Tanshinone IIA Promotes Apoptosis by Down-Regulating BCL2 and Up-Regulating TP53 in Triple-Negative Breast Cancer

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Research Article

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Abstract

Background/Objective: Tanshinone IIA, which is mainly used for the treatment of cardiovascular diseases, has been shown to inhibit the progression of a variety of cancers in recent years. In this study, we showed that tanshinone IIA could inhibit the proliferation of triple-negative breast cancer cells and promote their apoptosis. However, the specific mechanisms were not clear. Our research aims to clarify its mechanism.

Method: At the first, the effects of tanshinone IIA on cell viability of triple-negative breast cancer cell lines were analyzed. Then a mouse transplanted tumor model was used to study the anti-cancer effect *in vivo*. The related targets and mechanisms of tanshinone IIA were predicted through network-based systems biology and molecular docking analysis. At last, molecular docking and the molecular biological techniques were used to evaluate the predicted target.

Results: Tanshinone IIA could inhibit the cell proliferation after incubation for 24 h. At a concentration of 50 μM, Tanshinone IIA could half-inhibit the proliferation of 4T1 cells. Consistent with the results of the cell viability assays, Tan IIA induced the cancer cell apoptosis and resulted in a significant reduction in tumor volume. Then we analyzed the possible mechanisms with network-based systems biology, and predicted TP53, NF-κB, AKT, MYC and BCL-2 were the hub targets of Tan IIA for triple-negative breast cancer. P53 signaling pathway, PI3K/Akt pathway, the MAPK signaling pathway and the mTOR signaling pathways were the possible involved mechanisms. Using molecular docking analysis, we found Tan IIA has a high affinity for p53, Bcl-2 and NF-κB1, the binding energy were − 6.92, -6.07 and − 6.28 kcal/mol, respectively. Through western blotting assays, we further found the p53 protein expression was increased while Bcl-2 protein was decreased after Tan IIA treatment. These results verified that Tan IIA promotes apoptosis by down-regulating BCL2 and up-regulating TP53 in triple-negative breast cancer.

Conclusion: Our findings revealed the mechanism of Tan IIA in promoting apoptosis and inhibiting proliferation in triple-negative breast cancer cells, which provides a theoretical and experimental basis for further research into the anticancer effects of Tan IIA.

Introduction

Breast cancer has the highest incidence rate among all kinds of cancers worldwide based on the latest data released by the International Agency for Research on Cancer in 2020 [1]. Triple-negative breast cancer (TNBC) is the subgroup of breast cancers lacking estrogen receptor (ER), progesterone receptor (PR) and HER2 expression. It accounts for 15% of total breast cancer patients with a high biological aggressive nature, high rate of proliferation and invasion [2, 3]. Despite the improvement of treatment strategies, the prognosis of TNBC patients remains poor. Therefore, searching for effective regimens with minimal side effects remains the top priority in breast cancer research.

Dan-shen (*Salvia Miltiorrhiza*) is one of the most popular herbal remedies in China and have been widely used for clinical treatment of coronary heart diseases, cerebral ischemia and early cirrhosis. Tanshinones

are the major lipophilic ingredients of Dan-shen and have been shown to have diverse pharmacological activities including anti-inflammation, anti-oxidation and anti-cancer. Tanshinone IIA ($C_{19}H_{18}O_3$, 14, 16-epoxy-20-nor-5(10), 6, 8, 13, 15-abietapentaene-11, 12-dione, Tan-IIA) (Fig. 1), is one of important tanshinones. For the well-reported anti-inflammatory and antiangiogenic activities, the possible antitumor effects of Tan-IIA have received more attention in gastric cancer, acute leukemia, colorectal cancer, lung cancer, etc. [4–7]. The anti-TNBC effects of Tan-IIA has been reported [8, 9], but the mechanisms are currently unclear.

Network pharmacology, which clarifies the synergistic effects and the underlying mechanisms of multicomponent and multi-target agents through the analysis of networks, provides an understanding of the underlying complex relationships between CHM and diseases through the bridge “target” [10–13]. The aim of the present study was to investigate the mechanisms of Tan IIA on the TNBC cells. We proved Tanshinone IIA could induce apoptosis by down-regulating BCL2 and up-regulating TP53 in triple-negative breast cancer.

