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Furanodiene Enhances Tamoxifen-Induced Growth Inhibitory Activity of ERa-Positive Breast Cancer Cells in a PPARγ Independent Manner

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ABSTRACT

Herbal plants are enriched with compounds with a wide range of biological activities. Furanodiene is a sesquiterpene isolated from *Rhizoma Curcumae*. Growing evidence shows furanodiene exhibits diversified activities of hepatoprotection, anti-inflammation, anti-angiogenesis, and anti-tumor. However, its biological activities against breast cancer have not been deeply understood, and its potential as an anti-breast cancer agent combined with tamoxifen (TAM) has not been evaluated so far. This study describes the combined effects of furanodiene and TAM in human breast cancer cells in vitro. The results showed that ERa-negative MDA-MB-231 cells were much more sensitive than ERa-positive MCF-7 cells to the growth inhibition due to furanodiene. Combined administration of furanodiene and TAM led to marked increase in growth inhibition, cell cycle arrest and pro-apoptotic activity in ERa-positive cells compared to individual agent, and enhanced the down-regulation of p-cyclin D1, cyclin D1, CDK2, CDK6, p-Rb, Rb and p-p44, and the up-regulation of p27, Bax and Bad, but did not show increased cytotoxicity in ERa-negative MCF-10A non-tumorigenic breast epithelial cells. Co-incubation induced the typical PARP cleavage or caspase 9 cleavages compared to individual agent. In addition, PPARγ activity inhibition by its antagonist T0070907 did not significantly reverse the enhanced effect of furanodiene and TAM suggesting that anti-cancer properties of combination were PPARγ independent. Our data indicated that furanodiene could enhance the growth inhibitory and pro-apoptotic activity of TAM by inducing cell cycle arrest and cell apoptosis via CDKs–cyclins and mitochondria-caspases-dependent, and PPARγ-independent signaling pathways in breast cancer cells, without contributions to the cytotoxicity of TAM. J. Cell. Biochem. 113: 2643–2651, 2012.

KEY WORDS: FURANODIENE; TAMOXIFEN; BREAST CANCER; CELL CYCLE; APOPTOSIS; PPARγ

Natural products have been regarded as important sources of potential anti-cancer agents. Curcumin is the main active flavonoid derived from the rhizome of *Curcuma* [Minami et al., 2009]. Epidemiological evidence shows that lower incidence rates of many types of cancer occur in India where curcumin is widely consumed, suggesting the role of curcumin in cancer prevention [Lopez-Lazaro, 2008]. Pharmacodynamic studies exhibit that curcumin not only potentially inhibits cell proliferation and survival in several types of cancer [Goel et al., 2008], but also demonstrates synergistic effects with many chemotherapeutic agents such as 5-FU, paclitaxel, doxorubicin, and reduces the toxicity induced by anti-cancer drugs [Nautiyal et al., 2011]. However, the bioavailability of curcumin is relatively poor in clinical trials [Dhillon et al., 2008]. β-elemene is a main sesquiterpene isolated from the essential oil of *Curcuma* [Li et al., 2010], and it has been approved by China's State Food and Drug Administration as a secondary class innovative anti-cancer drug. β-elemene also exerts anti-cancer potential in many types of cancer [Li et al., 2005; Wang et al., 2005], especially in brain carcinomas due to the capacity of passing through the blood-brain barrier [Wu et al., 2009], which also indicated its potential for treating cerebral malignancy. Furthermore, it shows synergistic effects with other

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chemotherapeutic drugs [Zhao et al., 2007; Zhang et al., 2011]. Actually, besides the well-known curcumin and β-elemene, there is a rich source of compounds with a wide range of biological activities in herbal plants [Tan et al., 2011]. Furanodiene is another sesquiterpene isolated from the essential oil of *Curcuma wenyujin* [Yang et al., 2005] exhibiting hepatoprotective, anti-inflammatory, anti-angiogenic and anti-tumor activities [Matsuda et al., 1998; Makabe et al., 2006; Xiao et al., 2007; Zhong et al., 2011b]. Previous studies reported that furanodiene significantly inhibited Hela, Hep-2, HL-60, U251 cells proliferation and also suppressed the proliferation of uterine cervix (U14) tumor induced in mice [Xiao et al., 2007; Ma et al., 2008; Sun et al., 2009]. Furanodiene induced apoptosis of HL60 and HepG2 cells, characterized by DNA fragmentation, cleavage of caspases and poly (ADP-ribose) polymerase (PARP), which were involved in mitochondria-caspases apoptotic pathway [Xiao et al., 2007; Ma et al., 2008]. Moreover, furanodiene caused cell cycle arrest at G2/M, accompanied with activation of p38 and inhibition of ERK mitogen-activated protein kinase (MAPK) signaling in HepG2 cells [Xiao et al., 2007]. HL60 cells exposed to furanodiene resulted in the up-regulation of tumor necrosis factor receptor 1 (TNFR1), the formation of TNFR1 complex and remarkable production of TNF-α [Ma et al., 2008]. And recently, we also found that furanodiene exhibits a potential anti-angiogenic effect through suppression of endothelial cell growth, invasion, migration and tube formation via regulation of the PI3K pathway [Zhong et al., 2011b].

