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Antroquinonol, a natural ubiquinone derivative, induces a cross talk between apoptosis, autophagy and senescence in human pancreatic carcinoma cells☆

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Abstract

Pancreatic cancer is a malignant neoplasm of the pancreas. A mutation and constitutive activation of K-ras occurs in more than 90% of pancreatic adenocarcinomas. A successful approach for the treatment of pancreatic cancers is urgent. Antroquinonol, a ubiquinone derivative isolated from a camphor tree mushroom, *Antrodia camphorata*, induced a concentration-dependent inhibition of cell proliferation in pancreatic cancer PANC-1 and AsPC-1 cells. Flow cytometric analysis of DNA content by propidium iodide staining showed that antroquinonol induced G1 arrest of the cell cycle and a subsequent apoptosis. Antroquinonol inhibited Akt phosphorylation at Ser^{473}, the phosphorylation site critical for Akt kinase activity, and blocked the mammalian target of rapamycin (mTOR) phosphorylation at Ser^{2448}, a site dependent on mTOR activity. Several signals responsible for mTOR/p70S6K/4E-BP1 signaling cascades have also been examined to validate the pathway. Moreover, antroquinonol induced the down-regulation of several cell cycle regulators and mitochondrial antiapoptotic proteins. In contrast, the expressions of K-ras and its phosphorylation were significantly increased. The coimmunoprecipitation assay showed that the association of K-ras and Bcl-xL was dramatically augmented, which was indicative of apoptotic cell death. Antroquinonol also induced the cross talk between apoptosis, autophagic cell death and accelerated senescence, which was, at least partly, explained by the up-regulation of $p21^{Waf1/Cip1}$ and K-ras. In summary, the data suggest that antroquinonol induces anticancer activity in human pancreatic cancers through an inhibitory effect on PI3-kinase/Akt/mTOR pathways that in turn down-regulates cell cycle regulators. The translational inhibition causes G1 arrest of the cell cycle and an ultimate mitochondria-dependent apoptosis. Moreover, autophagic cell death and accelerated senescence also explain antroquinonol-mediated anticancer effect.

Keywords: Akt; mTOR; K-ras; Pancreatic cancer; Bcl-xL

1. Introduction

Pancreatic cancer is a malignant neoplasm of the pancreas. From the report of the National Cancer Institute, each year in the United States, more than 43,000 people are diagnosed with cancer of the pancreas. By the end of 2010, it is estimated that there will be about 43,140 cases and 36,800 deaths from the disease. The prognosis is poor. Without active treatment, patients with metastatic pancreatic cancer have a median survival of 3 to 5 months and 6 to 10 months for locally advanced disease [1]. Surgical operation is a major treatment of pancreatic cancer, and chemotherapy is used to gain a survival benefit and to improve the quality of life. In recent years, the molecular mechanism of pancreatic cancer has been better understood than ever before, leading to new approaches of the treatment.

The phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathway is not only a major signaling for cell survival, growth, motility and metabolism, but also a resistant mechanism against a wide variety of cancer chemotherapeutic drugs [2,3]. Currently, therapeutics targeting PI3-kinase/Akt pathways are being developed against numerous types of cancers. Several lines of preclinical and early clinical evidence support this strategy [3,4]. However, the general role of PI3-kinase signaling in diverse biologic function raises concerns about its therapeutic use. Further strategies for selectivity issues are needed to diminish its impact in normal cellular function. Recently, PI3-kinase/Akt/the mammalian target of rapamycin (mTOR) signaling pathway has been highlighted since the pathway is activated in various types of cancers [5,6]. The mammalian target of rapamycin is a serine/threonine protein kinase that regulates cell growth by integrating nutrient- and growth-factor-derived signals [7,8]. Currently, it is known that mTOR exists in two function complexes, mTORC1 and mTORC2. Rapamycin and its analogs are selective for mTORC1 as anticancer agents reported in numerous preclinical and clinical studies [9]. Because of the constitutively activated PI3-kinase/mTOR pathway in cancers, selective dual PI3-kinase/mTOR inhibitors have been developed [6,10].

