Combined effects of furanodiene and doxorubicin on the migration and invasion of MDA-MB-231 breast cancer cells *in vitro*

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Received February 19, 2016; Accepted March 11, 2016

DOI: 10.3892/or.2017.5435

Abstract. Furanodiene is one of the major bioactive components isolated from the natural product of the plant, *Curcuma wenyujin* Y.H. Chen et C. Ling. Furanodiene has been found to exert anticancer effects in various types of cancer cell lines, as well as exhibit antimetastatic activities. However, the antimetastatic capacity of furanodiene in combination with the common chemotherapy drug doxorubicin has not been investigated. We found that doxorubicin at a non-toxic concentration induced cell migration and cell invasion in highly metastatic breast cancer cells. Combinational treatments with furanodiene and doxorubicin blocked the invasion and migration of MDA-MB-231 breast cancer cells *in vitro*. We also clarified the effects of the combination on the signaling pathways involved in migration, invasion, and cytoskeletal organization. When combined with doxorubicin, furanodiene downregulated the expression of integrin αV and β-catenin and inhibited the phosphorylation of paxillin, Src, focal adhesion kinase (FAK), p85, and Akt. Moreover, combinational treatments also resulted in a decrease in matrix metalloproteinase-9 (MMP-9). Further study demonstrated that the co-treatments with furanodiene did not significantly alter the effects of doxorubicin on the tubulin cytoskeleton, represented by no influence on the expression levels of RhoA, Cdc42, N-WASP, and α/β tubulin. These observations indicate that furanodiene is a potential agent that may be utilized to improve the anticancer efficacy of doxorubicin and overcome the risk of chemotherapy in highly metastatic breast cancer.

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Abbreviations: Dox, doxorubicin; Fur, furanodiene; MMP-9, matrix metalloproteinase-9; TGF-β, transforming growth factor-β; PI3K, phosphoinositide 3-kinase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PS, penicillin-streptomycin; PI, propidium iodide; TPA, tetradecanoylphorbol acetate; DMSO, dimethyl sulfoxide; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; PFA, paraformaldehyde

Key words: furanodiene, doxorubicin, combination treatment, breast cancer, migration, invasion

Introduction

Chemotherapy is an effective approach by which to treat various types of cancer. However, chemotherapy regimens also carry the risks of side-effects, drug resistance, and serious complications (1-3). Thus, adjuvant chemotherapy is a rational way to overcome the side-effects (4,5). Numerous natural products have been associated with cancer prevention and treatment, and the bioactive compounds derived from natural products are always applied in combination with chemotherapy drugs (6-8). Doxorubicin is a first-line chemotherapy drug for the treatment of breast cancer, hepatocellular carcinoma and gastric cancer. In addition to its therapeutic activities, doxorubicin also enhances the malignant properties of cancer cells in clinical chemotherapy. Recent studies claim that doxorubicin induces serious side-effects, such as cardiomyopathy (9) and metastatic potential (10). Numerous studies have shown that many bioactive compounds have the capability to improve the anticancer activity of doxorubicin and reduce its side-effects (11,12). However, there have been few studies focusing on the metastatic potential induced by doxorubicin in chemotherapy. Therefore, it is significant to

explore effective compounds derived from natural products to reverse the metastatic potential induced by doxorubicin.

Curcuma wenyujin Y.H. Chen et C. Ling is a commonly used Chinese herb, which contains many bioactive components such as curcumin, β-elemene, and furanodiene (13,14). Curcumin exhibits antimetastatic properties via reducing the expression levels of α5β1 and α5β3 integrin and inhibiting the phosphorylation of focal adhesion kinase (FAK), Src, and PI3K/Akt (15-17). In adjuvant cancer treatment, curcumin suppressed the epithelial-mesenchymal transition induced by doxorubicin via the regulation of TGF-β and PI3K/Akt signaling pathways in triple-negative breast cancer cells (18). In addition, a previous study reported that there were no significant changes induced by curcumin in the Wnt/β-catenin pathway (19). β-elemene, another well-known bioactive component, was also found to exhibit antimetastatic properties in melanoma and breast cancer *in vitro* or *in vivo* (20,21). Growing evidence shows that furanodiene exerts pro-apoptotic, anti-angiogenic, and antimetastatic effects in several cancer cell lines (22,23). Furanodiene inhibited the adhesion, migration, and invasion of breast cancer cells through the regulation of integrin αV, β-catenin, FAK, PI3K/Akt, and MMP signaling pathways (24). However, the antimetastatic capacity of furanodiene in combination with the common chemotherapy drug doxorubicin has not been well investigated. Therefore, we initially studied the combined effects of furanodiene and doxorubicin against cancer cell migration and invasion with the view of improving treatment efficacy and overcoming side-effects.

