Annonacin induces cell cycle-dependent growth arrest and apoptosis in estrogen receptor-α-related pathways in MCF-7 cells

Yu-Min Ko [a], Tung-Ying Wu [b], Yang-Chang Wu [b], Fang-Rong Chang [b], Jinn-Yuh Guh [c,d,*], Lea-Yea Chuang [e,*]

[a] Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC
[b] Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC
[c] Department of Internal Medicine, Faculty of Medicine, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan, ROC
[d] Department of Internal Medicine, Kaohsiung Medical University Hospital, Kaohsiung Medical University, 100 Zihyou 1st Road, Kaohsiung, Taiwan, ROC
[e] Department of Biochemistry, College of Medicine, Kaohsiung Medical University, 100 Zihyou 1st Road, Kaohsiung, Taiwan, ROC

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ABSTRACT

Ethnopharmacological relevance: Tamoxifen resistance is common in estrogen receptor-α (ERα)-positive breast cancers. Pawpaw and soursop are anticancer annonaceous plants in complementary medicine. Thus, we studied the effects of annonacin, an annonaceous acetogenin, in breast cancer cells.
Materials and Methods: Cell growth and ERα-related pathways were studied. The effects of annonacin were tested in MCF-7 xenografts in nude mice.
Results: In ERα-positive MCF-7 cells, annonacin (half-effective dose $ED_{50} = 0.31\,\mu M$) and 4-hydroxytamoxifen ($ED_{50} = 1.13\,\mu M$) decreased cell survival whereas annonacin ($0.5-1\,\mu M$) increased cell death at 48 h. Annonacin and 4-hydroxytamoxifen were additive in inhibiting cell survival. Annonacin ($0.1\,\mu M$) induced G_0/G_1 growth arrest while increasing $p21^{WAF1}$ and $p27^{kip1}$ and decreasing cyclin D1 protein expression. Annonacin ($0.1\,\mu M$) decreased cyclin D1 protein expression more than 4-hydroxytamoxifen ($1\,\mu M$). Annonacin ($0.1\,\mu M$) increased apoptosis while decreasing Bcl-2 protein expression. The combination of annonacin ($0.1\,\mu M$) and 4-hydroxytamoxifen ($1\,\mu M$) decreased Bcl-2 protein expression and ERα transcriptional activity more than annonacin ($0.1\,\mu M$) did alone. Annonacin, but not 4-hydroxytamoxifen, decreased ERα protein expression. Moreover, annonacin decreased phosphorylation of ERK1/2, JNK and STAT3. In nude mice, annonacin decreased MCF-7 xenograft tumor size at 7–22 days. Moreover, annonacin decreased ERα, cyclin D1 and Bcl-2 protein expression in the xenograft at 22 days.
Conclusions: Annonacin induced growth arrest and apoptosis in ERα-related pathways in MCF-7 cells. Annonacin and 4-hydroxytamoxifen were additive in inhibiting cell survival and ERα transcriptional activity. Moreover, annonacin attenuated MCF-7 xenograft tumor growth while inhibiting ERα, cyclin D1 and Bcl-2 protein expressions in nude mice.

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1. Introduction

The pathogenesis of breast cancer includes estrogen and estrogen receptor-α (ERα)-related pathways (Osborne and Schiff, 2011) whereby nuclear ERα activates target genes via the estrogen-response elements (ERE) (Osborne and Schiff, 2011). Additionally, nonnuclear ERα rapidly activates growth factor downstream signals such as extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and signal transducers and activators of transcription 3 (STAT3) (Musgrove and Sutherland, 2009; Osborne and Schiff, 2011). Interestingly, STAT integrates cytoplasmic and nuclear estrogen actions (Fox et al., 2009).

Selective estrogen receptor modulators (SERM, such as tamoxifen) or selective estrogen receptor down-regulators (SERD, such as fulvestrant) are effective treatments for ERα-positive breast cancers commonly limited by resistance (Osborne and Schiff, 2011). Endocrine resistance may be induced by the loss of ERα, increased nonnuclear ERα or growth factor receptor signaling, deranged signal transducers (such as ERK1/ERK1, JNK and STAT3), cell cycle regulators (such as cyclin D1 and the cyclin-dependent kinase inhibitors $p21^{WAF1}$ and $p27^{kip1}$) or apoptosis regulators (such as

the anti-apoptotic Bcl-2 (Musgrove and Sutherland, 2009; Osborne and Schiff, 2011).

Pawpaw (*Asimina triloba*) and soursop (graviola, *Annona muricata*) are annonaceous plants used as anticancer folk therapies in (North, Central and South) America and Southeast Asia and have been studied in a few observational clinical studies (Cassileth, 2008; Coothankandaswamy et al., 2010; Liaw et al., 2010; McLaughlin, 2008). Annonaceous acetogenins are cytotoxic to multidrug-resistant MCF-7 cells (Oberlies et al., 1997). Annonacin ($C_{35}H_{64}O_7$), an annonaceous acetogenin containing a mono-tetrahydrofuran ring with two flanking hydroxyls, also inhibits growth in MCF-7 cells (Yuan et al., 2003). However, the molecular mechanisms are not understood.

Thus, we studied the growth-inhibitory mechanisms of annonacin in terms of ERα-related pathways (p-ERK1/2, p-JNK, p-STAT3, cyclin D1, Bcl-2, p21^{WAF1} and p27^{kip1}) in MCF-7 cells. Moreover, the effects of annonacin on MCF-7 xenografts in nude mice were also investigated.

2. Materials and methods

2.1. Cell culture and reagents

ERα-positive MCF-7 cells and ERα-negative MDA-MB-231 cells were purchased on February 18, 2009 from Bioresource Collection and Research Center (Hsinchu, Taiwan), where cells were authenticated by DNA fingerprints of short tandem repeat profiling. Cells were cultured in DMEM/F-12 (1:1) medium supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA) in a humidified 5% CO_2 incubator at 37 °C. Cells were fasted for 24 h before adding fresh medium containing 10% FBS and various concentrations of annonacin or 4-hydroxytamoxifen.