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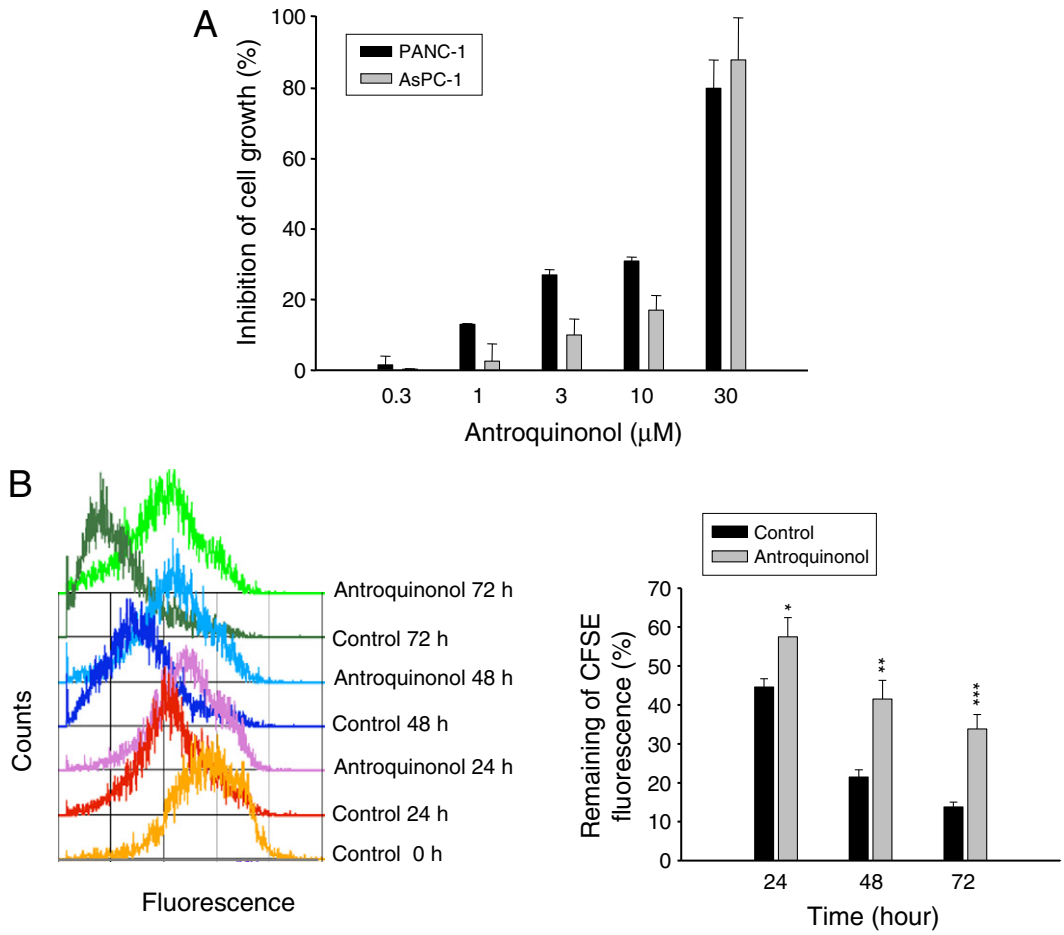

Fig. 1. Effect of antroquinonol on cell proliferation in AsPC-1 cells. (A) The graded concentrations of antroquinonol were added to cells for 48 h. The cells were fixed and stained with SRB, and the data were analyzed. Data are expressed as mean±S.E.M. of five determinations (each in triplicate). (B) Cells were labeled with CFSE and treated with vehicle (0.1% DMSO, control) or antroquinonol (30 μM) for the indicated times. The fluorescence intensity was determined by flow cytometric analysis. Data are expressed as mean±S.E.M. of three independent experiments. *P<.05, **P<.01 and ***P<.001 compared with the respective control.

