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ORIGINAL ARTICLE

Furanodienone induces cell cycle arrest and apoptosis by suppressing EGFR/HER2 signaling in HER2-overexpressing human breast cancer cells

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Abstract
Purpose Overexpression of EGFR and HER2 is seen in breast cancers and results in poor prognosis and decreased patient survival. Clinically, EGFR and HER2 are effective therapeutic targets. The objective of this study is to investigate the in vitro effects of furanodienone, an active chemical component isolated from Rhizoma Curcumae, on the activation of EGFR/HER2 signaling, cell cycle, and apoptosis in HER2-overexpressing BT474 and SKBR3 cells.
Methods Cell growth was assessed by SRB protein assay. Cell cycle analysis was carried out by flow cytometry, and apoptosis was observed by Annexin V and DAPI staining. Effects of furanodienone on the activation of EGFR/HER2 signaling-related proteins were analyzed by western blotting.
Results Furanodienone inhibited cell growth in BT474 and SKBR3 cells. Furanodienone caused G1 arrest in BT474 cells and induced apoptosis in SKBR3 cells. Furanodienone interfered with EGFR/HER2 signaling in treated cells as shown by decreases in phosphorylated EGFR, HER2, Akt, Gsk3β and an increase in $p27^{kip1}$ protein. Accordingly, furanodienone inhibited EGF-induced phosphorylation of EGFR, HER2, Akt, and Gsk3β. EGFR-specific siRNA knockdown did not affect the cell growth inhibitory effect of furanodienone. On the contrary, specific siRNA knockdown of HER2 increased cellular resistance to furanodienone toxicity. In HER-2-deficient MDA-MB-231 cells, the transfection and expression of HER2 increased the sensitivity of cells to furanodienone toxicity.
Conclusion Furanodienone inhibited EGFR/HER2 signaling pathway in BT474 and SKBR3 cells. More importantly, the effect of furanodienone was specifically dependent on HER2, but not EGFR, expression.

Keywords Furanodienone · EGFR · HER2 · Breast cancer

Introduction

Rationally designed, target-directed chemotherapies interfere with specific molecular events that are important in tumor growth, progression, or survival. Targeted therapies have been demonstrated to be effective in experimental models and in clinical situations [1]. One well-known molecular target of high promise in oncology is the ErbB (epidermal growth factor receptor EGFR) family that may activate various signaling pathways responsible for cell survival and proliferation and is frequently overexpressed, dysregulated, or mutated in epithelial tumors [2, 3].

The ErbB family consists of four closely related receptor tyrosine kinases: namely EGFR/HER1/ErbB-1, HER2/ErbB-2/Neu, HER3/ErbB-3, and HER4/ErbB-4. These receptors are composed of an extracellular ligand binding domain, a transmembrane lipophilic segment, and an intracellular receptor tyrosine kinase (RTK) domain [4]. These receptors exist at the plasma membrane in an inactivated state that can be activated upon ligand binding. Specific ligands include epidermal growth factor (EGF), amphiregulin,

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and transforming growth factor (TGF)-α. Other ligands such as HB-EGF, β-cellulin, and epiregulin have dual specificity for binding to EGFR and HER4. HRG-α binds HER3 and HER4. HER2 has no known direct activating ligand and may be in an activated state constitutively. The ligand-bound receptor undergoes a conformational change that induces the formation of homodimer or heterodimer. Dimerization induces phosphorylation of highly conserved tyrosine residues within the receptor cytoplasmic domain with subsequent initiation of several downstream signaling events such as the mitogen-activated protein kinase/extracellular-related kinase 1/2 (MAPK/ERK1/2) and phosphatidylinositol 3-kinase (PI3 K)/Akt signaling pathways which regulate cell proliferation, survival, migration, angiogenesis, and metastasis [1, 5–8]. Since HER2 has no known direct activating ligand, it may become activated upon heterodimerization with other family members such as EGFR.

Breast cancer is the most common cancer diagnosed and second most common cause of cancer-related deaths in women [9]. One of the frequent causes of breast cancers is the dysregulation of the HER-mediated signaling network. One well-known example of this is the amplification of the HER2 gene and the overexpression of HER2 protein which occur in 20–30% of breast cancers [10–17]. The efficacy of targeting HER2 receptor has been demonstrated by the clinical use of monoclonal antibody trastuzumab and a dual tyrosine kinase inhibitor lapatinib. Trastuzumab was introduced in 1998 and inhibits cell proliferation by binding to the extracellular domain of the HER2 receptor and has been becoming an important component of first-line treatment of patients with HER2-positive metastatic breast cancer. Lapatinib has been US FDA approved and is a small-molecular, dual tyrosine kinase inhibitor (TKI) of EGFR and HER2. The drug works by competing with ATP for binding sites on intracellular portions of HER1 and HER2 and interferes with downstream ERK1/2 and PI3 K/Akt pathways that regulate cell proliferation and cell survival [18–21]. Another monoclonal antibody, pertuzumab, binds to a different epitope on HER2 than trastuzumab, blocks dimerization of HER2 with EGFR and HER3, and is under clinical trials. The drug would be expected to inhibit signaling initiated from EGFR/HER2 and HER2/HER3 heterodimers [13]. In anticipation to future needs and developments, identification of novel agents that can inhibit EGFR/HER2 signaling remains important.

