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Original Article

ERα is a target for butein-induced growth suppression in breast cancer

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Abstract: Breast cancer (BCa) has the highest incidence and mortality among malignant diseases in female worldwide. BCa is frequently caused by estrogen receptor α (ERα), a ligand-dependent receptor that highly expressed in about 70% of breast tumors. Therefore, ERα has become a well-characterized and the most effective target for treating ERα-expressing BCa (ERα$^+$ BCa). However, the acquire resistance was somehow developed in patients who received current ERα signaling-targeted endocrine therapies. Hence, discovery of novel anti-estrogen/ERα strategies is urgent. In the present study, we identified butein as a potential agent for breast cancer treatment by the use of a natural product library. We showed that butein inhibits the growth of ERα$^+$ BCa both *in vitro* and *in vivo* which is associated with cell cycle arrest that partially triggered by butein-induced ERα downregulation. Mechanically, butein binds to a specific pocket of ERα and promotes proteasome-mediated degradation of the receptor. Collectively, this work reveals that butein is a candidate to diminish ERα signaling which represents a potentially novel strategy for treating BCa.

Keywords: Breast cancer, growth, estrogen receptor α, butein, degradation

Introduction

The incidence and mortality of breast cancer (BCa) rank the first in female cancers according to the world statistics in 2018 [1]. Based on the status of hormone receptors, BCa has been divided into four major subtypes, including progesterone receptor positive BCa, human growth factor receptor-2 positive BCa, estrogen receptor positive BCa and triple negative BCa [2, 3]. Estrogen receptor α (ERα) is overexpressed and drives the occurrence and progression in about 70% of BCa [4]. ERα is a ligand-dependent receptor and functions as a nuclear transcription factor upon the binding of estrogen [5]. The activation of ERα signaling promotes the G1-S phase transition through increasing the expression of cell cycle drivers, such as Cyclin D1 and c-myc, and inhibiting cyclin-dependent kinase inhibitors, such as p21 [6, 7]. Therefore, ERα has been proposed as the preferential target for treating ERα positive BCa (ERα$^+$ BCa) as well as a critical biomarker for monitoring prognosis of patients with ERα$^+$ BCa. Meanwhile, the efficacies and sensitivities of endocrine therapies targeted on the ERα signaling are closely related to the status of ERα [8, 9]. The clinical application of tamoxifen and fulvestrant, two well-established antagonists of estrogen, displays outstanding effects in prolonging survival and improving life quality of patients with ERα$^+$ BCa [10, 11]. However, the emergence of acquired resistance to tamoxifen and fulvestrant limits the effectiveness of current endocrine therapies [12-14]. Hence, the current situation raises several urgent requirements for BCa therapy, such as developing new ERα-targeted agents and enhancing the sensitivities of BCa to antiestrogens.

Butein, 2',3,4,4'-tetrahydroxychalcone, is a plant polyphenol that can be extracted from many Chinese herbal medicine, such as *Rhus veriniciflua Stokes, Caragana jubata, stem bark of cashews and Dalbergia odorifera*. Butein is classified as the chalcone family of flavonoids which are considered to possess multiple biological properties, such as antioxidation, anticancer, anti-hyperlipidemia, anti-inflammation [15, 16]. In Asian countries, butein has been approved to be a traditional herbal medicine for the treatment of liver tuberculosis, hypertension, diabetes and obesity [17]. It has also been reported that butein inhibits cancer cell proliferation in various cancers, including prostate cancer, hepatocellular cancer, lung cancer and bladder cancer [18-21]. Interestingly, butein suppresses the growth of several subtypes of BCa in different manners [22-24]. These findings indicate that Butein may possess multiple targets, which is critical to the development of anticancer agents for overcoming drug resistance in tranditional medicine. However, whether butein is effective in ERα$^+$ BCa models and the underlying mechanisms remain to be elucidated.

In the present study, we conducted a large screening by the use of a natural product library. We found that butein suppresses the growth of ERα$^+$ BCa cells and xenografts via inhibiting cell cycle progression. Mechanically, butein decreases the protein level and transcriptional activity of ERα through increasing its proteasome-mediated degradation. Moreover, butein enhances the anti-cancer activity of fulvestrant in both MCF7 and T47D cells. Together, our findings provide a promising compound for breast cancer treatment which is associated with its activity in promoting ERα degradation.

Materials and methods

Reagents

Butein (S8036, at a purity of 99%), MG132 (S2619, at a purity of 97%) and fulvestrant (S1191, at a purity of 99.86%) were purchased from Selleckchem (Houston, TX, USA). Cycloheximide (CHX) and Estrogen (E8875) were obtained from Sigma-Aldrich (Sigma-Adrich, Louis, MO). The above reagents were dissolved into DMSO and stored at -20°C. Antibodies were from as follows: anti-CDK4 (12790), anti-estrogen receptor alpha (8644), anti-Ubiquitin (3936), anti-Cyclin D1 (2922), anti-Bax (5023), anti-K48-ub (12805), anti-p21 (2947) and anti-p27 (3686) (Cell Signaling Technology, USA); anti-GAPDH (MB001) and anti-Ki67 (BS1454) (Bioworld Technology, USA). Co-IP assay kit (14311D) was obtained from Life Technologies (Carlsbad, CA). MTS (catalog no. G111) was purchased from Promega Corporation. Cell-Light^{TM} EdU Apollo 567 In Vitro Kit (C10310-1) was from RiboBio (Guangzhou, China).

Cell lines and cell culture

The ERα$^+$ BCa cell lines MCF7 and T47D were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were maintained in HyClone DMEM supplemented with 10% FBS and cultured at the atmosphere of 37°C and 5% CO_2.

Cell viability assay

The assay was applied as previously reported [25, 26]. Cells were seeded into 96-well plates for 24 h. Cells were exposed to butein at several dose for 24, 48 and 72 h. After adding 20 μl MTS in each well and culturing for 2 or 3 h, the OD values were detected to determine the cell viability.

EdU staining

As we previously described [27, 28], an EdU kit was applied to test the cell proliferative ability. Cells were plated into chamber slide and culture overnight. The cells were exposed to butein. After 24 h, cells were incubated with 50 μmol/L EdU for additional 2 h. After the incubation, 2 mg/ml glycine was added into the cells and then 0.5% Triton X-100 was used to incubate for 10 min. Cocktail carrying apollo reaction and annexing agent buffer was used to incubate cells. Images were captured to calculate the positive cells.

Cell cycle assay

We performed this assay as previously reported [29, 30]. BCa cells were seeded into a six-well plate at the coverage of 1×10^6 cells per well. After culturing the cells for 24 h, Cells were treated with butein for 24 h and then collected. The collected cells were suspended

with 500 μl PBS and 2 ml 70% ethanol for one night. Cells were incubated with 50 μg/ml PI, 100 μg/ml RNase and 0.2% Triton-X-100 complex for half an hour at dark. The stained cells were subjected to FACS analysis to measure the proportion of cells in each stage.

Cell clonogenic assay

The assay was applied to evaluate the ability of cell proliferation as we previously reported [31, 32]. The BCa cells in exponential growth period were digested and resuspended. The harvested cells were plated on a 6-well plate for 24 h and then exposed to butein for an additional 24 h. The treated cells were digested and resuspended at a coverage of 500 cells/ml medium. Then the cells were seeded in a new 6-well plate and cultured in DMEM supplemented with 10% FBS at an atmosphere of 5% CO_2 for 10-14 days. After then, cells were fixed with 4% paraformaldehyde for 15 min and washed with PBS thrice. Crystal violet solution was applied to stain fixed cells for 10 min. Colonies > 60 were counted.

