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Asiatic acid, a triterpene, induces apoptosis and cell cycle arrest through activation of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways in human breast cancer cells

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Abbreviations: ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; phospho-JNK, phosphorylated c-Jun NH_2-terminal kinase, phospho-p38, phosphorylated p38, phospho-ERK1/2, phosphorylated extracellular signal-regulated kinase; SB203580, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole; U0126, 1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene; FCS, Fetal calf serum; FasL, Fas ligand; mFasL, membrane–bound Fas Ligand; sFasL, soluble Fas Ligand; ELISA, enzyme-linked immunosorbent assay; LEHD-CHO, Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Leu-Glu-His-Asp-CHO; MEK1, mitogen-activated protein kinase kinase; siRNA, small interfering RNA.

Abstract

This study first investigates the anticancer effect of asiatic acid in two human breast cancer cell lines, MCF-7 and MDA-MB-231. Asiatic acid exhibited effective cell growth inhibition by inducing cancer cells to undergo S-G2/M phase arrest and apoptosis. Blockade of cell cycle was associated with increased p21/WAF1 levels, and reduced amounts of cyclinB1, cyclinA, Cdc2 and Cdc25C in a p53-independent manner. Asiatic acid also reduced Cdc2 function by increasing the association of p21/WAF1/Cdc2 complex and the level of inactivated phospho-Cdc2 and phospho-Cdc25C. Asiatic acid treatment triggered the mitochondrial apoptotic pathway indicated by changing Bax/Bcl-2 ratios, cytochrome *c* release and caspase-9 activation, but did not act on Fas/Fas ligand pathways and the activation of caspase-8. We also found that mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK1/2) and p38, but not c-Jun N-terminal kinase (JNK), are critical mediators in asiatic acid-induced cell growth inhibition. U0126 or SB203580, specific inhibitors of MEK and p38 kinase activities, significantly decreased or delayed apoptosis. Asiatic acid was likely to confine the breast cancer cells in the S-G2/M phase mainly through the p38 pathway, because both SB203580 and p38 siRNA inhibition significantly attenuated the accumulation of inactive phospho-Cdc2 and phospho-Cdc25C proteins and the cell numbers of S-G2/M phase. Moreover,

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U0126 and ERK siRNA inhibition completely suppressed asiatic acid-induced Bcl-2 phosphorylation and Bax upregulation, and caspase-9 activation. Taken together, these results imply a critical role for ERK1/2 and p38 but not JNK, p53, and Fas/Fas ligand in asiatic acid-induced S-G2/M arrest and apoptosis of human breast cancer cells.

Introduction

Breast cancer is one of the most common malignancies in women, and is the leading cause of death worldwide for women between the ages of 40 and 55 years in the world (Baselga et al., 1994). This pathology is currently controlled by surgery and radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies (Bange et al., 2001). However, breast cancer is highly resistant to chemotherapy, and there is still no effective cure for patients with advanced stages of the disease, especially in cases of hormone-independent cancer (Bange et al., 2001; Chopin et al., 2004). Effective chemopreventive treatment for breast cancer would have an important impact on breast cancer morbidity and mortality. A series of triterpenes are widely distributed in composite plants, and their biologic activities have attracted a great deal of attention. Many triterpenoids have shown promising effects when applied as antineoplastic agents (Haridas et al., 2004; Saleem et al., 2004). Asiatic acid, a plant-derived triterpenoid compound, was extracted from the tropical medicinal plant *Centella asiatica* (Coldren et al., 2003). It has been found to prevent UVA-mediated photo-aging, to inhibit β-amyloid-induced neurotoxicity, and to possess anti-ulcer and anti-hepatofibric activities (Dong et al., 2004; Jew et al., 2000; Lee et al., 2000; Soo Lee et al., 2003). It also has been reported to exhibit a cytotoxic effect against Hep G2 cells by Ca^{2+} release and p53 upregulation (Lee et al., 2002).

However, the anticancer effect and mechanism of asiatic acid in human breast cancer remains unknown.

Eukaryotic cell cycle progression involves the sequential activation of Cdks, whose activation is dependent upon their association with cyclins. The complex formed by the association of Cdc2 and cyclinB1 plays a major role at entry into mitosis (Sancar et al., 2004). The phosphorylation of Tyr15 of Cdc2 suppresses activity of Cdc2/cyclinB1 kinase complex. Dephosphorylation of Tyr15 of Cdc2 is catalyzed by Cdc25C phosphatases, and this reaction is believed to be the rate-limiting step for entry into mitosis (De Souza et al., 2000). Cell cycle progression is also regulated by the relative balance between the cellular concentrations of cyclin-dependent kinase inhibitors (CKIs), such as members of the cyclin-dependent kinase-interacting protein/cyclin-dependent kinase inhibitory protein (CIP/KIP) and inhibitor of cyclin-dependent kinase (INK) families, and that of cyclin-CDK complexes. The Cip/Kip family, including p21/WAF1, and p27/KIP, bind to cyclin-CDK complexes and prevent kinase activation, subsequently blocking the progression of the cell cycle at the G0/G1 or G2/M phases (Sancar et al., 2004).

The mitogen-activated protein kinases (MAPKs), a family of serine/threonine kinases, are mediators of intracellular signals in response to various stimuli. JNK (c-Jun NH_2-terminal protein kinase), p38 and ERK1/2 (extracellular signal-regulated

kinase) are the three main members of three different MAPK pathways that can be activated by growth factors, DNA damage, cytokines, oxidant stresses, UV light, anticancer drugs, and osmotic shock (Johnson et al., 2002; Olson et al., 2004). All three MAPK pathways can be differentially activated, and their involvement in apoptosis is highly context and model dependent. JNK and p38 are activated by cellular stress and both have been associated with apoptosis (Huh et al., 2004; Xiao et al. 2004). However, there have also been reports indicating that the JNK is required for IL-3-mediated cell survival, and that p38 is associated with the development of chemoresistance by activating NF-κB (Hendrickx et al., 2003; Yu et al., 2004). In contrast, the activation of the ERK1/2 pathway is generally considered to be a survival signal induced by mitogenic stimuli or growth factors against apoptotic signals (Johnson et al., 2002; Olson et al., 2004; Park et al., 2003). However, some of the literature contradicts ERK1/2's essential role in several types of chemotherapeutic or preventive agent-induced apoptosis, such as taxol, resveratrol, and quercetin (Bacus et al., 2001; Nguyen et al., 2004; She et al., 2001). This may be due to the cell type and cell content specificity of apoptosis inducers and their subsequent signaling transduction pathways.

In this study, we determined the cell growth inhibition activity of asiatic acid, and examined its effect on cell cycle distribution and apoptosis in the two human breast

cancer cell lines, MCF-7 and MDA-MB-231. Furthermore, to establish asiatic acid's anticancer mechanism, we assayed the levels of cell cycle control and apoptosis related molecules, which are strongly associated with the signal transduction pathway of apoptosis and affect the chemosensitivity of tumor cells to anticancer agents.

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Materials and Methods

Reagents

Fetal calf serum (FCS), nonessential amino acids, sodium pyruvate, insulin, and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO BRL (Gaithersburg, MD). Asiatic acid, dimethyl sulfoxide (DMSO), ribonuclease (RNase) and propidium iodide were purchased from Sigma Chemical Co. (St. Louis, MO). Nucleosome ELISA, WAF1 ELISA, Fas Ligand, Fas/APO-1 ELISA, and caspase-9, caspase-8 activity assay kits, caspase-9 inhibitor (LEDH-CHO) and p38 inhibitor SB203580(4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole) were purchased from Calbiochem (Cambridge, MA). The antibodies to β-actin, cyclinB1, cyclinA, Cdc2, Cdc25C, p21/WAF1, Bax, Bak, Bcl-2, phospho-Bcl-2 and Bcl-X_L were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The antibodies to JNK, p38, ERK, phospho-JNK, phospho-p38, phospho-ERK, phospho-Cdc2, phospho-Cdc25C and cytochrome *c*, and MEK inhibitor U0126 (1,4-Diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene) were obtained from Cell Signaling Technology (Beverly, MA).

Cell culture

Breast cancer cell lines MCF-7 (ATCC HTB-22) and MDA-MD-231(ATCC

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HTB-26) were obtained from the American Type Cell Culture Collection (Manassas, VA). MCF-7 cells were cultured in DMEM with nonessential amino acids, 0.1 mM sodium pyruvate, 10 μg/ml insulin, and 10% FCS. The MDA-MB-231 cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS and 1% penicillin-streptomycin solution (Life Technologies, Inc.).

Cell proliferation assay

Inhibition of cell proliferation by asiatic acid was measured by XTT (sodium 3'-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate) assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with asiatic acid (0, 2.5, 5, 10 and 20 μM) for 48 h. Fifty μl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 μl of electron coupling reagent, was then added to each well. After 4 h incubation, absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. Data were calculated as the percentage of inhibition by the following formula: inhibition % = [100 - (ODt/ODs) × 100]%. ODt and ODs indicated the optical density of the test substances and the solvent control, respectively. The concentration of 50% cellular cytotoxicity of cancer cells (IC_{50}) of test substances was calculated based on 48 h absorbance values.

