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ARTICLE

Tricetin, a Dietary Flavonoid, Inhibits Proliferation of Human Breast Adenocarcinoma MCF-7 Cells by Blocking Cell Cycle Progression and Inducing Apoptosis

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This study is the first to investigate the anticancer effect of tricetin in human breast adenocarcinoma MCF-7 cells. Results reveal that tricetin inhibits MCF-7 cells by blocking cell cycle progression in the G2/M phase and inducing apoptosis. Cell cycle blockade is associated with increased activation of ataxia telangiectasia-mutated (ATM). Activation of ATM by tricetin phosphorylated p53 at serine 15, resulting in increased stability of p53 by decreasing p53 and murine double minute-2 (MDM2) interaction. In addition, tricetin-mediated G2/M phase arrest was also associated with decreases in the amounts of cyclin B, cyclin A, cdc2 and cdc25C, and increases in the phosphorylation of Chk2, cdc25C and cdc2. The specific ATM inhibitor caffeine significantly decreased tricetin-mediated G2/M arrest by inhibiting the phosphorylation of p53 (serine 15) and Chk2. Tricetin-induced apoptotic cell death is associated with changes in the expression of Bax and Bak, decreasing levels of Bcl-2 and Bcl-X_L, and subsequently triggering the mitochondrial apoptotic pathway. In addition, pretreatment of cells with caspase-9 inhibitor blocked tricetin-induced apoptosis, indicating that caspase-9 activation is involved in tricetin-mediated MCF-7 cell apoptosis. These findings suggest that tricetin may be a promising chemopreventive agent against human breast cancer.

KEYWORDS: Tricetin; ATM; p53; cell cycle; apoptosis

INTRODUCTION

Breast cancer is one of the most common human malignancies and the second leading cause of cancer-related deaths in women, and its incidence in the developing world is on the rise (*1*). Different treatment strategies have been employed to reduce the mortality rate, including surgery, radiotherapy, and adjuvant chemo- or hormonotherapies (*2*). Unfortunately, breast cancer is particularly challenging, because it is highly resistant to radiation and conventional chemotherapeutic agents, and such resistance is associated with a poor prognosis for this metastatic disease, particularly in hormone receptor-positive breast cancer (*1*, *3*). About 30−40% of women with this form of cancer will develop metastasis and eventually die from this disease (*4*). Novel therapeutic agents are therefore needed to deal with the increasing incidence of human breast cancer.

Ataxia telangiectasia-mutated (ATM) is phosphoinositide 3-kinase-related kinase (PIKK) that plays an important, central role in cellular biology, including cell proliferation and DNA repair (*5*, *6*). Kinase activity of ATM is induced in response to DNA damage-dependent or independent events and then targets several regulators of cell cycle checkpoints and cell death (*6*−*8*). During this process, ATM undergoes autophosphorylation on Ser1981 and is recruited to sites of DNA damage, where it initiates a signaling cascade through phosphorylation of multiple DNA damage response and cell-cycle proteins, including p53, Chk1/2, and breast cancer 1 and E3 ubiquitin ligases such as murine double minute-2 (MDM2) and constitutively photomorphogenic 1 (COP1) (*7*, *9*, *10*). The tumor suppressor protein p53 is targeted by a wide variety of intracellular and extracellular stimuli, such as withdrawal of growth factors, hypoxia, irradiation, chemicals, and defects in nucleotide synthesis (*11*, *12*). The activation of p53 leads, primarily through its transcriptional function, to either apoptosis, eliminating those cells harboring severely damaged DNA, or growth arrest, allowing damaged DNA to be repaired and thereby suppressing tumor formation (*11*, *12*). Stability and activity of p53 are believed to be regulated in part by posttranslational modifications, such as phosphorylation and acetylation (*7*, *11*, *13*). Phosphorylation

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on NH_2-terminal residues, especially Ser15, Thr18, Ser20, and Ser37, is believed to affect interaction with the negative regulator MDM2 and hence contribute to the stabilization of p53 (*7*, *13*). Phosphorylation on COOH-terminals Ser315 and Ser392 in particular is believed to enhance the specific DNA binding of p53 *in vitro* (*7*, *14*).

Tricetin (5,7,3′,4′,5′-pentahydroxyflavone), a flavonoid derivative found in Myrtaceae pollen and *Eucalyptus* honey (*15*−*18*), presents a potent antiinflammatory activity (*19*). Tricetin content ranges from 202 to 769.9 μg in 100 g of eucalyptus honey, varying from different geographic regions and different *Eucalyptus* species. (*17*). Tricetin-3-*O*-glycoside is the major form presented in eucalyptus honey, but other forms of glycosides, such as glycosides of methyl ethers of tricetin, also coexist in *Stachys scardica* Griseb (*20*). In this study, we employed human breast adenocarcinoma MCF-7 cells to assess the molecular mechanisms responsible for the antiproliferative effect of tricetin. We found that tricetin caused cell cycle arrest at the G2/M phase and induced an apoptotic response.

MATERIALS AND METHODS

Chemicals and Reagents. Tricetin (5,7,3′,4′,5′-pentahydroxyflavone) was purchased from Extrasynthese (Genay, France). Fetal bovine serum (FBS) and DMEM were obtained from GIBCO BRL (Gaithersburg, MD). Dimethyl sulfoxide (DMSO), ribonuclease (RNase) and propidium iodide (PI) were purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies to p53, phospho-p53, p21, ATM, phospho-ATM, phospho-H2A.X, NBS1, phospho-NBS1, Chk2, phospho-Chk2, cyclin B, cyclin A, cdc2, cdc25C, phospho-cdc2, phospho-cdc25C, Bax, Bak, Bcl-2, Bcl-X_L, and β-actin were obtained from Cell Signaling Technology (Beverly, MA).