Immunoblotting and co-IP assays

Western blotting assay was applied to test protein expression as we previously reported [33, 34]. Protein lysates were extracted from the treated cells by butein, subjected to resolve by SDS-PAGE, and then to PVDF membranes. 5% non-fatted milk was used to incubate the membrane for one hour. The membrane was washed with PBS-T in triple, followed by primary antibodies overnight. The washed membrane by PBS-T was incubated with secondary antibodies. Analysis protein expression used ECL detection reagents. For co-IP assay, dynabeads mixed with antibodies for 16-24 h. The mixtures were added into cell lysates extracted from T47D cells and then rotated. The new mixtures were suspended with SDS loading buffer, separating from dynabeads.

Real-time polymerase chain reaction analysis

The mRNA level was evaluated by RT-qPCR assay as we previously described [35]. RNAiso plus (TakaRa Biotechnology) was applied to extract RNAs in butein-treated cells. The dose of RNAs was tested and equal. 1000 ng RNAs synthesize cDNA using PrimeScript RT Master Mix kit (TaKaRa). We measured the mRNA levels of GAPDH and ERα by Real-time quantitative PCR. In this study, primers for PCR as follow: GAPDH: F: 5' TCCCATCACCATCTTCCA3'; R: 5' CATCACGCCACAGTTTCC3'. ERα: F: 5' TCTTGGACAGGAACCAGGGA3'; R: 5' CAGAGACTTCAGGGTGCTGG3'.

BCa xenograft in mice

The nude balb/c female mice (18-22 g) were obtained from Guangzhou University of Chinese Medicine. The Institutional Animal Care and Use Committee of Guangzhou Medical University approved animal protocols and housed the animals. Mice were housed in barrier facilities for 5 days and then subcutaneously implanted with MCF-7 cells for one months. Mice were located into two groups which are control group and butein treatment group. Mice were administrated with butein (10 mg/kg/2 day) by intraperitoneal injection for 27 days. Tumor volumes and body weight were measured each other day.

Luciferase reporter promoter assay

Dual Luciferase Reporter Assay was purchased from Promega Corporation (Madison, WI, USA), and ER-luciferase reporter plasmid was from Yesen Company (Shanghai, China). Cells were plated into plates. 1000 ng estrogen receptor respond element (EREs) was applied to transfected with cells. Then cells were exposed to butein. According to the manufacture's instructions, the activity was detected by dual luciferase assays kit. The value was calculated by firefly to Renilla luciferase.

Immunohistochemical staining

As we reported in a previous study [36], tumor tissues were fixed and embedded in paraffin, subjected to section. The sections were incubated with MaxVision kits (Maixin Biol), followed by immunohistochemical staining for Bax, Ki67, Cyclin D1 and ERα. MaxVisionTM reagent was chosen, subjected to diaminobenzidine and H_2O_2 in 50 mM tris-HCl. Measuring primary antibodies used DAB.

Molecular simulation

The binding mechanism between ERα (PDB ID: 5FQT) and Butein (CID: 5281222) was per-

formed by molecular docking. The crystal structure of ERα was co-crystallized with ERα antagonist. The interaction between Butein and ERα was confirmed in the antagonist binding domain. YASARA was applied to Energy minimization of ligands. Autodock Vina was used for molecular docking [37]. The best conformations were applied to as the starting conformation for MD simulation. YASARA was chosen to molecular dynamics simulation [38]. Simulations run by AMBER 03 forcefield. The receptor-ligand complex was disolved by 0.9% NaCl in a dodecahedron box. 298 K was set for initiation of simulated annealing minimizations. Berendsen thermostat was applied and then minimize the influence of temperature control. What is more, velocities were regulated only every 100 simulation steps. Lastly, 100 ns MD simulations were treated at a rate of 2 fs.

Statistical analysis

Unpaired Student's t-test or one way ANOVA is used where appropriate. *P* value of less than 0.05 was considered to be significant. The data in this study were from three independent experiments as mean ± SD.

Results

Butein induced the growth inhibition of ERα positive BCa cells

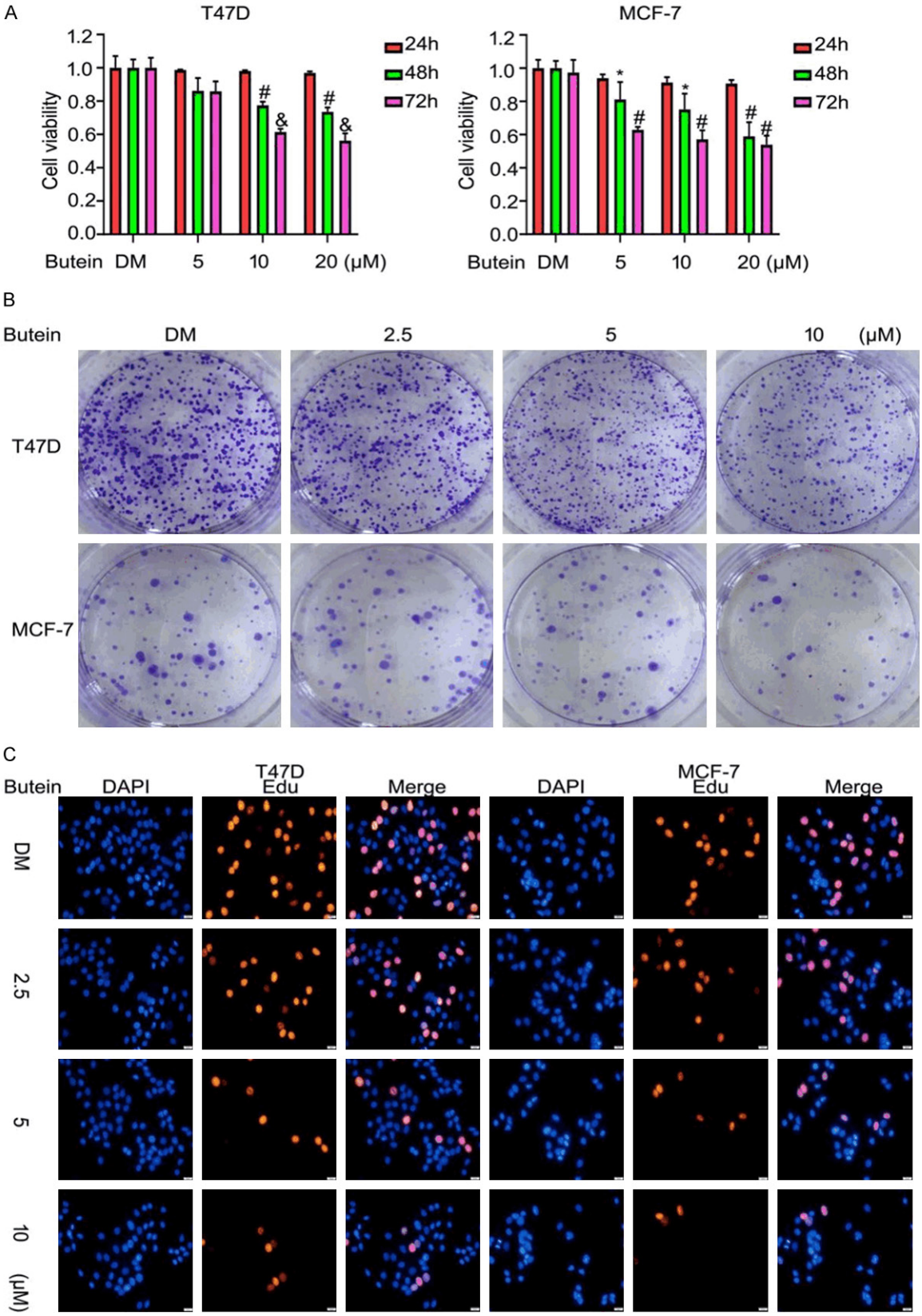
On the basis of different characteristics, various BCa cells are categorized as $HER2^+$, PR^+, $ER\alpha^+$ and triple negative breast cancer. To explore the effects of natural products on $ER\alpha^+$ BCa cells, two $ER\alpha^+$ BCa cells, MCF-7 and T47D, and a natural product library were chosen in the study. Cell viability analysis was applied to test the growth of breast cancer cells under the treatment of various natural products. The results showed that cell viability was significantly suppressed with the escalating dose of butein at three different time (**Figure 1A**). In addition, ERα negative BCa cells were applied to evaluate the anti-cancer activity of butein. The results showed that cell viability of these cells was also inhibited by butein, indicating that butein has additional targets beyond ERα (Supplementary Figure 1). We further confirmed the proliferative ability of BCa in the presence of butein using colony formation assay. We found that butein inhibited the colony formation after the treatment of butein for 24 h in MCF-7 and T47D cells (**Figure 1B**). To ensure the anti-proliferative effect of butein on $ER\alpha^+$ BCa cells, we performed the EdU staining assay and found that the stained cells were more in the control group than that of in the treatment of butein (**Figure 1C**, **1D**). Taken together, these results confirmed that butein has a potent anti-growth ability on $ER\alpha^+$ BCa cells.