Augmented activation of PI3-kinase/Akt/mTOR pathway has been reported in more than 50% of pancreatic cancers and has been associated with a poor prognosis [11,12]. To this end, dual PI3-kinase/mTOR inhibitors have been developed. Cao and colleagues reported that dual PI3-kinase/mTOR inhibitors produced significant antitumor activity in orthotopic xenografts inoculated with primary human pancreatic cancers [13]. Besides, the combination of both kinases inhibitors also demonstrated effective activity against pancreatic cancers [14]. These studies therefore suggest that targeting the PI3-kinase/mTOR pathway is a feasible strategy for pancreatic cancers that harbor activation of PI3-kinase/mTOR signaling pathway.

Antrodia camphorata, a camphor tree mushroom, is a precious traditional Chinese herbal medicine and shows pharmacological activities against several diseases. *Antrodia. camphorate* is rich in flavonoids, terpenoids, polyphenolics and polysaccharides and has been produced in agricultural manufacturing scales in Taiwan. Antroquinonol is a ubiquinone derivative isolated from *A camphorata*. Our previous study showed that antroquinonol displayed an anticancer activity against hepatocellular carcinoma (HCC) through activation of 5′adenosine-monophosphate-activated protein kinase (AMPK) and inhibition of mTOR pathway [15]. After further study, we found that antroquinonol is effective against pancreatic cancers through a distinct signaling pathway from that in HCC. It is of importance since pancreatic cancers are prone to be resistant to standard chemotherapies. Accordingly, several pharmacological and biochemical assessments have been used to delineate antroquinonol-mediated signaling cascade in pancreatic cancers.

2. Materials and methods

2.1. Materials

RPMI-1640 medium, fetal bovine serum (FBS), penicillin, streptomycin and all other tissue culture regents were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). Antibodies to cyclin D1, cyclin E, cyclin A, cyclin B1, Bcl-2, Bcl-xL, Bax, Bak, Mcl-1, $p21^{Waf1/Cip1}$, p53, N-ras, H-ras, K-ras and antimouse and antirabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Antibodies to phospho-4E-$BP1^{Thr37/Thr46}$, phospho-4E-$BP1^{Thr70}$, 4E-BP1, phospho-$p70S6K^{Thr389}$, phospho-$p70S6K^{Thr421/Ser424}$, p70S6K, AMPKα, phospho-$AMPK\alpha^{Thr172}$, phospho-Akt^{Ser473}, phospho-Akt^{Thr308}, Akt, phospho-$mTOR^{Ser2448}$, m-TOR, phospho-$eIF4E^{Ser209}$, eIF4E, LC3 and α-tubulin were from Cell Signaling Technologies (Boston, MA, USA). Sulforhodamine B (SRB), propidium iodide (PI), phenylmethylsulfonyl fluoride (PMSF), leupeptin, dithiothreitol, EDTA, paclitaxel, trichloroacetic acid (TCA), Triton X-100, Rnase, sodium orthovanadate and aprotinin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Carboxyfluorescein succinimidyl ester (CFSE) was purchased from Molecular Probes Inc. (Eugene, OR, USA). Antroquinonol was purified from *A. camphorate.* The purification and structure identification of antroquinonol were demonstrated elsewhere [16].

2.2. Cell lines and cell culture

Cancer cell lines including PANC-1 and AsPC-1 were from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in RPMI-1640 medium with 10% FBS (vol/vol) and penicillin (100 U/ml)/streptomycin (100 μg/ml). Cultures were maintained in a humidified incubator at 37°C in 5% CO_2/95% air.

2.3. SRB assays

Cells were seeded in 96-well plates in medium with 5% FBS. After 24 h, cells were fixed with 10% TCA to represent cell population at the time of compound addition (T_0). After additional incubation of DMSO or antroquinonol for 48 h, cells were fixed with 10% TCA, and SRB at 0.4% (wt/vol) in 1% acetic acid was added to stain the cells. Unbound SRB was washed out by 1% acetic acid, and SRB bound cells were solubilized with 10 mM Trizma base. The absorbance was read at a wavelength of 515 nm. Using the following absorbance measurements, such as time zero (T_0), control growth (C) and cell growth in the presence of the compound (Tx), the percentage growth was calculated at each of the compound concentrations levels. Percentage growth inhibition was calculated as $100-[(Tx-T_0)/(C-T_0)]\times100$. Growth inhibition of 50% (IC_{50}) is determined at the compound concentration that results in 50% reduction of total protein increase in control cells during the compound incubation.