Materials And Methods

Drugs and Reagents

Tan IIA (Purity: 99.35%, Fig. 1) was purchased from Chengdu Mansite Biotech Co. Ltd (China, Lot. MUST-19090910). Doxorubicin was obtained from Beijing Solarbio Science & Technology Co., Ltd. (China, Lot. 730M024). Cell Counting Kit-8 was purchased from MedChemExpress (USA, Lot. #67666). Matrigel matrix was purchased from Corning (USA, Lot. 9266008). Antibodies against Bcl-2 (ab79849), NF-κB1(ab32360), P53 (ab26), phospho-P53(ab33889) and β-actin (ab8226) were purchased from from Abcam (UK).

Cell Lines And Cultures

Murine triple negative breast cancer 4T1 cell lines were from Tianjin Medical University Cancer Institute and Hospital provided. The Cells were maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 mg/mL) in a humidified atmosphere of 50 mg/mL CO_2 at 37°C.

Cell Proliferation Assays

CCK-8 viability assay was performed to evaluate the effects of TAN IIA on cell proliferation. For this, 4T1 cells were suspended with 0.25% trypsin and inoculated into 96-well plates at a density of 3×10^3 cells per well. 4T1 cells were treated with TAN IIA at concentrations of 10, 20, 30, 40, 50, 60, 70 or 80 μM for 24, 48, and 72 hours, respectively. Then the cells were incubated with 10 μl CCK-8 solution for 60 min. Amount of viable cells was estimated according to absorbance (Abs) at 450 nm using a microplate reader (IMark, Bio-Rad). The cell inhibition rate was calculated as the following: inhibition rate (%) = ((Abs

control group − Abs experiment group) / (Abs control group − Abs blank group)) × 100% [14]. The SPSS 18.0 software was adopted to calculate 50% inhibitory concentration (IC50) value. All experiments were performed in triplicate and repeated three times.

Colony Formation Assays

Transfected cells were reseeded into six-well plate with 500 cells per well by treating with TAN IIA for 48 hours. Cells were further treated with 0 (blank), 12.5, 25, or 50 μM TAN IIA. After approximately 12 days of cultivation at 37°C, the natural colonies were washed with 1 × PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. Then the colonies were stained with 0.5% crystal violet for approximately 20 min. Finally, the stained colonies were imaged and quantified. Colonies formed with over 50 cells were calculated and recorded for statistical analysis. All experiments were performed in triplicate and repeated three times.

Network Pharmacology Analysis

As in our previous researches [15–16], the possible targets of Tan IIA were predicted from the following two databased:BATMAN-TCM [17] (http://bionet.ncpsb.org.cn/batman-tcm/); TCMSP Database and Analysis Platform [18] (https://tcmspw.com/tcmsp.php). Candidate TNBC targets were obtained from CTD [19] (http://ctdbase.org/) and TTD databases [20] (http://db.idrblab.net/ttd/). The functions of predicted targets were analyzed using online enrichment analysis [21]. Protein–protein interactions (PPI) was constructed with Cytoscape based on the STRING database [22].

Docking Exercises Of Ingredients Binding To Main Targets

The crystal structure of p53 was obtained from the Protein Data Bank (PDB ID: 3D06) [23]. The crystal structure of Bcl-2 was also obtained from the Protein Data Bank (PDB ID: 6GL8) [24]. The crystal structure of NF-κB1 was also obtained from the Protein Data Bank (PDB ID: 1MDI) [25]. Other targets' crystal structures from Protein Data Bank include EGFR (5UG9), CASP3 (4JJE), KIT (3G0E), AKT2 (1O6L) and Bcl-2l1 (3SP7) [26–30]. The docking exercise was conducted through AutoDock 4.2 [31].

Western Blotting

After cell lysis, protein concentration was determined using a BCA protein quantitative kit. Protein lysates were resolved by SDS-polyacrylamide gel electrophoresis and then transferred to a PVDF membrane. Thereafter, membrane was blocked with 5% w/v nonfat dry milk in 1xTBST for 2 hours and then incubated with respective primary antibodies (1:1000 dilution) overnight at 4°C. Primary antibodies against the following proteins were used: BCL2, NFκB1, TP53, phospho-TP53 and β-actin (Abcam, Cambridge, UK). Afterward, the membrane was washed 2–3 times with 1xTBST and then incubated with

the respective secondary antibody (goat anti-rabbit horseradish peroxidase (HRP) conjugated antibody, 1 : 5,000 dilution) (Abcam, Cambridge, UK) for 1 hour at room temperature. After addition of HRP substrate, membranes were examined using an Image acquisition and analysis system (CHEMIDOC XRS+, Bio-Rad, USA). The band signal of each target protein was quantified by ImageJ and normalized according to respective β-actin levels [32].