TAM has been considered as a promising therapeutic approach against breast cancer by blockade of ERa and inducing cell cycle arrest. Cell cycle is a series of events involving cell division and replication, and is closely related with tumor development as well. It is widely accepted that cellular proliferation is driven by cell cycle specific complexes of cyclins and cyclin-dependent kinases (CDKs), which are regulated by CDK inhibitors (CDKIs) such as molecular markers, p21 and p27. CDK–cyclin complexes initiate a signaling cascade involving phosphorylation of retinoblastoma tumor suppressor protein (Rb) [Sherr, 1996; Srivastava et al., 2007]. Deregulation of a normal cell-cycle machinery is an integral part in neoplasia process. Previous reports suggested that overexpression of CDK 6 and cyclin D1, and downregulation of $p27^{Kip1}$ might be highly associated with development of carcinogenesis [Yamamoto et al., 2010; Zhong et al., 2010]. An early decline of Rb phosphorylation induced by anti-estrogen resulted in G1 cell cycle arrest and a reduced cyclin D1/Cdk4 activity leading to anti-proliferative effects through increasing CDK inhibitor abundance to repress Cdk2 and Cdk4 activities [Watts et al., 1995]. TAM could cause MCF-7 cell cycle arrest, accompanied with up-regulation of p21 and p27 levels, down-regulation of cyclins and CDKs levels, and a decrease in binding affinity [Cariou et al., 2000; Kilker and Planas-Silva, 2006].

However, not all tumor cells are sensitive to TAM, so there is an urgent need for exploring and overcoming the TAM-resistance [Cittelly et al., 2010; McClaine et al., 2010; Davies and Hiscox, 2011]. In the present study, we found that furanodiene treatment produced a concentration-dependent decrease in cell viability for both cell lines MCF-7 and MDA-MB-231, and ERa-negative MDA-MB-231 cells were more sensitive than ERa-positive MCF-7 cells on the growth inhibitory effect of furanodiene. Recently it has been shown that ERa binding to peroxisome proliferator-activated receptor (PPAR) response element negatively interferes with PPARγ signaling in breast cancer cells [Keller et al., 1995; Yu et al., 2008b]. PPARγ is expressed by cell lines derived from many phenotypic breast tumors, and it plays an important role in the growth characteristics of breast cancer cells [Colin et al., 2010; Rubin et al., 2000]. Indeed, PPARγ ligands, such as troglitazone can induce apoptosis both in vitro and in vivo [Elstner et al., 1998; Yu et al., 2008a]. Recent reports show that combining ERa inhibitor with PPARγ agonist could be a better therapeutic approach for ERa-dependent breast cancer [Yu et al., 2008b; Lee et al., 2009]. However, it is not yet known whether furanodiene is involved in PPARγ-dependent growth inhibition in breast cancer cells, and also whether ERa contributes to the different effects of furanodiene between MCF-7 and MDA-MB-231 cells. More importantly, the effect of furanodiene on the growth-inhibitory activity of TAM in breast cancer cells has not yet been studied. Firstly we determined the cytotoxic activity of furanodiene, and then compared the inhibition discrepancy between human breast cancer cell lines MDA-MB-231 and MCF-7 in combination of furanodiene and TAM treatments.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Furanodiene (>96%) was isolated and identified by Prof. Shaoping Li (University of Macau, Macau, China) as previously described [Yang et al., 2005]. MCF-7 cell line, MDA-MB-231 cell line, T-47D cell line and MCF-10A cell line were purchased from American Type Cell Culture (Manassas, VA). RPMI-1640, phenol red-free DMEM and Kaighn's modification of Ham's F-12 medium (F-12K), fetal bovine serum (FBS), Charcoal Stripped FBS, phosphate buffered saline (PBS), penicillin–streptomycin (PS) and 0.25% (w/v) trypsin/1 mM EDTA were purchased from Invitrogen (Carlsbad, CA). MTT, propidium iodide (PI), TAM, dimethyl sulfoxide (DMSO), epidermal growth factor (EGF), Hydrocortisone, cholera toxin, insulin, T0070907 and Hoechst 33342 were supplied by Sigma (St. Louis, MO). Cytotoxicity Detection Kit (LDH) was obtained from Roche Diagnostics (Mannheim, Germany). DNA extraction kit was purchased from Beyotime (Shanghai, China). Anti-PPARγ, CDK6, p27, p-cyclin D1 (Thr286), Cyclin D1, CDK2, p-Rb (Ser807/811), Rb, Bcl-2, Bax, Bad, caspase 9, PARP, β-actin and secondary antibodies and ChIP-Grade Protein G Agarose Beads were obtained from Cell Signaling (Danvers, MA).