Materials and methods

Chemicals and reagents. Furanodiene was purchased from the National Institutes for Food and Drug Control (Beijing, China). RPMI-1640 culture medium was purchased from Gibco (Rockville, MD, USA). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin-streptomycin (PS), Tubulin Tracker Green, 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and 0.25% (w/v) trypsin/1 mM EDTA were obtained from Invitrogen (Carlsbad, CA, USA). Doxorubicin and tetradecanoylphorbol acetate (TPA) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Radioimmunoprecipitation assay (RIPA) lysis buffer was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies against caspase 8, Bcl-2, Bax, caspase 7, PARP, integrin αV, β-catenin, RhoA, Cdc42, N-WASP, α/β tubulin, Akt, p-Akt (Ser473), p-paxillin (Tyr118), p-Src (Tyr416), p-FAK (Tyr925), p-p85 (Ser428), and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA), as well as the secondary antibodies. Matrigel and Transwell chamber systems were obtained from BD Biosciences (Bedford, MA, USA). MMP-9 immunoassay kit was purchased from R&D Systems (Minneapolis, MN, USA).

Cell culture and drug treatment. MDA-MB-231 cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured as previously reported (25). The stock solution of furanodiene (20 mM) and doxorubicin (100 μM) dissolved in dimethyl sulfoxide (DMSO) was diluted to different concentrations as designed.

Cell viability assay. Cell viability was assessed by MTT assay as previously described (26). Briefly, exponentially growing MDA-MB-231 cells ($1x10^4$) in 100 μl were seeded into 96-well tissue culture plates. Cells were treated with different concentrations of furanodiene (0, 5, 10 and 20 μM) in the presence or absence of doxorubicin (0.1 μM) for 12 h. The cell viability was determined by adding 100 μl of MTT (1 mg/ml). Following an incubation period (4 h), the absorbance values at 570 nm were recorded using a Multi-label Counter (PerkinElmer, Singapore).

Western blotting assay. Western blotting assay was performed according to a previous study (23). Briefly, MDA-MB-231 cells ($1x10^6$) were seeded in 60-mm Petri dish and were treated with different concentrations of furanodiene (0, 5, 10 and 20 μM) in the presence and absence of doxorubicin (0.1 μM). The cells were harvested after a 12-h treatment, and the total proteins were extracted with RIPA lysis buffer. Equal amounts of total protein were separated by appropriate SDS-PAGE followed by transferring onto a polyvinylidene fluoride (PVDF) membrane. Following blocking with non-fat milk for 1 h, the membrane was incubated with a specific primary antibody (1:1,000) for 2 h and the corresponding secondary antibody (1:1,000) for 1 h, respectively. The specific protein bands were visualized using an ECL Advanced Western Blotting Detection kit (Amersham Life Sciences, UK).

Cell cycle assay. Cell cycle distribution was determined according to a previous study (27). Briefly, MDA-MB-231 cells ($2x10^5$) were seeded into a 6-well plate and were treated with different concentrations of furanodiene (0, 5, 10 and 20 μM) in the presence and absence of doxorubicin (0.1 μM) for 12 h. Then, the cells were collected and fixed with ice-cold 70% ethanol at -20˚C overnight. The cells were procured again by centrifuging and were incubated with PI staining solution for 30 min protected from light. The cell cycle distribution was analyzed using flow cytometry (BD FACSCanto™; BD Biosciences, San Jose, CA, USA). The results were analyzed by ModFit LT software (version 3.0).

Transwell invasion assay. The invasive potential of the cells was assessed by a three-dimensional (3-D) cell invasion assay as previously described (28). Briefly, the upper and lower sides of the membrane were pre-coated with 1:30 (v/v) and 1:100 (v/v) of Matrigel, respectively. Cell density was adjusted to $1x10^5$/ml. The cell suspension (500 ml) containing serum-free RPMI-1640 medium and different concentrations of furanodiene (0, 0.1 and 20 μM) and doxorubicin (0.1 μM) (alone or in combination) was deposited into the upper chamber of each well. The lower chambers were loaded with RPMI-1640 medium containing 1% FBS (v/v, as a chemoattractant), different concentrations of furanodiene (0, 0.1 and 20 μM), and doxorubicin (0.1 μM) (alone or in combination). After a 12-h incubation, the non-invasive MDA-MB-231 cells were removed by gentle scraping. The invasive cells were fixed with paraformaldehyde (PFA; 3.7%/v/v) at room temperature for 30 min, and then stained with Hoechst 33342 for 15 min. Images of invasive cells were captured at a magnification of x100 using a digital camera. The number of invasive cells was counted using a MetaMorph Imaging Series software (Molecular Devices, Tokyo, Japan).

Transwell migration assay. MDA-MB-231 cells were treated with furanodiene (0 and 20 μM) in the presence or absence of doxorubicin (0.1 μM) for 12 h. Cell migration assay was performed as described in the previous section (Transwell invasion assay) with a slight modification in that both sides of the insert were not pre-coated with Matrigel.

Real-time migration assay. Real-time cell migration was continuously determined by the xCELLigence system (Roche, Mannheim, Germany) as previously described (24). The density of the cell suspension was adjusted to $2x10^5$ cells/ml. The upper chamber of each well was filled with 100 μl of cell suspension containing serum-free RPMI-1640 medium and different concentrations of furanodiene (0, 5, 10 and 20 μM) and doxorubicin (0.1 μM) (alone or in combination). The lower chambers were loaded with RPMI-1640 medium containing 1% FBS (v/v, as a chemoattractant), corresponding concentrations of furanodiene (0, 5, 10 and 20 μM), and doxorubicin (0.1 μM) (alone or in combination). Firstly, the CIM-Plate was placed in an incubator to allow cell sedimentation for 30 min. Then, the RTCA DP analyzer started to record in an incubator, and the duration of measurement was 12 h with an interval of 5 min.