The dried rhizome of *Curcuma phaeocaulis* Valeton (Rhizoma Curcumae), a common Chinese medicinal herb, is clinically used in China for the treatment of breast cancer, liver cancer, cervix cancer, and leukemia [22]. One of the main bioactive constituents of Rhizoma Curcumae, furanodienone (Fig. 1a), has been reported to have antiinflammatory and anticancer activities [23, 24]. The present study is the first to describe that furanodienone may interfere with EGFR/HER2 signaling in breast cancer cells. Very interestingly, results presented here also demonstrate that the cell growth inhibitory effects of furanodienone could be selectively affected by the expression level of HER2, but not EGFR.

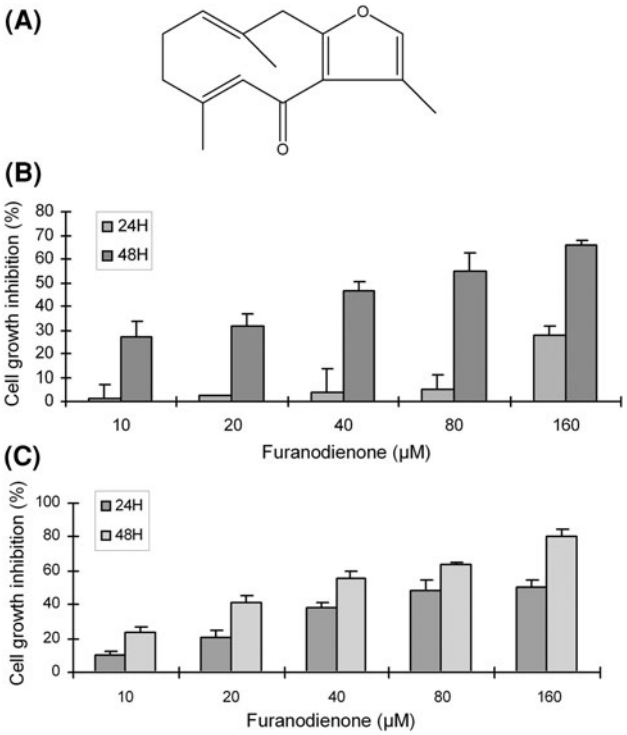

Fig. 1 Chemical structure of furanodienone and effects of furanodienone on HER2-overexpressing cells. **a** Chemical structure of furanodienone. **b** BT474 cells and **c** SKBR3 cells were treated with various concentrations of furanodienone (10–160 μM) for 24 and 48 h. Cell growth was assessed by SRB protein assay. Data shown are mean ± SD from three independent determinations

Materials and methods

Reagents and antibodies

Furanodienone was isolated in our laboratories from Rhizoma Curcumae. The chemical structure of furanodienone was verified by MS and NMR analysis, and the sample used in our studies had a purity >99% as determined by HPLC. Protocols for furanodienone isolation and identification are available upon request. A stock solution of furanodienone was prepared in DMSO to a final concentration of 160 mM and stored at −20°C. Ribonuclease A (RNase A), 4, 6-diamidino-2-phenylindole (DAPI), sulphorhodamine B (SRB), and propidium iodide (PI) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Control siRNA, EGFR siRNA, HER2 siRNA, $p27^{kip1}$, caspase-3, and

GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against HER2, p-EGFR, p-HER2, p-Akt, p-Gsk3β, p-Erk1/2, PARP, and cleaved caspase-3 were purchased from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit IgG antibodies labeled with horseradish peroxidase were purchased from Amersham Biosciences (Piscataway, NJ). Lipofectamine 2000 and pcDNA3.1 expression vector were obtained from Invitrogen (San Diego, CA). Epidermal growth factor (EGF) was purchased from Pepro Tech Inc (Rocky Hill, NJ). Annexin V-FITC Apoptosis Detection Kit was obtained from BD Pharmingen (San Diego, CA). HER2 plasmid was obtained from Addgene. HER2/ErbB2 Kinase Assay Kit was purchased from Cell Signaling Technology (Beverly, MA).

Cell culture and furanodienone treatment

Established human breast cancer BT474 and MDA-MB-231 cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The human breast cancer cell line SKBR3 was a generous gift from Dr. Richard K.W. Choy (Obstetrics and Gynecology Department, The Chinese University of Hong Kong). Cells were grown at 37°C in a humidified 5% CO_2 atmosphere in DMEM (for BT474 and MDA-MB-231) or McCoy's 5A medium (for SKBR3) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, NY, USA).

Furanodienone stock solution in DMSO was diluted with culture medium to various concentrations before adding to cultured cells. In all experiments, the final concentration of DMSO was less than 0.05% (v/v) that showed no significant toxicity.

Cell growth assay

Cells were seeded in 96-well plates at a density of 5×10^3 cells per well and cultured for 24 h. Furanodienone (10–160 μM) was added, and plates were incubated for another 24 or 48 h. Cell growth was evaluated by SRB protein assay [25].

To study the effects of furanodienone on EGF-induced cell proliferation, cells were plated, grown overnight, washed, and further incubated in serum-free medium for 24 h. After the addition of 50 ng/ml EGF and furanodienone (0–80 μM), cells were further incubated for 24 and 48 h. Cell growth was evaluated by SRB protein assay.

