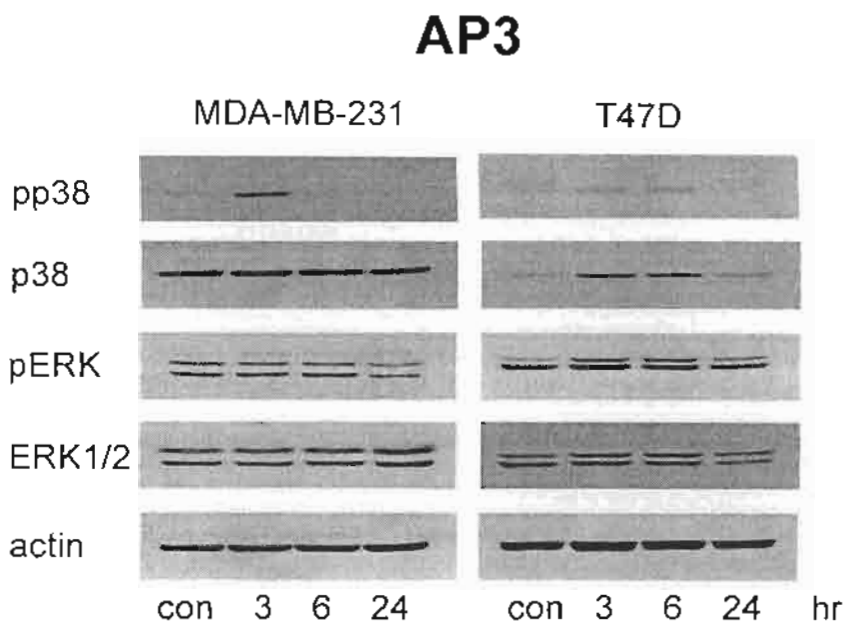

Figure 16 Western immunoblotting analysis of whole cell lysates from MDA-MB-231 and T47D cells cultured *in vitro* for pp38, p38, pERK1/2, ERK1/2 protein expression. Cells were treated with 25 μM of AP3 for 3, 6, and 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO). Blot were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. Results are shown from a single representative of three independent experiments.

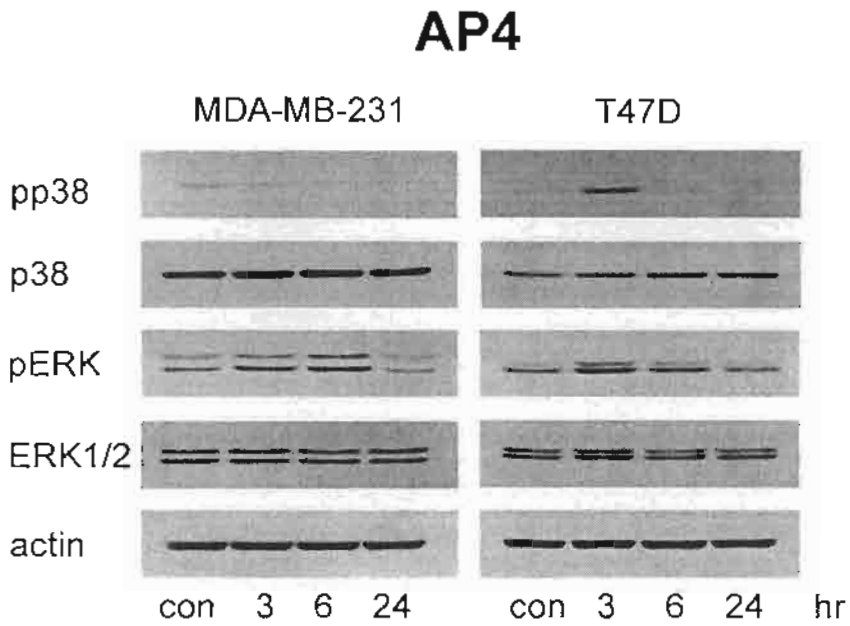

Figure 17 Western immunoblotting analysis of whole cell lysates from MDA-MB-231 and T47D cells cultured *in vitro* for pp38, p38, pERK1/2, ERK1/2 protein expression. Cells were treated with 25 μM of AP4 for 3, 6, and 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO). Blot were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. Results are shown from a single representative of three independent experiments.

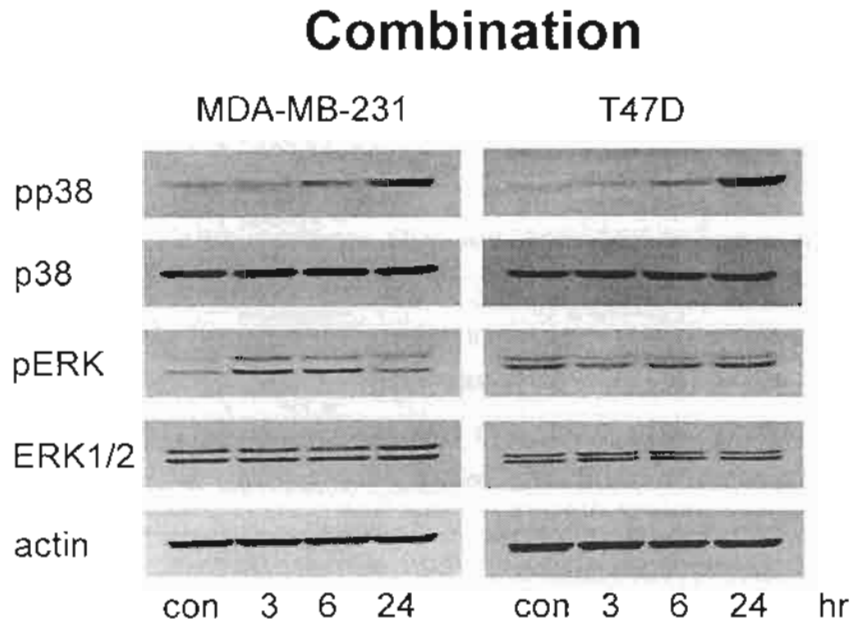

Figure 18 Western immunoblotting analysis of whole cell lysates from MDA-MB-231 and T47D cells cultured *in vitro* for pp38, p38, pERK1/2, ERK1/2 protein expression. Cells were treated with AP combination (25 μM of AP1+AP3+AP4) for 3, 6, and 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO). Blot were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. Results are shown from a single representative of three independent experiments.

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ภาคผนวก

Induction of Apoptosis and Alteration of Cell Cycle Regulators in Estrogen-Independent and –Dependent Human Breast Cancer Cells by Three Andrographolide Compounds

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Abstract

It has been reported that andrographolide (AP1) the major active compounds isolated from *Andrographis paniculata* inhibit growth of several cancer cell lines. Here, we investigated the anticancer mechanisms of AP1 and the other two diterpene compounds: 14-deoxy-11,12-didehydroandrographolide (AP3) and neoandrographolide (AP4). Estrogen-independent MDA-MB-231 and estrogen-dependent T47D human breast cancer cells were used for study anticancer activities of individual and combination of AP compounds. All AP treatments induced growth suppression with the order of potency combination > AP1 > AP3 > AP4. MDA-MB-231 cells exhibit higher sensitivity to growth inhibitory effect of AP compounds than T47D cells. Our results showed that AP compounds induced cell cycle arrest via down-regulation of cyclin D1, cyclin E, cyclin A, and cdk2 and by up-regulation of p21 and p27. AP compounds also induced cell death by apoptosis that was associated with increase of pro-apoptotic Bax and Bad, decrease of anti-apoptotic Bcl-xL protein levels, release of cytochrome C, and proteolytic of PARP. These results indicate that apoptosis induced by AP compounds is via mitochondria-mediated pathway. Additionally, the three AP compounds induced activation of p38MAPK as evidenced by increase in levels of phosphorylated p38MAPK protein which can lead to induction of apoptosis. Taken together, our study showed that AP compounds inhibit growth of breast cancer cells by mechanisms via cell cycle arrest, and induction of apoptosis. Although, the mechanisms involved appear to be similar, the most significant effects occurred with AP1. However, greater effects can be achieved by combination treatment. In conclusion, compound from *A. paniculata* especially AP1 and combination of AP1+AP3+AP4 have potential to be used for breast cancer therapy.

