1 blockade by Con A was due to interaction with cells' ERα. By analyzing the protein-ligand interaction fingerprints (PLIF), it was found that Leu A308, Thr C334, Val A368, Thr A371 were the most important amino acids in ERα protein involved in the interaction with lectin protein (Figure 5).

At the same time, the most observed interaction between lectin and ERα was recorded for pose **15** with a binding score of -44.76 kcal/mol and rmsd_refine value of 2.49. Pose **15** showed a very large area of interaction between lectin and ERα protein, proposing greatly the promising antagonistic effect of Con A on the ERα and its expected

synergistic effect with TAM in suppressing the ERα as well (Figures 6 and 7).

Conclusion

In summary, the combination of Con A and TAM showed synergistic antiproliferative, and apoptotic effects, which were approved through *in vitro* studies using MTT assay, cell cycle analysis, and Annexin V-PI apoptosis assay. The proposed mechanisms of the aforementioned effects were downregulation of the ERα mRNA that was accompanied by the downregulation of cyclin D1, which regulates the G1 phase cell cycle and the antiapoptotic gene *Bcl-2*. Meanwhile, the Con A/TAM combination is accompanied by a significant increase in the expression of the proapoptotic/autophagic gene *BNIP3*. Furthermore, docking studies suggested that Con A antagonizes greatly the ERα. These findings may explain the potential synergistic antitumor activity of Con A/TAM combination
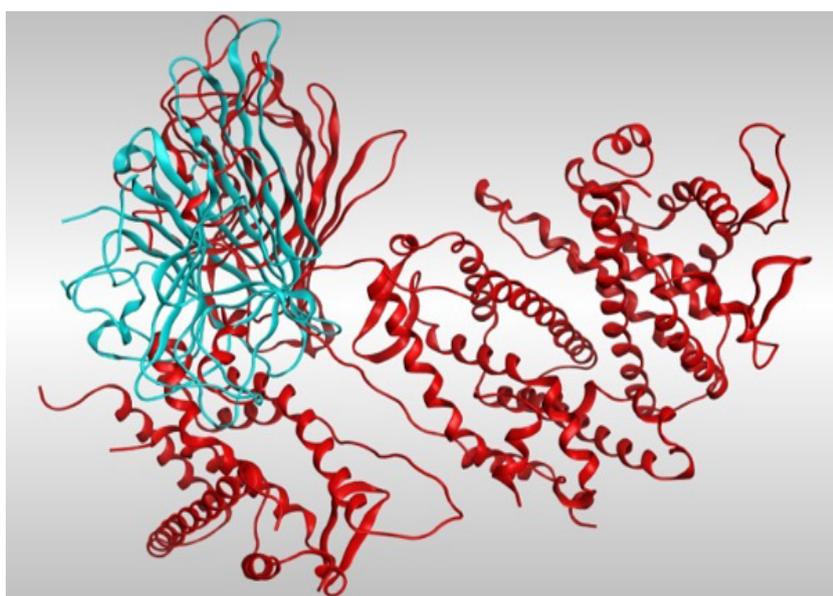

Figure 6. 3D representation showing the large area of interaction between lectin (turquoise) and ERα (red) proteins.
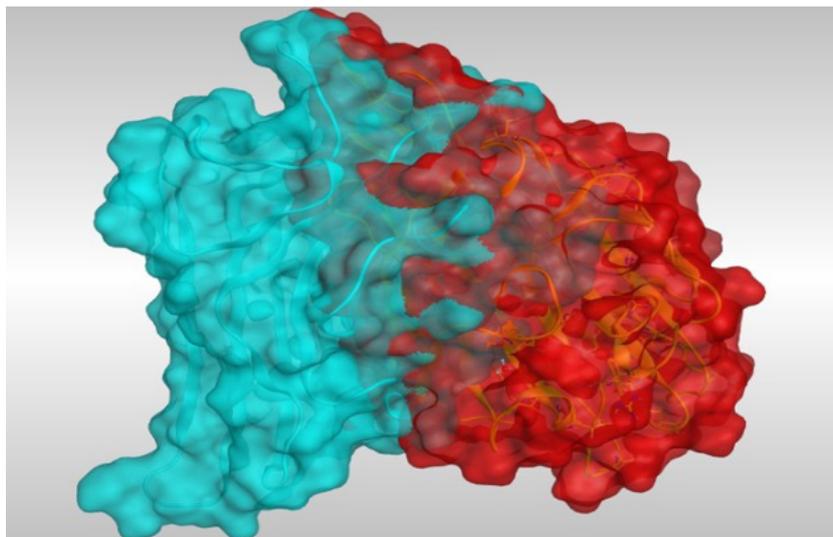
Figure 7. Lectin (turquoise) and ERα (red) as protein pocket representation showing the large surface area of interaction between them.

on ERα MCF-7 BC cells. This synergistic action of the Con A/TAM combination is proposed to be through achieving two crucial outcomes. First, reducing the therapeutic dose of TAM as indicated by DRI and hence decreases its side effects which hinder its use in many cases. Second, it will decrease the recently prominent chemo-resistance of cancerous cells to TAM through blocking ERα. Therefore, we suggest that Con A in combination with TAM is a new potential strategy for treating the ERα positive subtype of breast cancer.

Author Contributions
MFE: Conception and design of the work, acquisition, analysis, interpretation of data for the work, and drafting the work. NME: Analysis and interpretation of data for the work. IE: Analysis and interpretation of data for the work. WE: Drafting the work. AAA: Analysis and interpretation of data for the work, drafting the work, and revising it. All authors read and approved the final manuscript.

Conflict of Interest
The authors report no conflicts of interest.

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