Butein suppressed cell cycle progression via arresting the G0/G1 to S phase transition

Because numerous cancers are characterized by rapid cell cycle progression, inhibiting cell cycle transition represents an effective therapeutic schedule for cancer. To evaluate the role of butein on cell cycle progression of $ER\alpha^+$ BCa, we detected the cell cycle distributions of $ER\alpha^+$ BCa cells treated with butein for 24 h. We found that butein remarkably upregulated the ratio of G0/G1 phase in MCF-7 and T47D cells (**Figure 2A**, **2B**). To determine the molecular mechanism of cell cycle arrest induced by butein, we measured various checkpoint proteins associated with G0/G1 to S phase transition. Western blot analysis was applied to detect the protein expression of CDK4, Cyclin D1 and p27 in BCa cells post butein treatment. The results showed that CDK4 and Cyclin D1 expressions were decreased under butein treatment in MCF-7 and T47D cells. While the expression of p27 which inhibits G0/G1 to S phase progression was increased by butein (**Figure 2C**). These findings collectively indicated that butein arrested G0/G1 phase to S phase transition through altering CDK4, Cyclin D1 and p27 protein expression in $ER\alpha^+$ BCa. To address whether butein plays a role in migration and invasion in BCa cells, wound healing and transwell invasion assays were performed. The results showed that butein did not alter cell migration and invasion in $ER\alpha^+$ BCa cells (Supplementary Figure 2A, 2B).

Butein downregulated ERα protein level in $ER\alpha^+$ BCa

ERα promotes cell growth on breast cancer and thus targeting ERα is an important scheme for breast cancer therapy. Several inhibitors targeting ERα are used for clinical treatment in breast cancer patients, such as tamoxifen and fulvestrant. Considering the critical role of ERα


A
T47D
MCF-7
Cell viability
24h
48h
72h
Butein DM 5 10 20 (μM)
B
Butein
DM
2.5
5
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C
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DAPI
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DM
2.5
5
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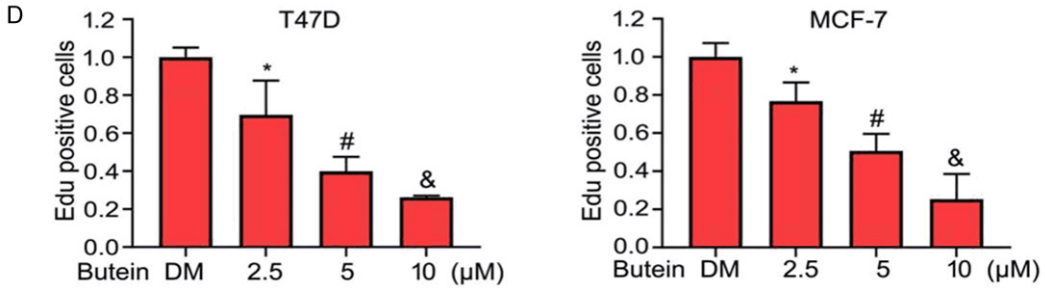

Figure 1. Butein induces growth inhibition of ERα positive breast cancer cells. A. Cells were exposed to butein (0, 5, 10, 20 μmol/L) for 24, 48, 72 h. Adding 20 μl MTS for cell viability for 3 h. In addition, Cells were exposed to butein (0, 2.5, 5, 10 μmol/L) for 24 h for the followed experiments. B. The treated cells were observed for colony formation for 2 weeks. The showed images were from three independent experiments. C, D. The indicated cells were stained with Edu for the additional 2 h. DAPI was used for nuclear staining. Scale bar, 20 μm. The showed images and pooled data were from three independent experiments. $^{*}P<0.05$, $^{\#}P<0.01$, $^{\&}P<0.001$ *versus* each vehicle control.

protein in breast cancer, we speculated whether butein decreases cell proliferation of ERα^{+} BCa by diminishing ERα protein expression or signaling pathway. To test this hypothesis, we further determined the level of ERα protein after the treatment of butein using western blot analysis. Consequently, ERα protein level was decreased in ERα^{+} BCa cells exposed to butein in dose- and time-dependent manner (**Figure 3A-D**). ERα is a nuclear transcription factor. The treatment of E2, an ERα-oriented ligand, can promote the nuclear import of ERα and thereby activating its transcriptional activity. Hence, we sought to study whether butein translocates with ERα into cytoplasm. Immunofluorescent staining assay was used to observe the expression in the nucleus and cytoplasm. The results showed that ERα expression was decreased in the nucleus by the treatment of butein, but butein did not change the translocation of ERα in MCF-7 and T47D cells (**Figure 3E**, **3F**).

Molecular simulations for the interaction of butein with ERα

To clarify the relation between butein and ERα, we conducted molecular docking analysis by using Autodock vina. The binding energy of ERα-butein complex was -8.424 kcal/mol. The three dimensional binding conformation of ERα-butein complex is showed in **Figure 4A**. We found four hydrogen bonds were formed between ARG-394, GLU-353, VAL-534 of ERα and butein. The distance of hydrogens bond between ERα and butein were 2.3, 2.7, 2.2, and 2.1 Å, respectively. It was also observed that butein interacted with LEU-391, MET-388, TRP-383, LEU-525, MET-343, LEU-349, THR-347, ALA-350, and VAL-533 *via* van der Waals force (**Figure 4B**). The start conformation for MD simulation is from the best conformation of ERα-butein via YASARA [38]. In addition, we showed the surface visualization models of ERα-butein complex in **Figure 4C**. The center of ERα is the binding site for butein until the end of MD simulation. Moreover, this finding demonstrates the evolution of heavy atoms root-mean-square deviation (RMSD) of the complex concerning the minimized structure. The heavy atoms RMSD track of ERα in ERα-butein complex raised from 0.6 Å to 2 Å during the first 5 ns, fluctuated between 1.9 to 2 Å during 5 to 40 ns, and then the RMSD rose from 2 Å to 2.3 Å during 40 to 50 ns, then fluctuated around 2.2 Å during last 50 ns (**Figure 4D**, red line). The heavy atoms RMSD track of unbound ERα raised from 0.6 Å to 3 Å during the first 25 ns, then reduced and fluctuated between 2.7 during last 75 ns (**Figure 4D**, blue line). These results suggest a strong binding between the kinase domain of ERα and butein, indicating that butein could directly target ERα.