Introduction

Cancer is a leading cause of death that claims the lives of more than six million people globally each year. Among all types of cancer, breast cancer is found to be the number one cancer observed in women worldwide. Despite good impact obtained from current breast cancer therapies, many concerns and downsides still remained. For example, an increase in the incidence of endometrium carcinoma in long-term tamoxifen treated patients due to the partial antagonistic effect of drug (Fornander et.al 1993). Moreover, resistance was found to develop after standard treatments. Therefore, the search for novel chemotherapeutic compounds is still a need. Medicinal plants are an importance source for novel drug development. The use of pure compounds or extracts from plants as substitution or combination with modern medicine might improve the efficacy and/or decrease the side effects of conventional compounds.

Andrographis paniculata (Burm) NEES, commonly known in Thai as Fa Tha Lai Jon, is a well-known traditional medicine in several countries. This herbal has been used for treatment of common cold, fever, and non-infectious diarrhea. Twenty-three compounds in the group of diterpenes, flavanoids, and steroids have been isolated and characterized from the whole plant, of which diterpene lactones are the major components (Siripong et al., 1992; Rao et al., 2004). Collective evidences reveal that the extracts possess a variety of beneficial activities such as anti-inflammatory (Shen et al., 2002), antiviral (Calabrease et al., 2000), antithrombotic (Shao and Fang, 1991), immunostimulatory (Kumar et al., 2004), hypoglycaemic (Zhang and Tan, 2000), hypotensive (Zhang and Tan, 1996), and anticancer (Matsuda et al., 1994; Kumar et al., 2004). Most therapeutic activities of this plant are attributed to andrographolide (AP1) and its related diterpene compounds such as 14-deoxy-11,12-didehydroandrographolide (AP3), and neoandrographolide (AP4) (Fig 1). AP1 has board antiproliferative effect on a panel of human cancer cell lines (Siripong et al., 1992; Kumar et al., 2004; Satyanarayana et al., 2004) and this compound inhibit growth of MCF-7 breast cancer cells by causing cell cycle arrest at G0/G1 phase (Rajagopal et.al., 2003). There was no report for anticancer mechanism of AP3 and AP4.

Tissue homeostasis is controlled by balance between cell growth and cell death. Cell cycle machinery consists of regulatory proteins which play critical role in controlling cell growth. Apoptosis is a morphologically and biochemically distinct form of cell death. Deregulation of the balance between cell growth and apoptosis lead to tumor development. Because of their pivotal roles, apoptosis and cell cycle are being the important targets for anticancer drug development. Previous study has showed that cell cycle arrest and induction of apoptosis are the molecular mechanisms for anticancer activity of aromatase inhibitors and antiestrogens, the two groups of breast cancer hormonal therapy currently available (Thiantanawat et al., 2003). Although the anticancer efficacy of AP1 has been reported, no

study has fully investigated for responsible molecular mechanisms. Likewise, there is no intensive study for anticancer activity of AP3 and AP4. Traditionally, medicinal plants are used as crude extract composed of more than one active compound. Indeed, many times these combinations bring out the better therapeutic effect. Therefore, much remain to be study for the cellular and molecular mechanisms of the effects of the three active diterpenoids AP1, AP3, and AP4 and their combinations when used in breast and other types of cancer. In this application, we set up the study to investigate anticancer molecular mechanisms of AP1, AP3, AP4, and their combinations by targeting their effect on cell cycle machinery and apoptosis signaling pathway. Our studies specifically accentuate on their anti-cancer therapeutic effect on both hormone-independent and hormone-dependent breast cancers. We utilized two different breast cancer cell lines MDA-MB-231 and T47D to represent hormone-independent and hormone-dependent breast cancer cells, respectively.

In addition to cell cycle and apoptosis, tissue homeostasis is also under influence of molecules in the mitogen-activated protein kinases (MAPK) signal transduction network. This network comprise of highly interactive series of protein kinases. These proteins are serine/threonine protein kinases that convey, amplify, and integrate signals from diverse stimuli such as growth factors, neurotransmitters, and cellular stresses. The net result includes array of cellular responses such as proliferation, differentiation, development, inflammation, or apoptosis (Dong and Bode, 2003). Several studies suggest an important role of MAPK signaling pathway in breast cell proliferation and development of drug resistance in breast cancer (Kato et al., 1995; Saten et al., 2002; Jelovac et al., 2005). Therefore, this study is also examine the effects of the three diterpenoids and combinations on the MAPK signaling which will provide the better and inclusive understanding of molecular mechanisms of the tested compounds.

Materials and Methods

Plant Material and Active Diterpenoids Preparation

Leaves and stems of *A. paniculata* plant were collected, air-dried, and powdered. The diterpenoids AP1, AP3, and AP4 were isolated and purified by Laboratory of Pharmacology and Laboratory of Medicinal Chemistry, Chulabhorn Research Institute using column chromatography and identified by UV, IR, and NMR spectra data following method by Matsuda *et al.* (1994).

Cell Lines

Cell lines used in this study were obtained from American Type Culture Collection. Estrogen-independent MDA-MB-231 cells were maintained in a standard medium of DMEM with 2 mM L-glutamine, non-essential amino acid, 4.5 g/L glucose, and 10% fetal bovine serum (FBS). Estrogen-dependent T47D cells were maintained in RPMI 1640 medium with 2 mM L-glutamine, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, 0.2 Units/ml

bovine insulin (90%), and 10% FBS. Cells were cultured at 37 °C in a humidified incubator with 5% CO_2 and 95% air.

Cell Growth Assay

Plates of 80% confluent cells were harvested by trypsinization. Cells were seeded in triplicate into 24-wells plates at 5,000 cells/well (for MDA-MB-231 cells) or 10,000 cells / well (for T47D cells) and were allowed to attach overnight. The medium was replaced with fresh medium containing the pure compounds AP1, AP3, or AP4 (single or in combination) at various concentrations as indicated to test for their effects on the cell growth. Control cells were treated with vehicle (0.2% DMSO). After 48 hours of treatment, cells were harvested by trypsinization and total cell number in each well was determined using a Coulter Counter model Z-1. Concentrations of each compound and their combinations that yield 50% cell number (IC_{50}) compared to control were calculated using linear equation.

Detection of Apoptosis by Annexin V/Propidium Iodide (PI) Assay

Apoptosis was assessed by measuring membrane translocation of phosphatidylserine using an annexin V-FITC apoptosis detection kit I (BD Pharmingen™, BD Biosciences USA). According to the manufacturer's protocol, after treatments, cells were collected and washed twice with cold PBS, resuspended in biding buffer at a concentration of $1x10^6$ cells/ml. Then 100 µl of the solution was transferred to new tube before added with 5 µl of FITC-conjugated annexin V antibody and 5 µl of PI (250 µg/ml stock solution). After incubation at room temperature for 15 min, 400 µl of binding buffer were added and cells were analyzed by flow cytometry. The percentage of cells undergoing apoptosis was determined by three independent experiments.

Preparation of Total Cell Lysates

Adherent and floating cells were harvested after treatment. The floating cells were collected by centrifugation at 500xg (4°C) for 15 min, and the cells that remained attached to the culture plate were detached by scraping. Both the floating and attached cells were mixed together, centrifuged, and washed in 10 ml of ice-cold DPBS. The cell pellet was resuspended in chilled cell lysis buffer [0.1 M Tris HCl, 0.5% Triton X-100, protease inhibitor mixture (Complete TM; Boehringer, Indianapolis, IN) with or without phosphatase inhibitors (1 mM Na_3VO_4, 100 mM NaF, and 100 µM PMSF) and sonicated for 20 sec. The homogenates were transferred to new Eppendorf tubes and were incubated on ice for 30 min. The homogenates were spun at 10,000xg (4°C) for 30 min, and supernatants were separated and stored at -80°C. Protein concentrations were determined by Bradford method using a Bio-Rad kit (Bio-Rad, Hercules, CA).