Cyclin D1, cyclin-dependent kinase 4 (cdk4), cyclin E, p21^{WAF1}, p27^{kip1}, Bcl-2, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ERα, STAT3 and JNK antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz Co., CA). α-tubulin antibody was purchased from Lab Vision Corporation (Fremont, CA). Serine 118 phosphorylated ERα (pSer118ERα), p-STAT3, ERK1/2, p-ERK1/2, JNK and p-JNK antibodies were purchased from Cell Signaling Technology (Danvers, MA). We had isolated, purified and characterized annonacin from the leaves of Formosan *A. muricata* (Liaw et al., 2002) (supplementary Methods and supplementary Table 1), which has a different molecular structure from that of tamoxifen (supplementary Fig. 1). 4-hydroxytamoxifen, an active metabolite of tamoxifen and other chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Annonacin was dissolved in dimethyl sulfoxide (DMSO, 0.1% final concentration). Because annonacin has not been used in humans clinically, the effective concentration was chosen based on our previous in vitro study (Yuan et al., 2003). 17β-estradiol and 4-hydroxytamoxifen were dissolved in ethanol (0.1% final concentration).

2.2. Measurement of cell survival and cell death

Cell survival was performed in quadruplicate similar to our previous study (Chou et al., 2008). Briefly, viable cell numbers were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. MTT was purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). Briefly, MCF-7 cells (6500 cells/well) or MDA-MB-231 cells (3500 cells/well) were plated and incubated in 96-well plates. MTT (1 mg/ml) was added into each well. Production of insoluble formazan by viable cells was measured at 540 nm.

Dose–response curves for various combinations of 4-hydroxytamoxifen and annonacin and the half-effective dose (ED_{50}) for cell survival at 48 h were analyzed by the four-parameter log-logistic model (Sørensen et al., 2007). The dose ranges used were: 0% 4-hydroxytamoxifen (i.e. annonacin, 0.01, 0.05, 0.1, 0.5 and 1 μM), 50% 4-hydroxytamoxifen (0.1, 0.5, 1, 5 and 10 μM), 91% 4-hydroxytamoxifen (0.1, 0.5, 1, 5 and 10 μM) and 100% 4-hydroxytamoxifen (0.1, 0.5, 1, 5 and 10 μM), respectively. The concentration addition model and the isobologram method (Sørensen et al., 2007) was used to assess the synergy, additivity or antagonism of the combinations of annonacin and 4-hydroxytamoxifen.

Cell death was assessed by lactate dehydogenase release through using the CytoTox 96 nonradioactive cytotoxicity assay kit according to the manufacturer's instructions (Promega Corp., Madison, WI).

2.3. Cell cycle analysis

Cell cycle analysis was performed similar to our previous study (Chuang et al., 2006). Briefly, cells were trypsinized and suspended in cold phosphate-buffered saline (PBS). Suspended cells were washed twice with cold PBS and fixed with 70% ice-cold ethanol and placed at −20 °C overnight. Cells were then centrifuged and resuspended with nuclear staining buffer (0.1% Triton X-100 in PBS, 200 μg/ml RNase, and 100 μg/ml propidium iodide) for 30 min at 37 °C. For each sample, at least 1×10^4 events were recorded. Cell cycle profiles were obtained with a FACScan flow cytometer (Becton Dickinson Co., San Jose, CA) and data were analyzed with WinCycle software (Phoenix Flow Systems Inc., San Diego, CA).

2.4. Immunoblotting

Immunoblotting was performed similar to our previous study (Guh et al., 2003). Briefly, a 30 μg sample of cell lysates was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes. After blocking, blots were incubated with antibody in blocking solution overnight (phospho-antibodies) or for 2 h (other antibodies) followed by 5 min wash twice in PBS containing 0.1% Tween 20 and then incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnologies Inc., Santa Cruz, CA) for 1 h. Enhanced chemiluminescence reagents were employed to depict protein bands on the membrane.

2.5. Measurement of apoptosis

Apoptosis was measured by flow cytometry by using the Vybrant^{TM} Apoptosis Assay Kit #2 (Alexa Fluor® 488 annexin V/Propidium Iodide kit #2) supplied by Molecular Probes Inc. (Eugene, OR) according to the manufacturer's instructions. This kit detects the externalization of phosphatidylserine in apoptotic cells using the green-fluorescent Alexa Fluor® 488 annexin and the red-fluorescent propidium iodide nucleic acid stain. Propidium iodide stains necrotic cells with red fluorescence. After treatment with both probes, apoptotic cells show green fluorescence, dead cells show red and green fluorescence, and live cells show little or no fluorescence.