Butein promoted ERα degradation and inhibited its transcriptional activity

Given that butein regulated the proliferation of ERα^{+} BCa cells and downregulated the protein levels of ERα, we sought to explore how butein mediates the downregulation of ERα protein. Firstly, we speculated whether butein induced the decrease of ERα protein resulting from

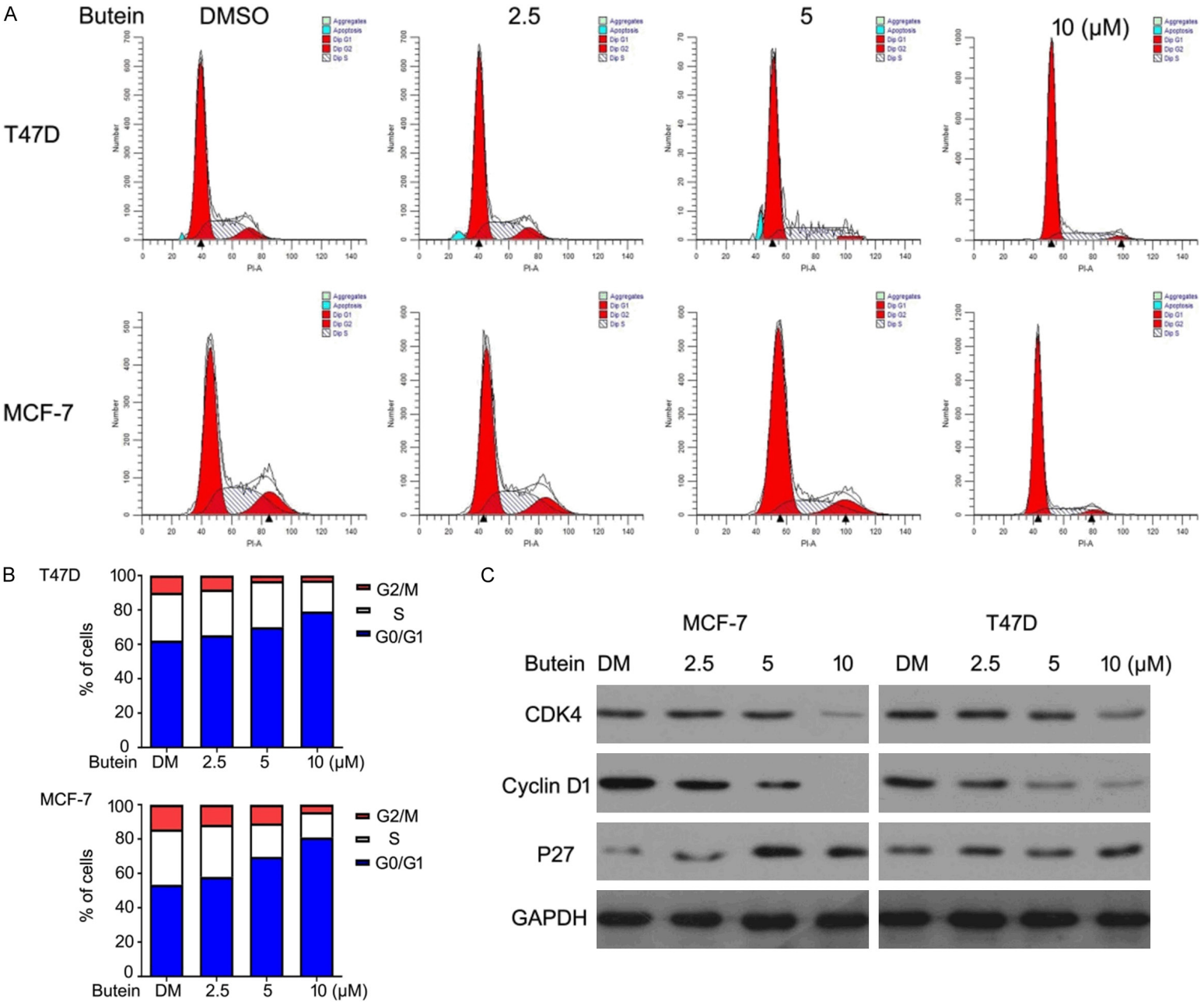
A
Butein
DMSO
2.5
5
10 (μM)
T47D
MCF-7
B
T47D
% of cells
G2/M
S
G0/G1
Butein DM 2.5 5 10 (μM)
MCF-7
C
MCF-7
T47D
Butein DM 2.5 5 10 DM 2.5 5 10 (μM)
CDK4
Cyclin D1
P27
GAPDH

Figure 2. Butein suppresses cell cycle progression via arresting the G0/G1 to S phase transition. MCF-7 and T47D cells were exposed to butein at the different dose for 24 h. A. We tested cell distribution using FACS. B. Calculated the percentage of cell number. C. The cells treated by butein were extracted to western blotting analysis for Cyclin D1, CDK4 and P27 proteins. GAPDH was a loading control.

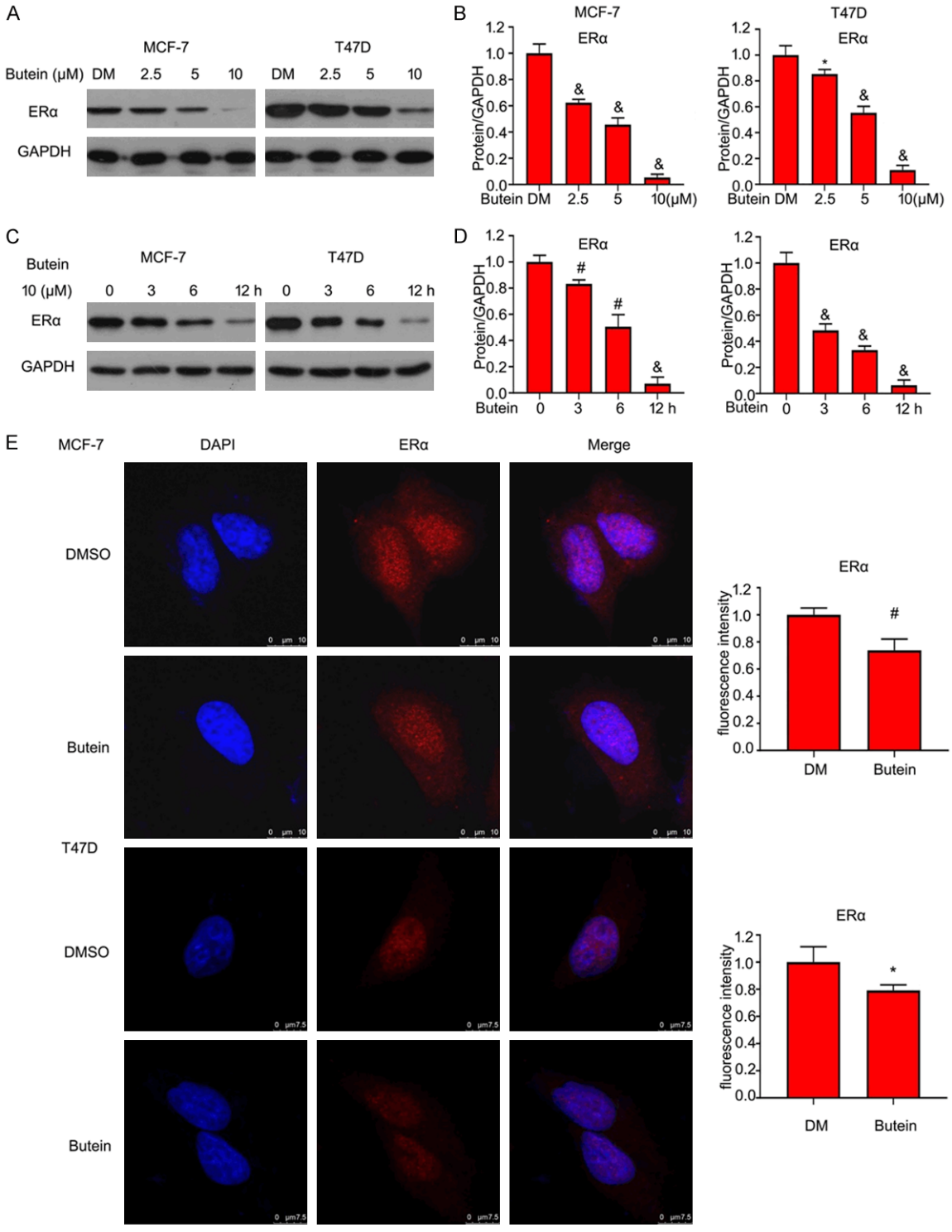

Figure 3. Butein regulates ERα protein expression in ERα⁺ BCa. A. The cells were treated with butein for 24 h. Protein lysates were extracted, subjected to western blotting analysis for ERα expression. C. Cells were exposed to

butein (10 μmol/L) for 0, 3, 6, 12 h. Protein lysates were prepared for ERα protein expression. GAPDH was a loading control. B, D. Densitometry with Image J was applied to quantify the bands of ERα. E. MCF-7 and T47D cells were treated with butein (10 μmol/L) for 24 h and then stained with anti-ERα. DAPI was for nuclear staining. Scale bar, 10 μm. The represent images were from three independent experiments. *P<0.05, #P<0.01, &P<0.001 *versus* each vehicle control.