Cytosolic Fractionation

Cells were incubated with extraction buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.02% digitonin, 1 mM sodium orthovanadate, 50 mM sodium

fluoride, 1 mM PMSF, and protease inhibitor cocktail) for 10 min on ice. The cell extract was centrifuged at 700 x g for 10 min at 4°C, resulting in a pellet (P1) containing nuclei and supernatant (S1) retaining mitochondria and cytosol. The S1 supernatant was recentrifuged at 16,000 x g for 30 min at 4°C. The resulting supernatant (S2) was further centrifuged at 100,000 x g at 4°C for 1 h to obtain the supernatant cytosolic fraction. Protein concentrations were determined by Bradford method using a Bio-Rad kit. Ten μg of fractionated cytosolic protein extract was subjected to SDS-polyacrylamide gels, and transferred to nitrocellulose membranes, and the protein levels of cytochrome C (cyt C), F1-ATPase, and actin were measured by Western immunoblot analysis.

Gel Electrophoresis and Western Blotting

Equal amount of total protein (20-30 μg) were subjected to SDS-PAGE (20 mA/gel), using the mini-Protean3 electrophoresis modules assembly (Bio-Rad) and transferred (100 V, 1 h) to nitrocellulose membranes (Hybond ECL; Amersham, UK). Immunodetections were performed using mouse monoclonal antibodies against human cyclin D1, p21, p38MAPK, phospho-p44/42MAPK (Cell Signaling Technology, USA), Cdk2, cyclin A, cyclin E (BD Transduction Laboratories, USA), Bcl-2 (Upstate, USA), cytochrome C, β-actin (Sigma, USA) or rabbit polyclonal antibodies against Bad, Bax, Bcl-xL, PARP, p27, phospho-p38MAPK, p44/42 MAPK (Cell Signaling Technology, USA). Immunoreactive bands were visualized using the enhanced chemiluminescence detection reagents (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions and quantitated by densitometry using Bio-Rad software (Quantity One).

Statistical Analysis

The statistical differences were analyzed using the student's *t* test. All statistical tests were two sided and differences were considered to be statistically significant when $P<0.05$.

Results

Pure compounds from *A. paniculata* inhibit growth of human breast cancer MDA-MB-231 and T47D cells

The first experiment is to examine antiproliferative effects of the three diterpenoids from *Andrographis paniculata* (AP1, AP3, and AP4. Two human breast cancer cell lines MDA-MB-231 (hormone-independent, ER –ve) and T47D (hormone-dependent, ER +ve) were utilized. In addition to effect of individual compound, the combinatory effect of AP1+AP3, AP1+AP4, AP3+AP4, and AP1+AP3+AP4 were also studied. Our results showed that the three diterpenoids compounds decreased *in vitro* growth of both cell lines. Among the three compounds, AP1 was the most potent growth inhibitor for with the IC_{50} values of 4.39 ± 0.11 μM and 22.62 ± 4.41 μM for MDA-MB-231 and T47D cells, respectively. AP4 was a less effective growth inhibitor with the IC_{50} >100 μM. As shown in Table 1, combination treatments induced a slight increase in growth inhibitory effect compared to single compound. This was

except for AP3+AP4 combination which IC_{50} value was markedly reduced from that of each compound alone. The greatest growth inhibiting effect was obtained from AP1+AP3+AP4 combination treatment. Interestingly, the hormone dependent T47D cells exhibited lesser sensitivity (3 to 5 fold) than the hormone independent MDA-MB-231 breast cancer cells.

AP compounds induced alteration of key proteins in cell cycle machinery

In attempt to investigate whether antiproliferative effect of AP compounds and their combinations observed in both cancer cell lines are related to cell cycle control, several key cell cycle regulatory proteins are studied. As demonstrated in figure 2A and 2B, expression of cyclin D1 was significantly down-regulated in AP1 treated cells. Levels of cyclin D1 protein after cells were treated with AP1 25 μM for 24 hours were decreased to 0.08 fold and 0.47 fold of control in MDA-MB-231 and T47D cells, respectively. There was a slight decrease in expression levels of this protein in AP 3 and AP4 treated cells. However, greater down-regulatory effects on cyclin D1 protein expression are observed after AP combination (AP1+AP3+AP4) treatment. This profile of cyclin D1 expression after AP treatments was similar for both cell lines.

There was no alteration in levels of cyclin A protein in T47D cells after AP1, AP3, or AP4 single compound treatment. However, eighty percent decrease in cyclin A protein levels were evidenced in cells treated with AP1+AP3+AP4 when compared to the control vehicle-treated cells (figure 2B). In addition to cyclin D1 and cyclin A, expression levels of cyclin E protein are slightly decreased (0.9 to 0.7 fold of control) after AP treatments.

Moreover, AP treatments caused decreases in expression levels of cdk2 protein which can be observed in both MDA-MB-231 and T47D cells (figure 2A and 2B, respectively). There were 20 to 30 percent decreases of cdk2 protein expression levels in MDA-MB-231 cells while no change of cdk2 protein expression was evidenced in T47D cells after AP1, AP3, or AP4 single compound treatment. However, there was a significant decrease in cdk2 protein expression levels of 50 to 70 percent reduction in MDA-MB-231 cells (figure 2A) and 30 to 40 percent reduction in T47D cells (figure 2B) after the AP combination treatments.

After found that AP treatments induced decrease in expression levels of cyclins and cdk2, we then further investigated whether AP treatments caused any effect on the cyclin-dependent kinase inhibitors. As expected, up-regulation in expression levels of p21 protein a cyclin-dependent kinase inhibitor were evidenced in all AP treatments in T47D cells (figure 2B). However, we could not detect p21 protein expression in MDA-MB-231 cells. Unable to detect p21 protein in MDA-MB-231 cells we then turn to p27 another cdk inhibitor. As shown in figure 2A, expression levels of p27 protein are slightly up-regulated (1.1 to 1.42 fold of control) in MDA-MB-231 cells after AP combination treatments.

Our results demonstrate that AP compounds from *A. paniculata* possess inhibitory effect on *in vitro* growth of MDA-MB-231 and T47D cells in part by interfere with the cell cycle

machinery. The mechanism is via down-regulates the expression of cyclin D1, cyclin A, and cyclin E protein and induces a reduction of their enzyme partner cdk2 protein level. Moreover, AP compounds also induce up-regulation of cdk inhibitors p21 and p27 protein expression.

AP compounds induce apoptosis in MDA-MB-231 and T47D cells

In addition to their effects on cell cycle progression, the reduction in cell number observed after AP treatments could be the result of induction of cell death. To investigate whether AP1, AP3, and AP4 induce apoptosis in the tested cell lines, the annexin V assay was performed by flow cytometry analysis. In the hormone-independent MDA-MB-231 breast cancer cells, percentage of apoptotic cell was significantly ($p<0.05$) increased to 23.3 ± 5, 18.5 ± 6, and 14.1 ± 2.5 after 48 hr treated with 25 μM of AP1, AP3, and AP4, respectively when compared to 7.1 ± 2.1 percent in control vehicle-treated cells (figure 3A). Treatment with AP combination induced 39.7 ± 4.8 percent cell death by apoptosis which is significantly greater than the effect of each individual compound ($p<0.05$).

The same profile was observed in T47D cells that all the three compounds induced apoptosis which the potency of apoptosis induction AP1>AP3>AP4. In T47D cells, AP1 induced apoptosis by 36.7 percent compared to 7.6 percent of the control cells (figure 3B). AP3 and AP4 treatments induced apoptosis in T47D cells by 20.3 and 15.5 percent, respectively. As in MDA-MB-231 cells, treatment with combination of AP1+AP3+AP4 in T47D cells also resulted in more potent apoptosis induction effect. The percentage of apoptotic cells was increase to 45.7 percent after combination treatment.