2.4. Cell proliferation assay with CFSE labeling

The cells were adjusted to a density of 10^6 cells/ml and were treated with CFSE at a final concentration of 10 μM. After incubation at 37°C for 10 min, labeling was blocked by the addition of RPMI medium with 10% FCS. Tubes were placed in ice for 5 min and then washed. After centrifugation, the cells were seeded in RPMI medium with 10% FCS for 24, 48 and 72 h at 37°C under 5% CO_2/95% air. After the treatment, the fluorescence intensity was determined by flow cytometric analysis.

2.5. FACScan flow cytometric assay

After the treatment of cells with vehicle (0.1% DMSO) or antroquinonol for the indicated times, the cells were harvested by trypsinization, fixed with 70% (vol/vol) alcohol at 4°C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 ml of phosphate-citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml PI solution containing Triton X-100 (0.1% vol/vol), RNase (100 μg/ml) and PI (80 μg/ml). DNA content was analyzed with FACScan and CellQuest software (Becton Dickinson, Mountain View, CA, USA).

2.6. Western blotting

After the treatment, cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml of lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF and 100 μM sodium orthovanadate. Total protein was quantified, mixed with sample buffer and boiled at 90°C for 5 min. Equal amount of protein (30 μg) was separated by electrophoresis in 8% or 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to PVDF membranes and detected with specific antibodies. The immunoreactive proteins after incubation with appropriately labeled secondary antibody were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.7. Measurement of mitochondrial membrane potential ($\Delta\Psi_m$)

Cells were treated with or without antroquinonol. Thirty minutes before the termination of incubation, a rhodamine 123 solution (final concentration of 5 μM) was added to the cells and incubated for the last 30 min at 37°C. The cells were finally harvested, and the accumulation of rhodamine 123 was determined using FACScan flow cytometric analysis.

2.8. Senescence-associated β-galactosidase detection

The senescent cells were observed by morphological changes under light microscopy. Besides, the cellular β-galactosidase activity, a well-known marker of senescent cells, was examined by using a Senescence β-Galactosidase Staining Kit (Cell Signaling, Beverly, MA, USA) according to the manufacturer's protocol. The senescent cells were observed by a positive staining of blue color.

2.9. Data analysis

The compound was dissolved in DMSO. The final concentration of DMSO was 0.1% in cell culture media. Data are presented as the means±S.E.M. for the indicated number of separate experiments. Statistical analysis of data was performed with one-way analysis of variance followed by a t test, and P values less than .05 were considered significant.

3. Results

3.1. Effect of antroquinonol on antiproliferative activity

To determine the antiproliferative activity of antroquinonol in human pancreatic carcinoma cells, the SRB assay developed by Skehan and colleagues for the measurement of cell proliferation [17] was used in PANC-1and AsPC-1 cell models. Incubation of the cells with antroquinonol resulted in a concentration-dependent inhibition of cell proliferation in both cell lines with IC_{50} values of 18.6 and 20.2 μM, respectively (Fig. 1A). The cell proliferation was also detected by using a CFSE assay. The dye CFSE couples to cellular proteins. After cell division, CFSE labeling is allocated evenly to two daughter cells, which have half the fluorescence intensity of the parents. As a result, the control AsPC-1 cells showed a time-related