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Cell cycle analysis

To determine cell cycle distribution analysis, 5×10^5 cells were plated in 60mm dishes and treated with asiatic acid (0, 5, and 10 μM) for 12 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in phosphate-buffered saline (PBS), resuspended in 1 ml of PBS containing 1 mg/ml RNase and 50 μg/ml propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed by EPICS flow cytometer. The data were analyzed using Multicycle software (Phoenix Flow Systems, San Diego, CA).

Assaying the levels of p53, p21/WAF1, Fas/APO-1 and Fas ligand (mFasL and sFasL)

p53 pan ELISA, WAF1 ELISA, Fas/APO-1 ELISA and Fas Ligand ELISA kits were used to detect p53, p21/WAF1, Fas/APO-1 receptor and FasL. Briefly, cells were treated with 0, 5, and 10 μM asiatic acid for the indicated times. The samples of cell lysate were placed in 96 well (1×10^6 per well) microtiter plates coated with monoclonal detective antibodies, and were incubated for 1 h (Fas/APO-1), 2 h (p53 or p21/WAF1) or 3 h (Fas ligand) at room temperature. It was necessary to determine the soluble Fas ligand in cell culture supernatant by using Fas Ligand ELISA kit. After removing unbound material by washing with washing buffer (50 mM Tris, 200 mM NaCl, and 0.2% Tween 20), horseradish peroxidase conjugated streptavidin was added

to bind to the antibodies. Horseradish peroxidase catalyzed the conversion of a chromogenic substrate (tetramethylbenzidine) to a colored solution, with color intensity proportional to the amount of protein present in the sample. The absorbance of each well was measured at 450 nm, and concentrations of p53, p21/WAF1, Fas/APO-1 and FasL were determined by interpolating from standard curves obtained with known concentrations of standard proteins.

Assay for caspase-8 and -9 activities

The assay is based on the ability of the active enzyme to cleave the chromophore from the enzyme substrate: Ac-IETD-pNA (Ac-Ile-Glu-Thr-Asp-*p*NA) for caspase-8, and LEHD-pNA (Ac-Leu-Glu-His-Asp-*p*NA) for caspase-9. Cell lysates were incubated with peptide substrate in assay buffer (100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) for 2 h at 37□. The release of *p*-nitroaniline was monitored at 405 nm. Results are represented as the percentage of change of activity compared to the untreated control.

Immunoprecipitation/immunoblot, and ERK1/2 and p38 MAPK kinase activity assays

Cells were treated with 10 μM asiatic acid in the absence or presence of MAPK inhibitors for specified intervals of time. Mitochondrial and cytoplasmic fractions

were separated using Cytochrome *c* Releasing Apoptosis Assay Kit (BioVision, California, USA). For immunoblotting, the cells were lysed on ice for 40 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EGTA, 12 mM β-glycerolphosphate, 10 mM NaF, 16 μg/ml benzamidine hydrochloride, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. The cell lysate was centrifuged at 14,000 × *g* for 15 min, and the supernatant fraction was collected for immunoblotting. Equivalent amounts of protein were resolved by SDS-PAGE (10-12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1-16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham, USA) according to the manufacturer's instructions.

For association of p21/WAF1 with Cdc2, cell lysates (300 μg) were incubated with 10 μl anti-Cdc2 for 1 h at 4°C. Immunocomplexes were resolved by 7.5% SDS–PAGE. Association of p21/WAF1 with Cdc2 was detected by incubating the blots with anti-p21/WAF1 and anti-Cdc2 antibodies as described above.

The ERK1/2 and p38 MAPK activities were determined using kits from Cell

Signaling Technology (Beverly, MA) according to the manufacturer's instructions.

siRNA knockdown of p38 and ERK expression

Breast cancer cell monolayers were transfected with SMARTpool™ p38/SPAK or MAPK1(ERK2) siRNA duplexes or non-specific control siRNA duplexes (Upstate Biotechnology Inc, NY) by using Lipofectamine 2000 (Invitrogen). At these times, immunoblot analysis showed that expression of p38 and ERK1/2 remained low but detectable, and expression of β-actin was unaffected by siRNA treatment.

Statistical analysis

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

Results

Asiatic acid induces cell cycle arrest and apoptosis in breast cancer cell lines

As shown in Figure 1A, asiatic acid inhibited cell growth in two human breast cancer cell lines in a concentration-dependent manner, with MCF-7 being more sensitive to asiatic acid-induced cell growth inhibition than MDA-MB-231. The IC_{50} values of asiatic acid were 5.95 μM for MCF-7 and 8.12 μM MDA-MB-231.

To examine the mechanism responsible for asiatic acid-mediated cell growth inhibition, cell cycle distribution was evaluated using flow cytometric analysis. The results showed that treating cells with asiatic acid caused a significant inhibition of cell cycle progression in both MCF-7 and MDA-MB-231 cells at 12 h (Figure 1B), resulting in a clear increase of the percentage of cells in the S-G2/M phase when compared with the control.

We next assessed the effect of asiatic acid on the induction of apoptosis in MCF-7 and MDA-MB-231 cells by DNA fragmentation assay. The results showed that asiatic acid treatment results in the formation of DNA fragments in both MCF-7 and MDA-MB-231 cells, as determined by agarose gel electrophoresis at 48 h (Figure 1C). Additionally, a quantitative evaluation was then sought using ELISA to detect histone-associated oligonucleosome DNA fragments. Compared to vehicle-treated cells, 10 μM asiatic acid induced a 4.5- and 7.7-fold increase of oligonucleosome in

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MCF-7 cells, whereas oligonucleosome only increased 3.5- and 6.2-fold in MDA-MB-231 cells at 24 and 48 h respectively (Figures 1D and E).

Asiatic acid increases p21/WAF1 expression through a p53-independent pathway

Because our studies showed that asiatic acid treatment of breast cancer cells results in S-G2/M phase cell cycle arrest, we examined the effect of asiatic acid on cell cycle-regulatory molecules, including p21/WAF1, cyclinB1, cyclinA, Cdc25C, and Cdc2. Previous reports have indicated that MCF-7 cells have a normal tumor suppression gene, p53, whereas in MDA-MB-231 cells the major protein of the p53 gene has mutated and is accompanied by the absence of p53 function (Amellem et al., 1990; Negrini et al., 1994). As shown in Figure 2A, asiatic acid failed to affect the expression of p53 at any of the examined time points in MCF-7 cells, but increased the expression of p21/WAF1 in both MCF-7 and MDA-MB-231 cells (Figures 2B and 2C).

Asiatic acid treatment of the cells resulted in a time-dependent decrease in the protein expression of cyclinB1, cyclinA and Cdc25C as well as Cdc2 in both MCF-7 and MDA-MB-231 cells (Figure 2D). In addition, exposure of cells to asiatic acid for 3 h resulted in an increase in the levels of inactive phospho-Cdc2 (Tyr 15) and phospho-Cdc25C (Ser 216). Results from time-dependent studies have indicated that decreasing functional Cdc25C by increasing phosphorylation was followed by an

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increase in phospho-Cdc2 (Figure 2D). In addition, the association of p21/WAF1 and Cdc2 increased in a time-dependent manner in asiatic acid-treated MCF-7 and MDA-MB-231 cells, as detected by immunoprecipitation assay (Figure 2E). We suggest that Cdc2 action was inhibited by a decrease in Cdc25C expression and an increase in the association of p21/WAF1 with Cdc2.

Fas/Fas ligand is not involved in asiatic acid-mediated apoptosis

To establish the sequence of events occurring during asiatic acid-induced apoptosis, we measured some of the molecular activity of the death receptor apoptotic pathway, including Fas/APO-1 receptor and its two ligands, mFas ligand and sFas ligand. However, treatment of either of these two cell lines with 5 or 10 μM asiatic acid failed to affect the levels of these proteins at any of the examined time points, including Fas/APO-1, mFas ligand and sFas ligand (data not shown). In addition, asiatic acid also failed to affect the activation of caspase-8 in both MCF-7 and MDA-MB-231 cells (data not shown).

Asiatic acid induces the execution of apoptosis through activation of the mitochondrial pathway

To investigate the mitochondrial apoptotic events involved in asiatic acid-induced apoptosis, we first analyzed the changes in the levels of pro-apoptotic protein Bax and anti-apoptotic proteins Bcl-2 and Bcl-X_L. Western blot analysis showed that treatment

of MCF-7 and MDA-MB-231 cells with asiatic acid increased Bax protein levels (Figure 3A). In contrast, asiatic acid decreased Bcl-2 and Bcl-X_L levels, which led to an increase in the proapoptotic/antiapoptotic Bcl-2 ratio (Figure 3A). In addition, asiatic acid also increased phosphorylation of Bcl-2 in both MCF-7 and MDA-MB-231 cells. However, asiatic acid failed to affect the Bak levels in either MCF-7 or MDA-MB-231 cells at any of the examined points in time.

Cytosolic extracts were prepared under conditions to preserve the mitochondria, and cytosolic cytochrome *c* protein levels were measured by immunoblotting analysis. Figure 3B shows that the cytosolic fraction from untreated MCF-7 and MDA-MB-231 cells contained no detectable amounts of cytochrome *c*, whereas it did become detectable after 12 h of 10 μM asiatic acid treatment in both MCF-7 and MDA-MB-231 cells.