Flow cytometric analysis of cellular DNA content

To investigate the effects of furanodienone on cell cycle distribution, BT474 cells were treated with furanodienone (20–80 μM) for 48 h. Cells were collected by centrifugation (500×*g* for 5 min at 4°C), washed twice with PBS, fixed in 70% ethanol, and stored at −20°C. Before flow cytometry cells were washed in PBS and stained in a PI (50 mg/l) solution containing RNaseA (2 mg/l) for 15 min at 37°C. Stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, USA) [26].

To observe the effects of furanodienone on EGF-induced cell cycle progression, BT474 cells were plated in six-well tissue culture dishes, grown overnight, washed, and further incubated in serum-free medium for 24 h. Furanodienone (0–80 μM) and 50 ng/ml EGF were added, and cells were further incubated for 48 h. Cells were collected and analyzed by flow cytometry as mentioned earlier.

Measurements of apoptosis

The percentage of cells undergoing apoptosis was evaluated using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen). SKBR3 cells were treated with furanodienone (0–80 μM) for 48 h, and cells remained attached were harvested and stained with Annexin V-FITC and propidium iodide (PI) according to manufacturer's protocol. Stained cells were analyzed using a FACScan flow cytometer (BD Biosciences, USA).

To verify the occurrence of apoptosis, SKBR3 cells were grown on glass slides, treated with furanodienone (0–80 μM) for 48 h, washed with PBS, and fixed with 4% paraformaldehyde for 10 min. Fixed cells were incubated with DAPI for 10 min and examined with a fluorescence microscope (Nikon, Japan).

Western blot analysis

Cells were treated with furanodienone as described earlier, washed with ice-cold PBS, and lysed in a modified RIPA buffer (in mM: 50 Tris-Cl, 150 NaCl, 1 EDTA, 1 EGTA, 1 phenylmethylsulfonyl fluoride, 1 NaF, 1 Na_3VO_4, and 1% v/v NP-40, 0.35% w/v sodium-deoxycholate, 10 μg/ml each of aprotinin, leupeptin, and pepstatin A, pH adjusted to 7.4) for 20 min at 4°C. Centrifugation (14,000×*g* for 15 min at 4°C) supernatant was collected and regarded as the whole-cell extract. Samples each containing 30–50 μg of proteins were separated by SDS–polyacrylamide gel electrophoresis, and protein bands were transferred onto nitrocellulose membranes (0.45 μm, Bio-Rad) that were subsequently immunoblotted with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (1:3,000). Labeled protein spots were visualized by ECL (Amersham Biosciences) according to manufacturer's instruction [27].

To study the effect of EGF on EGFR/HER2 signaling, BT474 and SKBR3 cells were plated at 1×10^5 per well in 6-well plates, grown overnight, washed twice with PBS,

maintained in serum-free medium for 24 h, and then stimulated with the 50 ng/ml EGF for the indicated times. Total proteins were extracted and analyzed as described above.

To study the effects of furanodienone on EGF-stimulated EGFR/HER2 signaling, BT474 and SKBR3 cells were seeded and grown as above, serum starved for 24 h, treated with furanodienone (0–80 μM) for 24 h, and treated with 50 ng/ml EGF for 5 min. Treatment was terminated by rapidly discarding the medium and washing cells three times with ice-cold PBS. Changes of proteins in the EGFR/HER2 signaling pathways were analyzed by western blotting as described above.

Transient transfection of siRNA

Control siRNA, EGFR siRNA, and HER2 siRNA were purchased from Santa Cruz Biotechnology, and RNA interference assay was performed according to manufacturer's protocol. Briefly, BT474 and SKBR3 cells were seeded in 6-well culture plates overnight and transfected with indicated siRNA using Lipofectamine 2000 (Invitrogen). At 48 h post-transfection, cells were treated with furanodienone at 20–40 μM for a further 48 h. The effect of furanodienone on cell growth was assessed by SRB protein assay [28].

Plasmid construction and transfection

A full-length cDNA encoding human HER2 was amplified by PCR using HER2-WT *(*Addgene plasmid 16257*)* as a template [29], and the resulting PCR fragment was subcloned into the *Hind* III/*Xho* I sites of vector pcDNA3.1 (Invitrogen), yielding pcDNA3.1-HER2. For transient transfection, MDA-MB-231 cells were seeded in 6-well culture plates overnight and transfected with pcDNA3.1-HER2 or pcDNA3.1 mockvector using Lipofectamine 2000 for 48 h. Cells were then treated with furanodienone at 40–80 μM for another 48 h. The effect of furanodienone on cell growth was evaluated by SRB assay.