Cell Culture. Breast cancer cell line MCF-7 was obtained from the American Type Cell Culture Collection (Manassas, VA). MCF-7 cells were maintained in monolayer cultures at 37 °C and 5% CO_2 in DMEM supplemented with 10% FCS, 5 μg/mL insulin, 100 units/mL of penicillin G, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B.

Cell Proliferation and Clonogenic Assay. Inhibition of cell proliferation by tricetin was measured by XTT (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well), and after 24 h incubation, treated with vehicle alone (0.1% DMSO) and various concentrations of tricetin for 48 h. 150 μL of XTT test solution, which was prepared by mixing 5 mL of XTT-labeling reagent with 100 μL of electron coupling reagent, was then added to each well. After 4 h of incubation, absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

To determine the long-term effects, cells were treated with vehicle alone (0.1% DMSO) and various concentrations of tricetin for 1 h. After being rinsed with fresh medium, cells were allowed to grow for 14 days to form colonies, which were then stained with crystal violet (0.4 g/L; Sigma).

Cell Cycle Analysis. To analyze cell cycle distribution, 5×10^5 cells were plated in 60 mm dishes and treated with vehicle alone (0.1% DMSO) and tricetin (40 and 60 μM) for 6 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), resuspended in 1 mL of PBS containing 1 mg/mL RNase and 50 μg/mL PI, incubated in the dark for 30 min at room temperature, and analyzed by EPICS flow cytometer. The data were analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Apoptosis Assay. Cells were treated with 40 and 60 μM tricetin for 48 h. Apoptotic cells were quantitatively carried out by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis by using BD ApoAlert DNA Fragmentation Assay kit. Briefly, cells were incubated with vehicle alone (0.1% DMSO) and tricetin (40 and 60 μM) for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37 °C. The stained cells were then analyzed with an EPICS flow cytometer and a fluorescence microscope at 20× magnification.

Assay for Caspase-9 Activity. The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate of caspase-9, LEHD-pNA (Ac-Leu-Glu-His-Asp-*p*NA) (Calbiochem, Cambridge, MA). Cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37 °C. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percentage of change in activity compared to the untreated control.

Mitochondrial Membrane Potential Assay. We used mitochondrial-specific cationic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′- tetraethylbenzimidazolylcarbocyanine iodide) (Molecular Probes, Inc.), which undergoes potential-dependent accumulation in the mitochondria. Cells were seeded in a 96-well plate. Following treatment with tricetin (40 and 60 μM) for the indicated times, cells were stained with 25 μM JC-1 for 30 min at 37 °C. Fluorescence was monitored with the fluorescence plate reader at wavelengths of 490 nm (excitation)/540 nm (emission) and 540 nm (excitation)/590 nm (emission) pairs. Changes in the ratio between the measurement at test wavelengths of 590 nm (red) and 540 nm (green) fluorescence intensities are indicative of changes in the mitochondrial membrane potential.

Immunoblot/Immunoprecipitation. Cells were treated with 60 μM tricetin at specified intervals. The cells were lysed on ice for 40 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EGTA, 12 mM β-glycerolphosphate, 10 mM NaF, 16 μg/mL benzamidine hydrochloride, 10 μg/mL phenanthroline, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 14000*g* for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by SDS−PAGE (8−12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1−16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham, USA) according to the manufacturer's instructions.

For association of p53 and MDM2, cell lysates (300 μg) were incubated with 10 μL anti-MDM2 for 1 h at 4 °C. Immunocomplexes were resolved by 7.5% SDS−PAGE. Association of MDM2 with p53 was detected by incubating the blots with anti-MDM2 and anti-p53 antibodies as described above.

The p53 Activity. p53 activity was determined by ELISA Trans-AM kit used according to the manufacturer's specifications (Active Motif, Carlsbad, CA). Briefly, the transcriptional factor of nuclear extracts, which were prepared by Nuclear Extract kit (Active Motif, Carlsbad, CA), were captured by binding to a consensus oligonucleotide (5′-CTTGGA-CATGCCCGGGCATGTCCCTC-3′) immobilized on a 96-well plate. The amount of p53 was determined in a colorimetric reaction using specific primary antibody and a secondary horseradish peroxidase-conjugated antibody. Spectrophotometric data were expressed as a ratio of absorbance of each experimental condition compared with control cells exposed to vehicle alone (*21*).

Stable Transfection. Transfection of MCF-7 cells was carried out using Lipofectamine 2000 reagent (Life Technologies). MCF-7 cells were exposed to the mixture of Lipofectamine 2000 reagent and pCMV-p53mt135 plasmid or empty vector for 6 h. After transfection, cells resistant to neomycin were selected by incubating with medium containing 1 mg/mL G418 (Geneticin) (Life Technologies), and then individual MCF-7 clones were isolated and tested for constitutive p53 expression. The p53-positive MCF-7 cells were selected and maintained in the presence of G418 (400 μg/mL), as were p53-negative control cells (*21*).

Statistical Analysis. Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of control and tricetin-treated cells were analyzed by Dunnett's test.

RESULTS

Chemical Structure of Tricetin. Figure 1 represents the chemical structure of tricetin (5,7,3′4′,5′-pentahydroxyflavone).

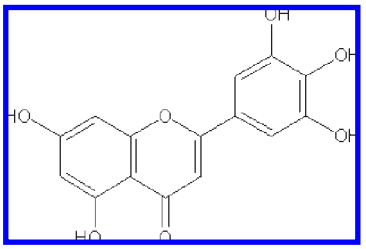

Figure 1. Chemical structure of tricetin (5,7,3′,4′,5′-pentahydroxyflavone), a flavonoid derivative, occurs naturally in Myrtaceae pollen and *Eucalyptus* honey.