CELL CULTURE AND DRUG TREATMENT

MCF-7, MDA-MB-231, T-47D, and MCF-10A cells were cultured as previously reported [Sivko and DeWille, 2004; Hong et al., 2009; Zhong et al., 2011a]. The stock solution of furanodiene (100 mM) and TAM (10 mM) was dissolved in DMSO and then diluted to various concentrations as needed.

CYTOTOXICITY ASSAY

Cells were trypsinized and seeded at 10^4 cells/well in 96-well plates. After a 24 h incubation at 37°C, the complete medium was removed,

and the cells were starved for 24 h for synchronization in low serum medium (0.5% FBS). Following these pre-incubations, different concentration of TAM and furanodiene (alone or in-combination) were added to the culture medium. After incubation for 48 h, cell viability was determined by adding 100 μl MTT (1 mg/ml). Then the MTT-containing medium was aspirated slightly after 4 h and 100 μl DMSO was added to solubilize the formazan followed by shaking 10 min under the dark. The absorbance at 570 nm was recorded using a Multilabel counter (Perkin Elmer, Singapore).

The LDH release from the cells was measured with a cytotoxicity detection kit according to a modification of the manufacturers' protocol. In brief, after 48 h of treatment with different concentration of TAM and furanodiene (alone or in-combination), 70 μl of supernatants (total perfect volume was 100 μl) from culture test or control wells was transferred to a new 96-well plate and then discarded the left supernatants. Cells were then lysed by adding 30 μl of 1% Triton-X lysis solution to each well through incubation for 30 min at 37°C in a humidified incubator with 5% CO_2 atmosphere, 70 μl of PBS solution was then added to the lysates. A 50 μl volume of supernatants was taken from each well for reaction with the LDH substrate after centrifugation at 350*g* for 5 min. The percentage of LDH release was calculated using the following formula:

$$\text{LDH Release Index} = \frac{\text{Spontaneous LDH Release}}{\text{Maximum LDH Release (Supernatants + Lysate Supernatants)}}$$

CELL CYCLE ASSAY

Cell cycle distribution was determined as previously described [Zhong et al., 2011a]. Briefly, cells were collected after treatment, washed twice with ice-cold PBS and harvested by centrifuging at 350*g* for 5 min followed by fixing in 1 ml ice-cold 70% ethanol at −20°C overnight. Then the cells was obtained again by centrifuging and then incubated with 100 μl PI stain solution (20 μg/ml PI, 8 μg/ml RNase) for 30 min protection from light. The cell apoptosis and cell cycle distribution were analyzed using a flow cytometry (BD FACS Canto™, BD Biosciences, San Jose) based on the sub-G1 cell population and DNA content, respectively. The results were analyzed by Mod Fit LT software (version 3.0).

APOPTOTIC MORPHOLOGICAL OBSERVATION

Cells were plated in cover slips and treated with different concentration of TAM and furanodiene (alone or in-combination) for 48 h. And then, cells were fixed with 3.7% paraformaldehyde for 30 min and stained with Hoechst 33342 for 30 min. Photographs were taken at 1,000× magnification using a fluorescence microscope (Olympus MVX10, Japan) equipped with a digital camera (ColorView II, Soft Imaging System, Olympus). Apoptotic cells were distinguished through their characteristic patterns of cytoplasmic rounding, membrane blebbing and nuclear condensation.

AGAROSE GEL ELECTROPHORESIS FOR ANALYSIS OF DNA FRAGMENTATION

Fragmented DNA was isolated by DNA extraction kit according to the manufacturer's instructions. After indicated treatment, the washed-cells were fully suspended in 500 μl of lysis buffer containing with 20 μl of 10 mg/ml proteinase K, and then the samples were incubated overnight in a 37°C water bath. DNA was extracted twice with 500 μl Tris balanced phenol and extracted once with chloroform, supernatants were collected. DNA fibers were obtained by adding 600 μl of cold absolute ethanol (EtOH) containing with 60 μl of 10 mM ammonium acetate and then incubated at −20°C over night. After centrifuging for 10 min at 12,500*g*, DNA fibers were washed once with 600 ml of −20°C 70% EtOH and then dissolved in 50 μl of TE buffer containing 0.2 mg/ml of RNase A for incubation at −4°C over night. Finally, equal amounts of DNA were subjected to electrophoresis on a 1% agarose gel at 90 V for 1 h to observe the appearance of DNA ladder and visualized by a UV light and photographed.

WESTERN BLOT ANALYSIS

Expression of relevant proteins was assessed as previously described [Zhong et al., 2011ab]. Briefly, after washing with PBS and harvesting, cell pellets were lysed using RIPA lysis buffer (Santa Cruz, CA) supplemented with protease inhibitors (1% phosphatase inhibitor cocktail and 1% phenylmethylsulfonyl fluoride). Following a 30-min incubation on ice, cellular debris was removed by centrifugation at 12,000*g* for 20 min at 4°C. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). An equivalent amount of protein from each group was separated by SDS–PAGE and then transferred onto a PVDF membrane. The membrane was probed with primary antibodies, followed by further incubation with HRP-conjugated secondary antibodies. Proteins bands were visualized using enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington Heights, IL).