HER kinase assay

A HER2/ErbB2 Kinase Assay Kit (Cell Signaling Technology) was used, and the kinase assay was performed in triplicates. Various concentrations of furanodienone were added to the HER2 kinase reaction mixture in 96-well polystyrene round-bottomed plates. The reaction mixture contained $MgCl_2$ (5 mM), $MnCl_2$ (5 mM), Na_3VO_4 (3 μM), DTT (1.25 mM), ATP (20 μM), peptide substrate (1.5 μM), and HER2 kinase (50 ng) in HEPES buffer (50 μl, 60 mM, pH 7.5). The reaction was initiated by adding the peptide substrate, allowed to proceed for 30 min at room temperature, and terminated by adding EDTA (50 μl, 50 mM, pH 8.0). Each reaction mixture (25 μl) was diluted with deionized water (75 μl), transferred to a 96-well streptavidin-coated yellow plate (PerkinElmer Life Sciences), incubated at room temperature for 60 min, and washed 3 times with PBS containing Tween 20 (0.1%; PBST). The primary antibody (100 μl; phospho-Tyrosine mAb, p-Tyr-100, 1:1,000 in PBST with 1% BSA) was then added, and plates were incubated at room temperature for 60 min and washed 3 times with PBST. Europium-labeled anti-mouse IgG (100 μl; 1:500 in PBST with 1% BSA) was added, and plates were incubated at room temperature for 30 min, washed 5 times with PBST, and then the Enhancement Solution (PerkinElmer Life Sciences) was added. Plates were incubated at room temperature for 5 min and were read by Envision 2104 Multilabel Reader at 615 nm.

Statistical analysis

All values are expressed as means ± SD. Student's *t* test was carried out using Microsoft Excel software. Results are considered statistically significant at $P < 0.05$.

Results

Furanodienone treatment induced G1 arrest in BT-474 cells and apoptosis in SKBR3 cells

The growth of BT474 and SKBR3 cells was inhibited by furanodienone. After exposure to furanodienone (10–160 μM) for 24 or 48 h, the relative protein mass of cells was assessed by SRB assay (Fig. 1b, c). Furanodienone inhibited cell growth in BT474 and SKBR3 cells in a time- and dose-dependent manner.

The effects of furanodienone on cell cycle were studied by flow cytometry. In BT474 cells treated with furanodienone (20–80 μM) for 48 h, a dose-dependent increase in G1-arrested cells and a parallel decrease in S-phase cells were observed (Fig. 2a, b). These were not observed in SKBR3 cells receiving the same furanodienone treatments (Supplemental Figure 1), but rather there were dose-dependent increases in Annexin V-FITC-labeled apoptotic cells (Fig. 2c) and DAPI-stained apoptotic bodies (Fig. 2d). Western blot analysis revealed that the furanodienone treatments increased the levels of cleaved caspase-3 and cleaved PARP proteins, and decreased the level of pro-caspase-3 (Fig. 2e).

Furanodienone inhibited EGF-stimulated cell cycle progression and cell proliferation in BT-474 and SKBR3 cells

To verify the involvement of the EGFR/HER2 signaling pathways, we evaluate the effects of furanodienone on