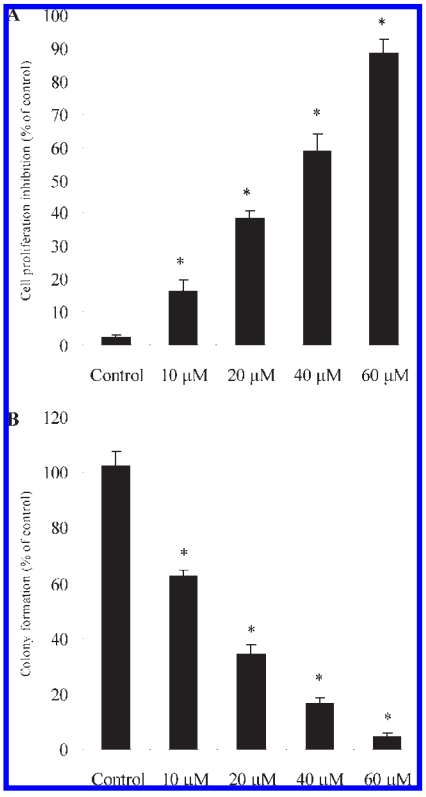

Figure 2. The effects of tricetin on cell proliferation inhibition and colony formation in human breast adenocarcinoma MCF-7 cells. (**A**) Inhibition effect of tricetin on MCF-7 cell proliferation. (**B**) Influence of MCF-7 on the number of colony-forming cells, as evaluated by clonogenic assay. Cell growth inhibition activity of tricetin was assessed by XTT. For colony-forming assay, the clonogenic assay was performed as described in Materials and Methods. Results are expressed as the percentage of cell proliferation relative to the proliferation of control. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and tricetin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

Tricetin Inhibits Cell Proliferation and Clonogenic Survival in Human Breast Adenocarcinoma Cells. To investigate the potential cell proliferative inhibition activity of tricetin in human breast adenocarcinoma cells, we first examined the effect of tricetin on cell proliferation and clonogenic survival in MCF-7 cells. As shown in **Figure 2A**, exposure of MCF-7 cells to tricetin for 48 h inhibited the growth of MCF-7 cells in a dose-dependent manner. The IC_{50} values of tricetin were 32.17 μM.

The anticancer activities of tricetin were assessed by clonogenic assays. MCF-7 cells showed the ability to form clones in the untreated control wells. However, with the addition of tricetin, a dose-dependent inhibition in clonogenicity was observed, with a >90% inhibition at a dosage of 60 μM tricetin (**Figure 2B**).

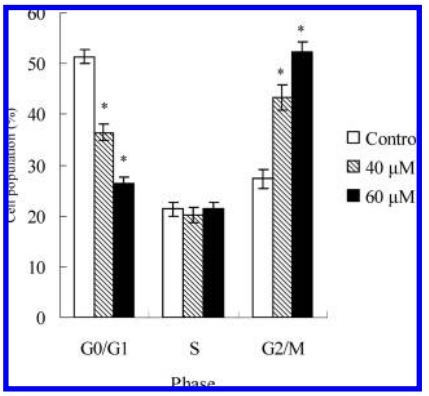

Figure 3. The effects of tricetin on cell cycle distribution in MCF-7 cells. MCF-7 cells following treatment with vehicle alone (0.1% DMSO) and tricetin (40 and 60 μM) for the indicated times were fixed and stained with PI, and cell cycle distribution was then analyzed by flow cytometry. The data indicate the percentage of cells in G0/G1, S, and G2/M phases of the cell cycle. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and tricetin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

Tricetin-Induced Cell Cycle Arrest and Apoptosis in MCF-7 Cells. To investigate the mechanisms leading to loss of cell proliferation by tricetin, we tested whether the observed inhibition effects of tricetin on cell proliferation are due to induction of cell cycle arrest. As shown in **Figure 3**, treatment of MCF-7 cells with 40 and 60 μM tricetin increased the percentage of cells in the G2/M phase after 6 h exposure (**Figure 3**).

We next assessed the effect of tricetin on the induction of apoptosis in MCF-7 cells by DNA fragmentation assay. A quantitative evaluation was also made using TUNEL to detect DNA-strand breaks. Compared to vehicle-treated cells, tricetin induced 30.47% and 45.98% of apoptotic cells in MCF-7 cells at concentrations of 40 and 60 μM at 48 h respectively (**Figure 4A**). TUNEL-positive cells were also made visible using a fluorescence microscope (**Figure 4B**).

The Effect of Tricetin on G2/M Phase-Related Factors. Because our studies showed that tricetin treatment of MCF-7 cells results in G2/M phase cell cycle arrest, we examined the effect of tricetin on cell cycle-regulatory molecules. We first assessed the status of p53 in tricetin-treated MCF-7 cells. Exposure of cells to 60 μM tricetin enhanced the phosphorylation of p53 on Ser15 and Ser392 (**Figure 5**). Phosphorylation of p53 on serine residues 6, 9, and 46 were undetectable. Tricetin treatment was also associated with an increase in p53's downstream target, p21 (**Figure 5**). Similarly, tricetin caused a significant time-dependent increase in the phosphorylation (Ser1981) of ATM protein in cells. However, tricetin treatment did not cause any change in the protein levels of total ATM. Exposure of MCF-7 cells to tricetin resulted in increased levels of the phosphorylated (activated) form of H2A.X (Ser139) (a variant form of histone H2A) and NBS1 (Ser343), which are directly influenced by activated ATM kinase (*8*, *9*) (**Figure 5**). Furthermore, treatment of MCF-7 cells with 60 μM tricetin resulted in rapid and sustained activation of Chk2 (phosphorylation at Ser345) (**Figure 5**).