2.6. Transient transfection and luciferase assay

Transient transfection was performed similar to our previous study (Chou et al., 2008). Briefly, MCF-7 cells were plated into 6-well plates at density of 1.2×10^5 cells/well in DMEM/F12 medium and grown overnight. Cells were transfected with 0.2 μg of the
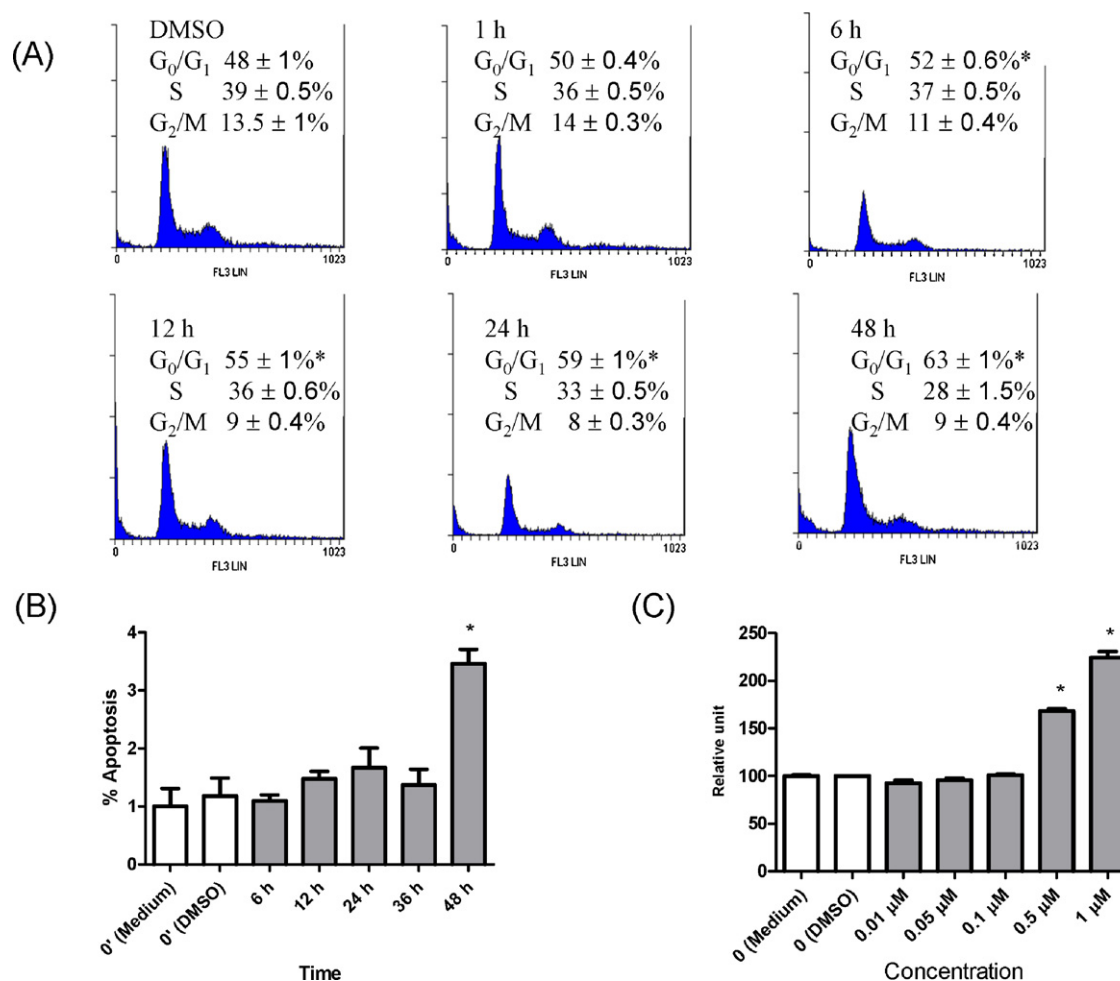
Fig. 1. Time-dependent effects of annonacin on cell cycle distribution and cell death in MCF-7 cells. MCF-7 cells (2.5×10^5 cells/dish) were plated and incubated in 6 cm dishes. Cells were fasted for 24 h before adding fresh medium (10% FBS) containing 0.1% DMSO or 0.1 μM annonacin. Cell cycle was measured by flow cytometry. Apoptosis was measured by flow cytometry by using the Vybrant™ Apoptosis Assay Kit #2 (Alexa Fluor® 488 annexin V/Propidium Iodide kit #2). (A) Time-dependent (1–48 h) effects of annonacin (0.1 μM) on cell cycle distribution. (B) Time-dependent (6–48 h) effects of annonacin (0.1 μM) on apoptosis. (C) Dose-dependent effects of annonacin on cell death. MCF-7 cells (6500 cells/well) were plated and incubated in 96-well plates. Cells were fasted for 24 h before adding fresh medium (10% FBS, open bars) containing 0.1% DMSO (open bars) or various concentrations of annonacin (gray bars). Cell death was measured by lactate dehydrogenase release. The results were expressed as the mean ± SEM of three independent experiments. *$P < 0.05$ versus DMSO.

ERα transcriptional activity reporter plasmid containing 3X ERE-TATA luc (Addgene Inc., Cambridge, MA) with LipofectAMINE 2000 (Life Technology, Gaithersburg, MD) and luciferase activities were assayed by integrating the total light emission over 10 s by using the Dynatech ML1000 luminometer.

2.7. In vivo tumor xenograft study

Female nude mice (4 weeks old; BALB/cA-nu [nu/nu]) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and were housed in specific pathogen-free conditions for 2 weeks. Afterwards, MCF-7 cells (5×10^6 cells in 200 μL PBS) were injected subcutaneously into the flanks, and tumors were allowed to develop for 30 days. Then eleven mice were randomly divided into two groups. Annonacin-treated mice ($N = 6$) were intraperitoneally injected daily with annonacin (50 mg/kg/day) in 200 μL of 25% polyethylene glycol. Control mice ($N = 5$) were intraperitoneally injected daily with 200 μL of 25% polyethylene glycol. Tumor volume was measured using calipers at 0, 3, 7, 11, 15, 19 and 22 days. Tumor volume was estimated by the following formula: tumor volume (mm^3) = $L \times W \times W$/2 (L: length; W: width). The xenografts of the annonacin-treated and the control mice were harvested and fixed in 4% formaldehyde, embedded in paraffin

for immunohistochemistry at 22 days. All animal procedures were approved and done in accordance with the national guidelines and the guidelines by the Kaohsiung Medical University Animal Experiment Committee.

2.8. Immunohistochemistry

Paraffin-embedded tumor tissues were cut to 4 μm sections for immunohistochemistry. The sections were treated with microwave at 100 °C for 30 min, and blocked nonspecific response. The sections were incubated at 4 °C overnight with primary antibodies (ERα, cyclin D1 and Bcl-2). After washing twice 10 min with PBS containing 0.2% Tween 20, the sections were incubated with biotinylated secondary antibodies for 1 h. After washing twice with PBS contain 0.2% Tween 20 for 10 min, sections were stained by Universal DAB + kit/HRP (Dako Corp., Carpinteria, CA) and counterstained with hematoxylin.