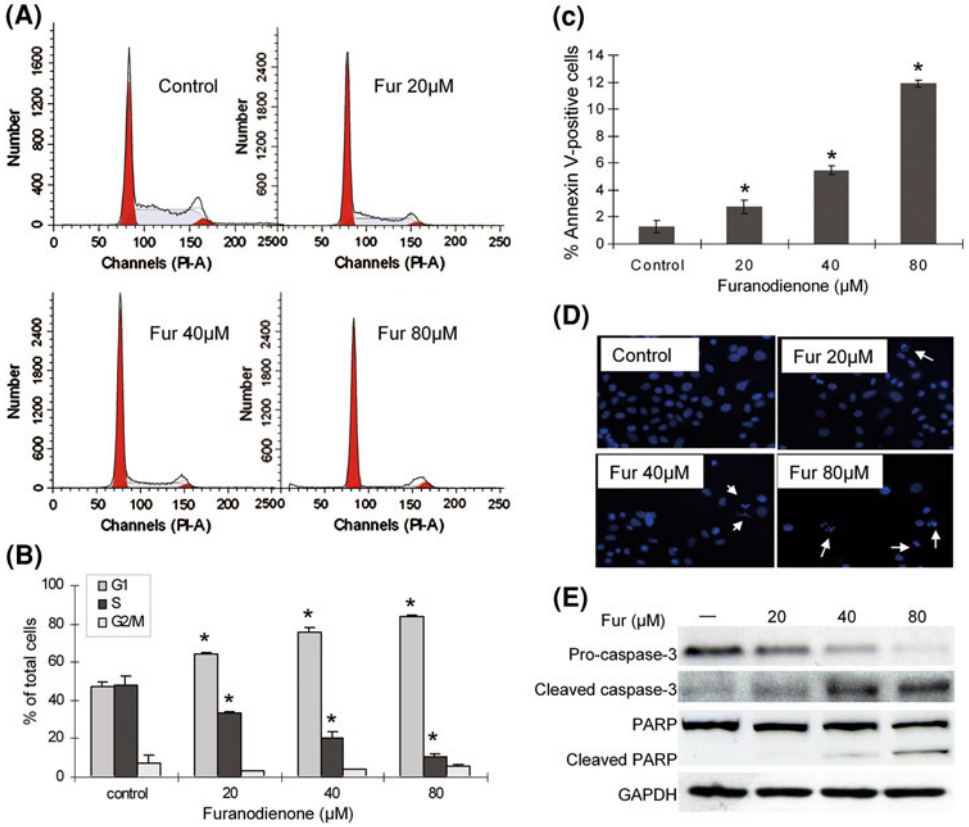

Fig. 2 Furanodienone induced G1 arrest in BT474 cells and apoptosis in SKBR3 cells. **a** Cell cycle analysis by flow cytometry of BT474 cells treated with furanodienone (20–80 μM) for 48 h. Results shown are representative of three independent experiments. **b** Quantified data from the three independent experiments above are collectively analyzed. $*P < 0.05$ versus the control. **c** SKBR3 cells were treated with furanodienone (20–80 μM) for 48 h, stained with FITC-labeled Annexin V and propidium iodide, and analyzed by flow cytometry to evaluate apoptosis. Data shown are mean ± SD from three independent determinations. $*P < 0.05$ versus the control. **d** Morphological changes in apoptotic cells. SKBR3 cells were treated with furanodienone (20–80 μM) for 48 h. and stained with DAPI. Pictures shown are representative from three independent experiments. *Arrows* indicate apoptotic bodies. **e** Furanodienone activated caspase-3 and increased cleaved PARP. SKBR3 cells were treated with furanodienone (20–80 μM) for 48 h, and protein extracts were analyzed by western blotting with the probing antibodies indicated on the *left*

EGF-stimulated BT474 and SKBR3 cells. As shown in Fig. 3a and b, in BT474 cells, 50 ng/ml of EGF reduced the number of G1-phase cells and significantly increased S-phase cells. Furanodienone (20–80 μM) treatments blocked the EGF-induced increase in S-phase BT474 cells and the decrease in G1-phase cells in a dose-dependent manner. Furanodienone inhibited EGF-induced cell proliferation in both BT474 (Fig. 3c) and SKBR3 cells (Fig. 3d).

Furanodienone inhibited EGFR/HER2 signaling in BT474 and SKBR3 cells

BT474 and SKBR3 breast cancer cells are HER2-over-expressing and also express moderate levels of EGFR. Expression levels of EGFR/HER2 signaling-related proteins were monitored with western blotting in furanodienone (20–80 μM)-treated BT474 and SKBR3 cells under log-phase growth conditions (Fig. 4a, b). In both BT474 and SKBR3 cells, furanodienone treatments significantly inhibited the phosphorylation of EGFR, HER2, Akt, and Gsk3β and increased the protein level of $p27^{kip1}$.

Furanodienone blocked EGF-activated EGFR/HER2 downstream signaling

EGF stimulated target cell growth by inducing EGFR/HER2 downstream signaling in BT474 and SKBR3 cells, and thus, we investigated the effects of furanodienone on EGF-stimulated cells. As shown in Fig. 5a and b, the addition of EGF induced the phosphorylation of EGFR, HER2, and other downstream signaling molecules including Akt, Gsk3β, and Erk1/2. Incubation of BT474 and SKBR3 cells with furanodienone for 24 h before EGF stimulation markedly inhibited EGF-induced phosphorylation of EGFR, HER2, Akt, and Gsk3β. In contrast, furanodienone failed to block EGF-stimulated phosphorylation of Erk1/2 (Fig. 5c, d).