Tricetin treatment of MCF-7 cells resulted in a time-dependent decrease in the protein expression of cyclin B, cyclin A, cdc2, and cdc25C (**Figure 5**). In addition, exposure of these cells to tricetin for 2 h resulted in increased levels of inactive phospho-cdc2 (Tyr15) and phospho-cdc25C (Ser216). Results from time-dependent

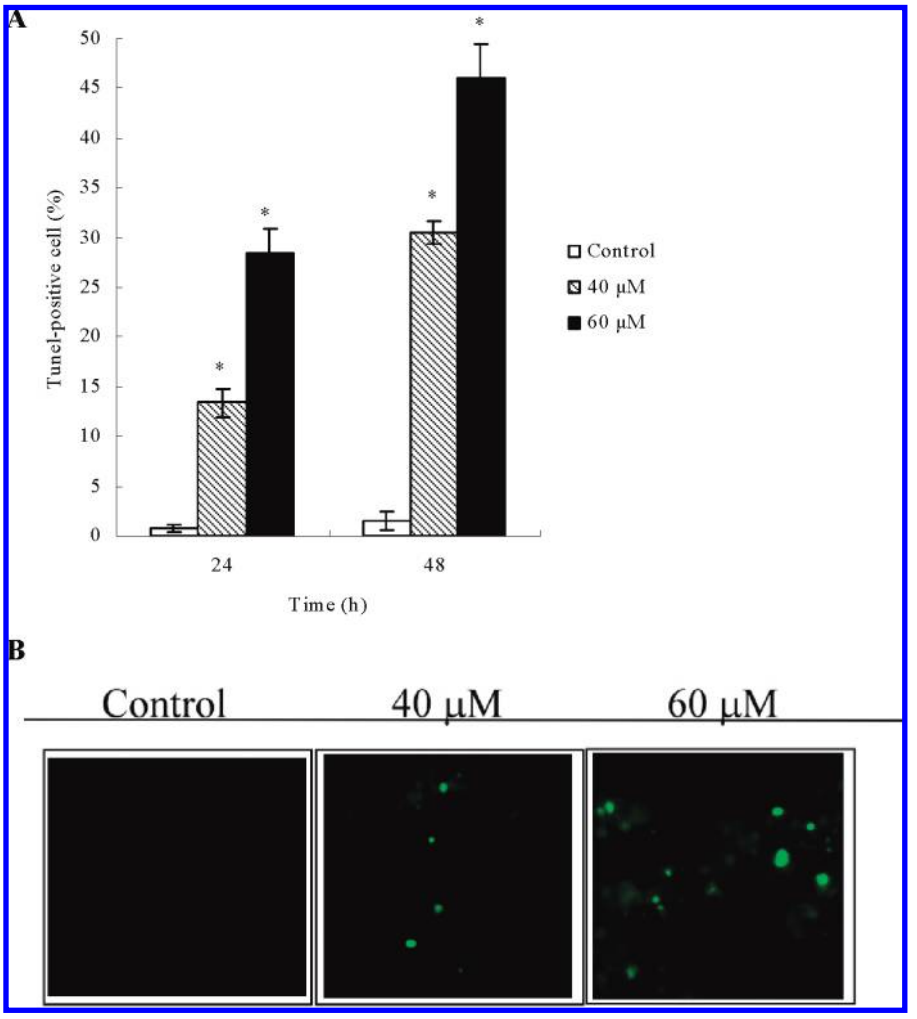

Figure 4. Tricetin induced apoptosis. Quantitative evaluations of TUNEL assay by flow cytometry (**A**) and fluorescence microscope (**B**). For **A** and **B**, TUNEL-positive cells were examined by flow cytometry, and were visible by means of fluorescence microscope. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and tricetin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

studies have indicated that increasing phosphorylated Chk2 is followed by an increase in phospho-cdc25C, which in turn increased phospho-cdc2 (**Figure 5**).

Tricetin Decreases the Interaction of p53 with MDM2. Previous studies have shown that the function and stability of p53 is principally regulated by phosphorylation at various sites (*11*). We assessed the DNA binding activity of p53 using the ELISA-based method, and the interaction of p53 with MDM2 by immunoprecipitation assay. As shown in **Figure 6A**, tricetin treatment resulted in the enhancement of p53 DNA binding activity. The enhancement of p53 transcriptal activity is correlated with the phosphorylation of p53 at Ser392. Furthermore, the association of p53 and MDM2 decreased in a time-dependent manner, which correlated with the phosphorylation of p53 at Ser15 (**Figure 6B**).

The Role of ATM on Tricetin-Mediated Cell Cycle Arrest. To verify the possible role of ATM in tricetin-mediated G2/M arrest, MCF-7 cells were pretreated for 1 h with caffeine, a specific inhibitor for ATM. Subsequently, the inhibitor-treated cells were exposed to tricetin, and then cell cycle distribution and associated events were examined. As shown in **Figure 7A**, tricetin-mediated ATM activation was effectively inhibited by 2.5 mM of caffeine. Flow cytometric analysis of MCF-7 cells exposed to tricetin for 6 h showed that caffeine blocked tricetin-mediated G2/M arrest (**Figure 7B**). In addition, pretreatment of cells with caffeine also decreased the tricetin-mediated phosphorylation of p53 (Ser15) and Chk2 (**Figure 7A**).

The Role of ATM on Tricetin-Mediated Cell Cycle Arrest and Apoptosis. To further define the role of p53 in tricetin-induced cell cycle arrest and apoptosis, we transfected pCMV-p53mt135 plasmid containing a gene encoding a dominant negative mutation of p53 that blocks normal p53 activity (*21*). Overexpression of mutant p53 protein in cells transfected with the dominant negative p53 mutant plasmid was verified by immunoblot using antibodies for human p53 (recognizing both wild and mutant types of p53) (**Figure 8A**). Cells expressing the p53 mutant were subsequently used to document tricetin-mediated cell cycle arrest and apoptosis. As shown in **Figure 8B**, the inhibition of p53 activity was accompanied by a reduction in the sensitivity of MCF-7 cells to tricetin-mediated G2/M arrest. Furthermore, compared to vehicle-treated cells, induction of apoptosis by 60 μM tricetin decreased from 36.3% in MCF-7 cells to 14.2% in p53 mutant cells after a 48 h treatment (**Figure 8C**).