Previously, cell growth study showed that the IC_{50} values of all AP treatments were higher in hormone-dependent T47D breast cancer cells than those in MDA-MB-231 cells. In agreement with the cell growth study, concentration 50 μM of AP compounds was needed in T47D cells to induce the same levels of apoptosis by 25 μM of AP compounds in MDA-MB-231 cells. The profiles of apoptotic induction observed in annexin V assay in both MDA-MB-231 and T47D cells were correlated well with the profile of cell growth inhibiting and IC_{50} values obtained in previous study (table 1).

Taken together, these results indicated that the growth inhibiting effects of AP1, AP3, and AP4 on MDA-MB-231 and T47D cells are mediated via disrupting cell cycle progression and by inducing apoptotic cell death.

Involvement of Bcl-2 family of protein in apoptosis activated by AP compound treatments

After observing induction of apoptosis by AP1, AP3, and AP4, we further examine the mechanisms involved. We first investigated the effect of the three compounds and their combination on protein members in Bcl-2 family. As shown in figure 4A, expression levels of pro-apoptotic Bax and Bad proteins were increased in MDA-MB-231 cells treated with AP compounds at the concentration of 10 μM for 6 hours. Among the three compounds, the

greatest effect in up-regulation of Bax and Bad proteins were by AP1 and AP combination treatment.

In the hormone-dependent T47D breast cancer cells, the significant up-regulation in expression of Bax and Bad proteins were evidenced in all treatments. As shown in figure 4B, expression levels of Bax protein in T47D cells were increased to 4.49, 3.96, and 2.26 fold of control after treated with AP1, AP3, and AP4, respectively. A potentiated effect (6.2 fold of control) in induction of Bax protein expression was exhibited in T47D cells after combination treatment. In addition, all treatments significantly induced up-regulation in expression of Bad protein at 4.2, 4.55, 3.18, and 4.72 fold of control in AP1, AP3, AP4, and combination treatments, respectively (figure 4B).

No alteration in expression levels of the anti-apoptotic Bcl-2 and Bcl-xL were observed in MDA-MB-231 cells after treatment with AP compounds. However, a forty percent reduction in expression levels of Bcl-xL was detected after AP combination treatment (figure 4A). In contrast, treatment with AP1 or combination (AP1+AP3+AP4) in T47D cells significantly decreased the levels of anti-apoptotic Bcl-2 protein to forty and thirty percent of control, respectively (figure 4B). A slight down-regulation in expression of Bcl-2 protein was induced by AP3 and AP4 with the decrease of fifteen percent from control levels in both treatments.

Induction of cytochrome C release and PARP cleavage by AP compound treatments

The involvement of Bax, Bad, Bcl-xL, and Bcl-2 implied that apoptosis induced by AP compounds might be via the mitochondria-mediated apoptotic pathway. Therefore, the next question was whether AP compounds induced cytochrome C release from mitochondria into cytosol. To assess this question cell fractionation was performed. Because AP1 and combination treatment were the most effective in inducing apoptosis in MDA-MB-231 and T47D cells, we tested effect of these two treatments on release of cytochrome C. Twenty-five μM of AP1 significantly induced release of cytochrome C into cytosol. This was evidenced as early as 3 hours in both cell lines (data not show) with the maximum cytochrome C release at 6 hours after treatments (figure 5). In MDA-MB-231 cells, a markedly increase in levels of cytochrome c release was detected in AP combination treatment. Quantitative analysis revealed 5.7 and 10.7 fold increase of cytochrome c release in AP1 and AP combination treatment when compared to control vehicle-treated cells. Release of cytochrome c was also observed in T47D cells. However, cytochrome c release after treatments with 25 μM of AP1 or AP combination were in the same levels of 2.9 fold of control.

The hallmark characteristic of apoptosis is DNA fragmentation. Poly (ADP-ribose) polymerase (PARP) is a key protein involved in DNA-repair system. Proteolysis of PARP is common in cell undergoing apoptosis induced by various stimuli (LazebniK et al., 1994). Therefore, we investigated whether mechanism of apoptosis induced by the three AP

compounds involved PARP proteolysis. Our previous experiments in determination of IC_{50} values and annexin V assay for apoptotic index of the three AP compounds and their combination were performed after 48 hours of treatments. However, alteration in members of Bcl-2 protein family and cytochrome c release induced by our treatments can be observed earlier at 3 and 6 hours. Therefore, we set up a time course assay (3, 6, and 24 hr) to determine the effect of AP1, AP3, AP4, or AP combination on PARP protein expression. As showed in figure 6A and 6B, the levels of proteolytic form of PARP were significantly increased ($p<0.05$) after treated with all treatments. Induction of PARP proteolytic can be observed as early as 3 hours after treatments and the levels of cleaved PARP were increased along the time courses of experiment.

Taken together, these data indicate that the growth inhibiting effect of AP compounds on MDA-MB-231 and T47D cells are mediated by inducing apoptosis. The mechanism involved up-regulation of pro-apoptotic Bax and Bad and down-regulation of anti-apoptotic Bcl-xL protein expression, induction of mitochondria cytochrome c release and proteolytic of PARP protein. Among the three AP compounds tested, the andrographolide (AP1) exhibit the greatest effect. Treatment with combination of the three compounds resulted in significant additional effect when compared to each compound alone.

Involvement of MAPK signaling pathway in antiproliferative effects of andrographolides

The levels of ERK1/2, p38 and their active phosphorylated pERK1/2 and pp38 proteins were monitored at 3, 6, and 24 hours after treatments with 25 μM of AP1, AP3, AP4, or AP combination. In MDA-MB-231 cells, no alterations in expression levels of p38 protein were observed in all treatments. However, expression levels of pp38 which is an active form of p38 were increased in all treatments except AP4 treatment (figure 7A). Our time-course study revealed that AP1 induce p38 activation at 6 hours after treatment while an activation induced by AP3 was evidenced at earlier time course of 3 hours after treatment (figure 16). Activation of p38 by AP1 and AP3 was in a transient manner.

In T47D cells, activation of p38 was detected after AP1 and AP4 treatment. As observed in MDA-MB-231 cells, induction of p38MAPK phosphorylation by AP1 and AP4 compounds in T47D cells were also short-lived. A significant increase in expression levels of pp38 can be observed at 24 hours after AP1 treatment and at 3 hours after AP4 treatment (figure 7B).

As showed in figure 7, combination treatment induced p38 activation with the same pattern in both cell lines. A significant increase in pp38 protein expression was evidenced at 6 hours after combination treatment. Unlike a transient activation as observed in single compound treatments, p38 activation induced by combination treatment was sustained for the duration of treatment. Moreover, levels of pp38 protein were further increased at 24 hours after treatment.

AP1 induced a significant decrease in pERK1/2 protein levels in both cell lines at 24 hours after treatment. Decrease in pERK1/2 was detected in MDA-MB-231 cells at 24 hours after AP3 treatment whereas an increase in pERK1/2 was observed in T47D cells at 3 and 6 hours before went back to the control levels at 24 hours (figure 7). Levels of pERK1/2 proteins were also increased after AP4 treatment in both cell lines. The activation was detected at 3 hours after treatment before it return to the control levels at 24 hours.

Despite the most potent condition for anti-growth effect, combination treatment did not inhibit activation of ERK1/2 in MDA-MB-231 cells. In contrast an induction in ERK1/2 activation was observed as the levels of pERK1/2 proteins were increased at 3 and 6 hours (figure 7). However, decrease in expression levels of pERK1/2 proteins were detected in T47D cells at 3 hours after combination treatment before return to the control levels at 6 hours.