Hallmarks of the apoptotic process include the activation of cysteine proteases, which represent both initiators and executors of cell death. Upstream caspase-9 activities increased significantly as shown by the observation that treatment with asiatic acid increased caspase-9 activity in both MCF-7 and MDA-MB-231 cells. This is consistent with the release of cytochrome *c* into the cytosol (Figures 3C and D). Furthermore, when cells were pre-treated with the specific caspase-9 inhibitor

LEHD-CHO before asiatic acid treatment, the apoptosis induction effect of asiatic acid decreased in both MCF-7 and MDA-MB-231 cells (Figure 3E).

Asiatic acid induces the activation of ERK and p38, but not JNK pathway

We assessed the level of MAPKs (JNK, p38, ERK1/2) in asiatic acid-treated MCF-7 and MDA-MB-231 cells. As shown in Figure 4A, we did not observe the levels and activation (phosphorylation) of JNK in the two breast cancer cell lines exposed to 10 μM asiatic acid for 1, 3, 6 and 12 h. Instead, we found that exposure of either line of breast cancer cells to 10 μM asiatic acid resulted in a rapid and sustained activation of p38 and ERK1/2 (Figure 4A). Activation (phosphorylation) of p38 and ERK1/2 was evident as early as 1 h after asiatic acid treatment and persisted for the duration of the experiment. On the other hand, the expression of p38 and ERK1/2 (unphosphorylated form) was not altered by asiatic acid treatment. Asiatic acid-mediated activation of p38 and ERK1/2 was additionally confirmed by determining phosphorylation of one of its substrates (ATF-2 and Elk-1 for p38 and ERK1/2, respectively.). As shown in Figure 4B, in comparison with the control, the Ser383 phosphorylation of Elk-1 increased after a 1 h exposure of MCF-7 and MDA-MB-231 cells to 10 μM asiatic acid. Phosphorylation of Elk-1 increased relative to the control at all 4 points in time (Figure 4B). Similarly, phosphorylation of ATF-2 at Thr 71 increased in both asiatic acid-treated MCF-7 and MDA-MB-231

cells at 1, 3, 6 and 12 h, in contrast to the control.

Decrease of asiatic acid-induced cell cycle arrest and apoptosis by ERK1/2 and p38 chemical inhibitors

To experimentally verify the possible role of p38 and ERK1/2 in asiatic acid-induced apoptosis, MCF-7 and MDA-MB-231 cells were pretreated for 1 h with specific inhibitor for MEK1 (mitogen-activated protein kinase kinase, an upstream kinase in ERK1/2 signaling pathway), U0126, or a potent specific inhibitor for p38, SB203580. Subsequently, the inhibitor-treated cells were exposed to asiatic acid, then the cell cycle distribution and apoptosis were determined. As shown in Figure 5A, the asiatic acid-mediated p38 and ERK activation was effectively inhibited by 20 μM SB203580 and 20 μM U0126 respectively. Flow cytometric analysis of MCF-7 and MDA-MB-231 cells exposed to asiatic acid for 12 h showed the effect of SB203580 (20 μM) or U0126 (20 μM) on S-G2/M progression. Pretreatment of SB203580 for 1 h reduced the S-G2/M populations, both of which were accumulated by the asiatic acid treatment (Figure 5B). Despite its ability to reduce the sub-G0 population, U0126 had no significant effect on the S-G2/M population (Figure 5B).

Figure 5C shows the effect of MEK and p38 inhibitor on asiatic acid-induced apoptosis in both MCF-7 and MDA-MB-231 cells. In comparison with control cells, the percentage of apoptotic cells was significantly higher in cultures exposed to 10

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μM asiatic acid, but this effect was blocked by MEK inhibitor U0126 at 24 and 48 h (Figure 5C). In contrast, co-treatment of cells with the p38 inhibitor SB203580 and asiatic acid resulted in a delay in the initiation of apoptosis. DNA fragmentation was not evident at 24 h, but started to become significant only after 48 h of treatment (Figure 5D).

The role of p38 in asiatic acid-mediated S-G2/M arrest

Previous studies have indicated that regulation of Cdc25C phosphorylation and accumulation of the inactive phosphorylated Cdc2 by p38 is a critical event for initiating the G2/M checkpoint. As shown in Figure 6A, pretreatment of cells with SB203580 decreased the levels of phospho-Cdc2 and blocked the degradation of Cdc2 observed after asiatic acid exposure. Similarly, SB203580 also abolished Cdc 25C degradation by increasing phospho-Cdc25 in both asiatic acid-treated MCF-7 and MDA-MB-231 cells. In contrast, U0126 failed to affect the asiatic acid-mediated changes in Cdc2 and Cdc25C expression and their phosphorylation in either MCF-7 or MDA-MB-231 cells. These data strongly suggest that the p38 MAPK pathway might plays an important role in asiatic acid-induced S-G2/M arrest.

The role of p38 and ERK1/2 in mitochondrial apoptotic pathways in asiatic acid-treated breast cancer cells

We further investigated the mechanism that accounts for the actions of p38 and

ERK1/2 in asiatic acid-induced apoptosis in breast cancer cells. We tested the involvement of the mitochondrial apoptotic pathways by examining the effect of p38 and ERK inhibitors on Bax, Bcl-2 and phospho-Bcl-2 expression, and caspase-9 activation. As shown in Figure 7A, co-treatment of MCF-7 and MDA-MB-231 cells with asiatic acid and U0126 completely blocked asiatic acid-mediated Bax upregulation, Bcl-2 downregulation and phosphorylation. In contrast, these effects were not affected when cells were co-treated with asiatic acid and SB203580. Furthermore, the activation of caspase-9 induced by asiatic acid was completely attenuated by pretreatment with U0126 (Figure 7B). Similarly, co-treatment of cells with asiatic acid and SB203580 failed to decrease the activity of caspase-9. These results suggest that activation of ERK1/2 plays an upstream role in asiatic acid-mediated mitochondrial apoptotic pathways.

Genetic inhibition of p38 and ERK blocks asiatic acid-mediated cell cycle arrest and apoptosis

Although SB203580 and U0126 are relatively selective inhibitors of p38 and ERK1/2, reports have suggested these compounds also interfere with G2 checkpoint-related proteins (Lali et al., 2000). We therefore also employed genetic inhibition to specifically inhibit p38 and ERK to assess the consequences of p38 and ERK inhibition on asiatic acid-mediated cell cycle arrest and apoptosis. To do so,

MCF-7 and MDA-MB-231 cells were transfected with a pool of siRNAs targeting p38 or ERK2, after which the cells were exposed to 10 μM asiatic acid for a specific time. As shown in Figure 8A and B, in comparison with oligonucleotide transfected control cells, transfection of cells with p38 and ERK2 siRNA reduced basal amounts of p38 and ERK1/2 and blocked the asiatic acid-induced activation of p38 and ERK1/2. Selective genetic inhibition of p38 not only blocked asiatic acid-induced S-G2/M phase arrest, but also abrogated phosphorylation of Cdc2 and Cdc25C as well as the degradation of two proteins (Figure 8C and D). On other hand, specific knock down ERK1/2 expression by ERK2 siRNA also inhibited asiatic acid-mediated apoptosis (Figure 8E). In addition, asiatic acid-induced phosphorylation of Bcl-2, upregulation of Bax and activation of caspase-9 were significantly prevented by specific siRNA inhibition of ERK1/2 in both MCF-7 and MDA-MB-231 cells (Figure 8F and G). These consequences of p38 and ERK1/2 inhibition by genetic inhibition on asiatic acid-mediated S-G2/M arrest and apoptosis induction coincide with chemical inhibitors, indicating that p38 and ERK1/2 may play important roles in molecular regulation.

Discussion

Breast cancer is the most common neoplasm in women in both developed and developing countries (Baselga et al., 1994). In this study, we first show that asiatic acid inhibits the growth of two breast cancer cell lines, MCF-7 and MDA-MB-231. Both MCF-7 and MDA-MB-231 cells treated with asiatic acid accumulated in the S-G2/M phase of the cell cycle, and underwent apoptosis in a dose and time-dependent manner.

In our study, we have found that asiatic acid decreases the expression of cyclinB1, cyclinA, Cdc25C, and Cdc2, while it increases the amount of p21/WAF1 and phosphorylation of Cdc2, as well as phospho-Cdc25C. The association of p21/WAF1 and Cdc2 also increased in asiatic acid-treated MCF-7 and MDA-MB-231 cells. Therefore, we suggest that asiatic acid may prove to be a valuable tool for inhibition of Cdc2/cyclinB1 and Cdc2/cyclinA complex in breast cancers for following reasons: (1) the downregulation of asiatic acid on cyclinB1 and cyclinA expression, (2) the induction of p21/WAF1 by asiatic acid in a p53-independent manner, which may subsequently inhibit the function of Cdc2 by forming Cdc2/p21/WAF complex, and (3) the increase of phospho-Cdc25C followed by an increase in inactivated phospho-Cdc2, suggesting that increased phosphor-Cdc25C levels may also decrease functioning phosphatase for dephosphorylating and activating Cdc2.