Tricetin Induced Apoptosis by Initiation of Mitochondrial Pathway. To investigate the mitochondrial apoptotic events involved in tricetin-induced apoptosis, we first analyzed the changes in the levels of the Bcl-2 family proteins. Immunoblot analysis showed that treatment of MCF-7 cells with tricetin increased Bax and Bak protein levels (**Figure 9A**). In contrast, tricetin markedly decreased Bcl-2 levels, which led to an increase in the Bax/Bcl-2

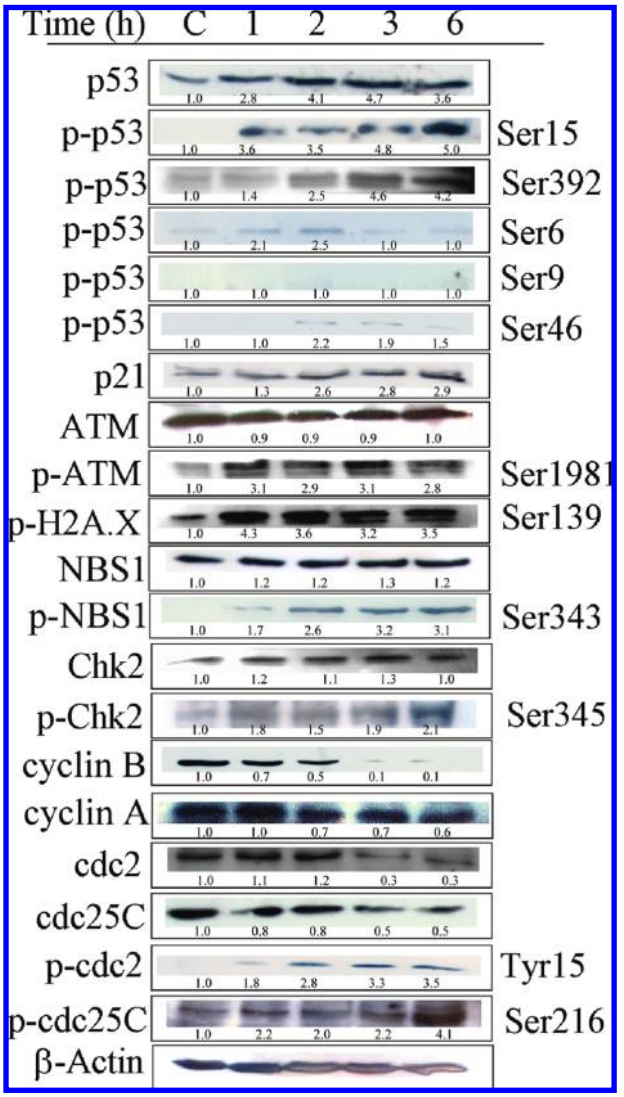

Figure 5. The effect of tricetin in cell cycle-related factor. Cells were treated with 60 μM tricetin. The levels of p53, phospho-p53, p21, ATM, phospho-ATM, phospho-H2A.X, phospho-NBS1, Chk2, phospho-Chk2, cyclin B, cyclin A, cdc2, cdc25C, phospho-cdc2, and phospho-cdc25C were assessed by immunoblot assay. Results shown are representative of three independent experiments.

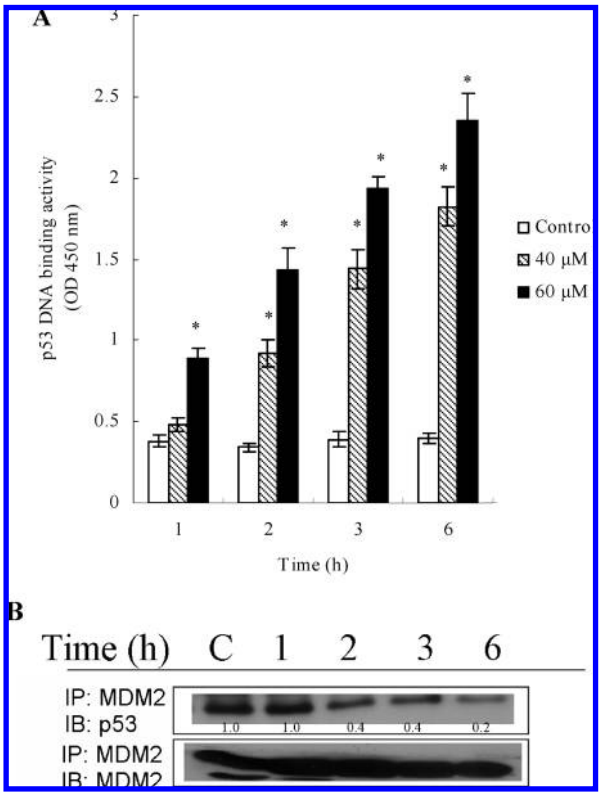

Figure 6. Tricetin increased the DNA binding activity and stability of p53. For **A**, cells were treated with vehicle alone (0.1% DMSO) and tricetin (40 and 60 μM) for the indicated times, and then DNA binding activity was assessed by TranAM p53 activity kit. For **B**, cells were treated with 60 μM tricetin and the interaction of p53 with MDM2 was assessed by immunoprecipitation. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and tricetin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

ratio (**Figure 9A**). Additionally, tricetin also decreased the expression of Bcl-X_L. These effects of tricetin on the Bcl-2 family proteins resulted in the decrease of mitochondrial membrane potential in MCF-7 cells (**Figure 9B**).