In Vivo Xenografts

2×10^6 4T1 cells were injected subcutaneously into the upper middle groin of 5 BALB/c female mice. Tumor volumes were determined every day (Tumor volume = $a*b^2/2$, a and b are the long and short diameters of the tumor, respectively). When the average length of the subcutaneous tumor reached 10 mm, the mice were sacrificed in a humane manner, and then the tumors were collected. The collected tumors were cut into small pieces with a length of 2 mm in the clean area and planted in the fat pads of the fourth pair of left breasts of the anesthetized (10% chloral hydrate) BALB/c female mice. Three days later, mice were randomly divided into 3 groups (n = 18): control group (0.9% saline); Tan IIA group (10 mg/kg Tan IIA); Dox group (2 mg/kg Dox). Drugs were given by intraperitoneal injection every other day, tumor volumes were determined every day. Starting from the 8th day, three mice were randomly selected from each group every 3 days and sacrificed in a humane manner. The tumors of the mice were isolated and stored at low temperature. All experiments were approved by the Experimental Animal Ethics Committee of Nankai University.

Tunel Assay

Apoptosis in tumor tissue was detected with the In Situ Cell Death Detection Kit (Roche Diagnostic Mannheim, Germany). Briefly, after fixing and permeabilization, the tissue was incubated with the TUNEL reaction mixture. Then was covered with mounting medium containing DAPI. Representative images were acquired using a confocal microscope (Olympus) [33].

Statistical Analysis

Graphs were generated using Excel, PowerPoint software program, GraphPad Prism 5 and ggplot2.

Statistical significance was determined using the Statistical Package for the Social Sciences (SPSS) 12.0. All experimental data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons, and there was no significant difference in the variance homogeneity test ($P > 0.05$).

Results

Effect of Tan IIA Treatment on the Proliferation of TNBC 4T1 Cells

We investigated the anti-cancer effects of Tan IIA by treating 4T1 cells at various concentrations (10, 20, 30, 40, 50, 60, 70 or 80 μM) for 24, 48, and 72 hours, respectively. The effects of Tan IIA on the viability of 4T1 were evaluated with CCK-8 assay. When treated with a more than 40 μM of Tan IIA for 24h, sustainable and stability inhibitory effects were showed (Fig. 2A, B). Tan IIA inhibited 4T1 cell proliferation *in vitro* in concentration- and time-dependent manners with IC50 values of 80, 50, 40 μmol/L at 24, 48, and 72 h, respectively.

Plate clone formation assay were further conducted to explore the effects of Tan IIA on cell proliferation. The results were consistent with cell viability results. Tan IIA significantly reduced the percentages of colony formation by 67.73%, 31.87% and 13.94% after administrated 12.5 μM, 25 μM and 50μM for 48h (Fig. 2C, D). These results suggested that Tan IIA could suppress proliferation and colony formation in 4T1 cells.

Tan Iia Suppresses Tumor Growth In Vivo

We next evaluated the in vivo effect of Tan IIA on 4T1 xenograft tumor growth. Tan IIA was given by intraperitoneal injection with 10 mg/kg; The chemotherapy drug, docetaxel (Dox), which is used in clinic for the treatment of TNBC cancer patients, were included in the experiments as positive control. Both Dox and Tan IIA treatment suppressed the growth of transplanted tumors ($P < 0.05$ for both, Fig. 2E). A significant decrease of the tumor weights was detected in mice of the Tan IIA group compared with the solvent control group. After the 23 days treatment, the relative tumor volumes in Tan IIA group was 877.67 ± 250.80, with 65.53% inhibition rates, and the relative tumor volumes in the solvent control group and Dox group were 1339.33 ± 56.58, and 656.33 ± 187.35, respectively (Fig. 2F), with 49.00% inhibition rates. Apoptosis levels in situ were analyzed by TUNEL assay, and the distribution of the TUNEL-positive nuclei was higher after the treatment of Tan IIA compared to that in the control group (Fig. 2H). Tan IIA treatment did not lead to toxicity in mice as it did not cause weight loss in animals (Fig. 2G).