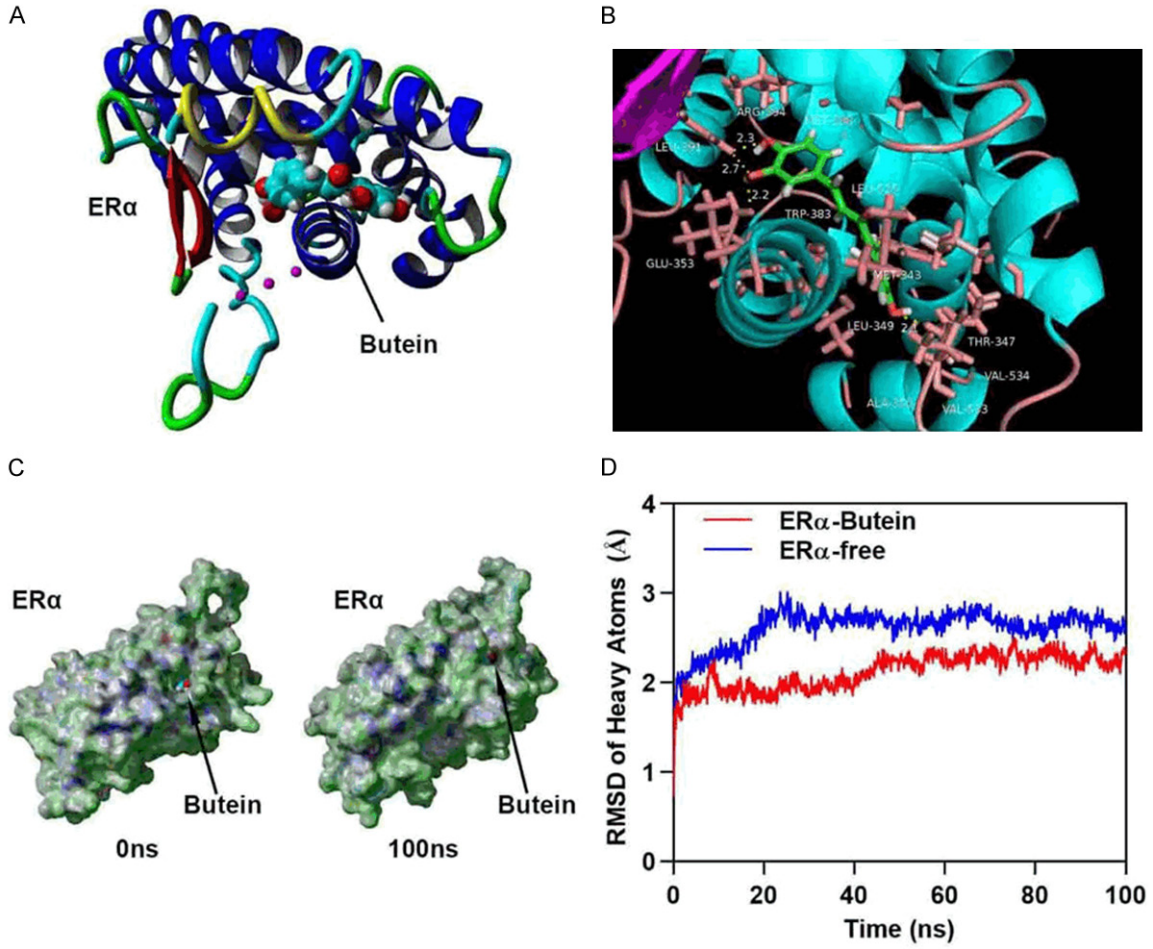

Figure 4. Molecular simulations for the interaction of Butein with ERα. A, B. Three dimensional crystal structure of Butein in complex with ERα (PDB ID: 5FQT). Green represents butein, and yellow line shows hydrogen bonds. C. Surface presentation of the ERα-Butein complex crystal structure at 0 ns and 100 ns. D. Plots of root mean square deviation (RMSD) of heavy atoms of ERα-Butein complex (red) and unbound ERα (blue).

inhibiting the transcriptional synthesis of ERα. Thus, we determined the mRNA level of ERα using RT-qPCR and the results showed that butein did not significantly inhibit ERα expression at mRNA level (**Figure 5A**). In addition, the transcriptional activity of ERα was detected by Dual luciferase reporter analysis. We found that butein remarkably suppressed transcriptional activity of ERα in MCF-7 cells (**Figure 5B**). Therefore, we further hypothesized whether butein increased the degradation of ERα protein. To our expected, the cycloheximide (CHX) chasing assays confirmed that and the half-life of ERα was decreased upon butein treatment (**Figure 5C**, **5D**). In addition, the proteasome inhibitor, MG132, significantly rescued the downregulation of ERα protein induced by butein (**Figure 5E**, **5F**), suggesting that butein decreased ERα protein level via the ubiquitin proteasome system. Moverover, the poly-ubiquitinated ERα was detected using co-IP assay, and the results showed that butein dramatically increased levels of pan-poly-ubiquitinated ERα and K48-poly-ubiqui-