One of the hallmarks of the apoptotic process is the activation of cysteine proteases, which represent both initiators and executors of cell death. Upstream caspase-9 activities also increase significantly when MCF-7 cells are treated with tricetin (**Figure 9C**). However, when cells are pretreated with the specific caspase-9 inhibitor LEHD-CHO before tricetin treatment, the apoptosis induction effect of tricetin on MCF-7 cells decreased (**Figure 9D**).

DISCUSSION

Breast cancer is the most common neoplasm in women of both developed and developing countries (*22*). Epidemiological studies suggest that consumption of flavonoid protects against cancer, but its absorption in human is controversial. Flavonoids are mainly present as glycosides in plants and foods, and it has been thought that the aglycons are the predominant form that is capable of being absorbed by the gastrointestinal tract (*23*). However, other studies have shown that flavonoid glycosides can be absorbed without preceding hydrolysis (*24*, *25*). In our study, we have found that tricetin effectively inhibits tumor cell growth, concomitant with induction of cell cycle arrest and apoptosis. Therefore, tricetin, a glycoside form of flavonoid, could have physiologic value taken in a natural food form.

Tumor suppressor gene p53 is a key element in the induction of cell cycle arrest and apoptosis following DNA damage or cellular stress in human cells (*11*, *26*). Cell-cycle arrest dependent on p53 requires transactivation of p21 or other cell cycle−related factors (*27*). The induction of p21 causes subsequent arrest in the G0/G1 or G2/M phase of the cell cycle by binding of the cyclin−cdk complex (*28*, *29*). In this study, we have shown that treatment of MCF-7 cells with tricetin resulted in the accumulation of p53 and phospho-p53 (Ser15). We have also found that suppression of normal p53 activity via dominant-negative p53 decreased tricetin-induced G2/M arrest, suggesting that p53 plays an important role in tricetin-triggered cell cycle checkpoint. Indeed, we also have found that tricetin decreases the expression of cyclin B, cyclin A, cdc25C, and cdc2, while it increases the amount of p21 and phosphorylation of cdc2, phospho-cdc25C and phospho-Chk2. Our results further indicated that tricetin induces phosphorylation of cdc25C (Ser216) through Chk2 activation while remaining cdc25C inactive. Further downstream, inactivated cdc2 was not dephosphorylated by cdc25C. Therefore, cdc2 accumulated in an inactive phosphorylated state (Tyr15), resulting in cells that were unable to proceed through the mitotic phase. These data suggest

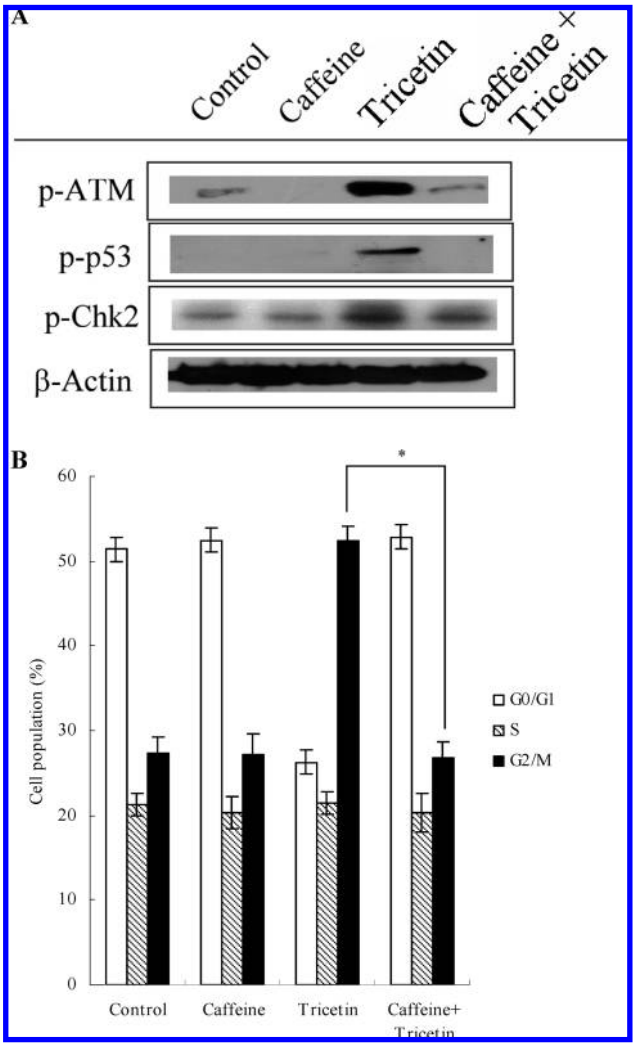

Figure 7. ATM inhibitor blocked tricetin-mediated cell cycle arrest and phosphorylation of p53 and Chk2. For all blocking experiments, cells were incubated for 1 h in the presence or absence of 2.5 mM caffeine, then 60 μM tricetin was added and incubated for 3 h for ATM, Chk2 and p53 phosphorylation (**A**), and 6 h for cell cycle analysis (**B**). Cell cycle distribution was assessed by flow cytometric analysis. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test groups, as analyzed by Dunnett's test ($p < 0.05$).