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Two major distinct apoptotic pathways have been described for mammalian cells. One involves caspase-8, which is recruited by the adapter molecule Fas/APO-1 associated death domain protein to death receptor upon Fas ligand binding (Hengartner, 2000). We did not observe any alteration of either Fas/APO-1 or Fas ligand (mFas ligand and sFas ligand) expression or caspase-8 activation in asiatic acid-treated MCF-7 and MDA-MB-231 cells. On the other hand, asiatic acid treatment resulted in a significant increase of Bax expression, and decreased the amount of Bcl-2 and Bcl-X_L, suggesting that changes in the ratio of proapoptotic and antiapoptotic Bcl-2 family proteins might contribute to the apoptosis-promotion activity of asiatic acid. In addition, elevation of phospho-Bcl-2 by asiatic acid treatment further helps to reduce its ability to bind with Bax, leading to an enhanced susceptibility of the cells to apoptosis (Hu et al., 1998). These regulatory effects of asiatic acid on the Bcl-2 family are correlated with the release of cytochrome *c* from the mitochondria into the cytoplasm and the activation of caspase-9. The importance of this pathway was further confirmed by the protection from programmed cell death that is conferred by caspase-9 inhibition.

Recent studies have shown that MAPKs signaling pathways regulate the eukaryotic cell cycle. p38 kinase has been demonstrated as essential for sustained G2 arrest induced by γ-irradiation, decitabine, vanadate and genistein (Frey et al., 2003; Lavelle

et al., 2003; Wang et al., 2000; Zhang et al., 2003). Reduced activity of Cdc25C and a subsequent increase in Cdc2 phosphorylation lead to cell cycle arrest at the G2/M phase (Bulavin et al., 2001). In this study, we found that activation of p38 was involved in the accumulation of inactive phospho-Cdc2, which may be due to the decrease of Cdc25 activation by phosphorylation, leading to subsequent G2 arrest. These effects however were abolished in MCF-7 and MDA-MB-231 cells that were co-treated with asiatic acid and SB203580. In addition, selectively known down p38 expression by p38 siRNA-based inhibition approach also decreased the effects of asiatic acid on the regulation of Cdc2 and Cdc25C and cell cycle arrest. Moreover, p38 inhibition by SB203580 also delayed apoptosis until 48 h, suggesting that the S-G2/M arrested cells were sensitive to apoptosis induced by asiatic acid treatment

In general, the JNKs and p38 kinase pathways are associated with increased apoptosis, whereas the ERK1/2 pathway is shown to suppress apoptosis (Johnson et al., 2002; Olson et al., 2004; Sah et al., 2004). Surprisingly, our results show that the activation of ERK1/2 is involved in asiatic acid-mediated apoptosis. This difference may be due to dissimilar species of cell types, or to different extracellular stimuli. Previous studies have documented the involvement of ERK1/2 in induction of apoptosis by RRR-α-tocopheryl succinate, quercetin, resveratrol and taxol (Bacus et al. 2002; Nguyen et al., 2004; She et al., 2001; Yu et al. 2001). Furthermore, several

downstream targets of ERK1/2, including Fas/APO-1 receptor and p53 have been discovered to be involved in ERK1/2-triggered apoptosis (Goillot et al., 1997; Ko et al., 2004). However, these two proapoptotic factors were not the downstream target of ERK1/2 in our study, because their expression did not increase expression when ERK1/2 was activated by asiatic acid treatment. In contrast, ERK1/2 activation is involved in the events of asiatic acid-mediated mitochondrial apoptotic pathway, which is completely inhibited by using chemical inhibitor U0126 and ERK1 siRNA-based inhibition, including Bax augmentation, Bcl-2 reduction, Bcl-2 phosphorylation, and caspase-9 activation. However, since activating ERK1/2 to commit suicide is not a universal feature of mammalian cells, the actual mechanism by which asiatic acid converts the outcome of ERK1/2 signaling from mitogenesis to apoptosis requires further investigation.

In conclusion, the present study demonstrates that: (*a*) the breast cancer cell lines MCF-7 and MDA-MB-231 are highly sensitive to growth inhibition by asiatic acid, (*b*) reduced survival of either of breast cancer cell lines after exposure to asiatic acid is associated with S-G2/M phase cell cycle arrest and apoptosis induction, (*c*) asiatic acid can inhibit cell cycle progression at the S-G2/M phase by increasing p21/Cdc2 interaction and decreasing the expression of Cdc2, Cdc25C, cyclinB1 and cyclinA, and (*d*) asiatic acid-induced cell growth inhibition in the MCF-7 and MDA-MB-231

cell lines is mediated by activation of p38 and ERK1/2 kinases, but not JNKs, p53 or Fas/Fas ligand pathway. Finally, *(e)* it has also been demonstrated that the p38 pathway may operate in cell cycle arrest induced by asiatic acid, and that the ERK1/2 cascade of events plays a role in the apoptosis, but not in the cell cycle regulation, of these cells. These data provide a basic mechanism for the chemopreventive properties of asiatic acid in breast cancer cells. Future *in vivo* studies using animal models and human patients would will ascertain whether this pro-apoptotic effect of asiatic acid might contribute its overall chemopreventive effects in the fight against breast cancer, and possibly have future therapeutic applications.

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Footnotes

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Figure legends

Figure 1. The effect of asiatic acid on cell growth inhibition, cell cycle arrest and apoptosis in MCF-7 and MDA-MB-231 cells. (A) Cell growth inhibition of asiatic acid. (B) The distribution of cell cycles in asiatic acid -treated cells. The induction of apoptosis in asiatic acid-treated cells by electrophoresis assay (C) and cytoplasmic nucleosome analysis in MCF-7 (D) and MDA-MB-231 cells (E). For (A), cell growth inhibition of asiatic acid was assessed by XTT. For (B), cells were treated with vehicle and asiatic acid for 12 h, and cell cycle distribution was assessed by flow cytometry. For (C), cells were treated with vehicle and asiatic acid for 48 h, and then DNA fragmentation was assessed by agarose gel electrophoresis. For (D) and (E), the cytoplasmic oligonucleosome of asiatic acid-treated cells at the indicated times was estimated by Nucleosome ELISA kit. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and asiatic acid-treated cells, as analyzed by Dunnett's test ($p<0.05$).

Figure 2. The effect of asiatic acid on cell cycle-related molecules. The effect of asiatic acid on p53 in MCF-7 cells (A). The effect of asiatic acid on p21/WAF1 in MCF-7 (B) and MDA-MB-231 cells (C). The effect of asiatic acid on cyclinB1, cyclinA, Cdc2, Cdc25C, phospho-Cdc2 and phospho-Cdc25C (D). The effect of

asiatic acid on association of p21/WAF1 and Cdc2 (E). For (A)-(C), the level of p53 and p21/WAF1 protein was measured by p53 pan ELISA and WAF1 ELISA kits. For (D), the cell cycle-related expression levels of 10 μM asiatic acid-treated MCF-7 and MDA-MB-231 cells were determined by Western blotting. For (E), cell lysates were subjected to immunoprecipiation with anti-Cdc2, followed by immunoblotting analysis with anti-p21/WAF1. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and asiatic acid-treated cells, as analyzed by Dunnett's test ($p<0.05$).

Figure 3. Asiatic acid induced apoptosis through the initiation of the mitochondrial pathway. The expression level of Bcl-2 family proteins (A) and release of cytochrome *c* (B) in asiatic acid-treated MCF-7 and MDA-MB-231 cells. The activation of caspase-9 by asiatic acid in MCF-7 (C) and MDA-MB-231 cells (D). The effect of caspase-9 inhibitor on asiatic acid -induced apoptosis (E). For (A) and (B), cytoplasm and mitochondria were separated from the cell pellets by lysis buffer and centrifugation. Western blotting analysis assessed protein expressions. For (C)-(D), caspase-9 activity was assessed by a caspase-9 activity assay kit. Each value is the mean ± SD of three determinations. For blocking experiments, cells were pre-incubated with LEHD-CHO (20 μM) for 1 h before the addition of 10 μM asiatic acid for an additional 48 h. Each value is the mean ± SD of three determinations. The

asterisk indicates a significant difference between control and asiatic acid -treated cells, as analyzed by Dunnett's test ($p<0.05$).

Figure 4. Effect of asiatic acid on expression and activation of MAPKs (A), and kinase activity of p38 and ERK1/2 (B). For (A), the cells were treated with 10 μM asiatic acid at different time points. The control cells received an equal volume of DMSO. The cell lysates were prepared, and Western blotting was performed using antibodies against MAPKs and phospho-MAPKs. For (B), the p38 and ERK1/2 kinase activity was determined using p38 and ERKs activity kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions.

Figure 5. Effect of treatment of MCF-7 and MDA-MB-231 cells with the p38 inhibitor SB203580 or MEK1 inhibitor U0126 on asiatic acid-mediated activation of p38 and ERK1/2 (A). The S-G2/M arrest of asiatic acid was inhibited by SB203580, but not U0126 (B). The induction of apoptosis was inhibited by U0126 (C), but only delayed by SB203580 in asiatic acid-treated cells. (D). For all blocking experiments, cells were incubated for 1 h in the presence or absence of 20 μM SB203580 or U0126, then 10 μM asiatic acid was added and incubated for specific times (3 h for p38 and ERK1/2 activity, 24 h and 48 h for apoptosis assay, and 12 h for cell cycle analysis). The activation of p38 and ERK1/2 were measured as described in the legend to Figure 4. The induction of apoptosis was estimated by Nucleosome ELISA kit. Cell cycle

distribution was assessed by flow cytometric analysis. Each value is the mean ± SD of three determinations.