In conclusion, these results indicate that the three AP compounds caused different effect on activation of p38 and ERK1/2 proteins. AP1 induced activation of p38 and reduced activation of pERK1/2 in both cell lines. AP3 induced activation of p38 and reduce activation of pERK1/2 in MDA-MB-231 cells but not in T47D cells. Transient activation of pERK1/2 in both cell lines and a transient activation of p38 in T47D cells were observed after AP4 treatment. Combination treatment induced a prolong activation of p38 in both cell lines.

Discussion

Andrographis paniculata extract is traditionally used as a medicine to treat different diseases. The plant extract is known to contain diterpenes, flavanoids, and stigmasterols (Siripong et al., 1992). Recently, andrographolide (AP1) the major diterpenoid from this plant has been reported to have cytotoxic activity against panel of human cancer cell lines (Kumar et al., 2004). Additional studies report anticancer activity of AP1 against *in vitro* and *in vivo* growth of breast cancer MCF-7 cells. In this present study we investigate and compare the anticancer mechanisms of AP1 and other two active andrographolide compounds AP3 and AP4. Our studies were performed using two human breast cancer cell lines; hormone-independent MDA-MB-231 and hormone-dependent T47D cells. Our purpose to use these cells is to inclusive study the potential of AP compounds for breast cancer therapy in both estrogen-sensitive and estrogen-insensitive breast cancers. Despite difference in their respond to estrogen, both cell lines have been reported to express mutant p53 protein (Runnebaum et al., 1991). Mutation of this tumor suppressor protein has been found to be well correlated with several types of cancer. Therefore, in addition to represent two different types of breast cancer, the two cell lines that used in this study are also represent cancer that express mutated p53.

Our results showed that all three AP compounds inhibit growth of both cell lines. However, the growth inhibitory effect of AP1 was superior compared with AP3 or AP4. We

also observed an increase in growth inhibiting effect from combination treatment. This additive effect can be either the three compounds activate different anti-growth mechanisms or just the adding up of the same mechanism induced by more than one compound. Among the two cell lines, the hormone-independent MDA-MB-231 cells are more sensitive to antiproliferative effect of AP compounds. This is may be because estrogen that has growth stimulatory effect on T47D cells since we culture both cell lines in normal serum which contain physiological amount of estrogen. To investigate the anti-growth mechanisms of AP compounds, we examined the effect of these compounds on cell cycle machinery, apoptosis, and MAPK signaling which are the major systems that regulate cell growth and cell death. It has been reported that AP1 induced cell cycle arrest at G0/G1 phase (Cheung et al., 2005) Additional studies in MCF-7 cells have revealed that AP1 block cell cycle at the G0/G1 phase through the induction of p27 and reduction of cdk2 protein (Satyanarayana et al., 2004). Our results here demonstrate that AP compounds induce a decrease in expression of cyclin D1 and cyclin E which are the G1 cyclins. Down-regulation of these cyclins was accompanied by down-regulation of their partner cdk2. In addition to cyclin and cdk, cell cycle also regulated via another group of molecule called CKI which inhibits cdk activity. Our results showed that all three AP compounds induced an up-regulation of CKI protein p21 in T47D cells. However, we could not detect expression of this protein in MDA-MB-231 cells. It has been reported that the tumor suppressor gene p53 is mutated at the DNA binding domain in both MDA-MB-231 and T47D cells on R280K and L194F, respectively (Runnebaum et al., 1991). Transcription of p21 can be induced by p53. Mutation at R280K on DNA binding domain of p53 in MDA-MB-231 cell may affects transcription of p21 protein and may be the reason for no detection of p21 protein in this cell line. However, increase in expression of another CKI, p27 protein was observed in MDA-MB-231 cells.

In addition to induced cell cycle arrest, our studies showed that AP compounds also induce apoptotic cell death. Apoptotic index obtained from annexin V assay was well correlated to order of growth inhibiting potency and the IC_{50} value obtained from cell growth assay. Our further studies showed that AP compounds up-regulate expression of pro-apoptotic proteins Bax and Bad in both MDA-MB-231 and T47D cells. The potency in inducing Bax and Bad protein expression was combination > AP1> AP3 > AP4 in both cell lines. In contrast, AP compounds have lesser effect on anti-apoptotic members of Bcl-2 family of protein. However, combination treatment significantly induced down-regulation of the anti-apoptotic Bcl-2 and Bcl-xL in T47D and MDA-MB-231, respectively. The relative levels of pro- and anti-apoptotic Bcl-2 family proteins function as a "rheostat" regulating the apoptotic threshold of the cell (Yang and Korsmeyer, 1996). Therefore, a markedly increase in expression levels of pro-apoptotic proteins with a slight decrease in expression levels of anti-apoptotic proteins observed after AP compounds treatment should affect the regulation of

apoptotic threshold of the cells leading to apoptosis. Our results are in agreement with previous report by other that pro-apoptotic Bcl-2 family members (Bax and Bid) play a critical role in AP induced apoptosis in HepG2 and HeLa cells (Zhou et al., 2006). It is well established that Bcl-2 family members act upstream of mitochondria-mediated apoptotic pathway. Decreasing of pro-apoptotic/anti-apoptotic ratio leads to opening of mitochondria permeability transition pores and the release of cytochrome c (Susin et al., 1999). In this present study, reduce in pro/anti-apoptotic ratio induced by AP compounds was concurrent with release of cytochrome c into cytosol. Our results suggest that apoptosis induced by AP compounds is via the mitochondrial pathway. In addition, we also found that AP compounds induced proteolytic of PARP an important enzyme for DNA repair. Proteolysis of PARP is common in cell undergoing apoptosis induced by various stimuli. The profile of PARP proteolytic activated by AP compounds was in agreement with the IC_{50} value and apoptotic index data obtained from previous experiments. AP combination treatment activated more PARP cleavage than that induced by each compound alone.

Proliferation of breast cancer cells can be promoted via a MAPK-dependent pathway. MAPK phosphorylates Ser-118 in the estrogen receptor (ER), leading to ligand-independent ER activation (Kato et al., 1995). Moreover, it has been reported that MAPK is over active in tamoxifen resistant breast cancer cells (Kurokawa et al., 2000; Saten et al., 2002). These findings suggest an important role of MAPK signaling pathway in breast cell proliferation. Our results showed that the three AP compounds caused different effect on activation of p38 and ERK1/2 proteins. AP1 induced activation of p38 and reduced activation of pERK1/2 in both cell lines. AP3 induced activation of p38 and reduce activation of pERK1/2 in MDA-MB-231 cells but not in T47D cells. Transient activation of pERK1/2 in both cell lines and a transient activation of p38 in T47D cells were observed after AP4 treatment. Combination treatment induced a prolong activation of p38 in both cell lines. Activation of p38 and inactivation of ERK1/2 can lead to induction of apoptosis (Dong and Bode, 2003). However, whether their effects on p38MAPK and ERK1/2 are contributed to anti-growth actions of AP compounds is unknown and will require additional investigation. Taken together, our study showed that AP compounds inhibit growth of breast cancer cells both hormone-dependent and hormone-independent by mechanisms via cell cycle arrest, and induction of apoptosis. Although, the mechanisms involved appear to be similar, the most significant effects occurred with AP1. However, greater effects can be achieved after combination treatment.

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Figure Legends

Figure 1 The three active diterpenoids from *Andrographis paniculata*

Figure 2 Western immunoblotting analysis of whole cell lysates from A) MDA-MB-231 cells; B) T47D cells cultured *in vitro* for cyclin D1 cyclin E, cyclin A, cdk2, p21, and p27 protein expression. Cells were treated with 25 μM of AP1, AP3, AP4, or combination (AP1+AP3+AP4) for 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO) for the same period of time. Blots were stripped and probed for β-actin to verify that equal amounts of protein were loaded in each lane.