2.9. Statistical analysis

The results were expressed as the mean ± standard errors of the mean. Unpaired Student's t-tests were used for the comparison between two groups. $P < 0.05$ was considered as statistically
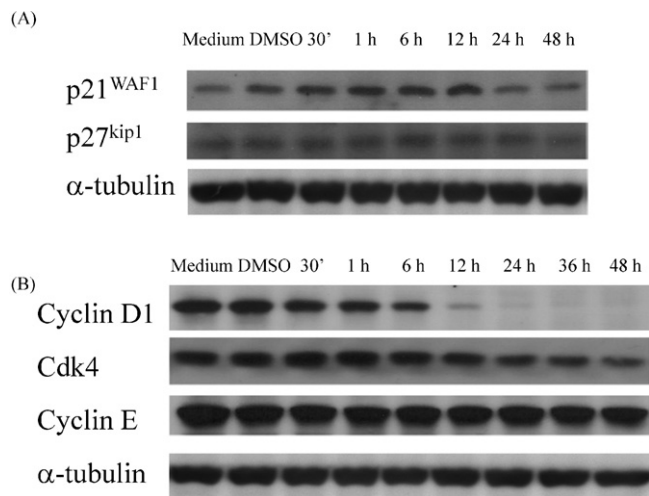
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Fig. 2. Effects of annonacin on cell cycle regulatory proteins in MCF-7 cells. MCF-7 cells (2.5×10^5 cells/dish) were plated and incubated in 6 cm dishes. Cells were fasted for 24 h before adding fresh medium (10% FBS) containing 0.1% DMSO or 0.1 μM annonacin. Cell cycle regulatory proteins (cyclin D1, cdk4, cyclin E, p21[WAF1] and p27[kip1]) were measured by immunoblotting. (A) Time-dependent effects of annonacin (0.1 μM) on p21[WAF1] and p27[kip1] protein expression. (B) Time-dependent effects of annonacin (0.1 μM) on cyclin D1, cdk4 and cyclin E protein expression. This figure is representative of three independent experiments.

significant. Concentration addition model and the isobologram method (Sørensen et al., 2007) were assessed by the drc (dose–response curves) package in the R statistical program (Knezevic et al., 2009).

3. Results

3.1. Effects of annonacin on cell cycle distribution and cell death in MCF-7 cells

Annonacin (0.1 μM) time-dependently (6–48 h) arrested cells in the G_0/G_1 phase of the cell cycle (Fig. 1A) while increasing apoptosis at 48 h (Fig. 1B). Additionally, annonacin dose-dependently (0.5–1 μM) increased cell death at 48 h (Fig. 1C). Annonacin also dose-dependently (0.05–1 μM) decreased cell survival at 48 h (supplementary Fig. 2A). Moreover, annonacin (0.1 μM) time-dependently (24–48 h) decreased cell survival (supplementary Fig. 2B). However, annonacin (0.1 μM) did not affect cell survival at 6–48 h in MDA-MB-231 cells (supplementary Fig. 2C).

3.2. Time-dependent effects of annonacin on cell cycle regulatory proteins in MCF-7 cells

Annonacin (0.1 μM) time-dependently (30 min to 12 h) increased p21[WAF1] but decreased p21[WAF1] protein expression at 24–48 h (Fig. 2A). In contrast, annonacin (0.1 μM) time-dependently increased p27[kip1] protein expression at 1–6 h (Fig. 2A). Additionally, annonacin (0.1 μM) time-dependently (1–48 h) decreased cyclin D1 and time-dependently (12–48 h) decreased cdk4 protein expression, but not that of cyclin E (Fig. 2B).

3.3. Effects of annonacin and 4-hydroxytamoxifen on cell survival and cyclin D1 or Bcl-2 protein expression in MCF-7 cells

Annonacin (0.1 μM), 4-hydroxytamoxifen (1 μM) and a combination of annonacin (0.1 μM) plus 4-hydroxytamoxifen (1 μM) decreased cell survival to a similar degree at 24 h (Fig. 3A). However, the combination of annonacin (0.1 μM) plus 4-hydroxytamoxifen

(1 μM) decreased cell survival more than annonacin (0.1 μM) did alone at 48 h (Fig. 3A).

The estimated half-effective doses from dose–response curves at 48 h were: 0% 4-hydroxytamoxifen (i.e. annonacin, $ED_{50} = 0.31$ μM), 50% 4-hydroxytamoxifen ($ED_{50} = 0.45$ μM), 91% 4-hydroxytamoxifen ($ED_{50} = 1.01$ μM), 100% 4-hydroxytamoxifen ($ED_{50} = 1.13$ μM), respectively. Isobologram analysis showed that the ED_{50} of the various combinations (50% and 91% 4-hydroxytamoxifen) coincided with the estimated concentration addition isobole (Fig. 3B). In other words, the combination effects of annonacin and 4-hydroxytamoxifen were additive instead of synergistic (ED_{50} of the various combinations shifted to left) or antagonistic (ED_{50} of the various combinations shifted to right).

As shown in supplementary Fig. 3A, annonacin (0.01–2 μM) evenly decreased cyclin D1 and Bcl-2 protein expression at 48 h. Additionally, annonacin (0.1 μM) time-dependently (1–48 h) decreased Bcl-2 protein expression (supplementary Fig. 3B). Annonacin (0.1 μM) decreased cyclin D1 protein expression more than 4-hydroxytamoxifen (1 μM) did at 48 h (Fig. 3C). In contrast, annonacin (0.1 μM) and 4-hydroxytamoxifen (1 μM) decreased Bcl-2 protein expression to a similar degree at 48 h (Fig. 3C). Moreover, the combination of annonacin (0.1 μM) plus 4-hydroxytamoxifen (1 μM) decreased Bcl-2 protein expression more than annonacin (0.1 μM) did alone at 48 h (Fig. 3C).

3.4. Effects of annonacin and 4-hydroxytamoxifen on ERα transcriptional activity, ERα protein expression and phosphorylation in MCF-7 cells

As shown in Fig. 4A, annonacin (0.1 μM) and 4-hydroxytamoxifen (1 μM) decreased ERα transcriptional activity to the same degree at 24–48 h. Moreover, the combination of annonacin (0.1 μM) plus 4-hydroxytamoxifen (1 μM) decreased ERα transcriptional activity more than annonacin (0.1 μM) did alone at 24–48 h (Fig. 4A).