Gelatin zymography assay. A gelatin zymography assay was performed according to a previous study (24). In brief, MDA-MB-231 cells ($2x10^5$) were seeded into a 6-well plate, and treated with different concentrations of furanodiene (0, 5, 10 and 20 μM) in the presence and absence of doxorubicin (0.1 μM) in RPMI-1640 medium (containing 1% FBS and 20 ng/ml TPA) for 12 h. The culture medium was centrifuged at 350 x g for 5 min at 4˚C. In addition, 20 μl of the supernatant was mixed with 5 μl of the non-reducing sample buffer [5X, 62.5 mM Tris-HCl, pH 6.8, 2% (v/v) SDS, 25% (v/v) glycerol, 0.01% (v/v) bromophenol blue]. Then, the mixed sample (20 μl) was separated on a 7% polyacrylamide gel (0.1% gelatin) at 100 V for 75 min and was rinsed with renaturing buffer for 1.5 h at room temperature. After a 42-h incubation in developing buffer at 37˚C, the gel was stained with Coomassie blue and was destained with 15% acetic acid.

Quantitative immunoassay for matrix metalloproteinase-9 (MMP-9). Quantitative immunoassay for MMP-9 was performed according to a previous study (29). MDA-MB-231 cells ($5x10^4$) were seeded in each well of 24-well plates. The cells were treated with furanodiene (20 μM) and doxorubicin (0.1 μM) (alone or in combination) in the presence or absence of TPA (20 ng/ml) for 12 h. The conditioned culture medium was collected and the level of MMP-9 secreted from the cells was determined using immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The absorbance values at 450 and 570 nm were recorded using a Multi-label Counter (PerkinElmer).

Tubulin level assay. MDA-MB-231 cells ($2x10^5$) were seeded into 6-well tissue culture plates and were incubated in a humidified incubator for 24 h. The cells were treated with different concentrations of furanodiene (0, 5, 10 and 20 μM) in the presence and absence of doxorubicin (0.1 μM) for 12 h. The induced cells were harvested and washed with PBS, and were then re-suspended in serum-free RPMI-1640 medium containing 100 nM Tubulin Tracker Green probe for a further 30-min incubation at 37˚C. For quantitative assessment, the cells were collected and re-suspended in PBS, followed by analysis via flow cytometry (BD FACSCanto™). The fluorescence of 10,000 analyzed cells (corrected for autofluorescence) in each group represents the tubulin level.

Data analysis. All data represent the mean of 3 separately performed experiments and were represented as mean ± SEM. The significance of group differences was evaluated via one-way ANOVA using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, USA). Newman-Keuls multiple comparison tests were performed for post hoc pairwise comparisons. Statistical differences were considered significant at $P<0.05$. Data from at least 3 independent experiments were used for the statistical analysis.

Results

Co-treatments with furanodiene (0-20 μM) do not enhance the cytotoxic effect of doxorubicin (0.1 μM) in MDA-MB-231 cells. The cytotoxic effects of furanodiene and doxorubicin in the highly metastatic MDA-MB-231 human breast cancer cells were determined by MTT assay. As shown in Fig. 1A, co-treatments with furanodiene (0-20 μM) did not enhance the cytotoxicity of doxorubicin (0.1 μM). Accordingly, we used different methods to confirm cell status alteration after co-treatments with furanodiene (0-20 μM) and doxorubicin (0.1 μM). Our results showed that co-treatments with furanodiene (0-20 μM) and doxorubicin (0.1 μM) did not induce the cleavage of caspase 8 and 7, and PARP and also did not affect the expressions of Bcl-2 and Bax (Fig. 1B). These results confirmed that the co-treatments did not induce apoptosis in the MDA-MB-231 cells. Furthermore, the co-treatments did not induce cell cycle arrest at the corresponding concentrations, indicating that the cell status was normal after the co-treatments with furanodiene (0-20 μM) and doxorubicin (0.1 μM) for 12 h in the MDA-MB-231 cells (Fig. 1C).

Combined effects of furanodiene and doxorubicin on cell invasion and migration in MDA-MB-231 cells. Doxorubicin (0.1 μM) treatment alone induced a significant increase in invasion (Fig. 2A) and migration (Fig. 2B) of the MDA-MB-231 cells, with 1.36- and 3.92-fold, respectively. Furanodiene (0.1 μM) treatment alone did not significantly affect the cell invasion, but markedly attenuated the invasion of the MDA-MB-231 cells induced by doxorubicin (Fig. 2A). Furthermore, co-treatments with furanodiene (20 μM) and doxorubicin (0.1 μM) resulted in much more marked reduction in the invasion (Fig. 2A) and the migration (Fig. 2B) of MDA-MB-231 cells than that of furanodiene (20 μM) treatment alone. Fig. 2C and D are statistical results of the cell invasion and migration.