IMMUNOPRECIPITATION

Two hundred microgram of the cell lysates were mixed with 1 μg of antibody and incubated overnight at 4°C with constant rotation. To recover immunoprecipitated complexes, 50 μl of protein A-sepharose were then added to the lyastes and incubated on ice for additional 4 h of incubation with constant rotation. The beads were harvested by centrifugation and washed three times with RIPA solution. Then the bound proteins were eluted by incubation in 5× SDS loading buffer by boiling at 95°C for 5 min. The eluted proteins were analyzed by Western blot analysis.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. To analyze the data produced from experiments with two independent variables, one-way analysis of variance (ANOVA) was performed using GraphPad Prism software (GraphPad Software). Bonferroni multiple comparison tests were performed for post hoc pairwise comparisons. *P*-values <0.05 were considered statistically significant. Data from at least three independent experiments were used for statistical analysis.

RESULTS

EFFECT OF FURANODIENE IN MCF-7 AND MDA-MB-231 BREAST CANCER CELLS

MCF-7 and MDA-MB-231 cells exposed to furanodiene for 48 h showed a concentration-dependent decrease in cell viability, as measured by MTT assay. However, ERa-negative MDA-MB-231 cells were much more sensitive than ERa-positive MCF-7 cells on the growth inhibitory effect of furanodiene (Fig. 1A).

EFFECT OF FURANODIENE ON THE GROWTH INHIBITORY ACTIVITY OF TAM IN BREAST CANCER CELLS

The combined effects of furanodiene and TAM on breast cancer cell growth were determined after 48 h of treatment. TAM at concentrations of 2.5, 5 and 7.5 μM shows concentration-dependent suppression on MCF-7 cells. Furanodiene at the concentration (25 μM) was chosen as this was non-toxic dose, and also could enhance the growth inhibitory activity of TAM in a concentration-dependent fashion. Furthermore, combination of furanodiene