As shown in supplementary Fig. 4A, 17β-estradiol increased ERα transcriptional activity whereas annonacin (0.1 μM) attenuated ERα transcriptional activity at 12–48 h. Additionally, annonacin (0.1 μM) decreased ERα protein expression at 24 h and decreased ERα protein serine 118 phosphorylation at 12 h (supplementary Fig. 4B). Annonacin dose-dependently (0.01–2 μM) decreased ERα protein expression at 48 h (Fig. 4B). In contrast, 4-hydroxytamoxifen (1 μM) did not affect ERα protein expression at 48 h (Fig. 4C). Finally, the combination of annonacin (0.1 μM) plus 4-hydroxytamoxifen (1 μM) decreased ERα protein expression to a similar degree as annonacin (0.1 μM) did alone at 48 h (Fig. 4C).

3.5. Time-dependent effects of annonacin on phosphorylation of ERK1/2, JNK and STAT3 protein in MCF-7 cells

Annonacin (0.1 μM) time-dependently (1–48 h) decreased ERK1/2 protein phosphorylation (Fig. 5A). Annonacin (0.1 μM) also time-dependently (1–48 h) decreased JNK protein phosphorylation (Fig. 5B). Additionally, annonacin (0.1 μM) time-dependently (30 min to 48 h) decreased STAT3 protein phosphorylation (Fig. 5C).

3.6. Effects of annonacin on MCF-7 xenograft tumor size and expression of ERα, cyclin D1 and Bcl-2 proteins in nude mice

To confirm the growth-inhibitory effects of annonacin in vivo, MCF-7 cells were grafted into the flanks of nude mice as xenografts. As shown in Fig. 6A, annonacin decreased tumor size at 7–22 days. As shown in Fig. 6B, annonacin attenuated the expression of ERα, cyclin D1 and Bcl-2 protein in the nude mice at 22 days.
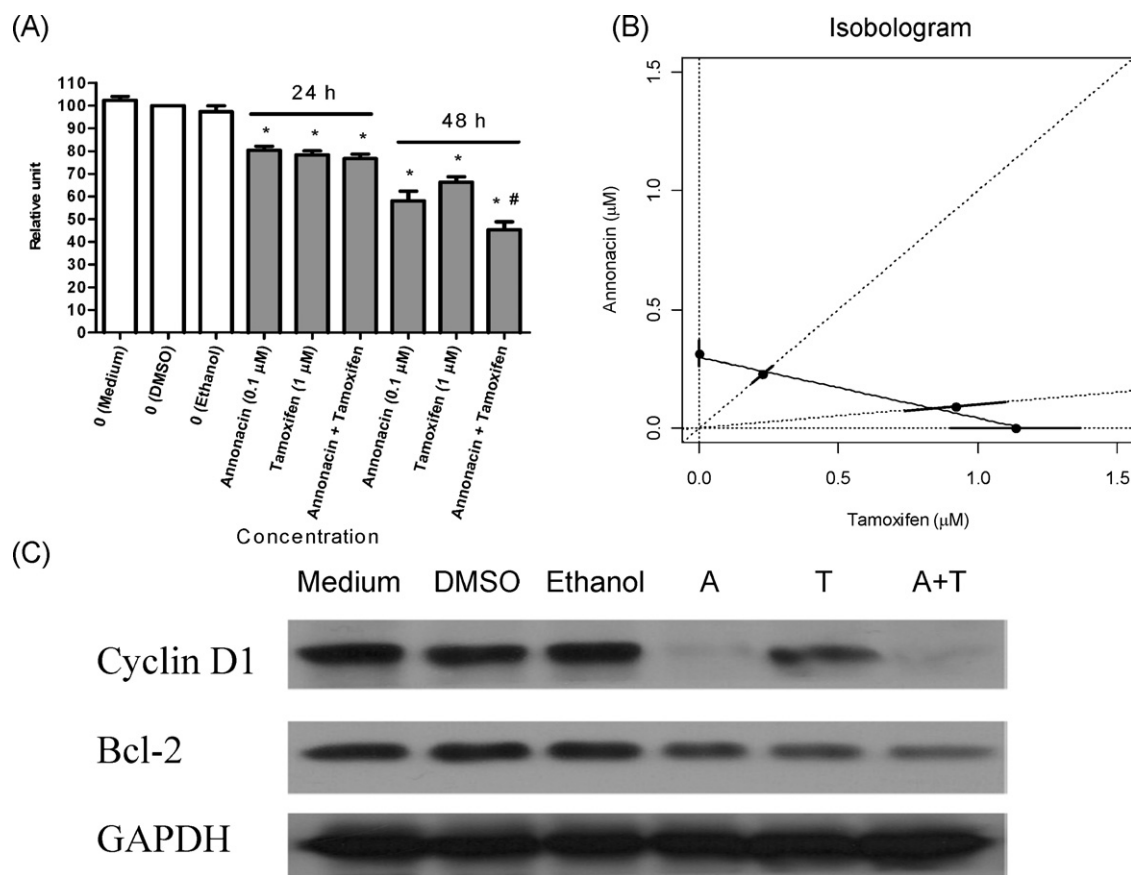
Fig. 3. Combination effects of annonacin and 4-hydroxytamoxifen on cell survival and cyclin D1 and Bcl-2 protein expression in MCF-7 cells. MCF-7 cells (6500 cells/well) were plated and incubated in 96-well plates. Cells were fasted for 24 h before adding fresh medium (10% FBS) containing 0.1% DMSO or 0.1% ethanol. Cell survival was measured by the MTT assay. (A) Effects of annonacin (A, 0.1 μM), 4-hydroxytamoxifen (T, 1 μM) or their combination ($A + T$) on cell survival at 24 h (lanes 4–6) or 48 h (lanes 7–9). $^*P < 0.05$ versus control. $^\#P < 0.05$ versus annonacin alone. (B) Isobologram analysis of various combinations (50% 4-hydroxytamoxifen and 91% 4-hydroxytamoxifen) of annonacin and 4-hydroxytamoxifen at 48 h. Dose combinations (dotted lines) achieving ED_{50} (dots) with standard errors together with the estimated isoboles are shown. The estimated concentration addition isobole is shown as the solid line connecting the 100% and 0% 4-hydroxytamoxifen ED_{50} dots. MCF-7 cells (2.5×10^5 cells/dish) were plated and incubated in 6 cm dishes. Expression of cyclin D1 and Bcl-2 protein was measured by immunoblotting and normalized to that of GAPDH. (C) Effects of annonacin (A, 0.1 μM), 4-hydroxytamoxifen (T, 1 μM) or their combination ($A + T$) on cyclin D1 and Bcl-2 protein expression at 48 h. This figure is representative of three independent experiments.