Meanwhile, our real-time results also demonstrated an increase in the migration of MDA-MB-231 cells following doxorubicin (0.1 μM) incubation, and furanodiene (0-20 μM) treatment attenuated the doxorubicin-induced migration gradually, even at the low concentration of 5 μM within 12 h (Fig. 3).

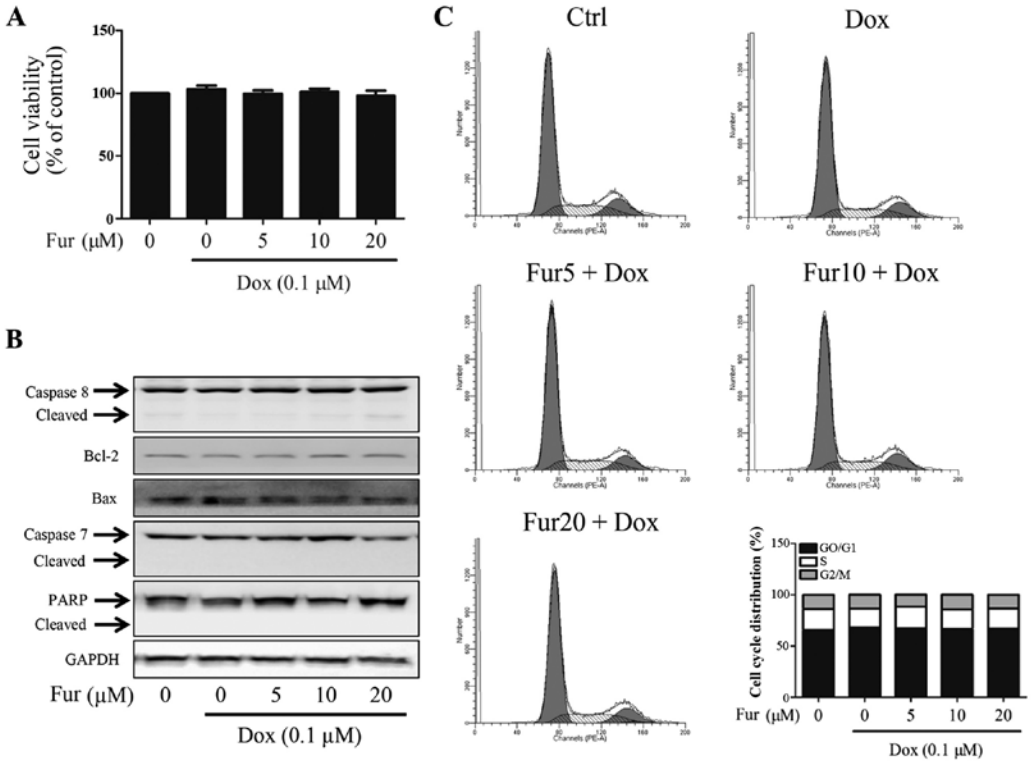


Figure 1. Combined effects of doxorubicin (Dox) and furanodiene (Fur) on cell viability, cell apoptosis and cell cycle distribution in MDA-MB-231 cells. Cells were co-treated with Dox (0.1 μM) and Fur (0, 5, 10 and 20 μM) for 12 h. (A) Cell viability was detected by MTT assay. (B) Biomarkers of cell apoptosis were detected by western blotting. (C) Cell cycle was analyzed by flow cytometry. Histograms represent the cell cycle distribution and the ratios were also calculated. Ctrl, control.

Combined effects of furanodiene and doxorubicin on the metastasis-related proteins in MDA-MB-231 cells. To explore the underlying mechanisms involved in the antimetastatic effects of furanodiene and doxorubicin in the MDA-MB-231 cells, the expression levels of several proteins related to the regulation of metastasis were determined by western blotting. Our results showed that doxorubicin (0.1 μM) treatment alone increased the expression of integrin αV and induced the phosphorylation of FAK, Src, paxillin, p85 and Akt. However, co-treatments with furanodiene (5-20 μM) and doxorubicin (0.1 μM) downregulated the expression levels of integrin αV and β-catenin and inhibited the phosphorylation of FAK, Src, paxillin, p85, and Akt (Fig. 4A).

Co-treatments with furanodiene and doxorubicin attenuate the MMP-9 release induced by TPA in MDA-MB-231 cells. The gelatin enzymography assay was performed to investigate whether co-treatments with furanodiene and doxorubicin suppress breast cancer cell invasion by affecting the activity of MMP-9. Our results showed that co-treatment with furanodiene (20 μM) and doxorubicin (0.1 μM) markedly attenuated TPA-induced MMP-9 activity. However, lower concentrations of furanodiene (5 and 10 μM) did not significantly affect the MMP-9 activity induced by TPA (20 ng/ml) and doxorubicin (0.1 μM) (Fig. 4B). Afterwards, the ELISA immunoassay was initially used to confirm the changes in MMP-9 release in the conditioned medium after co-treatment with furanodiene and doxorubicin. As shown in Fig. 4C, the MMP-9 release induced by TPA in the conditioned medium decreased to 50.50% with significance in response to co-treatment with furanodiene (20 μM) and doxorubicin (0.1 μM).