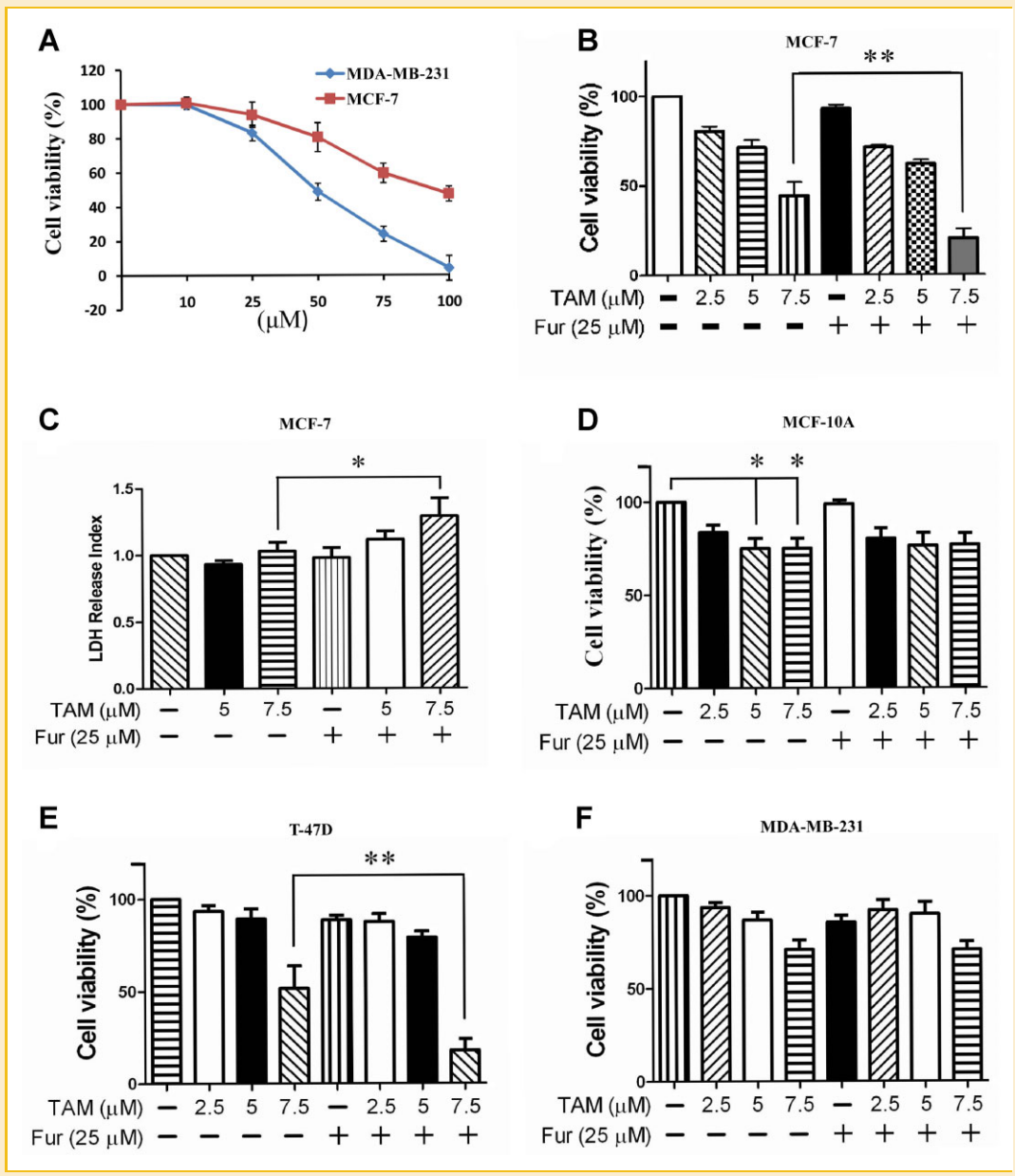

Fig. 1. The effect of furanodiene (Fur) and tamoxifen (TAM; single or combination) on MCF-7, MDA-MB-231, T-47D, and MCF-10A cells proliferation. A: MCF-7 and MDA-MB-231 cells were treated with various concentrations of furanodiene (0–100 μM) for 48 h. MCF-7 (B,C), MCF-10A (D), T-47D (E), and MDA-MB-231 cells (F) were treated with different concentration of TAM (0–7.5 μM) in the absence or presence of furanodiene (25 μM) for 48 h. A,B,D,E,F: Cell proliferation was assessed by MTT assay. C: The LDH release to culture medium was determined by a LDH assay kit. Data shown are expressed as mean ± SEM ($^{*}P < 0.05$, $^{**}P < 0.01$). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

(25 μM) and TAM (7.5 μM) enhanced the growth inhibition nearly 50% compared to TAM (7.5 μM) treatment alone on MCF-7 cells (Fig. 1B). LDH release assay also confirmed that furanodiene dramatically enhanced the growth inhibitory activity of TAM in MCF-7 cells (Fig. 1C).

Furthermore, furanodiene and TAM co-incubation also potently increased growth inhibition of the other ERa-positive breast cancer cell line T-47D (Fig. 1E). However, the same effect was not observed in ERa-negative breast cancer cell line MDA-MB-231 (Fig. 1F).

These results indicate that furanodiene has the capacity of enhancing the growth inhibitory activity of TAM (an anti-ER agent) in ERa-positive breast cancer cells.

EFFECT OF FURANODIENE ON TOXICITY IN MCF-10A NON-TUMORIGENIC HUMAN BREAST EPITHELIAL CELLS

TAM treatment alone induced toxicity to a small extent at the higher concentrations (5 or 7.5 μM) in ERα-negative MCF-10A cells, which is a non-tumorigenic human breast epithelial cell line. However, furanodiene treatment alone did not affect the proliferation of MCF-10A cells at 25 μM. Addition of furanodiene (25 μM) did not intensify cytotoxic activity of TAM on ERa-negative MCF-10A non-tumorigenic breast epithelial cells (Fig. 1D).

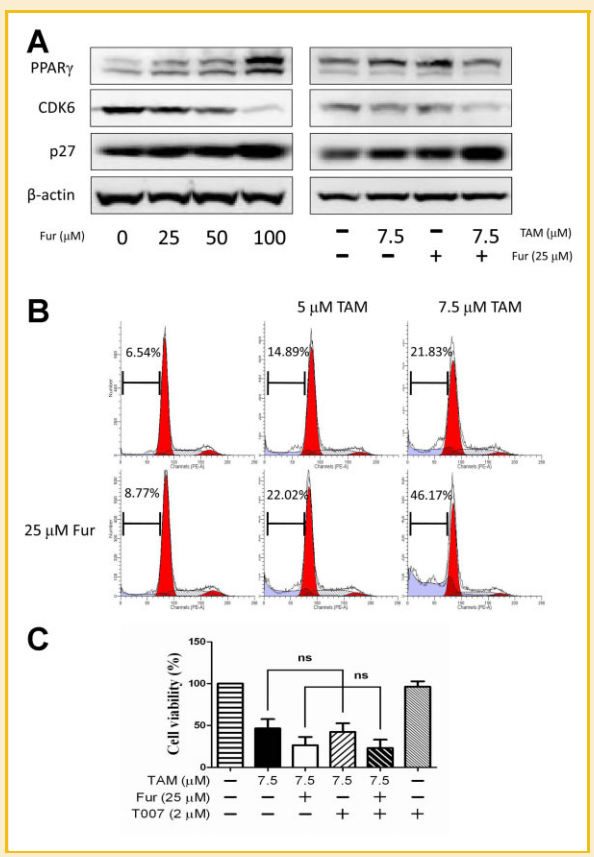

Fig. 2. The effect of furanodiene (Fur) and TAM (single or combination) on the expression levels of relevant proteins, cell cycle progression, and PPARγ-related signaling pathways in MCF-7 cells. MCF-7 cells were exposed to furanodiene and TAM (single or combination) for 48 h with or without T0070907, relevant proteins expression were determined by Western blotting (A), the various phases of the cell cycle were evaluated by flow cytometry (B), and cell viability was assessed by MTT assay (C). Data were representative of at least three independent experiments (ns, not significant). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