Predicted Targets And Mechanisms Of Tan Iia For Breast Cancer Treatment Based On Network Pharmacology

After searching the TTD and CTD databases, we obtained 1344 TNBC-related target candidates. Further, 215 drug targets were predicted from BATMAN-TCM and TCMSP databases. The potential TNBC targets for Tan IIA treatment were matched by combined the disease and drug targets. 45 common targets were obtained to construct an ingredient-target (cl-cT) network using Cytoscape (Fig. 3A). We further put the screened 45 targets into the STRING website to get the protein- protein interactions (PPI) among these proteins with the conditional effect score was set as > 0.9. After removing the targets without interactions,

a PPI with 39 targets was obtained (Fig. 3B), which indicated that these targets tend to form close associates among each other. TP53, NF-κB, AKT, MYC and BCL-2 were the hub targets among the PPI network. The functions of 39 hub targets were further analyzed with DAVID website, we found these targets major involved in cancer-related biological processes and pathways, such as cell apoptosis, P53 signaling pathway, PI3K/Akt pathway, the MAPK signaling pathway and the mTOR signaling pathway and so on (Fig. 3C, 3D).

To ascertain the interaction between Tan IIA and their hub targets, computational docking exercises were conducted to mimic the binding characteristics. It is generally believed that the binding energy value less than − 4.25 kcal/mol indicates a certain binding activity, less than − 5.0 kcal/mol indicates good binding activity, and less than − 7.0 kcal/mol indicates strong binding activity. The binding energies of Tan IIA with these hub targets P53, BCL-2, NF-κB, EGFR, CASP3, KIT, AKT2, and BCL-2L1 were shown in Table 1. Binding affinities to the first three targets were − 6.92, -6.07 and − 6.28, respectively, indicating a good or strong binding activity. Docking results were visualized in the Pymol software. Figure 3E showed Tan IIA docking with P53, BCL-2, NF-κB, EGFR, CASP3, KIT, AKT2, and BCL-2L1, which are mainly related with the cell apoptosis.

Table 1
Docking exercises of ingredients binding to p53, Bcl-2 and NF-κB1

Target	Gene ID	PDB ID	Binding energy (kcal/mol)
P53	7157	3D06	-6.92
Bcl-2	596	6GL8	-6.07
NF-κB1	4790	1MDI	-6.28
Egfr	1956	5UG9	-7.88
Casp3	836	4JJE	-5.70
Kit	3815	3G0E	-6.82
Akt2	208	1O6L	-6.40
Bcl-2l1	598	3SP7	-7.07

Changes In The Level Of Major Proteins In Tan Iia-treated 4t1 Cells

To investigate the mechanism by which Tan IIA induced apoptosis, Western blotting was utilized to detect Bcl-2, NF-κB1 and p53 proteins in 4T1 cells. Western blot results showed that the levels of Bcl-2 and NF-κB1 gradually reduced in response to increasing Tan IIA concentrations in vitro compared with control group after Tan IIA treatment ($P < 0.05$ for all, Fig. 4A, B). The levels of p53 and phosphorylated p53 also

showed an upward trend. These data showed that Tan IIA could inhibit tumor growth by activating apoptosis. Taken together, we mapped the hub targets to the canonical signaling pathways according to the results of the network-based systems and molecular-biological analyses, Tan IIA mediated its anti-cancer effects by reduced the expression of the Bcl-2 and upregulating the expression of P53 through NF-kB signaling pathway (Fig. 4C).

Discussion

Tan IIA is a herbal medicine extracted from the root or rhizomes of *S. miltiorrhiza* Bunge and has been reported to have potential chemo-preventative effects relevant to various human cancers. As a multi-target drug, the molecular targets of Tan IIA include apoptotic-regulating proteins, transcription factors and inflammatory mediators. In the present study, we demonstrated that Tan IIA could significantly suppress the growth of breast cancer cells via promoting cell apoptosis. We then applied a bioinformatics approach to detect the potential pharmacology of Tan IIA. Based on the network pharmacology analysis, we found that TP53, NF-κB, AKT, MYC and BCL-2 were the major hub putative targets of Tan IIA.