Figure 6. SB203580 inhibits the effect of asiatic acid on cell cycle related molecules. Cells were incubated for 1 h in the presence or absence of 20 μM SB203580, then 10 μM asiatic acid was added and incubated for 3 h. The levels of Cdc2 and Cdc25 were assessed by Western blotting analysis.

Figure 7. SB203580 and U0126 on asiatic acid-mediated mitochondrial apoptosis-related molecules (A) and activation of caspase-9 (B). Cells were incubated for 1 h in the presence or absence of 20 μM SB203580 or U0126, then 10 μM asiatic acid was added and incubated for 24 h. The expressions of Bax, cytochrome *c* and phospho-Bcl-2 were assessed by Western blotting analysis, and the activation of caspase-9 and caspase-3 was determined by caspase-9 activity kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and asiatic acid -treated cells, as analyzed by Dunnett's test ($p<0.05$).

Figure 8. Genetic suppression of p38 and ERKs inhibited asiatic acid-mediated p38 (B) and ERK1/2 activation (B). The effect of asiatic acid on S-G2/M arrest (C) and regulation of cell cycle related proteins were inhibited by p38 siRNA transfection (D). The effect of asiatic acid on apoptosis induction (E), Bcl-2 proteins regulation (F) and caspase-9 activation (G) was blocked by ERK2 siRNA transfection. MCF-7 and

MDA-MB-231 cells were transfected with control oligonucleotide, p38 or ERK2 siRNA by lipofectamine 2000 agents, then treated with asiatic acid for the indicated times (3 h for p38, ERK1/2 activity and cell cycle-related proteins, 12 h for cell cycle analysis, 24 h for Bcl-2 family protein and caspase-9 activity, and 48 h for apoptosis assay). The expressions of various proteins were assessed by Western blotting analysis. The cell cycle distribution was determined by flow cytometry analysis. The induction of apoptosis was assessed by Nucleosome ELISA kit. Caspase-9 activity was measured by a caspase-9 activity kit. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and asiatic acid -treated cells, as analyzed by Dunnett's test ($p<0.05$).

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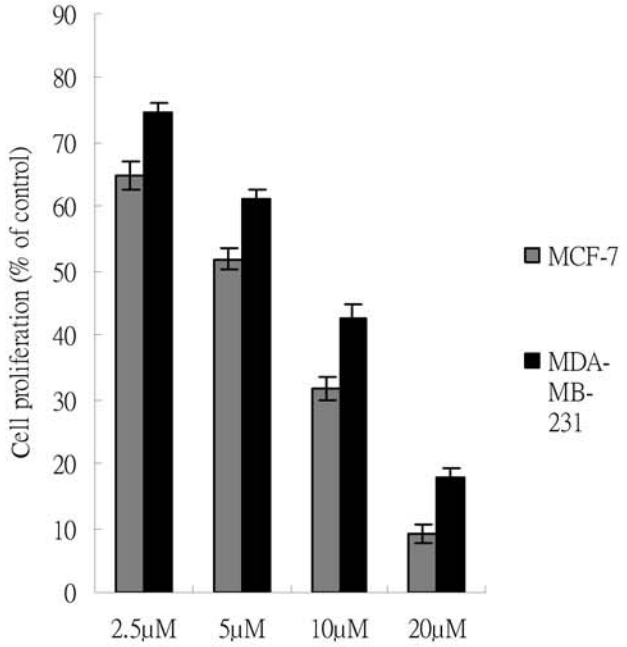

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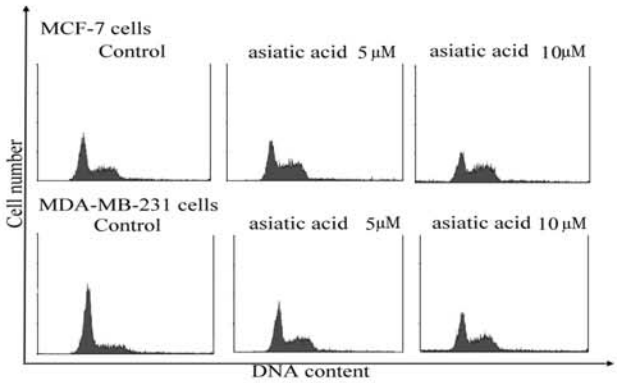
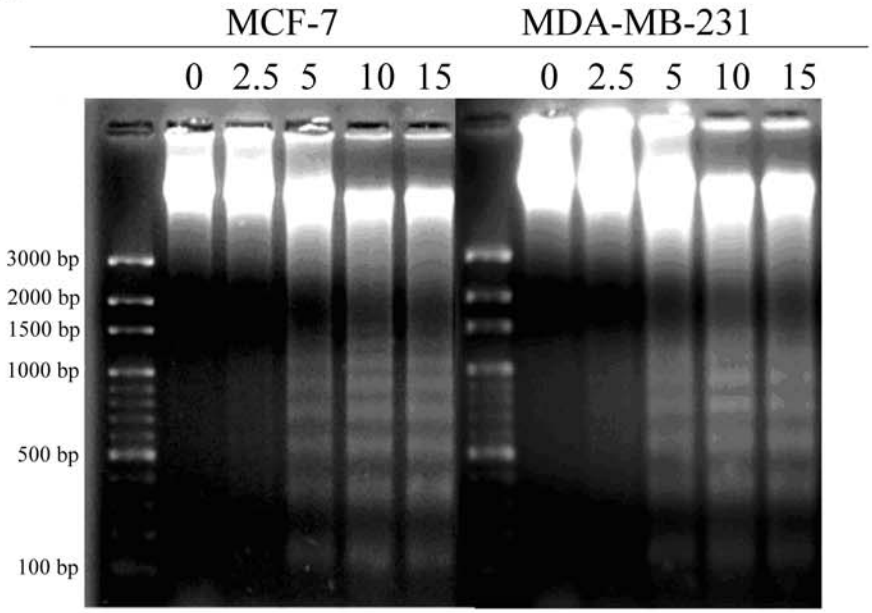


	MCF-7 (%)				MDA-MB-231 (%)			
	sub-G0	G0/G1	S	G2/M	sub-G0	G0/G1	S	G2/M
control	0.2	58.2	12.3	29.3	0.3	67.7	11.2	20.8
asiatic acid 5 μM	0.5	45.1	15.6	38.3	1.2	52.0	15.5	31.3
asiatic acid 10 μM	7.3	34.6	13.8	44.8	6.4	40.8	14.7	38.1

C.



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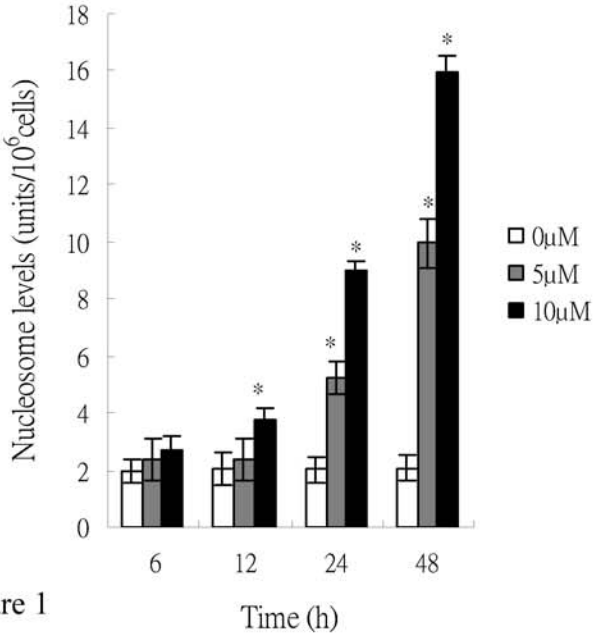

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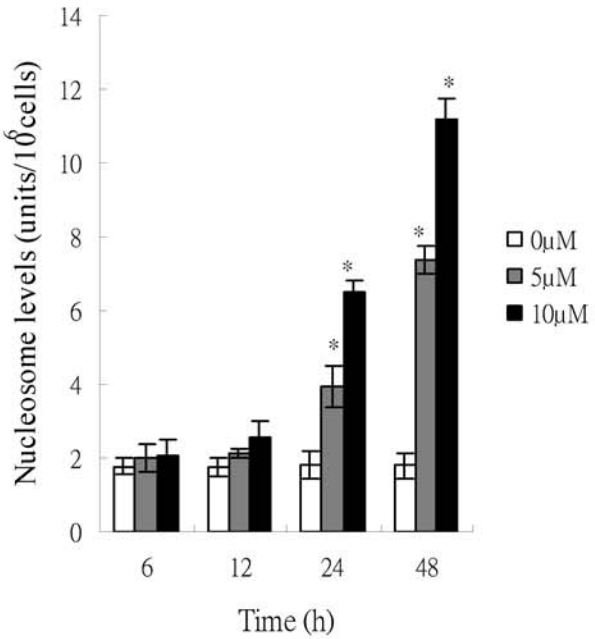