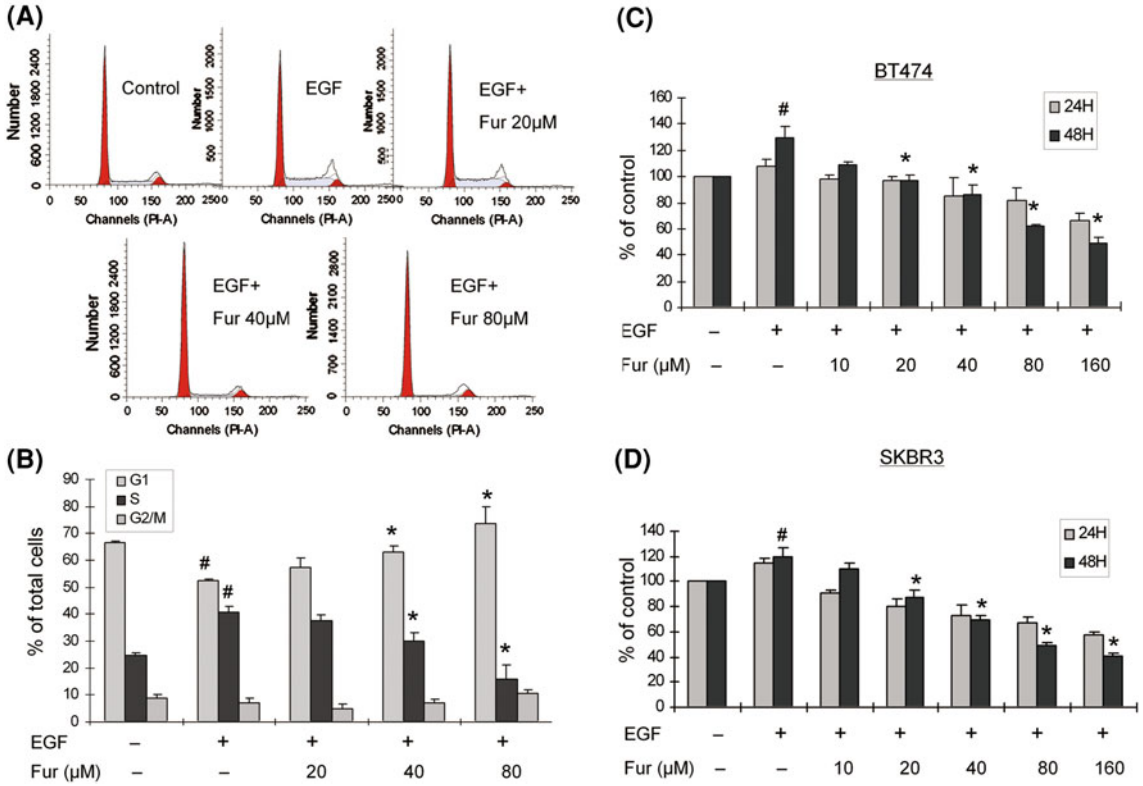

Fig. 3 Furanodienone inhibited EGF-stimulated cell cycle progression and cell proliferation in HER2-overexpressing breast cancer cells. **a** Representative flow cytometry cell cycle analysis results from three independent experiments are shown. BT474 cells were preconditioned for 24 h in serum-free medium and treated as indicated. **b** Quantified cell cycle distribution data from the three independent experiments above are collectively analyzed. $^{\#}P < 0.05$ versus the control; * $P < 0.05$ versus EGF treatment. **c** BT474 cells and **d** SKBR3 cells were preconditioned for 24 h in serum-free medium and treated as indicated. Cell growth was evaluated by SRB assay. $^{\#}P < 0.05$ versus the control; *$P < 0.05$ versus EGF treatment

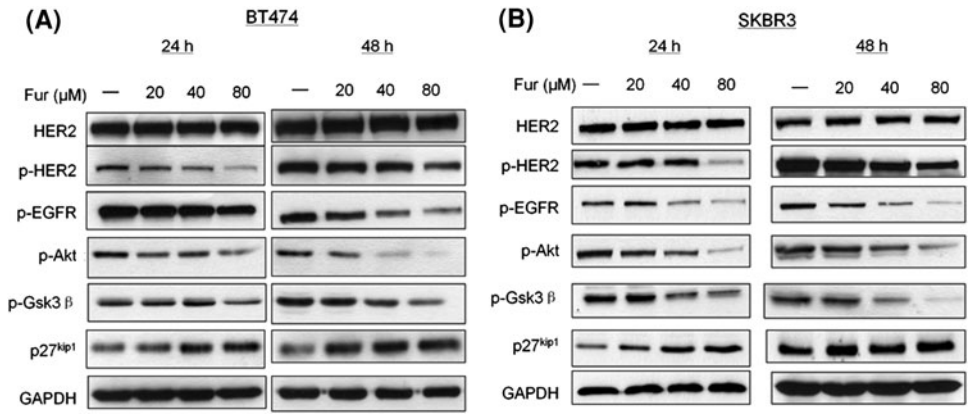

Fig. 4 Effects of furanodienone on the expression and/or activation of HER2, EGFR, and downstream Akt, Gsk3β, and p27^{kip1}. **a** BT474 cells and **b** SKBR3 cells were incubated with furanodienone (20–80 μM) for 24 and 48 h. Protein extracts were analyzed by western blotting with the probing antibodies indicated on the *left*

Antiproliferation effects of furanodienone on BT474 and SKBR3 cells were independent from EGFR expression level

EGFR expression was suppressed by the transfection of specific siRNA, and the role of EGFR in furanodienone action was examined in EGFR knockdown cells. Suppression of EGFR expression was confirmed by western blot analysis which showed significantly decreases in EGFR expression in siRNA-transfected BT474 and SKBR3 cells. At 48 h after knockdown, 20 or 40 μM furanodienone was added to the culture medium and cells were incubated for another 48 h. Knocking down EGFR expression did not affect the antiproliferation activity of furanodienone as compared to cells transfected with control siRNA (Supplemental Figure 3). Our observation indicated that the antiproliferation effect of furanodienone was not dependent on EGFR expression level.