Cell apoptosis is an important manifestation of cell death, which is implicated with the development of tumors. Bcl-2 is one of the anti-apoptotic proteins, which plays an important role in the regulation of the mitochondria-mediated pathway of apoptosis. Bcl-2 can inhibit pro-apoptotic proteins Bax [34]. In addition to playing an important role in regulating cell apoptosis, Bcl-2 can also regulate tumor migration, invasion, autophagy and angiogenesis [35, 36]. In our experiments, we found TanIIA could inhibit the 4T1 proliferation and induce the apoptosis by reducing the expression of the Bcl-2.

P53 is one of the most important genes to maintain homeostasis and genome integrity in the process of cell cycle arrest, DNA repair and apoptosis. In 65–80% of triple-negative breast cancer cases, P53 is the most commonly mutated gene [37]. Recent studies have shown that TNBC patients with reduced p53 protein function have a reduced overall survival and an increased risk of metastasis [38]. If the DNA damage stress is strong enough, the S46 of p53 will be additionally phosphorylated to induce p53-mediated apoptosis, and induce the expression of some pro-apoptotic genes [39]. According to the predicted results of the network-based systems, p53 was one of the hub targets of Tan IIA for breast cancer treatment. Western blotting also proved Tan IIA promotes 4T1 cell apoptosis by enhancing the expression of p53 in 4T1 cells.

NF-κB can regulate a variety of target genes and produce complex feedback effects, including inflammation, innate immunity, stress response, cell differentiation or proliferation, and cell death [40]. Our research found NF-κB was also one of the major targets of Tan IIA for breast cancer treatment. In the KEGG enrichment analysis, some cell proliferation signaling pathways such as PI3K/Akt/mTOR pathway and the MAPK signaling pathway were predicted for breast cancer treatment of Tan IIA. Most of these pathways involved in the transportation of NF-κB to the nucleus.

In conclusion, our present results demonstrated that Tan IIA inhibited cell proliferation and tumor growth in vitro and in vivo by inducing cell apoptosis; while the downregulation of Bcl-2 and NF-κB as well as the

upregulation of p53 were the possible mechanisms of Tan IIA in TNBC cell apoptosis. Our studies provide strong evidence to support further investigations on developing Tan IIA as effective and safe agent for the therapy and prevention of TNBC.

Declarations

Ethics approval and consent to participate: All experiments were approved by the Experimental Animal Ethics Committee of Nankai University.

Consent for publication: All authors have no objections and agree to publish.

Availability of data and materials: The datasets generated and/or analysed during the current study are not publicly available due subsequent experiments have not been completed but are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no conflict of interest.

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Authors' contributions: Jinfeng Liu,Chang Zhang and Shuang Liu have contributed equally to this studyand share first authorship.