Figure 1

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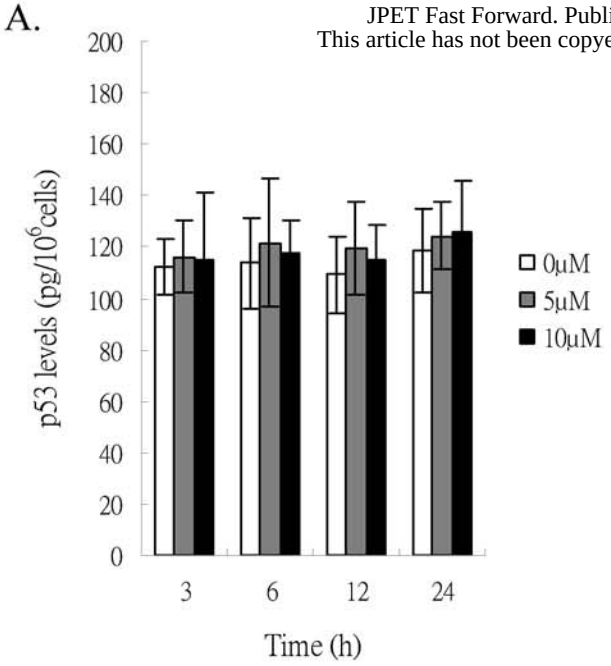

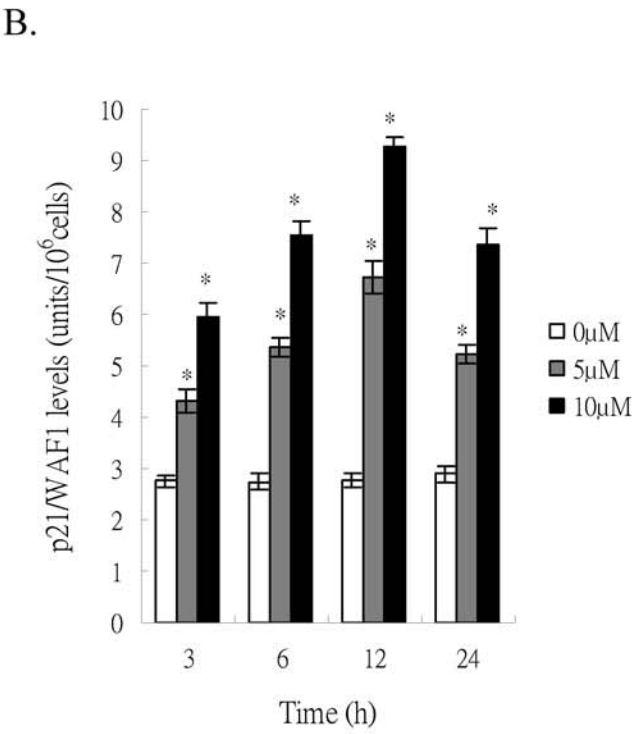

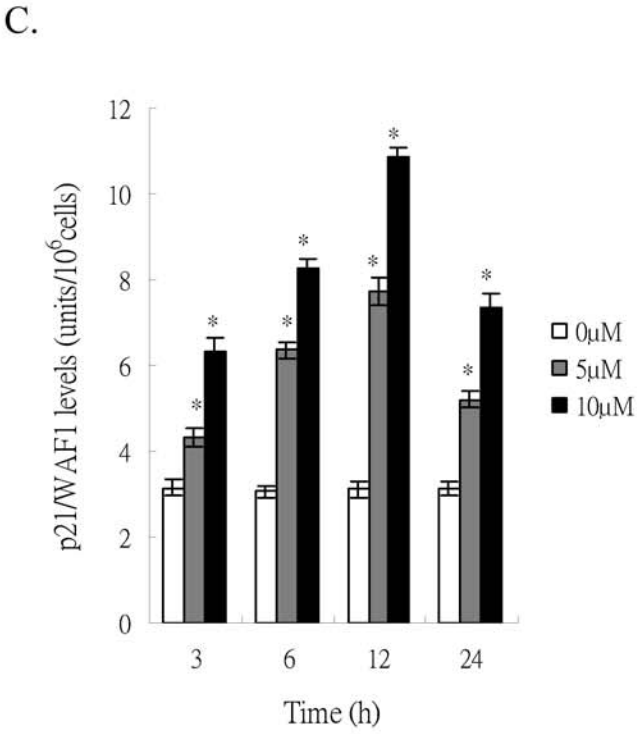

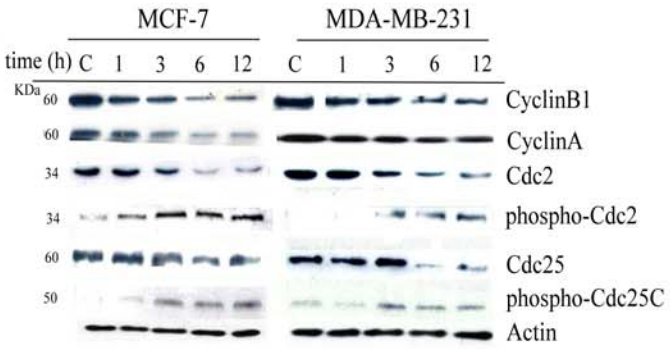

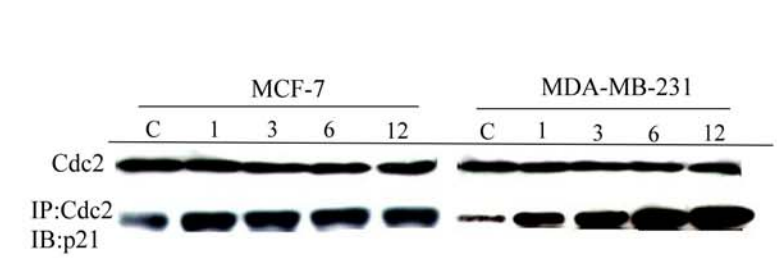

Figure 2

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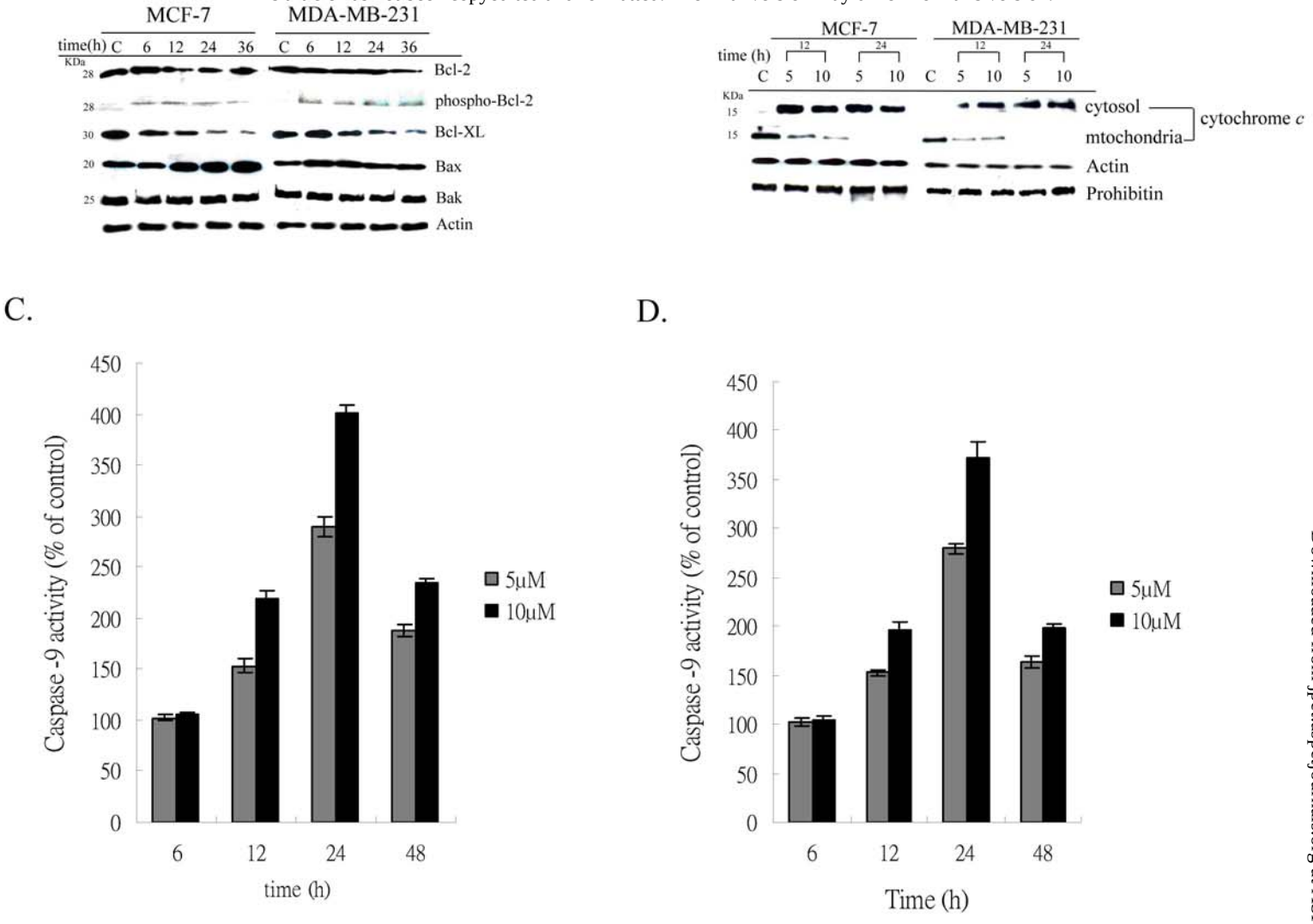