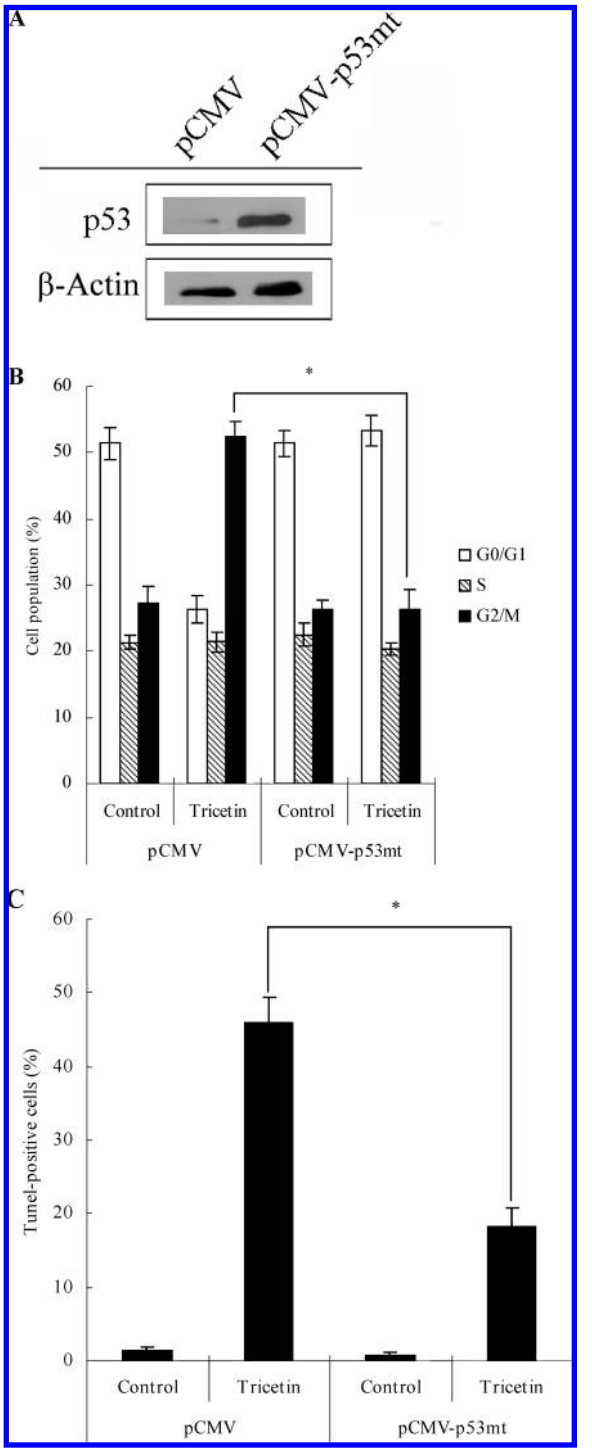

Figure 8. Effect of p53 inhibition on tricetin-mediated cell cycle arrest and apoptotic cell death. (**A**) The expression of p53mt in stable pCMV-p53mt transfected MCF-7 cells. The p53 inhibition decreased the effect of tricetin on cell cycle arrest (**B**) and apoptosis induction (**C**). MCF-7 cells and mutant p53-transfected MCF-7 cells were treated with 60 μM tricetin for 6 h (cell cycle assay) and 48 h (apoptosis assay). The induction of apoptosis was determined by TUNEL analysis. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between two test groups, as analyzed by Dunnett's test ($p < 0.05$).

that tricetin may prove to be a valuable tool for inhibition of cdc2/cyclin B and cdc2/cyclin A complex in breast cancers for the following reasons: (1) the downregulation of cyclin B and cyclin A by tricetin, (2) the induction of p21 by tricetin in a p53-dependent manner, which may subsequently inhibit the function of cdc2, and (3) the increase of activated Chk2 followed by an increase in inactivated phospho-cdc25C and phospho-cdc2, suggesting that increased cdc25C phosphorylation by Chk2 may also decrease functioning phosphatase for dephosphorylating and activating cdc2.

ATM pathway has been demonstrated to be associated with the triggering of cell cycle checkpoint by both DNA damage-dependent and independent manners (*6*−*8*, *30*). Once activated, ATM phosphorylates various downstream molecules such as p53, MDM2, Chk1, Chk2, H2A.X, and Nijmegen breakage syndrome (NBS1), resulting in cell cycle arrest or cell death (*8*−*10*). Phosphorylated NBS1 is an adaptor molecule for ATM-dependent phosphorylation of Chk2, which phosphorylates the cdc25 phosphatase and is responsible for S and G2/M phase checkpoints (*6*, *7*, *31*−*33*). ATM phosphorylates p53 at Ser15, resulting in prolonging of p53 half-life by inhibiting p53-MDM2 complex formation (*34*, *35*). In this report, we have shown that treatment of MCF-7 cells with tricetin resulted in the accumulation