Combined effects of furanodiene and doxorubicin on cell cytoskeleton reorganization. To determine whether the cell cytoskeleton reorganization may be involved in the antimetastatic effects of furanodiene and doxorubicin, the cytoskeletal regulatory proteins were detected by western blotting. As shown in Fig. 5A, our results showed that co-treatments with furanodiene (0-20 μM) and doxorubicin (0.1 μM) did not alter the expression levels of RhoA, Cdc42, N-WASP, and α/β tubulin in the MDA-MB-231 cells. Furthermore, the tubulin level was also detected with Tubulin Tracker Green probe by flow cytometry. In addition, the results showed that tubulin assembly was not significantly regulated by co-treatments with furanodiene (0-20 μM) and doxorubicin (0.1 μM) for 12 h (Fig. 5B and C).

Discussion

Although chemotherapy is an effective approach to prolong survival and reduce mortality, the chemotherapy-induced side-effects must not be overlooked (30-32). Combinational therapy with reduced drug dosages is commonly an effective approach to prevent side-effects. However, sometimes low dosage may increase the metastatic potential, which is related to cancer recurrence. Several first-line drugs, such as oxaliplatin, cyclophosphamide, and doxorubicin, were reported for conspicuous side-effects, namely chemotherapy-driven metastasis (33-35).

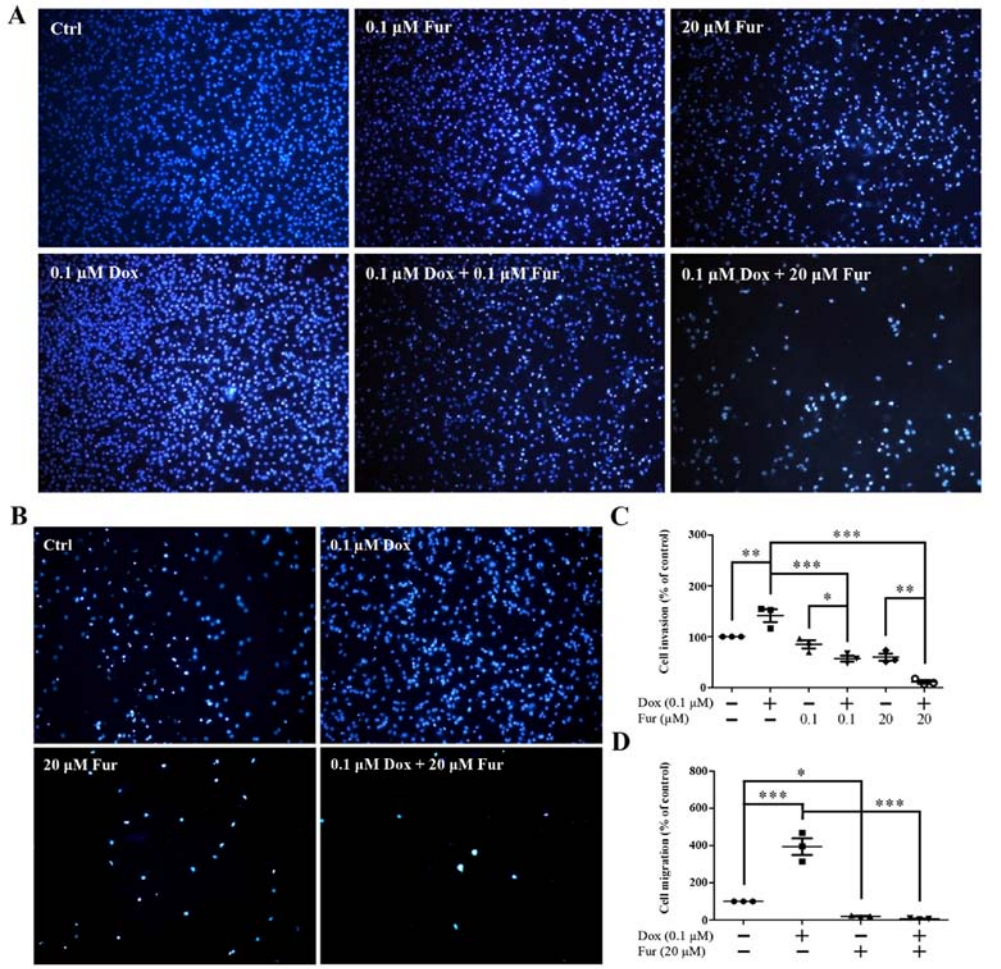
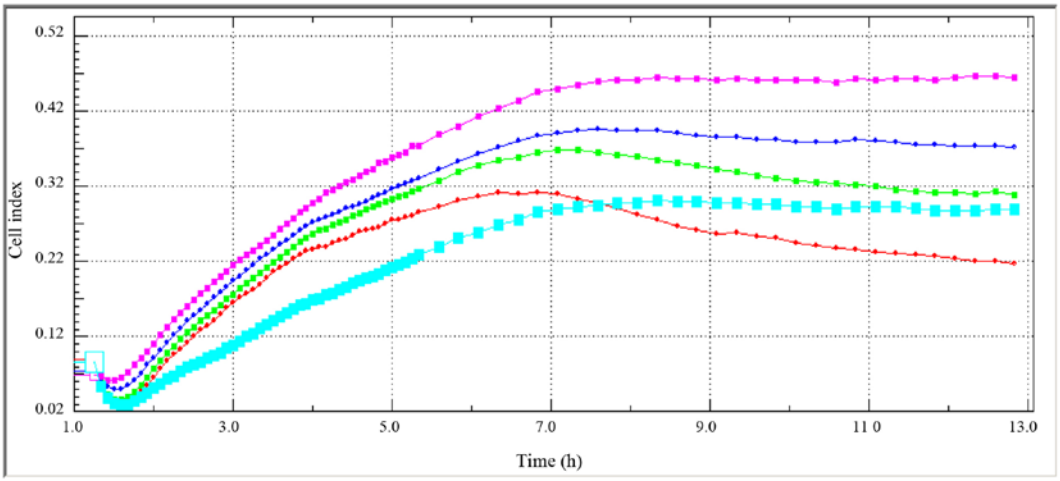