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Figures

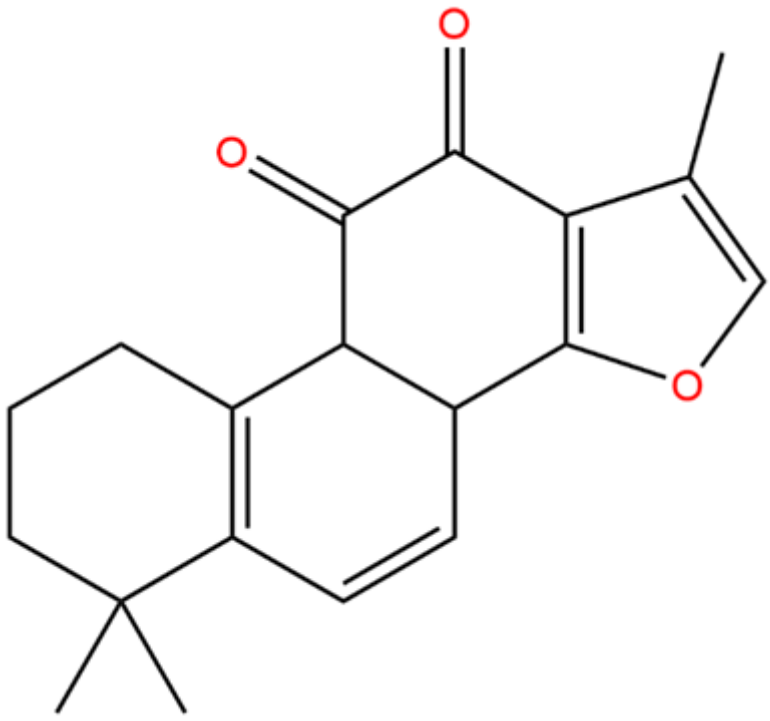

Figure 1

The molecular structure of Tanshinone IIA

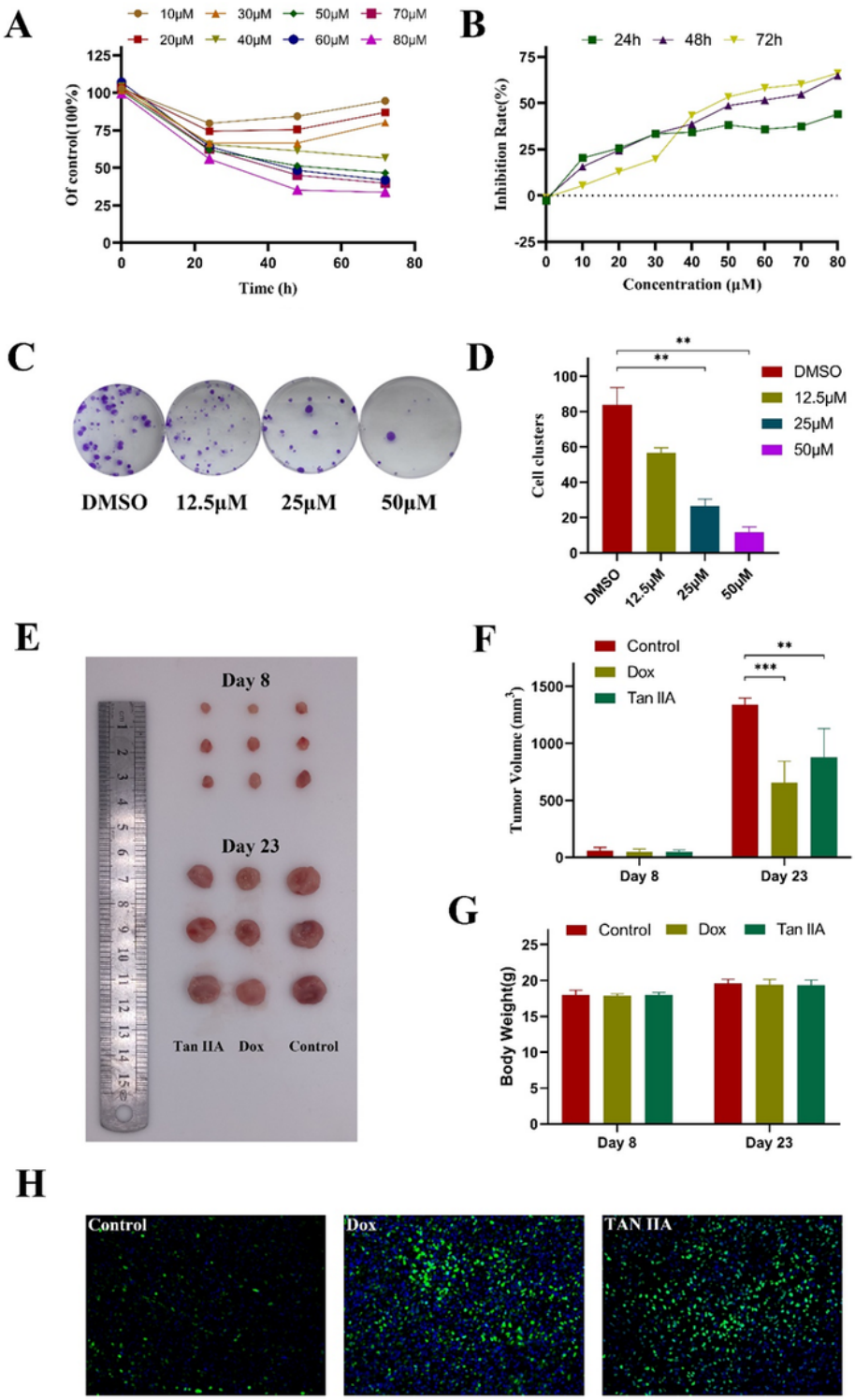

Figure 2

A, B:Effect of Tan IIA treatment on the proliferation of TNBC 4T1 cells (Tan IIA was able to decrease 4T1 cell viability in a dose- and time-dependent manner)