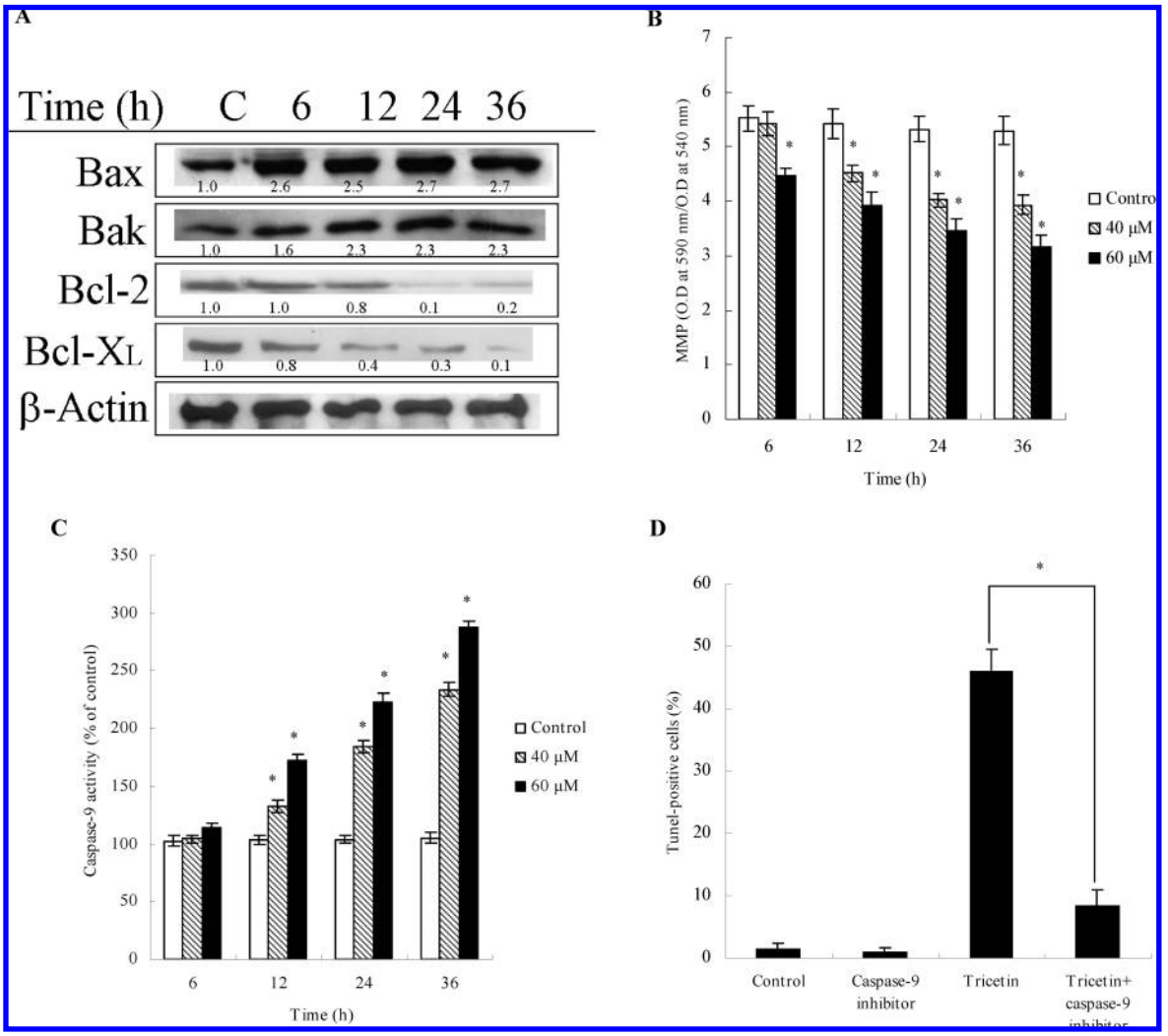

Figure 9. Tricetin induces apoptosis through the mitochondrial apoptotic pathway. (**A**) The effect of tricetin on expression of the Bcl-2 family protein. (**B**) Tricetin caused a decrease of mitochondrial membrane potential, but (**C**) increased caspase-9 activation. (**D**) Caspase-9 inhibitor decreased tricetin-induced apoptosis. Cells were treated with 60 μM tricetin, and the levels of Bcl-2 family proteins were assessed by immunoblot assay. The $\triangle\Psi$m was measured by JC-1 staining. Caspase-9 activity was measured by caspase-9 activity assay kit. For caspase-9 inhibitor blocking experiments, cells were preincubated with LEHD-CHO (20 μM) for 1 h before the addition of 60 μM tricetin for an additional 48 h. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and tricetin-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

of phospho-ATM at Ser1981. This ATM activation correlated well with the tricetin-induced increase of H2A.X and NBS1 phosphorylation. Furthermore, we observed that blocking the tricetin-induced activation of ATM by the specific inhibitor caffeine could prevent p53 phosphorylation (Ser15), suggesting that tricetin-induced ATM activation contributes to the stabilization of p53 function by Ser15 phosphorylation, which decreases the interaction of p53 and MDM2. In addition, our results reveal that exposure of MCF-7 cells to tricetin led to a concurrent phosphorylation of Chk2, whereas caffeine pretreatment inhibited Chk2 phosphorylation, suggesting that the activation of ATM induced by tricetin is involved in the activation of Chk2. Moreover, the ATM inhibitor caffeine prevented tricetin-induced G2/M arrest, further suggesting that cooperation of ATM with Chk2 and the p53-dependent pathway plays a crucial role in tricetin-induced G2/M arrest.

Mitochondrial apoptotic pathway has been described as an important signaling of apoptotic cell death for mammalian cells (*36*, *37*). Following the treatment of MCF-7 cells with tricetin, we observed that tricetin treatment resulted in a significant increase of Bax and Bak expression, and a decrease of Bcl-2 and Bcl-X_L, suggesting that changes in the ratio of the proapoptotic and antiapoptotic Bcl-2 family proteins might contribute to the apoptosis-promotion activity of tricetin. Our findings have also shown a collapse of $\triangle\Psi$m and the activation of caspase-9 after MCF-7 cells were treated with tricetin. These mitochondrial apoptotic events are correlated with the modulation of tricetin on Bcl-2 family proteins. These results confirm that tricetin-induced apoptosis is associated with regulation of Bcl-2 family proteins.

In conclusion, this study demonstrates that (a) human breast adenocarcinoma MCF-7 cells are highly sensitive to growth inhibition by tricetin, (b) reduced survival of MCF-7 cells after exposure to tricetin is associated with G2/M phase cell cycle arrest and induction of apoptosis, (c) tricetin can inhibit cell cycle progression at the G2/M phase by increasing p21 expression in a p53-dependent manner, and by decreasing the expression of cdc2, cdc25C, and cyclin B, (d) tricetin-induced cell growth inhibition in the MCF-7 cells is mediated by activation of ATM, which stabilizes p53 by phosphorylation of p53 at Ser15, and by decreasing the interaction of p53 and MDM2, (e) tricetin-mediated ATM activation also phosphorylates Chk2, and subsequently increases the accumulation of inactived cdc25C and

cdc2, and finally, (f) tricetin triggers mitochondrial apoptotic pathway by regulation of the Bcl-2 family protein expression. These findings suggest that tricetin may be a promising chemopreventive agent in the fight against human breast adenocarcinoma cells.

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