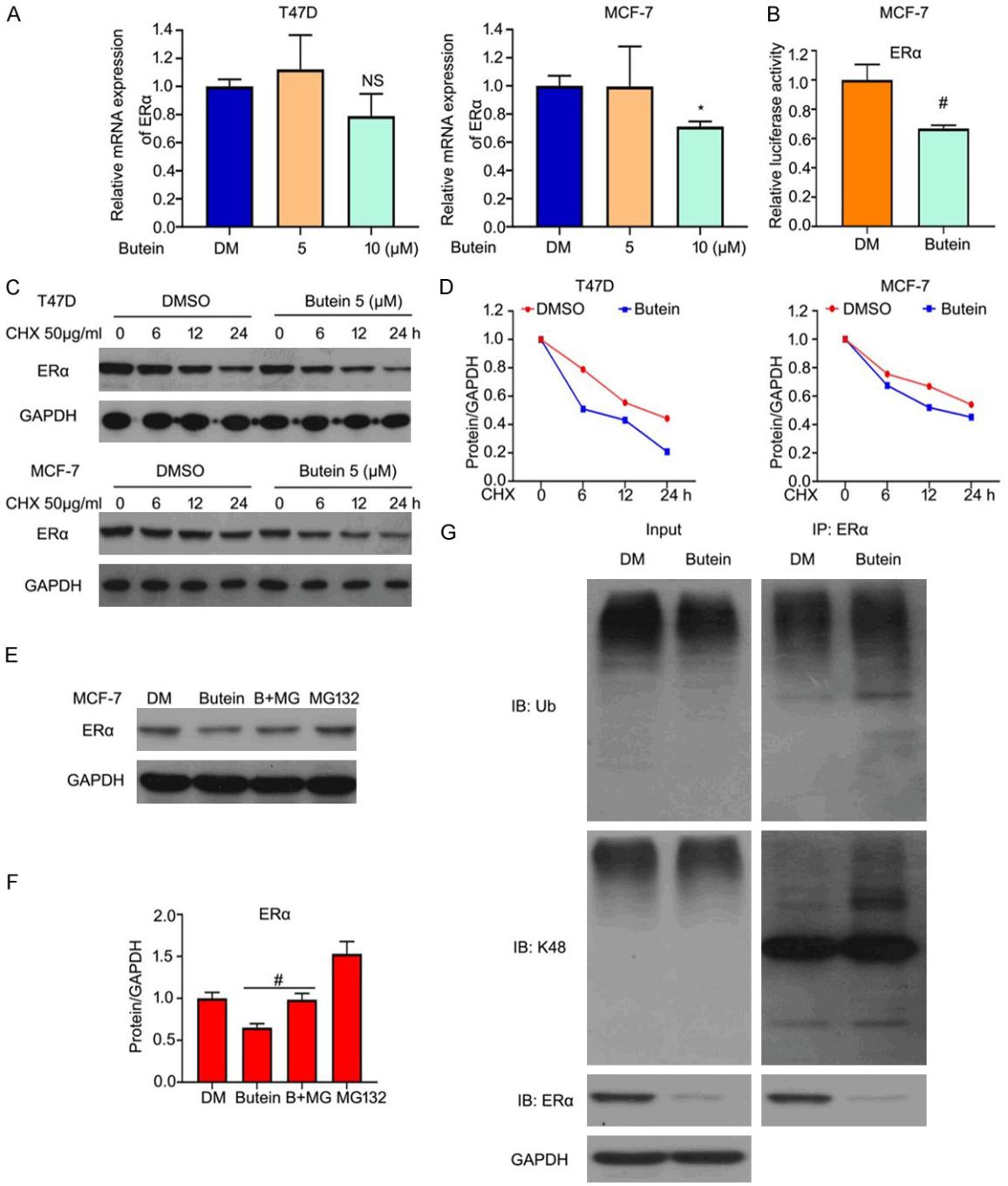

Figure 5. Butein promotes ERα degradation and mediates its transcriptional activity. A. Total RNA were collected from cells exposed to butein for 12 h. RT-qPCR was prepared to analyse mRNA level of ERα. NS (no significance) is $P > 0.05$ vs. each vehicle control. B. Cells were transfected with plasmid containing EREs for 24 h and then treated to butein for the additional 24 h. Protein lysates were extracted to dual-luciferase analysis for tanscriptional activity. C. Cells were treated with butein (10 μM) and CHX (50 μg/ml) for the different time. The protein expression of ERα were measured. D. The bands of ERα were quantify using Image J. E. Cells were pretreated with MG132 for 6 h and then with butein for an additional 24 h. Protein lysates were prepared for ERα expression. F. The bands of ERα were quantify. G. T47D cells were exposed to butein for 24 h and MG132 for 6 h. Immunoprecipitated with ERα beads and immunoblotted with ubiquitin (Ub), K48.

tinated ERα (**Figure 5G**). These findings indicated that butein promoted the ubiquitination and proteasome-mediated degradation of ERα in breast cancer.

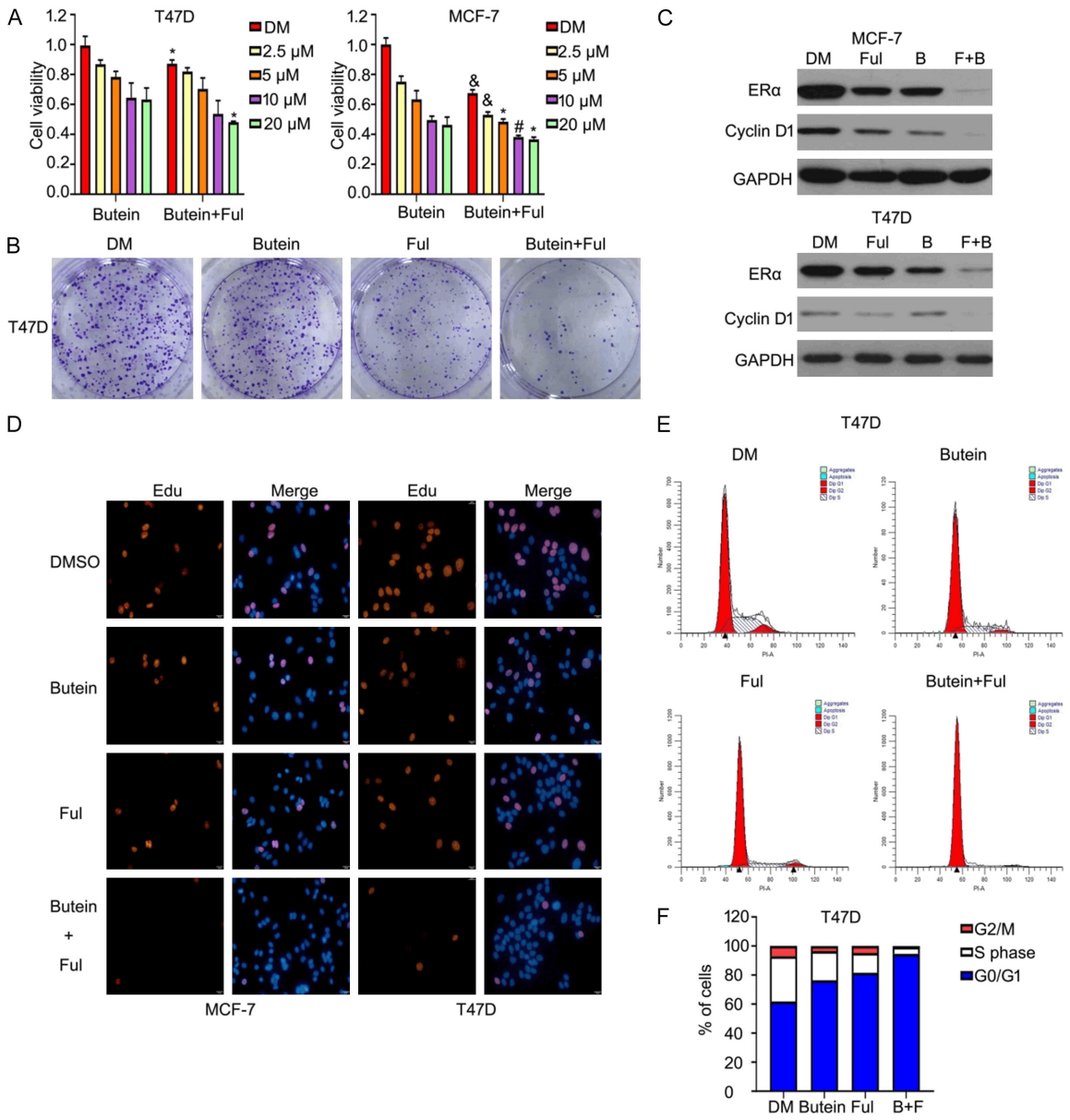

Figure 6. Butein enhances the anti-cancer effect of fulvestrant in ERα⁺ BCa. A. MCF-7 and T47D cells were exposed to butein (0, 2.5, 5, 10, 20 μM), fulvestrant (1 nM), or the combination of the both agents for 48 h in triple. 20 μl MTS were added to measure cell viability. *P<0.05, #P<0.01, &P<0.001 *versus* each vehicle control. B. T47D cells were exposed to butein, fulvestrant, or the both for 24 h. Colonic formation assay was performed. The showed images were from three independent experiments. C. MCF-7 and T47D cells were treated with butein, fulvestrant, or both for 24 h. Protein lysates were extracted for ERα and Cyclin D1 expression. D. Cells were treated with butein, fulvestrant, or both for 24 h. Edu stain assay were performed and images were captured using fluorescence microscope in triple. E. T47D cells were treated and collected, subjected to FACS for cell distribution. F. The percent of cell number was calculated.