Figure 2. Combined effects of doxorubicin (Dox) and furanodiene (Fur) on MDA-MB-231 cell invasion and migration in a 3-D model. Cells were co-treated with doxorubicin (0.1 μM) and furanodiene (0, 0.1 and 20 μM) for 12 h. (A) Furanodiene attenuated doxorubicin-induced MDA-MB-231 cell invasion compared with the negative control (Ctrl). (B) Furanodiene attenuated doxorubicin-induced MDA-MB-231 cell migration compared with the negative control (Ctrl). (C) Statistical result of A. (D) Statistical result of B (*P<0.05, **P<0.01, and ***P<0.001).



	Light blue	Pink	Dark blue	Green	Red
Dox (μM)	0	0.1	0.1	0.1	0.1
Fur (μM)	0	0	5	10	20

Figure 3. Combined effects of doxorubicin (Dox) and furanodiene (Fur) on MDA-MB-231 cell migration in a real-time model. Real-time cell migration was continuously determined by the xCELLigence system and recorded with RTCA DP analyzer. The duration of measurement was 12 h with an interval of 5 min. Furanodiene (0-20 μM) attenuated doxorubicin (0.1 μM)-induced migration in the MDA-MB-231 cells.

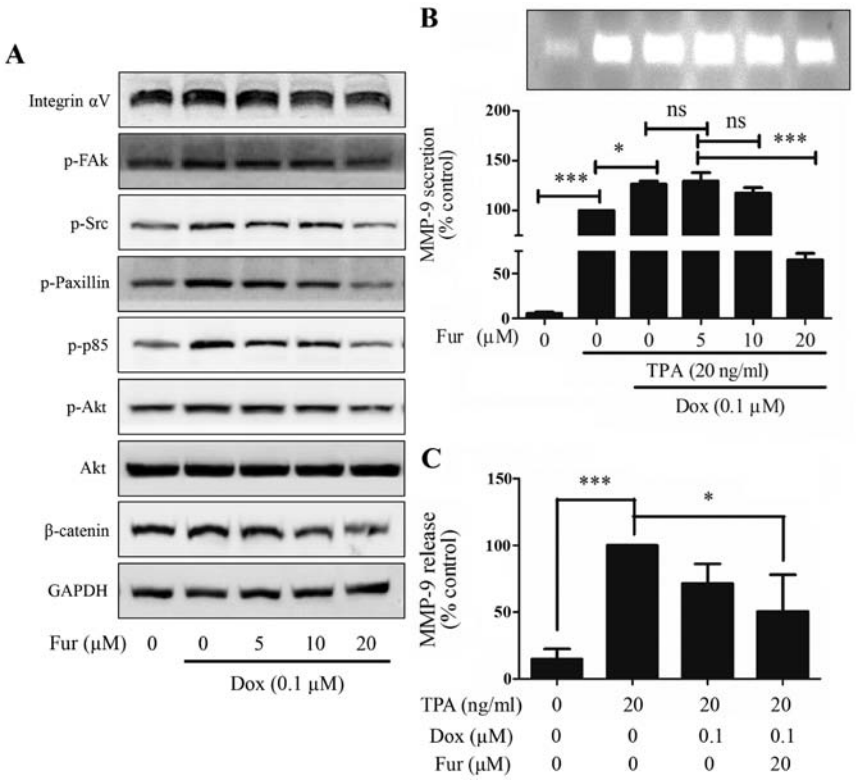


Figure 4. Combined effects of doxorubicin (Dox) and furanodiene (Fur) on the metastasis-associated pathways in MDA-MB-231 cells. Cells were co-treated with Dox (0.1 μM) and Fur (0-20 μM) for 12 h, or in the presence of TPA (20 ng/ml). (A) The metastasis-associated proteins were detected by western blotting. (B) MMP-9 activity was analyzed by gelatin enzymography assay. (C) MMP-9 secretion level was detected using the ELISA kit (*P<0.05 and ***P<0.001).