EFFECT OF FURANODIENE ON CELL CYCLE ARREST ACTIVITY OF TAM IN MCF-7 CELLS

Furanodiene treatment alone (0–100 μM) caused a concentration-dependent increase of PPARγ and p27 expression in MCF-7 cells, and decrease of CDK6 expression. Unexpectedly, furanodiene exposure (25 μM) could not enhance the PPARγ expression induced by TAM. However, furanodiene exposure (25 μM) could enhance the up-regulation of p27 expression and the down-regulation of CDK6 expression induced by TAM (Fig. 2A).

To further investigate the combined effects of furanodiene and TAM on cell cycle, MCF-7 cells were treated with different concentration of TAM and furanodiene (alone or in-combination) for 48 h. The DNA content of the cellular nuclei was analyzed by flow cytometry. TAM caused an increase in G1-phase and a reduction in G2-phase in a dose-dependent manner. However, combination of TAM and furanodiene, the percentage of cells in sub-G1 or S-phase was markedly different from those treated with TAM alone (Fig. 2B). These results suggest that the combination of furanodiene and TAM synergistically affected cell cycle progression of MCF-7 cells.

PARTICIPATION OF PPARγ IN THE COMBINED ANTI-CANCER EFFECT OF FURANODIENE AND TAM ON MCF-7 CELLS

Figure 2C shows the effects of a PPARγ antagonist, T0070907, on combination of furanodiene and TAM induced cell death in MCF-7 cells. The enhanced effect of furanodiene and TAM was not reversed by T0070907, indicating that the combined anti-cancer property of furanodiene and TAM was not characterized by PPARγ involvement.

EFFECT OF FURANODIENE ON CELL CYCLE ARREST RELEVANT PROTEIN OF TAM IN MCF-7 CELLS

TAM treatment alone (5 or 7.5 μM) decreased the expression of p-cyclin D1, cyclin D1, CDK2 and CDK6, and addition of furanodiene exposure (25 μM) could enhance the down-regulation of p-cyclin D1, cyclin D1, CDK2 and CDK6 expression. TAM treatment alone (5 or 7.5 μM) increased the expression of p27, p-Rb, Rb and p-p44, and addition of furanodiene exposure (25 μM) could enhance p27 expression. However, furanodiene exposure (25 μM) attenuated the expression of p-Rb, Rb, and p-p44 induced by TAM. The expression of p44 was not affected by TAM or furanodiene (alone or in-combination; Fig. 3A).

From Figure 3B, co-incubation of furanodiene and TAM resulted in less formation of the complexes of CDK2/p-cyclin D1, CDK2/p-cyclin D1, and also cyclin D1/Rb.

EFFECT OF FURANODIENE ON PRO-APOPTOTIC ACTIVITY OF TAM IN MCF-7 CELLS

To determine the combined effects of furanodiene and TAM on pro-apoptotic activity of MCF-7 cells, cells were treated with 25 μM

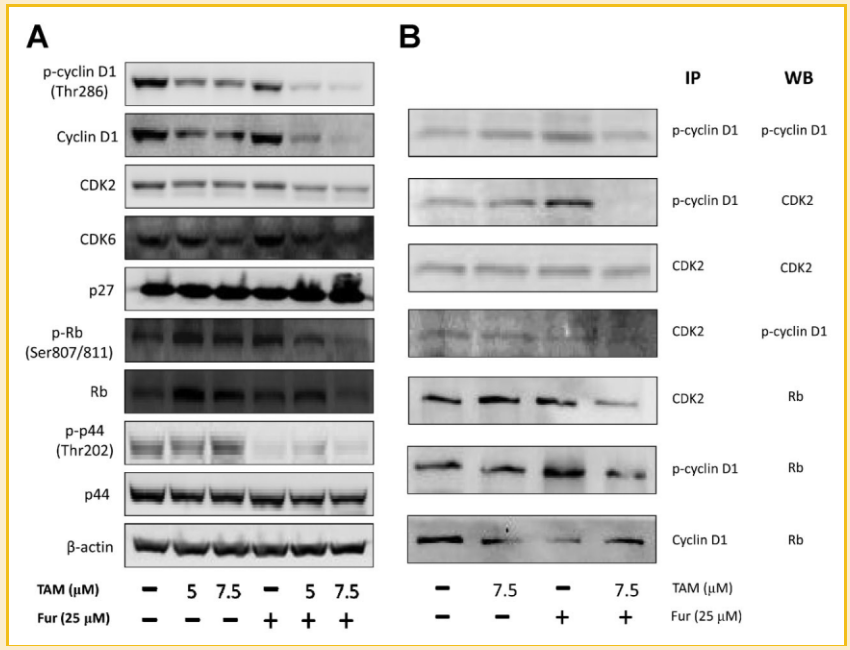

Fig. 3. The effect of furanodiene (Fur) and TAM (single or combination) on the expression levels of cell cycle regulatory proteins in MCF-7 cells. MCF-7 cells were treated with furanodiene and TAM (single or combination) for 48 h, cell cycle regulatory proteins expression were determined through Western blotting (A), and IP (B). All data presented are representative of at least three independent experiments.