4. Discussion and conclusions

This study adds mechanistic insights to our previous finding that annonacin inhibits growth in MCF-7 cells (Yuan et al., 2003). We found that annonacin induced cell death only at high doses (≥ 0.5 μM). In contrast, low-dose (0.1 μM) annonacin, like tamoxifen and fulvestrant (Cariou et al., 2000; Musgrove and Sutherland, 2009), induced cell-cycle-dependent (G_0/G_1) growth arrest concomitantly with the induction of p21^{WAF1} and p27^{kip} and the inhibition of cyclin D1 protein expression.

Cell cycle (G_0/G_1 transition) inhibitors p21^{WAF1} and p27^{kip} are implicated in the pathogenesis or prognosis of breast cancer (Caldon et al., 2006). For example, the loss of p21^{WAF1} or p27^{kip} may mediate tamoxifen resistance in breast cancer (Musgrove and Sutherland, 2009). Conversely, transfection of p21^{WAF1} or p27^{kip} genes inhibits MCF-7 cell growth (Jiang et al., 2009). Surprisingly, annonacin inhibited both p21^{WAF1} and cyclin D1 protein expression at 24–48 h. However, inhibition of p21^{WAF1} alone cannot attenuate cyclin D1 inhibition-induced growth arrest in cancer cells (Masamha and Benbrook, 2009).

Our finding that annonacin inhibited cyclin D1 protein expression is compatible with the notion that cyclin D1 overexpression is associated with poor prognosis and tamoxifen resistance in ERα-positive breast cancer (Butt et al., 2008; Musgrove and Sutherland, 2009). Interestingly, cyclin D1 overexpression induces whereas

cyclin D1 knockout inhibits breast cancer in mice (Butt et al., 2008). Similarly, cyclin D1 overexpression increases whereas cyclin D1 inhibition decreases proliferation in MCF-7 cells (Grillo et al., 2006).

Annonacin, like tamoxifen and fulvestrant (Lam et al., 2009; Musgrove and Sutherland, 2009), also induced apoptosis while inhibiting Bcl-2 protein expression. Note that cell survival, cell death and cell cycle pathways are interconnected in cancers (Maddika et al., 2007). For example, apoptosis is a cell-cycle checkpoint during cell injury and Bcl-2 is an anti-apoptotic protein which also inhibits the G_1/S checkpoint of the cell cycle (Maddika et al., 2007; Musgrove and Sutherland, 2009). Transfection of the Bcl-2 gene inhibits whereas inhibition of the Bcl-2 gene enhances chemosensitivity of breast cancer cells (Emi et al., 2005). Moreover, Bcl-2 protein expression is associated with tamoxifen resistance in breast cancer patients (Musgrove and Sutherland, 2009).