Figure 3

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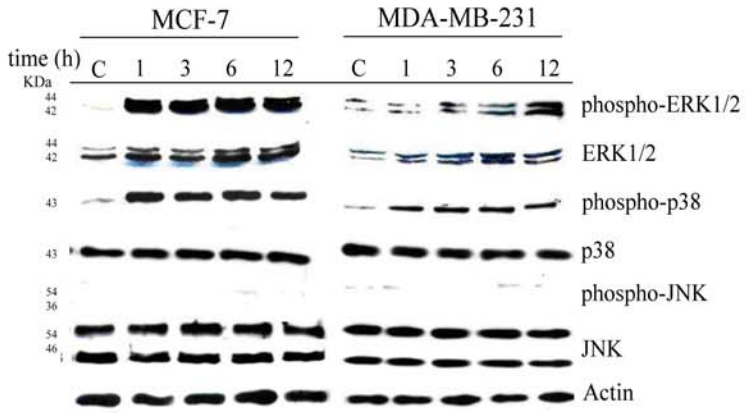

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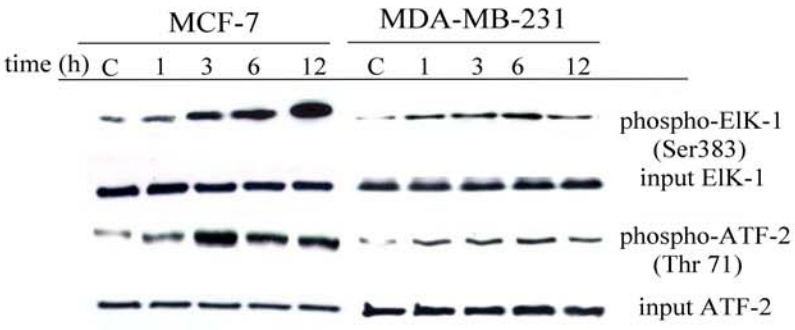

Figure 4

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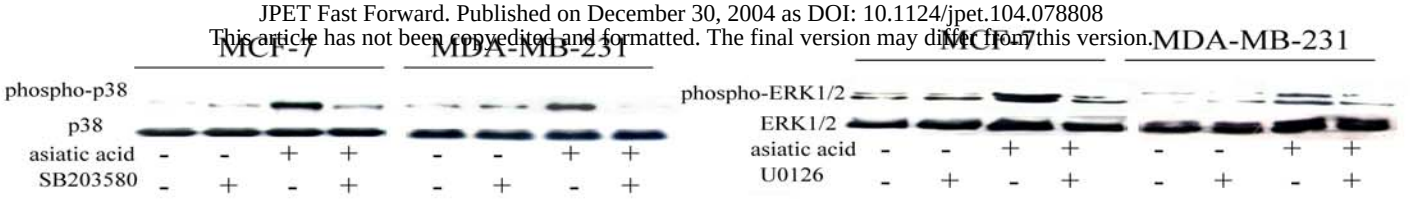

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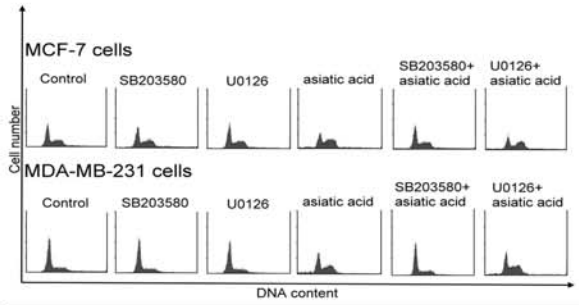

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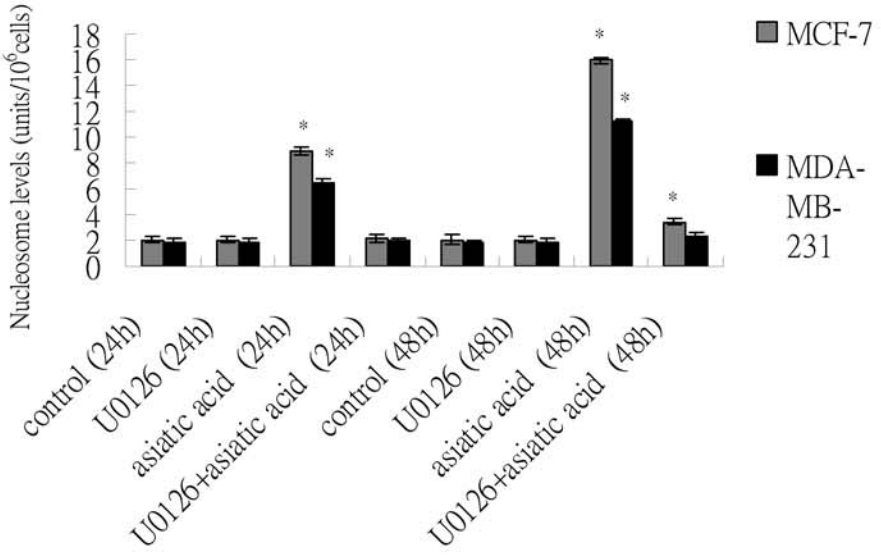
control	0.2	58.2	12.3	29.3	0.3	67.7	11.2	20.8
SB203580	0.5	55.7	11.8	32.0	0.3	67.8	12.2	19.8
U0126	0.6	56.2	12.5	30.8	0.2	66.8	11.7	21.3
asiatic acid	7.3	34.6	13.8	44.3	6.4	41.8	13.7	38.1
SB203580+ asiatic acid	0.8	54.4	11.7	33.1	0.5	68.4	11.6	19.5
U0126+ asiatic acid	3.8	36.4	11.3	48.5	3.3	44.4	14.5	37.8

C.



D.

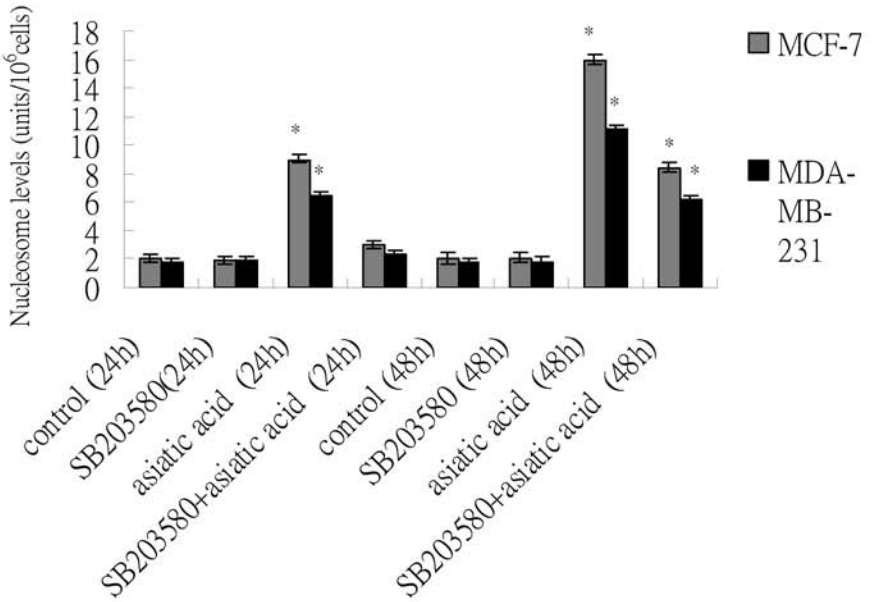


Figure 5.