Figure 3 Induction of apoptosis in A) MDA-MB-231 and B) T47D cells as determined by annexin V assay. Cells were treated with 25 μM (for MDA-MB-231) or 50 μM (for T47D) of AP1, AP3, AP4, AP1+AP3+AP4 (comb), or vehicle 0.2%DMSO (control). After 48 hours of treatments, cells were harvested and subjected to annexin V flow cytometry analysis as described in "Materials and Methods". Percentages of apoptotic cells were quantitated from three independent experiments. Data are presented as mean ± SE. *, statistically significant ($P<0.05$) versus control; +, statistically significant ($P<0.05$) versus AP1.

Figure 4 Western immunoblotting analysis of whole cell lysates from A) MDA-MB-231 cells B) T47D cells cultured *in vitro* for Bax, Bad, Bcl-xL and Bcl-2 protein expressions. Cells were treated with 25 μM of AP1, AP3, AP4, or combination (AP1+AP3+AP4) for 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO) for the same period of time. Blots were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane.

Figure 5 Western immunoblotting analysis of cytosolic fraction from MDA-MB-231 cells and T47D cells cultured *in vitro* for cytochrome c protein. Cells were treated with 25 μM of AP1, or combination (AP1+AP3+AP4) for 6 hr. Control (Con) lane is cytosolic fraction of cells treated with vehicle (0.2% DMSO) for the same period of time. Blots were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. A. Blots of cytochrome c and actin. B. Quantitative analysis from three independent experiments. Results represent fold of control; bars, ± S.E.

Figure 6 Induction of proteolysis of PARP in A) MDA-MB-231 B) T47Dcells. Western immunoblotting analysis of whole cell lysates from cells cultured *in vitro* for PARP protein expression. Cells were treated with 25 μM of AP1, AP3, AP4, or combination (AP1+AP3+AP4) for 3, 6, and 24 hr. Control (Con) lane is lysate from cells treated with

vehicle (0.2% DMSO) for 3 hr. Blots were probed with anti-PARP antibodies that recognized both intact PARP at M, 116 kDa and proteolytic PARP at M, 85 kDa. Blot were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. Results are shown from a single representative of three independent experiments.

Figure 7 Western immunoblotting analysis of whole cell lysates from A) MDA-MB-231 and 8) T47D cells cultured *in vitro* for pp38MAPK, p38MAPK, pERK1/2, ERK1/2 protein expression. Cells were treated with 25 μM of AP compounds for 3, 6, and 24 hr. Control (Con) lane is lysate from cells treated with vehicle (0.2% DMSO). Blot were stripped and probed for β-actin (bottom panel) to verify that equal amounts of protein were loaded in each lane. Results are shown from a single representative of three independent experiments.

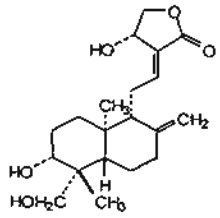
Table 1 IC_{50} values of AP1, AP3, AP4, and their combinations on *in vitro* growth of MDA-MB-231 and T47D cells

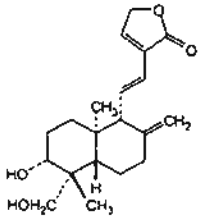
Treatments	IC_{50} (μM)	
	MDA-MB-231	T47D
AP1	4.39 ± 0.11	22.62 ± 4.41
AP3	15.67 ± 1.06	80.72 ± 9.02
AP4	> 100	> 100
AP1+AP3	3.26 ± 0.54	15.09 ± 0.76
AP1+AP4	3.22 ± 0.10	16.24 ± 0.52
AP3+AP4	16.05 ± 0.87	46.93 ± 2.53
AP1+AP3+AP4	2.62 ± 0.84	14.19 ± 0.93

MDA-MB-231 cells ($5x10^3$ cells/well) or T47D cells ($1x10^4$ cells/well) were cultured in 24 wells plate. The day later, media were refreshed and triplicate wells were treated with the indicated treatments of AP1, AP3, AP4, or their combinations at concentrations of 1, 5, 10, 25, 50, and 100 μM. At 48 hours of treatments, numbers of cell in each well were determined using Coulter Counter Model Z-1. Cell growth inhibition was calculated as percentage of control cells grew in vehicle (0.2%DMSO). Linear equation was utilized for IC_{50} values determination. Results are expressed in the mean ± SE of triplicate independent experiments.