C, D:Plate clone formation assay

E, F, G:Tan IIA suppresses tumor growth in vivo

H:TUNEL Assay (the distribution of the TUNEL-positive nuclei was higher after the treatment of Tan IIA compared to that in the control group)

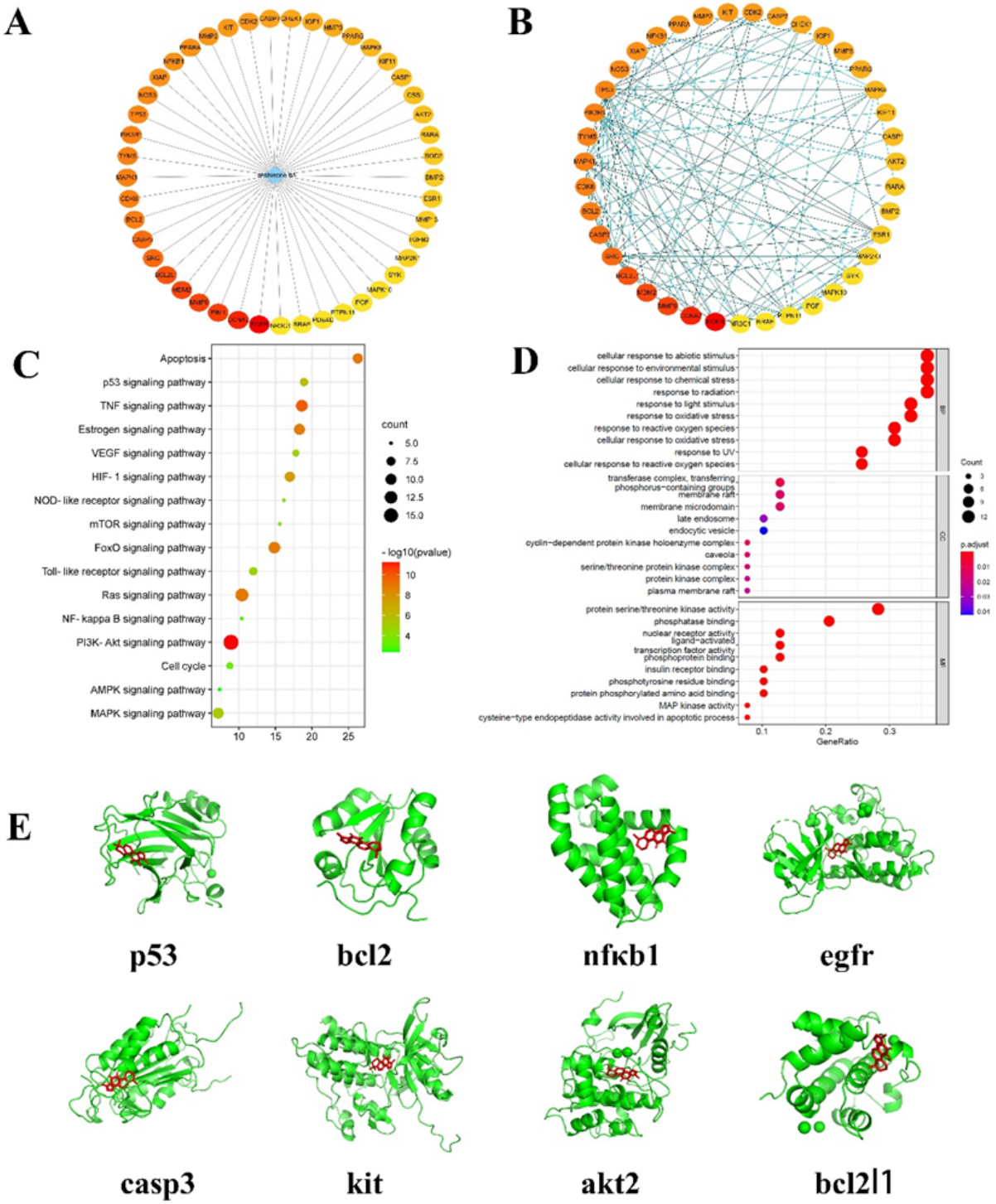

Figure 3

A: Prediction of TAN II-Related Targets (The more important the disease target is in triple-negative breast cancer, the redder the color of the node.)

B: Protein-protein interaction network (The higher the protein interaction score, the darker the color of the line.)