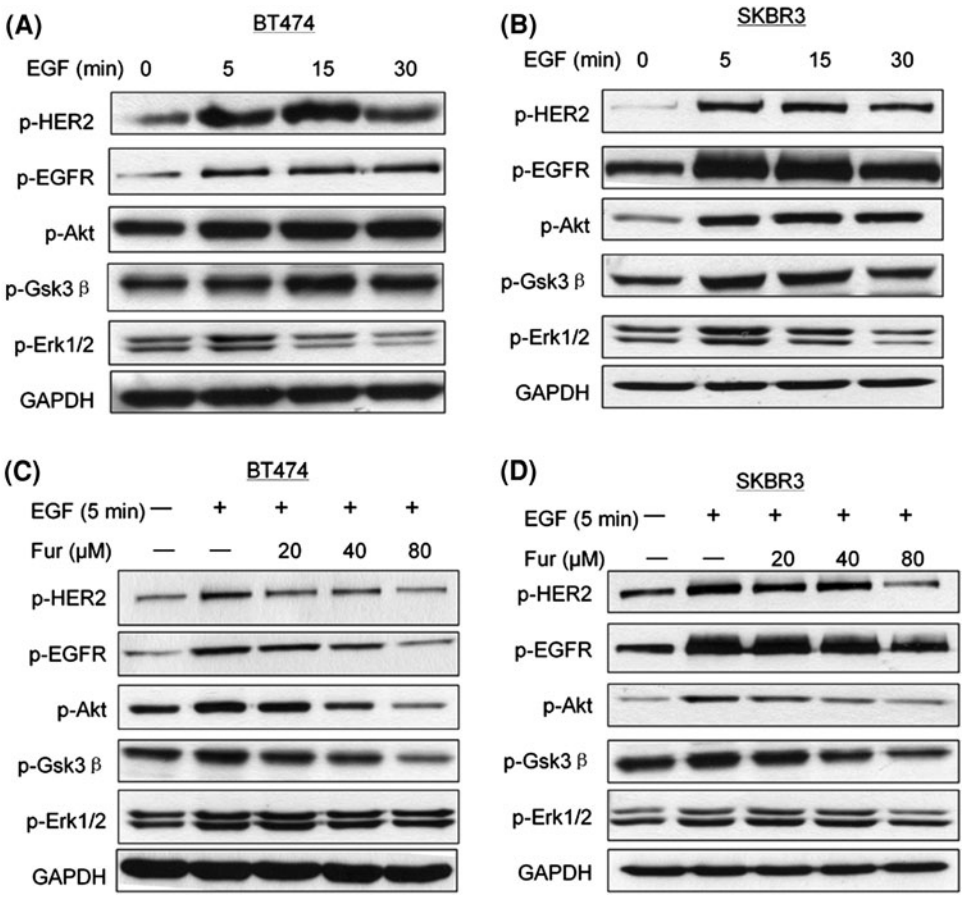

Fig. 5 Effects of EGF and furanodienone on HER2, EGFR, Akt, Gsk3β, and Erk1/2 in BT474 and SKBR3 cells. **a**, **b** EGF induced the activation of HER2, EGFR, and downstream Akt, Gsk3β, and Erk1/2 in BT474 and SKBR3 cells. After serum starvation, BT474 (**a**) and SKBR3 (**b**) cells were treated with 50 ng/ml EGF for indicated times, and protein extracts were analyzed by western blotting with the probing antibodies indicated on the *left*. **c**, **d** Furanodienone blocked EGF-induced activation of HER2, EGFR, and downstream Akt and Gsk3β in BT474 and SKBR3 cells. BT474 (**c**) and SKBR3 (**d**) cells were seeded and serum starved for 24 h. Cells were treated with furanodienone (20–80 μM) for 24 h followed by stimulation for 5 min with 50 ng/ml EGF. Protein extracts were analyzed by western blotting with the probing antibodies indicated on the *left*

HER2 may have a role in the antiproliferation action of furanodienone on BT474 and SKBR3 cells

Since EGFR seemed not to be involved in the action of furanodienone, we next examined the role of HER2. Similar as above, the effects of furanodienone on HER2-specific siRNA-transfected BT474 and SKBR3 cells were examined. HER2 knockdown was verified with western blot analysis (Fig. 6a, b). Forty-eight hours after siRNA transfection, 20 or 40 μM furanodienone was added to the culture medium and cells were incubated for another 48 h. Our results showed that HER2 knockdown cells became more resistant to furanodienone inhibition as compared to cells transfected with control siRNA. These results suggested that HER2 could play an important role in the antiproliferation action of furanodienone.

To verify our hypothesis, we studied the role of forced expressed HER2 in a HER2-deficient cell line, MDA-MB-231. Cells were transfected with control vectors or HER2 constructs that increased HER2 expression. The antiproliferation effects of furanodienone on the two cell populations were assessed. It was found that by increasing HER2 expression, MDA-MB-231 cells became more sensitive to furanodienone inhibition (Fig. 6c).

We also investigated whether furanodienone could inhibit HER2 kinase activity. Results showed that lapatinib, a dual inhibitor of EGFR and HER2 kinase, could effectively inhibit HER2 kinase activity, but furanodienone did not inhibit HER2 tyrosine kinase in vitro (Fig. 6d).

Discussion

Overexpression of EGFR and HER2 is seen in 20–80% and 25–30% of human breast cancers, respectively [11, 12]. It is well documented that overexpression of EGFR and HER2 is associated with a more malignant phenotype and poor prognosis. Clinically, EGFR and HER2 are emerging as promising targets for breast cancer therapy. The activation of the EGFR signaling pathway is initiated by the binding of EGF to the extracellular domain of EGFR which leads to receptor dimerization, kinase activation, and autophosphorylation of EGFR. HER2 does not have a known ligand but it is the preferred partner to form heterodimers with other ErbB family members and is phosphorylated by transactivation [30]. EGFR, HER2, and their downstream signal molecules including Akt, Gsk3β, and Erk are rapidly phosphorylated in response to EGF stimulation.