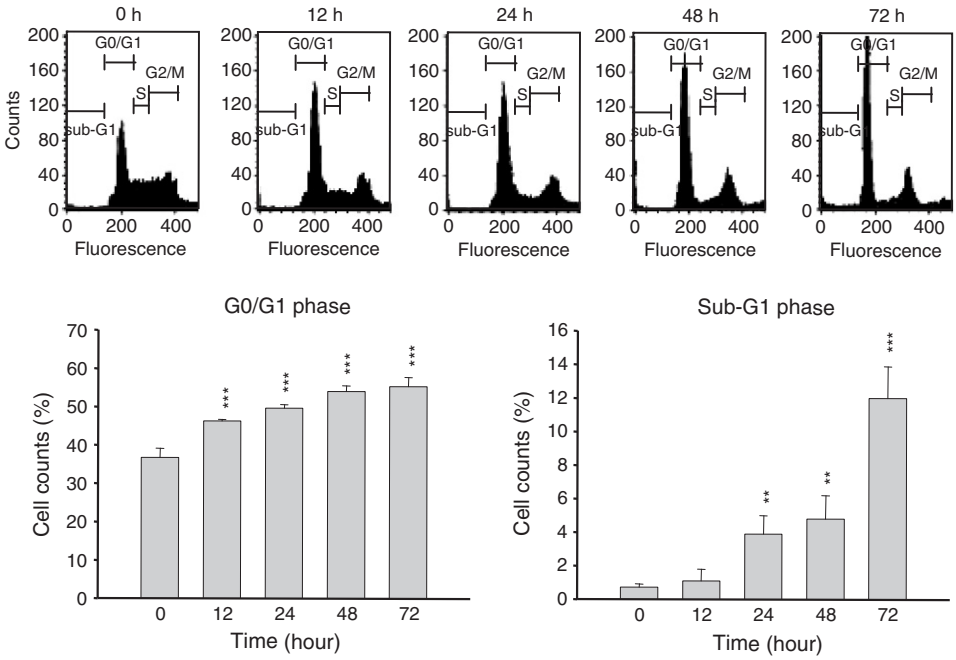

Fig. 2. Effect of antroquinonol on cell cycle progression. AsPC-1 cells were treated with antroquinonol (30 μM) for the indicated times. Cells were fixed and stained with PI for the analysis of DNA content by using FACScan flow cytometer. Data are expressed as mean±S.E.M. of five independent experiments. **P<.01 and ***P<.001 compared with the respective control.