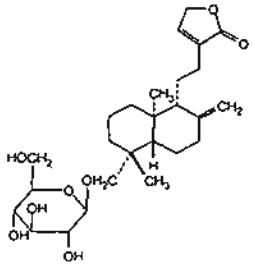
Table 1

AP1: Andrographolide



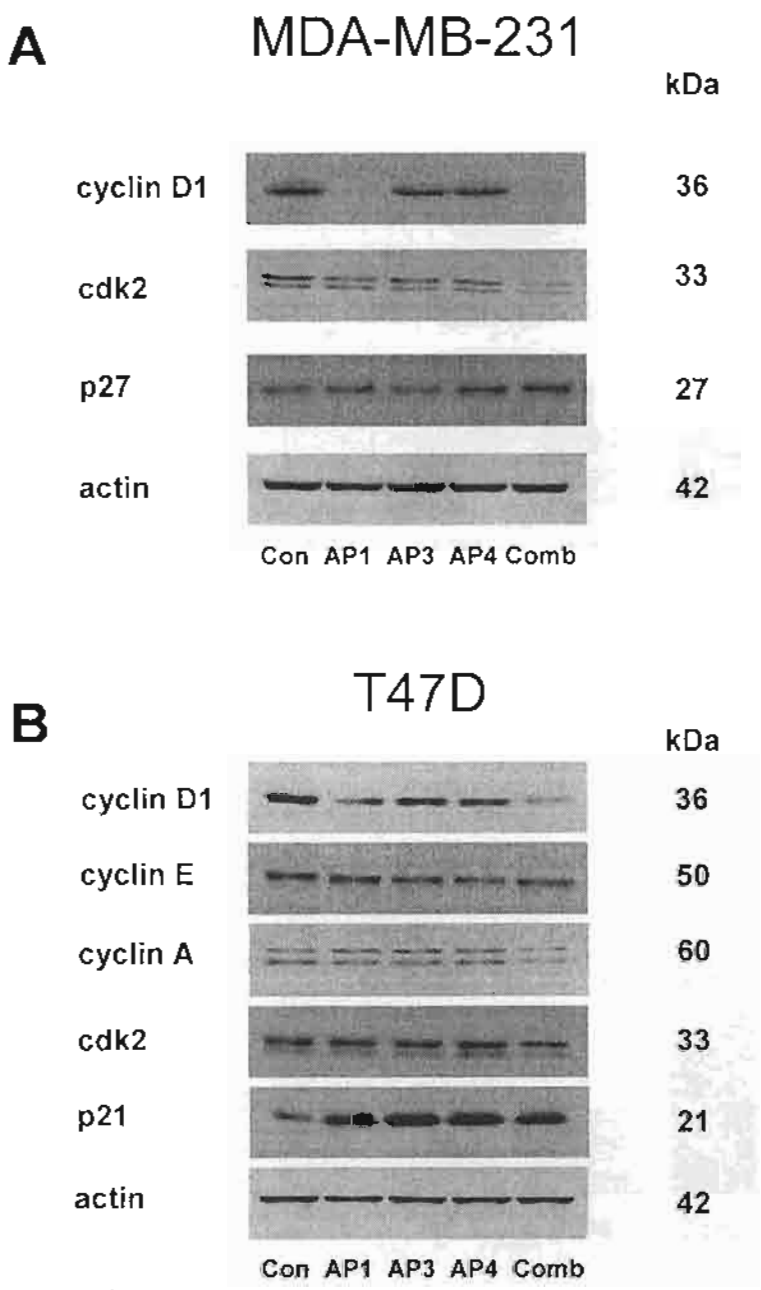


AP3: 14-Deoxy-11,12-didehydro andrographolide



AP4: Neoandrographolide

Figure 1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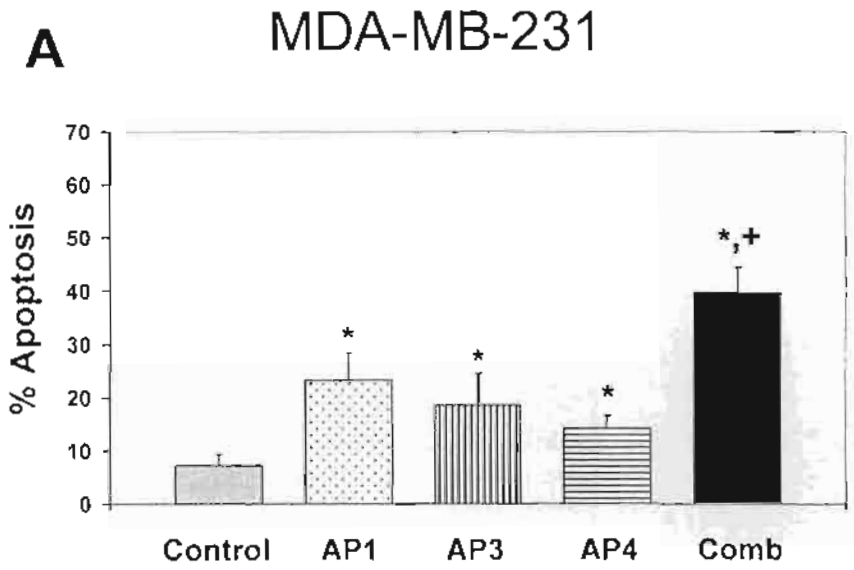
MDA-MB-231
kDa
cyclin D1
cdk2
p27
actin
36
33
27
42
Con AP1 AP3 AP4 Comb
B
T47D
kDa
cyclin D1
cyclin E
cyclin A
cdk2
p21
actin
36
50
60
33
21
42
Con AP1 AP3 AP4 Comb

Figure 2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MDA-MB-231
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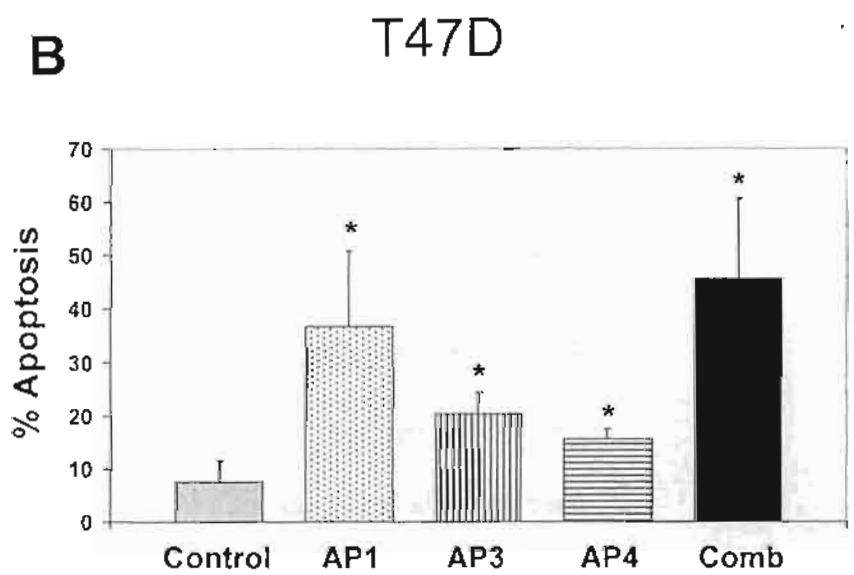
0
*
*
*
*,+
Control
AP1
AP3
AP4
Comb


B
T47D
% Apoptosis
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60
50
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30
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10
0
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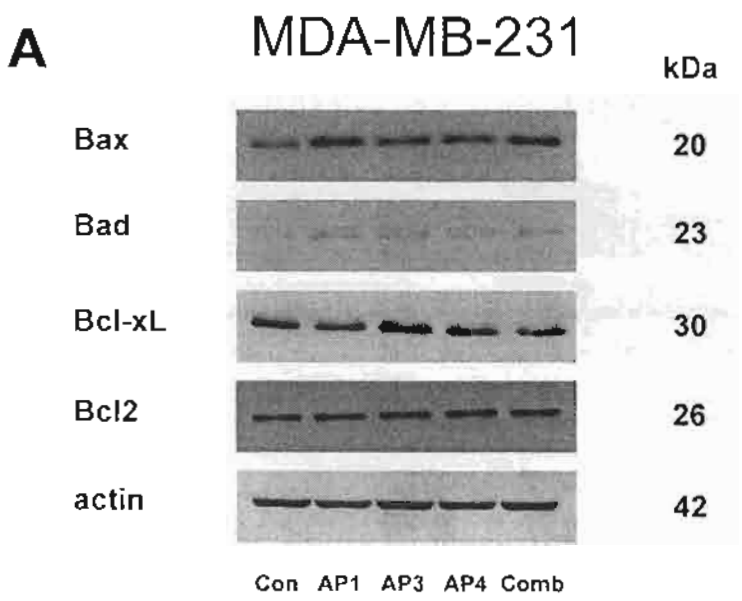
*
*
Control
AP1
AP3
AP4
Comb

Figure 3


A
MDA-MB-231
kDa
Bax
Bad
Bcl-xL
Bcl2
actin
20
23
30
26
42
Con AP1 AP3 AP4 Comb
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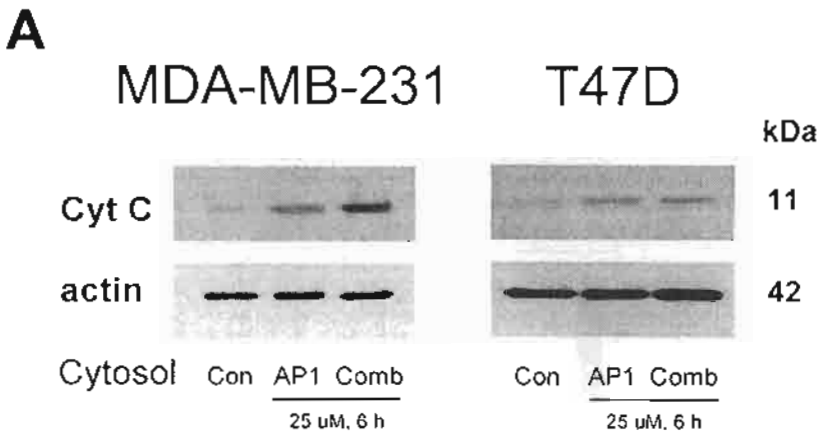
Con AP1 AP3 AP4 Comb