JPET Fast Forward. Published on December 30, 2004 as DOI: 10.1124/jpet.104.078808
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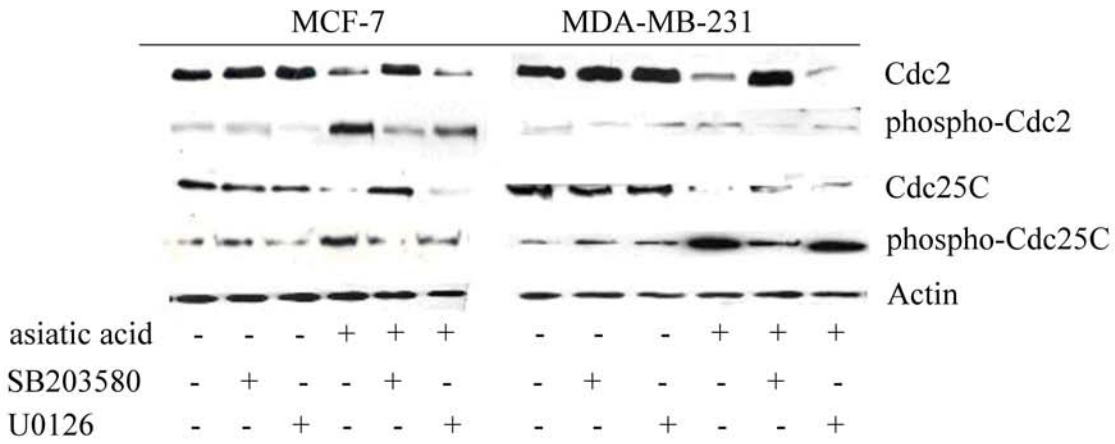
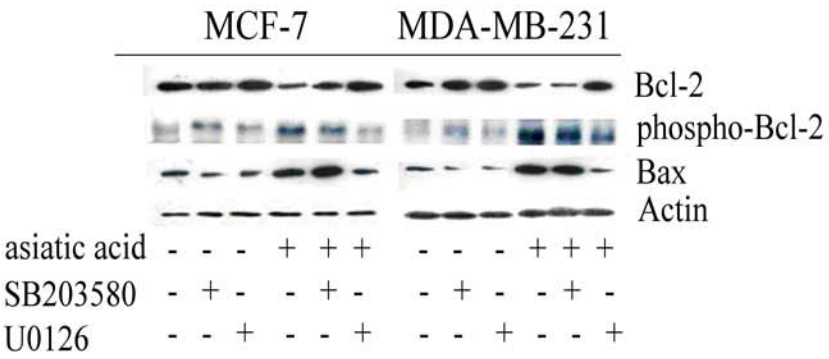


Figure 6

JPET Fast Forward. Published on December 30, 2004 as DOI: 10.1124/jpet.104.078808
This article has not been copyedited and formatted. The final version may differ from this version.

A.



B.

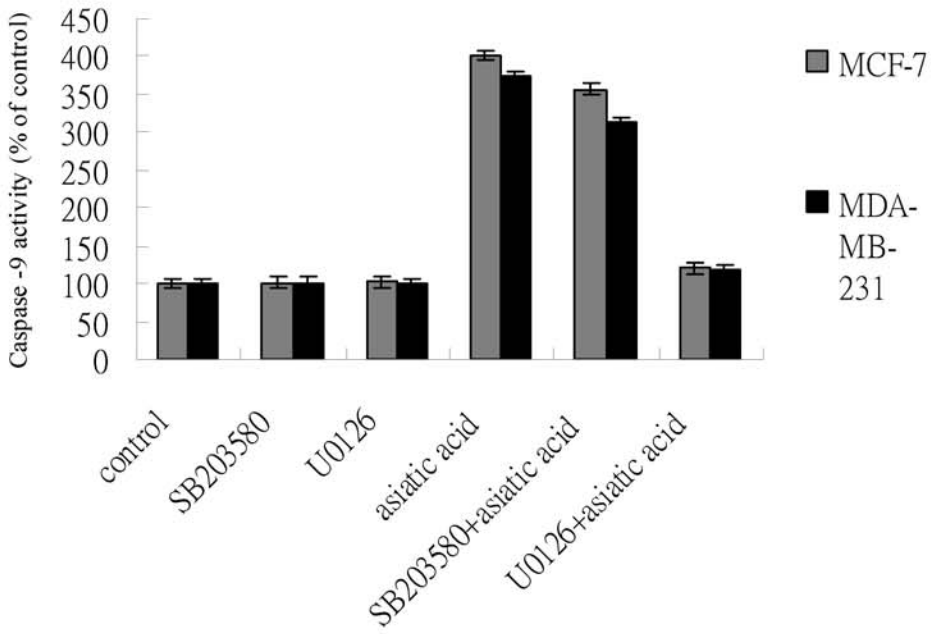
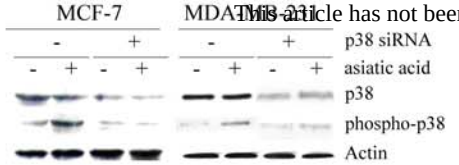


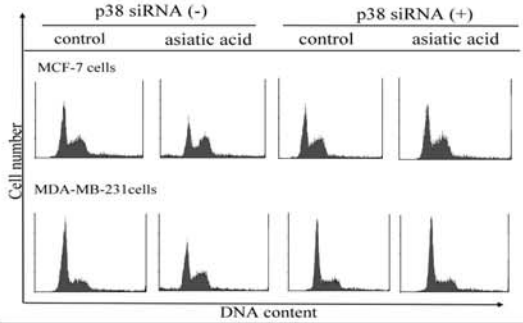
Figure 7

A.



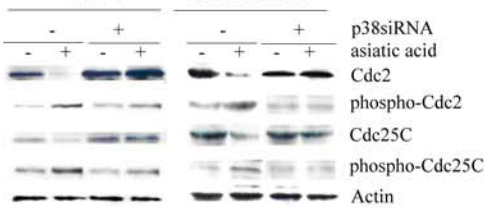
B.

C.

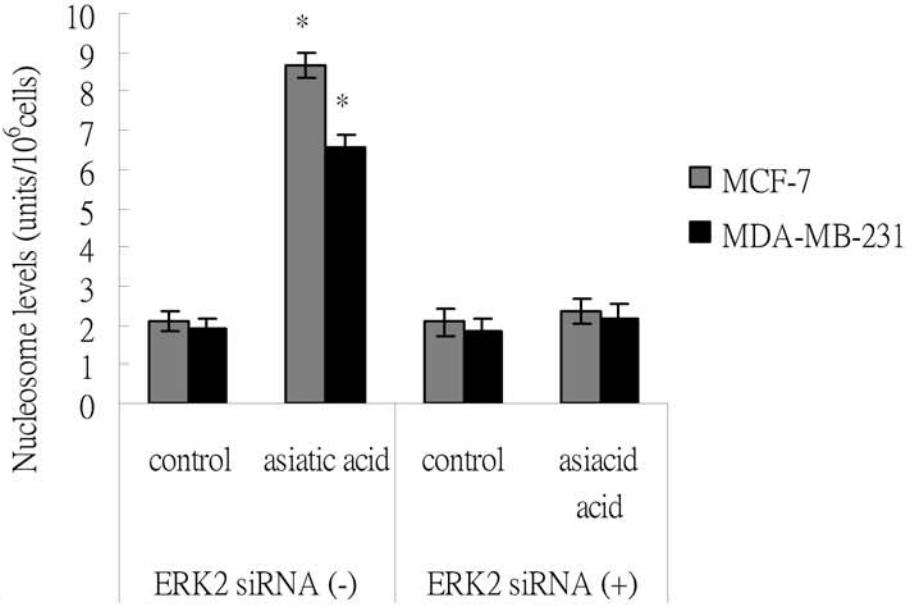


siRNA transfection		MCF-7(%)				MDA-MB-231(%)			
		Sub-G0	G0/G1	S	G2/M	Sub-G0	G0/G1	S	G2/M
p38siRNA(-)	control	0.2	60.2	13.9	25.7	0.3	66.6	10.8	22.3
	asiatic acid	8.1	38.1	16.6	37.2	6.4	48.8	13.3	31.5
p38siRNA(+)	control	0.3	58.1	13.1	28.5	0.2	67.2	10.2	22.4
	asiatic acid	0.8	56.9	15.3	27.2	0.4	65.9	10.8	22.9

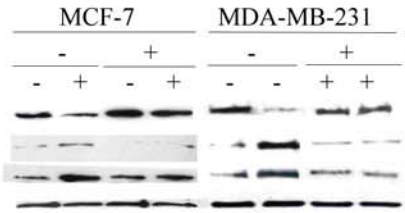
D.



E.



F.



G.

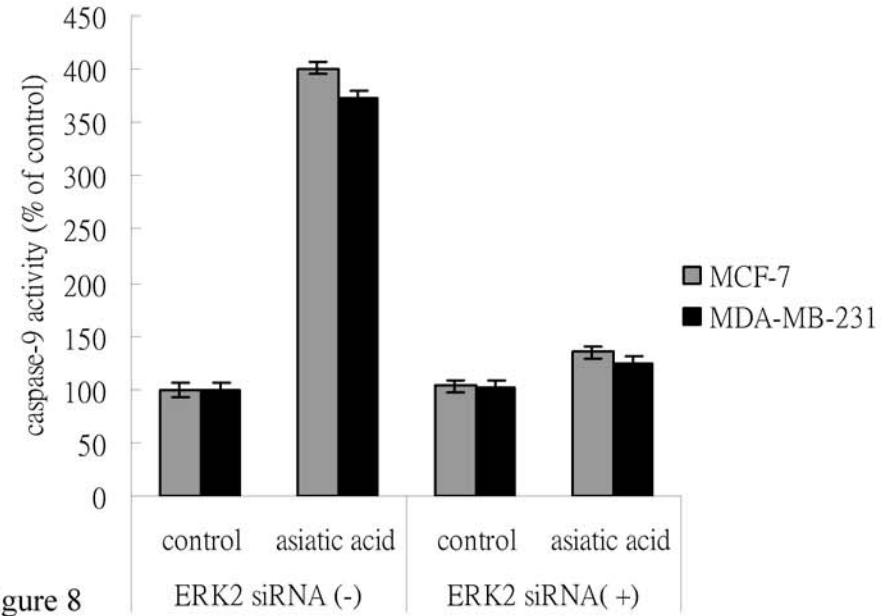


Figure 8