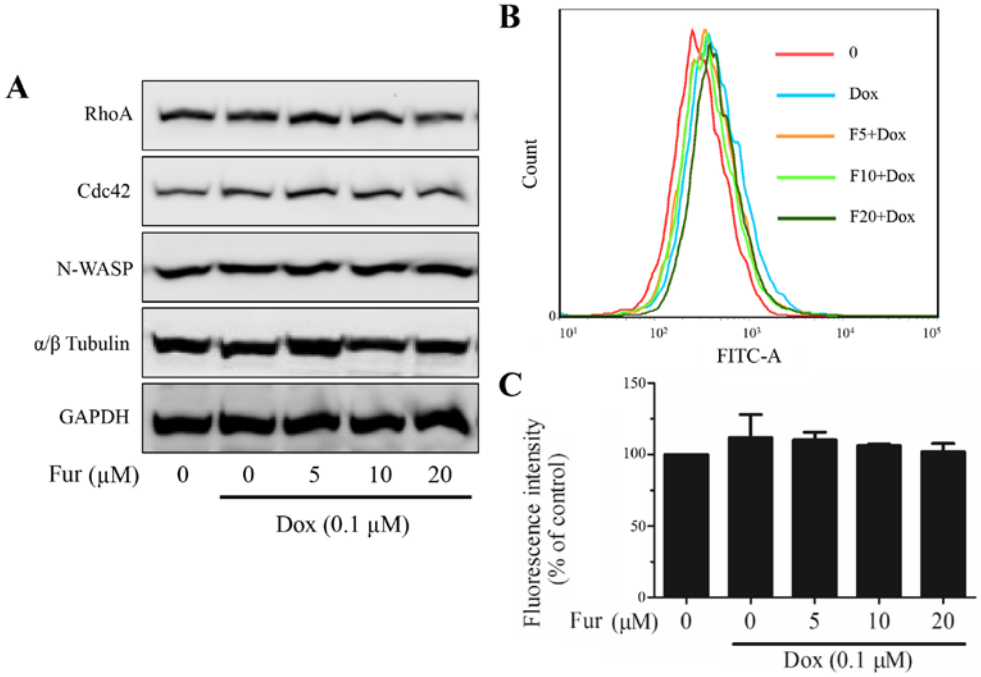


Figure 5. Combined effects of doxorubicin (Dox) and furanodiene (Fur or F) on cell cytoskeleton reorganization in the MDA-MB-231 cells. Cells were co-treated with doxorubicin (0.1 μM) and furanodiene (0-20 μM) for 12 h. (A) The cytoskeleton reorganization associated proteins were detected by western blotting. (B) The cytoskeletal regulatory proteins were detected with Tubulin Tracker Green probe using flow cytometry. (C) Statistical results of B.

Metastasis is highlighted as one of the main problems (34,36). Various studies have reported enhanced migration and invasion in MHCC97L and HepG2 cells *in vitro* and increased metastasis into the lung in hepatocellular carcinoma *in vivo* after oxaliplatin treatment (37). Cyclophosphamide was found to enhance pulmonary metastasis in T739 mice injected with

LA795 cells and contribute to bone metastasis in a mouse model of prostate cancer (35). Likewise, doxorubicin administration increased the TGF-β1 level and circulating tumor cells, thereby increasing lung metastasis in a MMTV/*PyVmT* transgenic model of metastatic breast cancer (38). In addition, doxorubicin treatment at certain dosages also enhanced metastasis to the lung in an MDA-MB-231 orthotopic xenograft model and metastasis to the bone in a 4T1 orthotopic xenograft model (10,39). In light of these studies, suppression of chemotherapy-driven metastasis is urgent and necessary.

As the major bioactive component isolated from *Curcuma wenyujin* Y.H. Chen et C. Ling, furanodiene exerts anticancer effects in various types of cancer cells as well as antimetastatic activities (24). Our previous study confirmed that furanodiene (0-25 μM) treatment alone suppressed migration and invasion without inducing cytotoxicity in highly metastatic breast cancer cells (24). However, the antimetastatic capacity of furanodiene combined with the common chemotherapy drug doxorubicin has not yet been investigated. Our present study confirmed that doxorubicin (0.1 μM) treatment caused a significant increase in the invasion and migration of highly metastatic breast cancer MDA-MB-231 cells. Expectably, furanodiene attenuated the invasion and the migration of MDA-MB-231 cells induced by doxorubicin even at nanomolar concentrations. Accordingly, furanodiene blocked doxorubicin-induced cell migration, which was indicated by real-time alterations of attachment and spreading of cells monitored by the xCELLigence system. Under the same conditions, this co-treatment with furanodiene did not enhance the cytotoxic effect of doxorubicin. Taking into account that no alteration in cell apoptosis and cell cycle distribution occurred following co-treatment, the attenuation of doxorubicin-induced migration and invasion by fuanodiene was independent of cell cytotoxicity. Collectively, the natural components extracted from *Curcuma wenyujin* Y.H. Chen et C. Ling may be potential natural products used to reduce the side-effects of chemotherapy agents and prevent cancer recurrence.