Figure 4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Comb
Con
AP1
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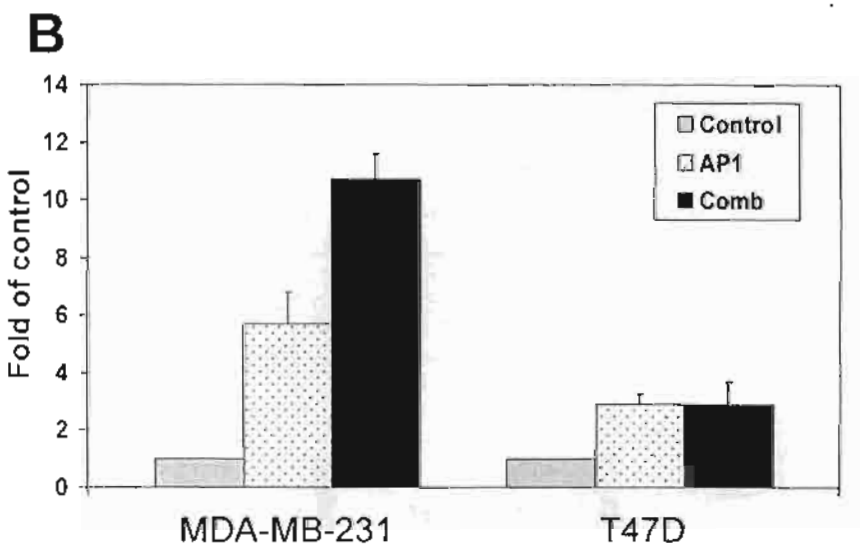
25 uM, 6 h
25 uM, 6 h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Fold of control
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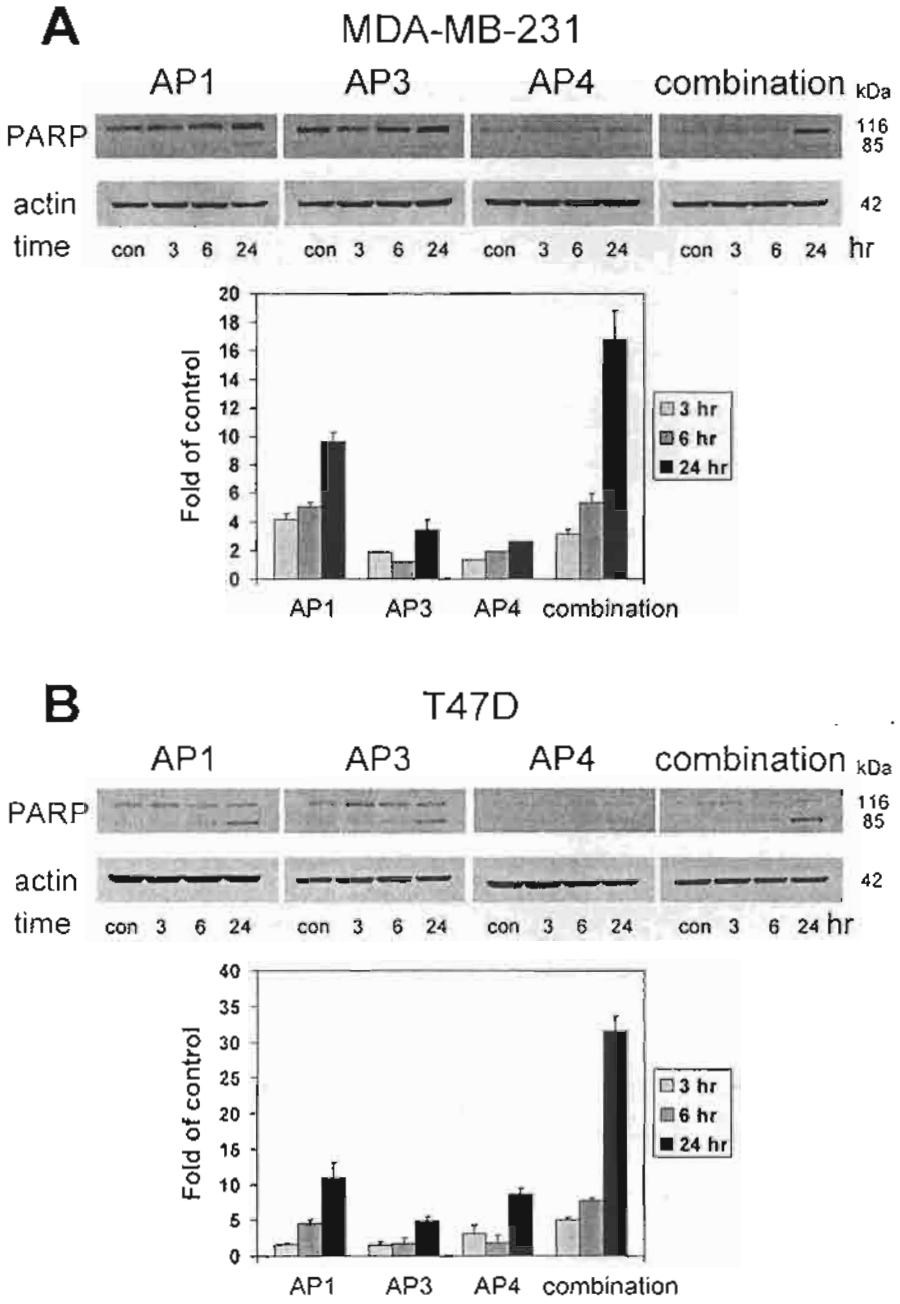
Control
AP1
Comb
MDA-MB-231
T47D

Figure 5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AP1
AP3
AP4
combination
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PARP
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85
actin
42
time
con 3 6 24 con 3 6 24 con 3 6 24 con 3 6 24 hr
Fold of control
3 hr
6 hr
24 hr
AP1 AP3 AP4 combination
B
T47D
AP1
AP3
AP4
combination
kDa
PARP
116
85
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time
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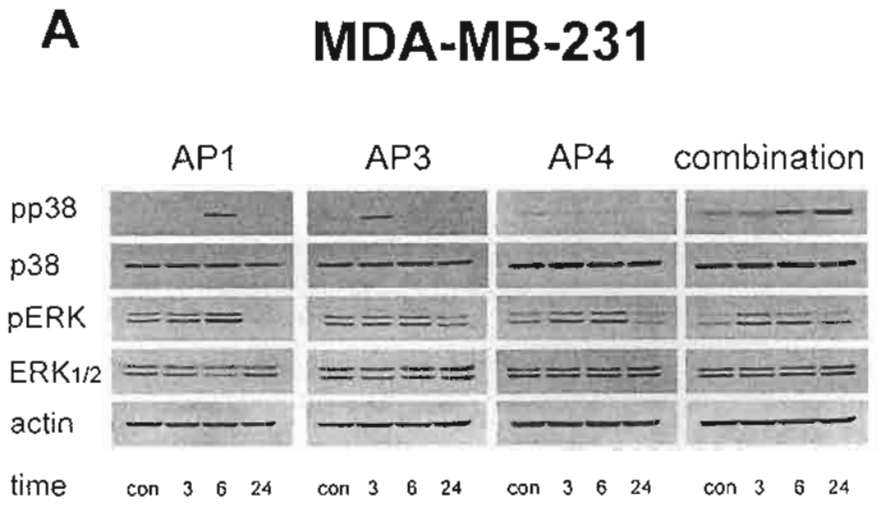
Fold of control
3 hr
6 hr
24 hr
AP1 AP3 AP4 combination

Figure 6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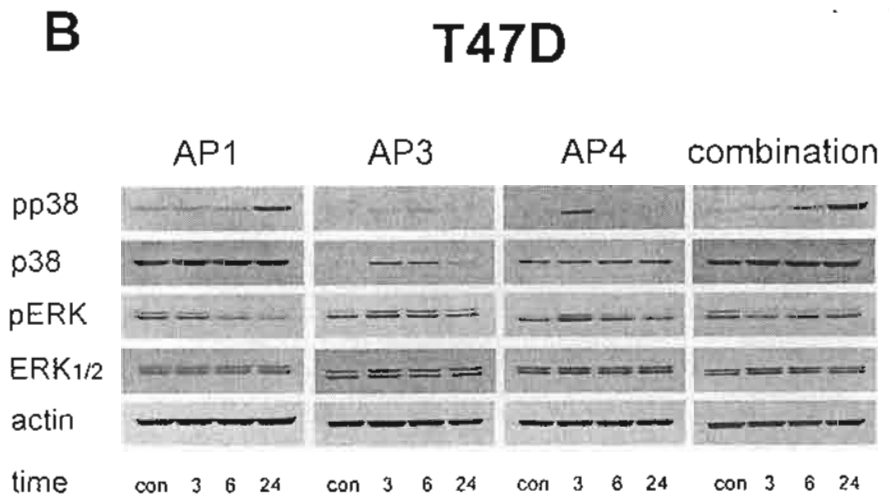
con 3 6 24
con 3 6 24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T47D
AP1
AP3
AP4
combination
pp38
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ERK1/2
actin
time
con 3 6 24
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con 3 6 24
con 3 6 24

Figure 7