The observation that annonacin decreased cell survival in ERα-positive MCF-7 cells, but not ERα-negative MDA-MB-231 cells, suggests that ERα is required for the effects of annonacin. Interestingly, annonacin ($ED_{50} = 0.31$ μM) was more potent than 4-hydroxytamoxifen ($ED_{50} = 1.13$ μM), while annonacin and 4-hydroxytamoxifen were additive, in inhibiting cell survival. Similarly, annonacin was more potent than 4-hydroxytamoxifen, and annonacin and 4-hydroxytamoxifen were additive, in inhibiting ERα transcriptional activity.
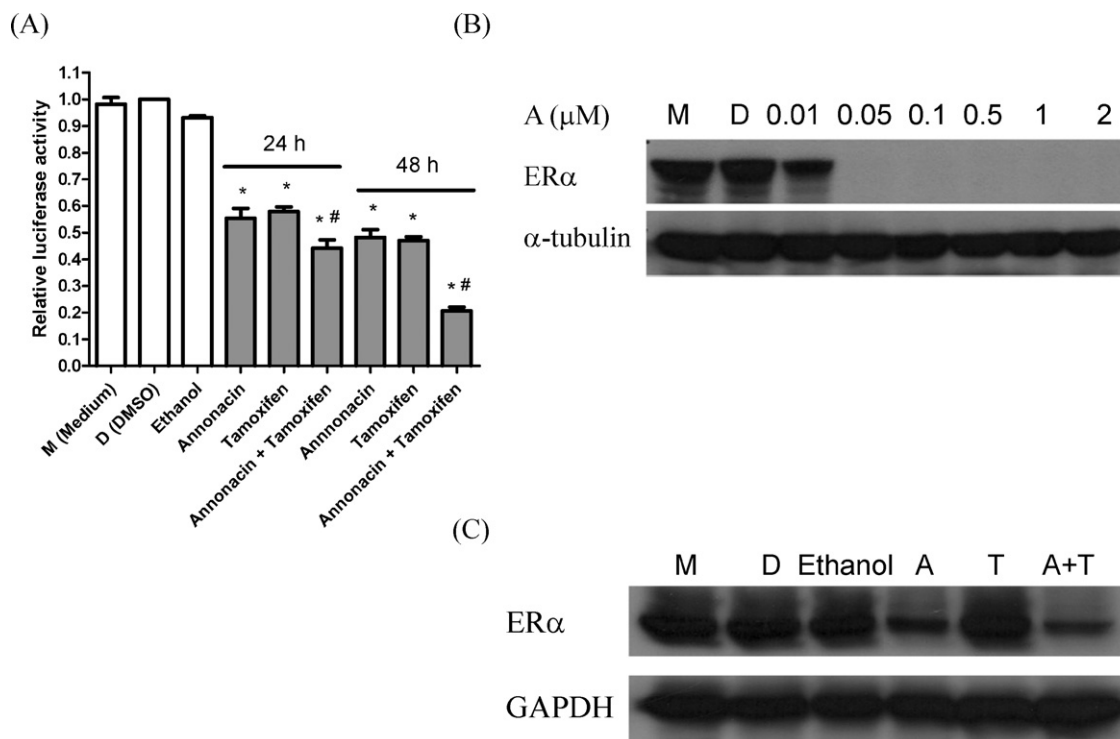
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Fig. 4. Effects of annonacin and 4-hydroxytamoxifen on ERα transcriptional activity and ERα protein expression in MCF-7 cells. MCF-7 cells (1.2×10^5 cells/well) were plated and incubated in 6-well plates. Cells were fasted for 24 h before adding fresh medium (M, 10% FBS) containing 0.1% DMSO (D) or 0.1% ethanol. (A) Effects of annonacin (0.1 μM), 4-hydroxytamoxifen (1 μM) or their combination on ERα transcriptional activity at 24 h or 48 h. Cells were transfected with 3X ERE-TATA luc with LipofectAMINE 2000 and luciferase activities were assayed by integrating the total light emission over 10 s by using the Dynatech ML1000 luminometer. *$P < 0.05$ versus control. #$P < 0.05$ versus annonacin alone. MCF-7 cells (2.5×10^5 cells/dish) were plated and incubated in 6 cm dishes. Cells were fasted for 24 h before adding fresh medium containing 0.1% DMSO, 0.1% ethanol, annonacin or 4-hydroxytamoxifen. Expression of ERα protein was measured by immunoblotting. (B) Dose-dependent effects of annonacin (A, 0.01-2 μM) on ERα protein expression at 48 h. Expression of ERα was normalized to that of α-tubulin. (C) Effects of annonacin (A, 0.1 μM), 4-hydroxytamoxifen (T, 1 μM) or their combination (A + T) on ERα protein expression at 48 h. Expression of ERα was normalized to that of GAPDH. Both figures B and C are representative of three independent experiments.
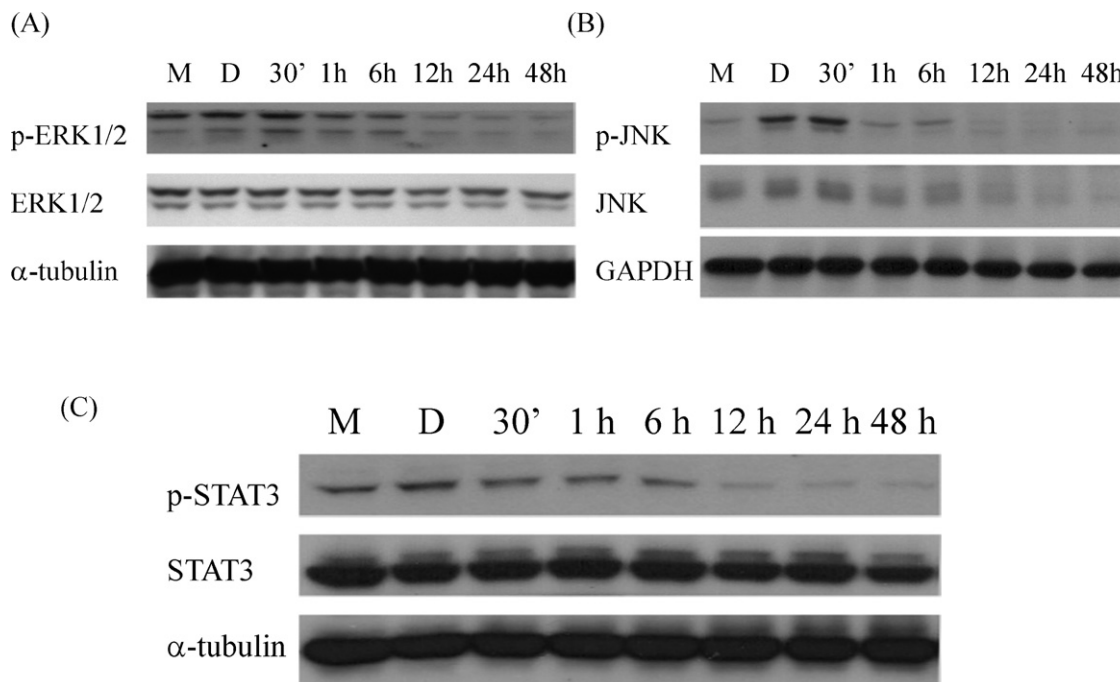
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Fig. 5. Time-dependent effects of annonacin on phosphorylation of ERK1/2, JNK and STAT3 protein in MCF-7 cells. MCF-7 cells (2.5×10^5 cells/dish) were plated and incubated in 6 cm dishes. Cells were fasted for 24 h before adding fresh medium (M, 10% FBS) containing 0.1% DMSO (D) or annonacin for 30 min to 48 h. Phosphorylation of ERK1/2, JNK and STAT3 protein was measured by immunoblotting and normalized to that of ERK1/2, JNK and STAT3. (A) Time-dependent effects of annnonacin (0.1 μM) on p-ERK1/2 protein phosphorylation. (B) Time-dependent effects of annnonacin (0.1 μM) on p-JNK protein phosphorylation. (C) Time-dependent effects of annnonacin (0.1 μM) on p-STAT3 protein phosphorylation. This figure is representative of three independent experiments.
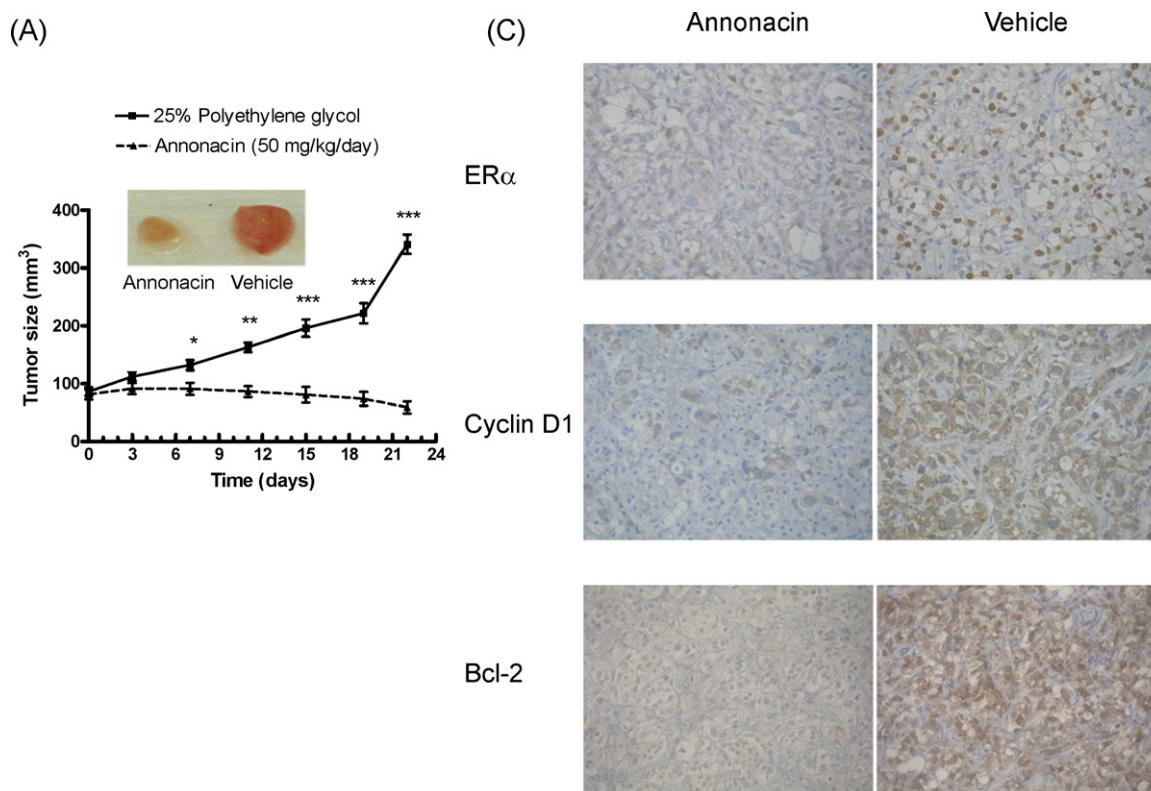
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(C)