We investigated the underlying mechanisms involved in the antimetastatic effects of furandiene and doxorubicin in MDA-MB-231 cells. Integrins are α/β heterodimeric cell surface receptors that may be involved in embryonic development, angiogenesis, and tumor metastasis (40). Focal adhesion kinase (FAK) plays a pivotal role in the control of cell survival, spreading, and migration, which is in response to integrin-mediated signal transduction (41). Clustering of integrins leads to FAK activation and the autophosphorylation of FAK at Tyr397. This relationship between integrins and FAK gives rise to a series of subsequent events, including activation of Src family kinases, regulation of adhesion disassembly through paxillin, and activation of PI3K through p85 binding (42,43). Additionally, PI3K activates the Akt cascade, which controls cell migration during the occurrence and development of cancer (44). In addition, the interaction of PI3K with β-catenin is necessary for the mediation of cell motility functions (45). All the proteins mentioned above compose signaling networks with antimetastatic effects, and furanodiene was found to interfere with these signaling networks to attenuate doxorubicin-induced migration and invasion. More specifically, doxorubicin-induced metastatic potential was confirmed by increased expression of integrin αV and phosphorylation of FAK, Src, paxillin, p85, and Akt after doxorubicin treatment alone. Afterwards, co-treatment with furanodiene and doxorubicin significantly reversed these increases in integrin αV and the phosphorylation of FAK. Both are a central part in the downstream signaling regulation of cell motility and cell adhesion, as mentioned above. In addition to the suppression of the central part, marked inhibition of downstream signaling was also observed, including inhibition in the phosphorylation of Src, paxillin, p85, and Akt. Notably, Akt phosphorylation and β-catenin expression, which are associated with PI3K and implicated in cell migration and cell motility, were also decreased after the co-treatment with furanodiene and doxorubicin. Therefore, furanodiene interfered with the signaling networks to improve doxorubicin-induced migration and invasion in the MDA-MB-231 cells.

Matrix metalloproteinase (MMP) activities are associated with cancer occurrence and development. Among the family members, MMP-9 is an important factor during tumor invasion (46). TPA promotes MMP-9 activity by regulating the integrin pathway and proteolytic cleavage in human breast cancer cells (47). Co-treatment with furanodiene and doxorubicin markedly attenuated the TPA-induced MMP-9 activity in the highly metastatic breast cancer MDA-MB-231 cells.

Rho GTPases, including Rac1, RhoA and Cdc42, play a critical role in the regulation of actomyosin contractility and focal adhesion assembly (48). High expressions of RhoA and Cdc42 are regarded as adhesion markers (49). As a downstream effector, N-Wiskott-Aldrich (N-WASP; syndrome protein) serves an essential role in many actin-based processes, including the regulation of filopodium formation and actin polymerization (50). N-WASP binding to the Arp2/3 complex is required for Cdc42-dependent actin polymerization in reorganization of the actin cytoskeleton (51). In addition, this reorganization of the actin cytoskeleton is the primary mechanism for cell motility and migration (52). Inhibition of Rho GTPases and N-WASP leads to suppression of cell cytoskeleton reorganization. However, in the present study, no alterations were observed after co-treatment with furanodiene and doxorubicin. This indicated that the antimetastatic effects of furanodiene and doxorubicin may be independent of cell cytoskeleton reorganization. Additionally, there are 3 types of cytosolic fibers in the cytoskeleton, including microtubules, actin and intermediate filaments (53). Many microtubule-targeting drugs bind to α/β tubulin and disrupt microtubule dynamics to block mitosis in cancer cells. Likewise, no alteration in α/β tubulin was observed after co-treatment of furanodiene and doxorubicin in the MDA-MB-231 cells, including protein expression and florescence levels. Thus, the effects of the co-treatment with furanodiene and doxorubicin may be independent of cytoskeleton reorganization.

In conclusion, furanodiene attenuated the metastatic capacity of highly metastatic breast cancer cells induced by doxorubicin through the regulation of integrin αV, FAK/Src/paxillin, PI3K/Akt, β-catenin, and MMP-9 signaling pathways. In addition, the effects were independent of cytotoxicity and cell cytoskeleton reorganization. These results also indicate that natural products derived from *Curcuma wenyujin* Y.H. Chen et C. Ling may be developed as agents to reduce the side-effects caused by chemotherapeutic agents and prevent cancer recurrence.

Acknowledgements

The present study was supported by the Macao Science and Technology Development Fund (077/2011/A3 and 048/2013/A2), the Research Fund of the University of Macau [CPG2014-00012-ICMS, UL016/09Y4/CMS/WYT01/ICMS and MYRG208 (Y3-L4)-ICMS11-WYT], the National Natural Science Foundation of China (NSFC, no. 81470170), and the Fundamental Research Funds for the Central Universities (lzujbky-2014-148). W.-A.Q. was supported by the Baskes Foundation and Robert H. Lurie Comprehensive Cancer Center at Northwestern University (Chicago, IL, USA).

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