C: Kyoto Encyclopedia of Genes and Genomesenrichment analysis

D: Gene Ontology enrichment analysis

E: Docking exercises of ingredients binding to P53, BCL-2, NF-κB, EGFR, CASP3, KIT, AKT2, and BCL-2L1

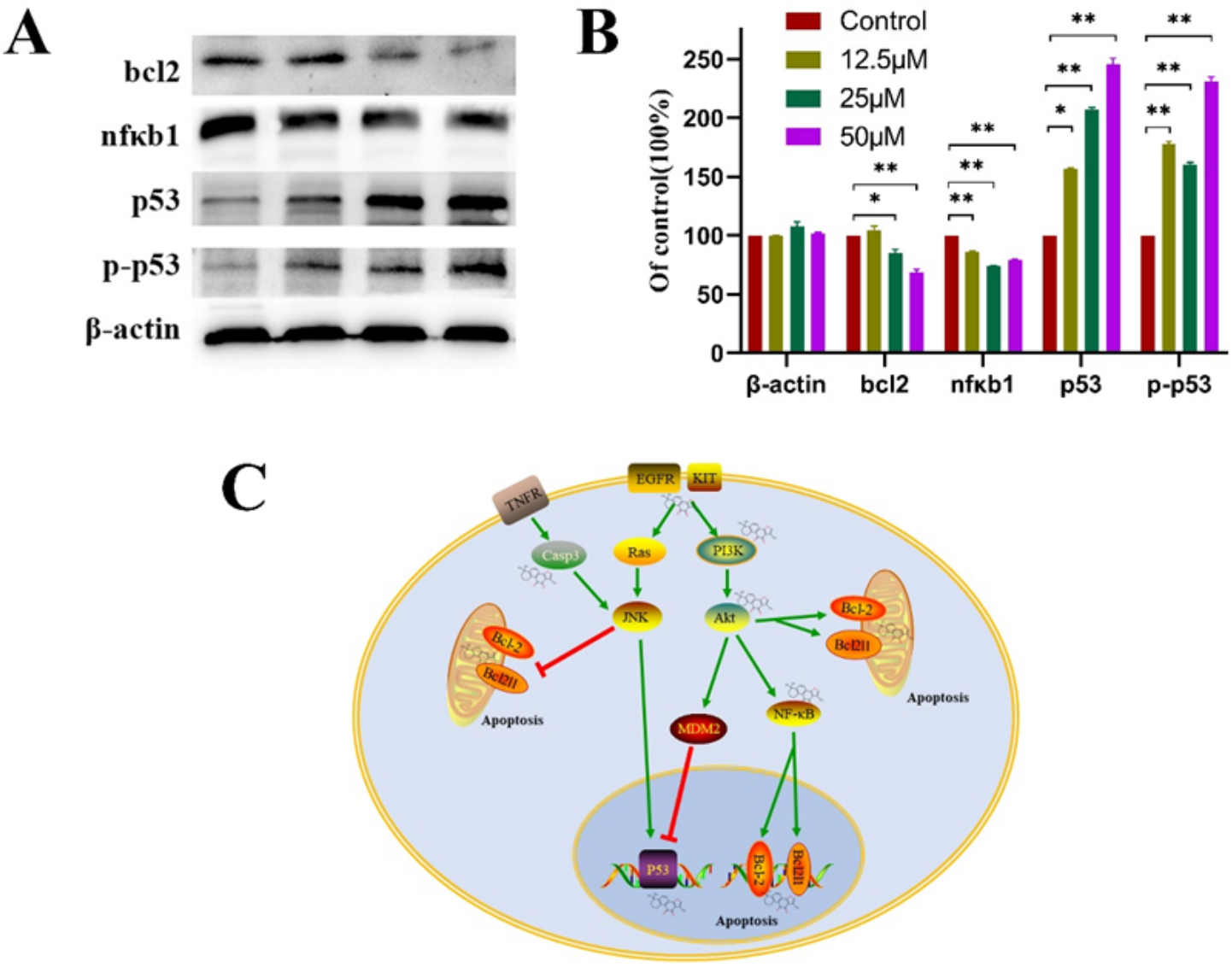

Figure 4

A, B:Changes in the level of major proteins in Tan IIA-Treated 4T1 Cells

C:Potential Pro-apoptotic Mechanism of Tan IIA