Fig. 6. Effects of annonacin on MCF-7 xenograft tumor size and expression of ERα, cyclin D1 and Bcl-2 protein in the nude mice. MCF-7 cells (5×10^6 cells in 200 μL PBS) were grafted into the flanks of the nude mice as xenografts, and tumors were allowed to develop for 30 days. Afterwards, annonacin (50 mg/kg/day) or vehicle (25% polyethylene glycol) was administered intraperitoneally for 21 days. (A) Tumor volume was measured every 3–4 days using calipers at 0, 3, 7, 11, 15, 19 and 22 days (inset: tumor at 22 days). (B) Tumor was excised at 22 days and immunohistochemistry was performed for the expression of ERα, cyclin D1 and Bcl-2 protein. The results were expressed as the mean ± SEM of 5 mice in each group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle.

The mode of action of annonacin can be studied by ERα binding assay and ERα transcriptional activation assay (Dang et al., 2011). For example, SERM and SERD usually act by inhibiting ERα ligand-binding. We did not study the ERα-binding ability of annonacin. However, we found that annonacin, like SERD, also decreases ERα protein expression. Thus, annonacin, like SERD, may be useful in tamoxifen resistance (Osborne and Schiff, 2011). We also found that annonacin decreased ERE-dependent ERα transcriptional activity. Note that ERE is indispensable for ERα-induced phenotypes in breast cancer cells (Nott et al., 2009). Moreover, annonacin decreased ERα serine 118 phosphorylation, which is required for some ERα-induced effects (Duplessis et al., 2011). In contrast, both tamoxifen and fulvestrant increase ERα serine 118 phosphorylation (Lipfert et al., 2006; Maggi, 2011). However, there are many other possible modes of action whereby annonacin can inhibit ERα-induced effects (Shapiro et al., 2011).

Annonacin, unlike tamoxifen (Ishii et al., 2008; Lam et al., 2009; Musgrove and Sutherland, 2009), inactivated ERK1/2, JNK and STAT3 in this study. Interestingly, inhibition of ERK1/2 attenuates tamoxifen resistance (Ghayad et al., 2010) and inhibition of JNK or STAT3 induces apoptosis in breast cancer cells (Kunigal et al., 2009; Mingo-Sion et al., 2004). In summary, annonacin differs from tamoxifen in terms of ERα protein abundance and phosphorylation, p-ERK1/2, p-JNK and p-STAT3. These observations may partly account for the finding that annonacin and tamoxifen were additive in inhibiting MCF-7 cell growth.

The in vitro effects of annonacin were corroborated by our findings that annonacin attenuated tumor size and the expression of ERα, cyclin D1 and Bcl-2 protein in MCF-7 cell-grafted nude mice. In this regard, cyclin D1 is overexpressed in 50% of breast cancers (Caldon et al., 2006). Breast epithelial cell-specific overexpression

of cyclin D1 induces breast cancer in mice, while cyclin D1-null mice are resistant to oncogene-induced breast cancer (Butt et al., 2008). Moreover, inhibition of Bcl-2 in combination with chemotherapy was effective in some breast cancer patients in a clinical trial (Fato et al., 2008).

In conclusion, annonacin induced cell-cycle-dependent growth arrest and induced apoptosis in ERα-related pathways (ERK1/2, JNK, STAT3, cyclin D1, Bcl-2, p21^{WAF1} and p27^{kip1}) in MCF-7 cells. Annonacin and 4-hydroxytamoxifen were additive in inhibiting growth and ERα transcriptional activity. Moreover, annonacin attenuated MCF-7 xenograft tumor growth while inhibiting ERα, cyclin D1 and Bcl-2 protein expression in nude mice.

Acknowledgement

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.07.056.

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