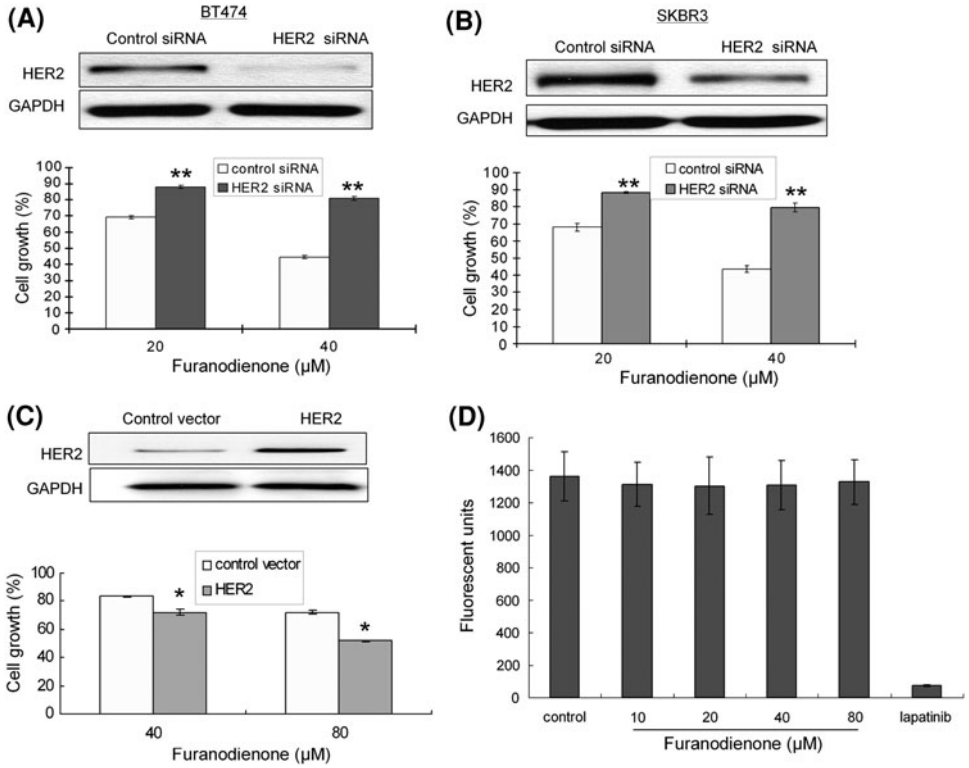

Fig. 6 Effects of furanodienone on HER2 knockdown BT474 and SKBR3 cells, HER2-transfected MDA-MB-231 cells, and HER2 kinase activity. **a**, **b** BT474 cells and SKBR3 cells were transfected with control or HER2 siRNA for 48 h, and cells were treated with furanodienone at 20–40 μM for another 48 h. HER2 expression was analyzed by western blotting. GAPDH was used as a loading control. The growth inhibitory effect of furanodienone on BT474 (**a**) and SKBR3 (**b**) cells were studied by SRB assay. Growth of HER2 knockdown cells was compared to that of control siRNA-transfected cells. Data shown are mean ± SD from three independent determinations. $^{**}P < 0.01$. **c** Cells were transfected with control or HER2 vectors. Forty-eight hours after transfection, cells were treated with furanodienone at 40–80 μM for another 48 h. HER2 expression was evaluated by western blotting. GAPDH was used as a loading control. Cell growth was assessed by SRB assay. Growth of HER2-transfected cells was compared to that of control vector-transfected cells. Data shown are mean ± SD from three independent determinations. $^{*}P < 0.05$. **d** Effects of furanodienone on HER2 kinase activity were assessed by measuring biotinylated peptide substrate phosphorylation. Data shown are mean ± SD from three independent determinations

In this study, we demonstrated that furanodienone induced cell cycle arrest and apoptosis in EGFR- and HER2-expressing BT474 and SKBR3 cells. Furanodienone inhibited the phosphorylation of HER2, EGFR, Akt, and Gsk3β and increased the expression of the cyclin-dependent kinase inhibitor $p27^{kip1}$ in both cell lines. The growth inhibition effect of furanodienone was unaffected in cells where EGFR expression was knocked down by transfected siRNA. On the contrary, siRNA-transfected and HER2 knockdown cells became more resistant to furanodienone toxicity than cells transfected with control vector constructs.

The HER2 dependence of furanodienone toxicity was further demonstrated in HER2-deficient MDA-MB-231 breast cancer cells. In this cell line, furanodienone showed only a minor effect on growth. The increase in HER2 expression in MDA-MB-231 cells by HER2 constructs transfection enhanced the growth inhibitory effects of furanodienone. This suggests that HER2 signaling is playing an important and specific role in the growth inhibitory action of furanodienone.

We have found that furanodienone did not affect the expression levels of HER2. Current target therapies directed toward HER2 either block dimerization of the receptor or inhibit the subsequent tyrosine kinase activity to disrupt intracellular signaling [31]. Furanodienone did not inhibit HER2 tyrosine kinase activity in vitro. An important question remains to be answered is whether furanodienone directly affects HER2 receptor dimerization.

In summary, this study reports for the first time that furanodienone, and possibly related compounds, may inhibit EGFR/HER2 signaling and induce cell growth inhibition. The growth inhibitory effect is being carried out in a HER2-, but not EGFR-, dependent manner. Our findings provide a rational and scientific basis for the further research and development on furanodienone-derived compounds as novel agents possibly useful in the treatment of HER2-positive breast cancers.

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