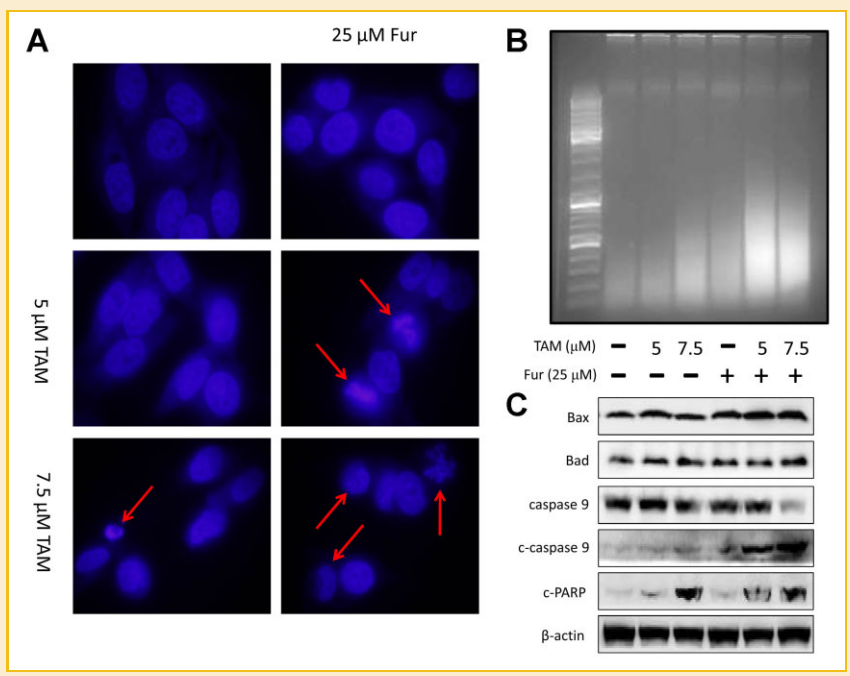

Fig. 4. The effect of furanodiene (Fur) and TAM (single or combination) on nuclear morphology, DNA fragmentation and apoptosis-relevant proteins. MCF-7 cells were treated with furanodiene and TAM (single or combination) for 48 h, the nuclear morphology was determined with Hoechst 33342 staining (A), analysis of DNA fragmentation was determined using agarose gel electrophoresis assay (B), and the expression levels of several apoptosis-relevant proteins were determined by Western blotting (C). All data presented are representative of at least three independent experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

furanodiene and 0–7.5 μM TAM alone or in combination for 48 h. The induction of apoptosis in MCF-7 cells was measured by Hoechst 33342 staining and DNA extraction kit. The combinatorial treatments of furanodiene and TAM caused more pronounced in cellular morphological changes than TAM treatment alone. Nuclear condensation, partition of cytoplasm and nucleus into membrane-bound vesicles (apoptotic bodies) were also increased by co-incubation of furanodiene and TAM compared with TAM treatment alone (Fig. 4A). As shown in Figure 4B, TAM treatment dose-dependently caused an increase in DNA fragmentation, and addition of furanodiene induced a much greater DNA fragmentation. These results suggest that the combination of furanodiene and TAM strongly enhanced apoptosis in MCF-7 cells. To determine whether these enhanced effects were involved in apoptotic signaling, we used Western blotting to show that MCF-7 cells exposed to combination of furanodiene and TAM exhibited the typical PARP cleavage and caspase 9 cleavages, Bax and Bad expression of apoptosis compared to cells exposed to TAM alone (Fig. 4C). However, the change of Bcl-2 expression was not significant in the presence or absence of furanodiene and TAM (data are not shown).

DISCUSSION AND CONCLUSIONS

Furanodiene treatment alone showed strong growth inhibition in both MDA-MB-231 and MCF-7 cells, and ERa-negative MDA-MB-231 cells were more sensitive to furanodiene than ERa-positive MCF-7 cells. These results indicate that ERa maybe inhibit the furanodiene-induced growth inhibitory activity in breast cancer cells. In order to investigate the effect of furanodiene on the growth inhibitory activity of TAM in ERa-positive breast cancer cells, MCF-7 cells were treated with different concentration of TAM (0–7.5 μM) in the presence or absence of 25 μM furanodiene.