Butein enhanced the anti-cancer effect of fulvestrant in ERα⁺ BCa

Fulvestrant, an estrogen receptor antagonist that can downregulate the expression of ERα protein, is clinically used for patients with breast cancer who did not respond to tamoxifen. We firstly confirmed that fulvestrant induced the inhibition of cell growth and ERα expression. In addition, we explored the effect of butein combined with fulvestrant in ERα⁺ BCa. The results showed that butein increased the sensitivity of MCF-7 and T47D cells to fulvestrant but there is no synergistic effect because the values of CI (combination index) are more than 1 (**Figure 6A**, **6B**). Besides, the

immunoblot results showed that butein notably strengthened fulvestrant-induced ERα downregulation (**Figure 6C**). Moreover, the EdU staining analysis and flow cytometry analysis were used to test DNA duplicate and cell cycle progression of breast cancer cells. The results showed that the ratio of DNA duplicate in breast cancer cells treated with butein in combination with fulvestrant was lower than that of cells in the alone treatment group, while the ratio of G0/G1 phase in breast cancer cells treated with butein in combination with fulvestrant was higher than that of cells in the alone treatment group (**Figure 6D-F**). These results demonstrated that butein enhances the sensitivities of ERα$^+$ BCa cells to endocrine therapy without synergistic effects possibly due to share a similar target.

Butein induced the inhibition of ERα$^+$ BCa growth in vivo

After observing the anti-proliferative effect of butein on cell growth in ERα$^+$ BCa cells *in vitro*, we sought to explore the role of butein *in vivo*. MCF-7 xenografts were established in nude mice models. We found that butein treatment for about one months induced xenograft shrinkage (**Figure 7A**). Consistently, butein significantly decreased tumor volumes and weight, but not influence the body weight (**Figure 7B-D**). In addition, IHC assays showed that the expression of Bax was increased and the levels of Cyclin D1, Ki67 and ERα were declined in the group of butein treatment (**Figure 7E**, **7F**). Moreover, TUNEL assay was used to detect apoptosis in the tissues of breast cancer. The results showed that the stained cells were upregulated in butein group (**Figure 7G**, **7H**). These findings indicated that butein inhibited tumor proliferation derived from MCF-7 cells *in vivo*.

Discussion

Breast cancer is the most common threat to the female all over the world. HER2, PR and ER are key drivers that activate the growth and progression of breast cancer and have become important biomarkers for breast cancer therapy. Blocking these receptors can significantly suppress a large of breast cancers. Among the three molecules, ER is the most frequently used target and indicator for breast cancer treatment and outcome predicting in clinic. Endocrine therapy targeting ERα has been developed as an irresistible trend because it exerts lower side cytotoxicity compared to traditional chemotherapy. The inhibitors of ERα, such as tamoxifen and fulvestrant, have been used as first-line drug for patients with ERα$^+$ BCa [39, 40]. However, due to the AF-2 domain mutations of ERα gene, resistance to the endocrine therapy developes in many patients who received the treatment [41, 42]. Therefore, identifying novel compounds targeting ERα and tackling acquired resistance have become urgent needs for the current science. In recent years, increasing compounds, such as Shikonin, Caffeine and Caffeic Acid, have been explored for breast cancer treatment which function to suppress breast cancer cell growth via modulating the expression and transcription of ERα [43, 44]. Besides, several proteins were identified for the regulation of breast cancer progression through modulating ERα ubiquitination and degradation [45-47]. In this study, we confirmed that butein, a Chinese herbal medicine which belongs to chalcone family of flavonoids, inhibits the growth of ERα$^+$ BCa *in vitro* and *in vivo*. Importantly, butein promotes the degradation of ERα via increasing the expression of ubiquitinated ERα, suggesting that butein may represent a potential medicine for breast cancer therapy.

We firstly found that butein can suppress cell viability and proliferation of ERα$^+$ BCa cells. Subsequently, we demonstrated that butein arrests cell cycle from G0/G1 to S phase transition. The proteins related to cell cycle promotion, such as CDK4, Cyclin D1, are downregulated post butein treatment. The cell cycle inhibitor, p27, are upregulated post butein treatment. We also used Annexin V-FITC staining assay to determine apoptosis upon the treatment of butein. The results showed that butein suppresses cell growth resulting from cell cycle arrest rather than apoptosis. Moreover, we demonstrated that butein exerts an anti-ERα$^+$ BCa activity *in vivo*. These findings suggest that butein has a potent anti-breast cancer activity through blocking cell cycle progression.

We further explored the molecular mechanisms underlying butein-induced proliferative suppression. It has been reported that Cyclin D1 and p27 are downstream effectors of ERα.

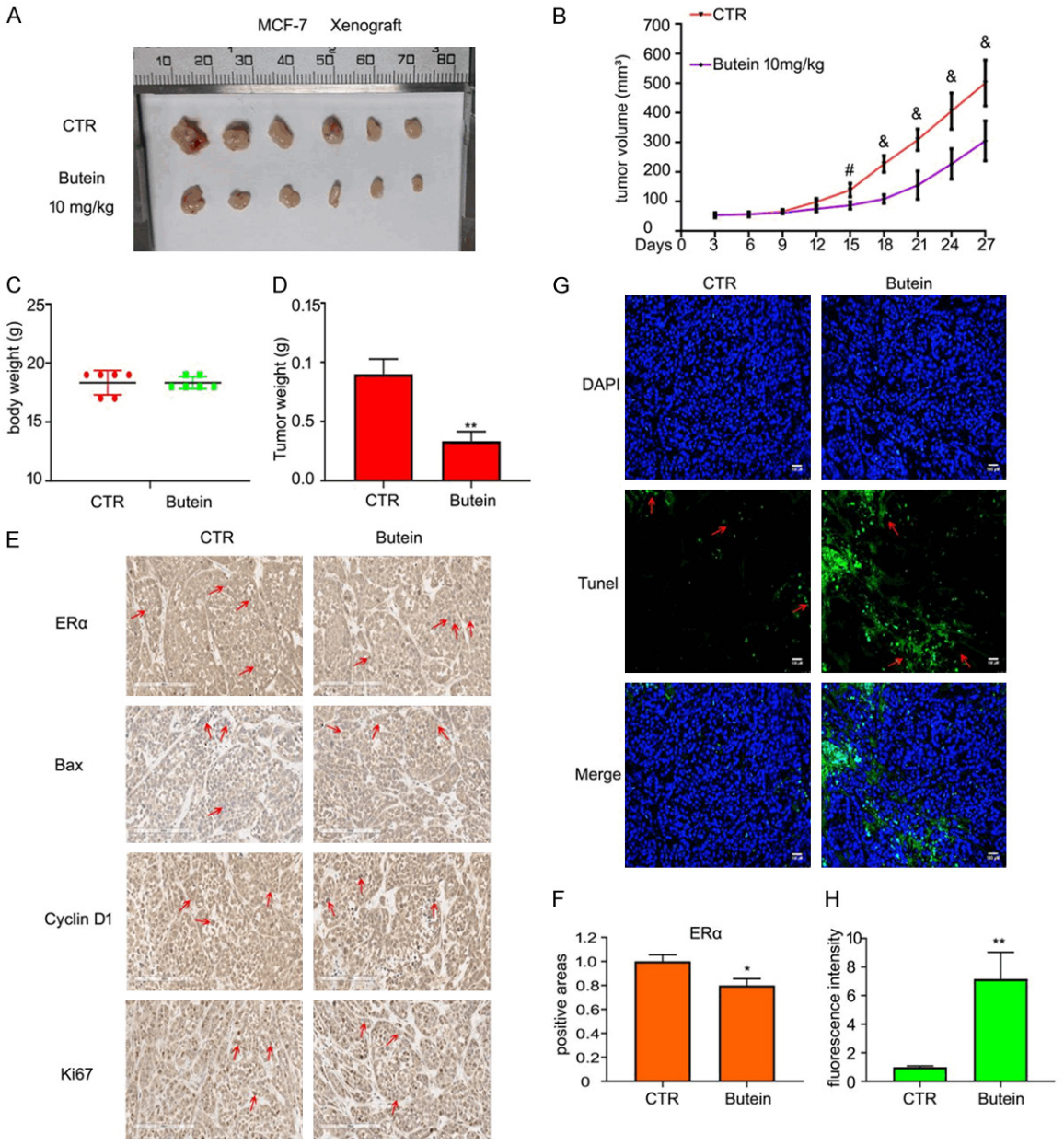

Figure 7. Butein induces growth inhibition of ERα⁺ BCa *in vivo*. MCF-7 cells were used to injected subcutaneously into BALB/c nude mice for 1 months. Mice were treated with butein via intraperitoneal injection for 27 days. The images (A) and volumn (B) of tumor were showed. The weight of body (C) and tumor (D) were measure. (E) Immunohistochemistry staining assay was performed for ERα, Bax, Cyclin D1 and Ki67 expression. Scale bar, 20 μm. (F) And positive areas of ERα from three images of IHC were quantified. *P<0.05 *versus* each vehicle control. TUNEL staining was performed to test apoptosis (G) and Fluorescence intensity (H) was quantitated, Scale bar, 100 μm. #P<0.01, &P<0.001 *versus* each treatment alone.