Interestingly, the combination of 7.5 μM TAM and 25 μM furanodiene enhanced the growth inhibitory rate by nearly 50% compared to the TAM (7.5 μM) treatment alone. These results indicate that furanodiene significantly enhanced the growth inhibitory activity of TAM in MCF-7 cells. The LDH release results also confirmed that combined treatment of furanodiene and TAM significantly increased growth inhibition of TAM in ERa-positive MCF-7 breast cancer cells. Furthermore, furanodiene exposure did not enhance the cytotoxicity of TAM in ERa-negative MCF-10A non-tumorigenic human breast epithelial cells, which indicates that this ''strengthening'' effect is specific to ERa-positive breast cancer cells without contribution to the cytotoxicity to non-tumorigenic cell line. As expected, combination of furanodiene and TAM also potently increased growth inhibition of the other ERa-positive breast cancer cell line T-47D. However; the same effect was not observed in ERa-negative breast cancer cell line MDA-MB-231. Therefore the additive effect of furanodiene with TAM against breast cancer cells might involve in ER signaling.

Previous study found that ERa inhibited PPARγ ligand signaling in breast cancer cells [Keller et al., 1995], but we found that furanodiene treatment still significantly increased PPARγ expression in ERa-positive MCF-7 cells. However, furanodiene treatment did not enhance PPARγ expression induced by TAM, moreover, inhibition of PPARγ activity by its antagonist T0070907 did not significantly reverse the enhanced effect of furanodiene and TAM, which indicated that the additive effect of furanodiene and TAM might be not involve PPARγ-related signaling pathways. However, we found that furanodiene and TAM co-treatment significantly reduced CDK6 expression and increased p27 expression which implied that the enhanced effect of furanodiene and TAM might involve cell cycle arrest. To confirm that co-incubation of furanodiene and TAM could induce cell cycle arrest, we investigated the combined effects of furanodiene and TAM on cell cycle. Interestingly, as we have expected, furanodiene exposure enhanced the sub-G1 phase arrest induced by TAM.

To further investigate the underlying mechanisms of cell cycle arrest induced by co-incubation of furanodiene and TAM, we investigated the expression levels of p-cyclin D1, cyclin D1, CDK2, CDK6, p-Rb, Rb, p-p44, p44. It is widely accepted that cellular proliferation is driven by cell cycle specific complexes of cyclins and cyclin-dependent kinases (CDKs), which are regulated by direct interaction with CDK inhibitors (CDKIs), such as p27. CDK–cyclin complexes initiate a signaling cascade involving phosphorylation of retinoblastoma tumor suppressor protein (Rb) [Sherr, 1996; Srivastava et al., 2007]. Cyclin D1 is frequently overexpressed in the pathogenesis of breast cancer, and evidence suggests the anti-cancer role of the CDK inhibitors [Barnes and Gillett, 1998; Hunt, 2008]. The early decline of Rb phosphorylation caused by anti-estrogen results in G1 cell cycle arrest and anti-proliferative effects [Watts et al., 1995]. Gefitinib (EGFR/HER2 inhibitor) treatment transmits mitogenic signaling through MAPK, p44/42, leading to cell cycle arrest through down-regulating cyclin D1, p-Rb but up-regulating p27 expression [Piechocki et al., 2007]. In our current study, we found that furanodiene exposure (25 μM) enhanced down-regulation of p-cyclin D1, cyclin D1, CDK2, CDK6, p-Rb, Rb, and p-p44 and up-regulation of p27 expression induced by TAM. Furthermore, the generation of the complexes of p-cyclin D1/CDK2, p-cyclin D1/CDK2, and cyclin D1/Rb were decreased, after the co-treatment of furanodiene and TAM.

Cell apoptosis can regarded as be one of the consequences for cell cycle arrest, therefore we investigated whether the combination of furanodiene and TAM together affected apoptosis of MCF-7 cells. Our results showed that furanodiene at non-toxic concentration induced the apoptosis of TAM-treated MCF-7 cells using nucleus staining and DNA ladder assays, whereas apoptosis was less severe than individual treatment. The cell apoptotic process which includes down-regulation of anti-apoptotic protein and/or up-regulation of pro-apoptotic protein molecules involves serious cascades of caspases activation, resulting in activating the nuclear enzyme poly (ADPribose) polymerase (PARP) [Thornberry and Lazebnik, 1998; Tong et al., 2002; Coultas and Strasser, 2003]. Therefore, the activation of PARP maybe an indicator for early apoptosis signal in human tumor [Bursztajn et al., 2000]. To further understand whether the enhanced effect of furanodiene and TAM were pro-apoptotic, we used Western blotting to display that MCF-7 cells exposed to the combination of furanodiene and TAM induced the typical PARP cleavage or caspase 9 cleavages, and the increased expression levels of Bax and Bad compared to cells exposed to TAM or furanodiene alone.

From these results, we concluded that furanodiene could enhance the growth inhibitory and pro-apoptotic activity of TAM through increasing cell cycle arrest and cell apoptosis via CDKs–cyclins, mitochondria-caspases and PPARγ-independent signaling pathways in breast cancer cells, without contributions to the cytotoxicity to non-tumorigenic cell line. Our findings provide a rational and scientific reference, which suggest co-administration of furanodiene with current chemopreventive agents in the treatment of human breast cancer.

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