ERα can increase the expression of Cyclin D1 and decrease p27 proteins in multiple breast cancer models. Given that butein decreased the expression of Cyclin D1 and increased p27, we subsequently explored whether butein regulates the expression or activity of ERα protein. We found that protein expression of ERα is inhibited by butein at dose- and time-dependent manners. The conformation assays demonstrated that butein can bind to the ERα, indicating that ERα is a critical target of butein. As previously reported, the K302 and K303 sites are necessary to the ubiquitination of ERα [48]. Hence, we assessed the conjugate site

between butein and ERα. However, the conjugate site is neither at K302 nor at K303. We speculated that butein induced ERα degradation possibly via modulating ERα co-regulators, such as deubiquitinases, E3 ligases and chaperones that are related to the ubiquitination of ERα, but it needs numerous statistics which will be acquired by further investigations. In addition, we explored whether butein regulates ERα expression at transcriptional and translational levels. The RT-qPCR assays confirmed that butein did not regulate ERα synthesis. We further supposed whether butein inhibits ERα expression at post-translational levels. The CHX chasing experiments and the MG132 rescue experiments showed that butein induces the proteasome-mediated degradation of ERα. Moreover, butein increased both pan-poly-linked and K48-linked ubiquitin chains on ERα. The results demonstrated that butein decreases the expression of ERα protein via increasing the ubiquitinated ERα.

Of note, ERα is a potential target, but not the exclusive one for butein-induced proliferative inhibition in breast cancer. Firstly, we demonstrated that butein promotes the degradation of ERα and modulates its downstream regulators, including Cyclin D1, CDK4 and p27. Secondly, butein sensitize ERα$^+$ BCa cells to the treatment of antiestrogens but without synergistic effects, indicating that butein and antiestrogens share a similar target. Moreover, the docking simulation assay showed that butein can bind to the ERα. These findings collectively suggest that ERα is a critical target of butein. However, several reports have demonstrated that butein displays anti-cancer effects in other subtypes, including HER2 positive breast cancer cells and ERα negative breast cancer cells [22-24], indicating that butein has multiple targets beyond ERα. Therefore, our findings not only uncover a promising compound for treating breast cancer, but strengthen a novel notion that appropriately multiple targets of anti-cancer chemicals are beneficial to avoid drug resistance.

In conclusion, this work strongly revealed an inhibitory property of butein on ERα$^+$ BCa and provided a novel regimen for ERα$^+$ BCa treatment via targeting ERα degradation.

Disclosure of conflict of interest

None.

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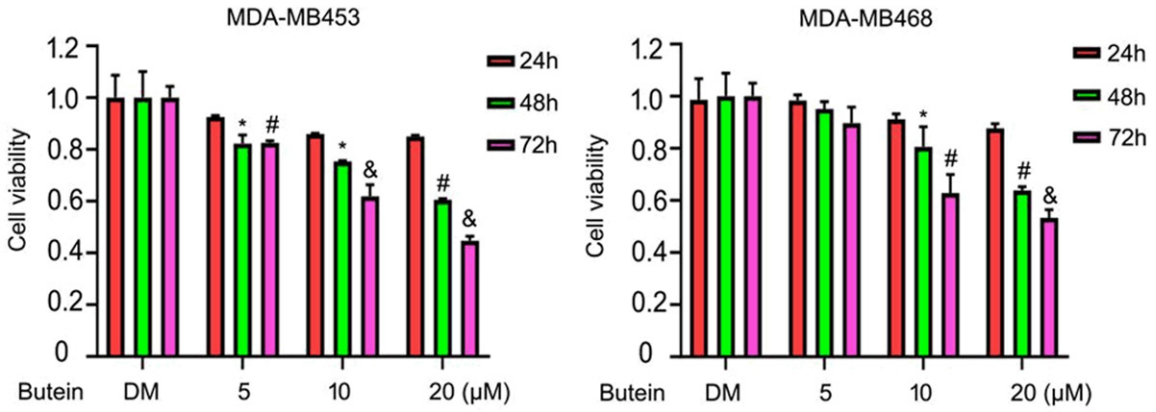
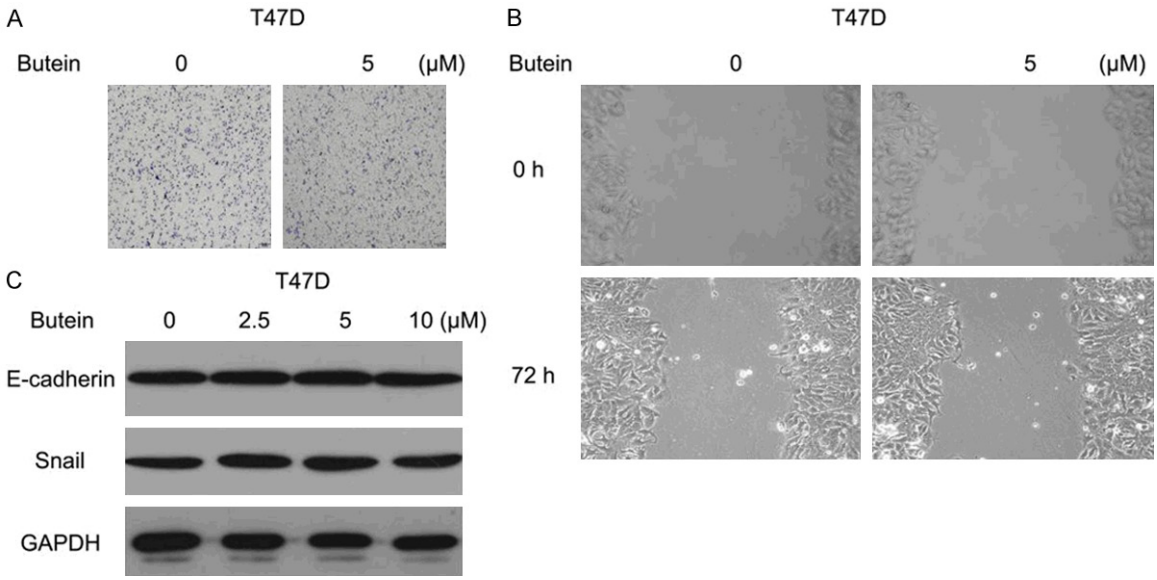


Supplementary Figure 1. The effect of butein on ERα negative BCa cells. MDA-MB453 and MDA-MB468 cells were treated to butein for the indicated time. Adding 20 μl MTS for cell viability for 3 h. *P<0.05, #P<0.01, &P<0.001 versus each vehicle control.



Supplementary Figure 2. The function of butein on migration and invasion of ERα+ BCa cells. A. Wound healing assay was performed on T47D cells exposed to butein. B, C. Transwell invasion assay was performed on T47D cells exposed to butein.