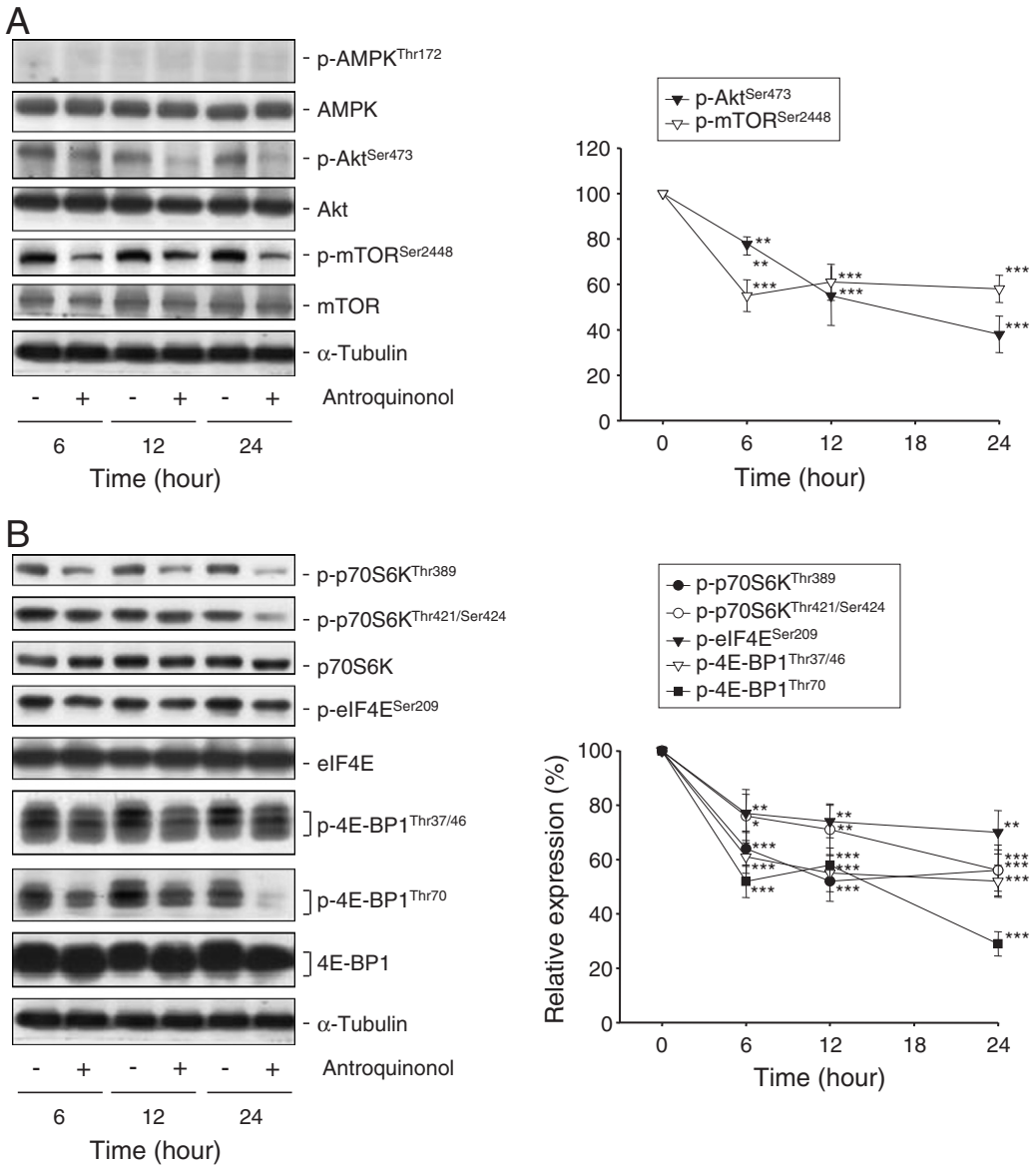

Fig. 3. Effect of antroquinonol on the expressions of several proteins. (A and B) AsPC-1 cells were incubated in the absence or presence of antroquinonol (30 μM) for 6, 12 and 24 h. Cells were harvested and lysed for the detection of the indicated protein expression by Western blot. The expression was quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). The data are expressed as mean±S.E.M. of three to five independent experiments. *P<.05, **P<.01 and ***P<.001 compared with the respective control.

decrease of fluorescence intensity revealing the proceeding of cell division. In contrast, antroquinonol significantly prevented the loss of fluorescence, confirming the antiproliferative effect (Fig. 1B).

3.2. Effect of antroquinonol on G1 arrest and subsequent apoptosis

The progression of cell cycle is sensitive to cellular stresses, being arrested at different phases depending on the stimuli and affected cellular regulators [18,19]. The aberrant induction of checkpoint arrest renders cells to apoptosis. The flow cytometric analysis of DNA content by PI staining showed that antroquinonol induced a time-dependent G1 arrest of the cell cycle and a subsequent apoptosis (sub-G1 phase) in AsPC-1 cells (Fig. 2).

3.3. Effect of antroquinonol on the regulation of mTOR activity

The mammalian target of rapamycin integrates mitogenic signals to regulate cell proliferation. The mTOR inhibitor rapamycin has been extensively reported to block cell cycle progression in a broad range of human cancer cell lines [20]. Our previous study demonstrated that antroquinonol exhibited anticancer activity against HCCs through AMPK-involved inhibition of mTOR translational pathway [15]. In this study, antroquinonol also inhibited the phosphorylation of mTOR at Ser^{2448}, a site dependent on mTOR kinase activity. However, the inhibition of mTOR activity was not regulated by AMPK in AsPC-1 cells (Fig. 3A). Next, the PI3-kinase/Akt signaling was examined since mTOR serves as a downstream effector of PI3-kinase/Akt pathway that phosphorylates mTOR at Ser^{2448} [21]. As a consequence, antroquinonol inhibited the phosphorylation of Akt at Ser^{473} (Fig. 3A), a phosphorylation site that is critical to generate a high level of Akt kinase activity [22]. The data suggest that antroquinonol inhibits mTOR activity through the blockade of PI3-kinase/Akt pathway.

To further determine mTOR/p70S6K/4E-BP1 signaling pathway in AsPC-1 cells, several phosphorylation sites responsible for the activity of these regulators were examined. The data demonstrated that antroquinonol significantly inhibited the phosphorylation of the

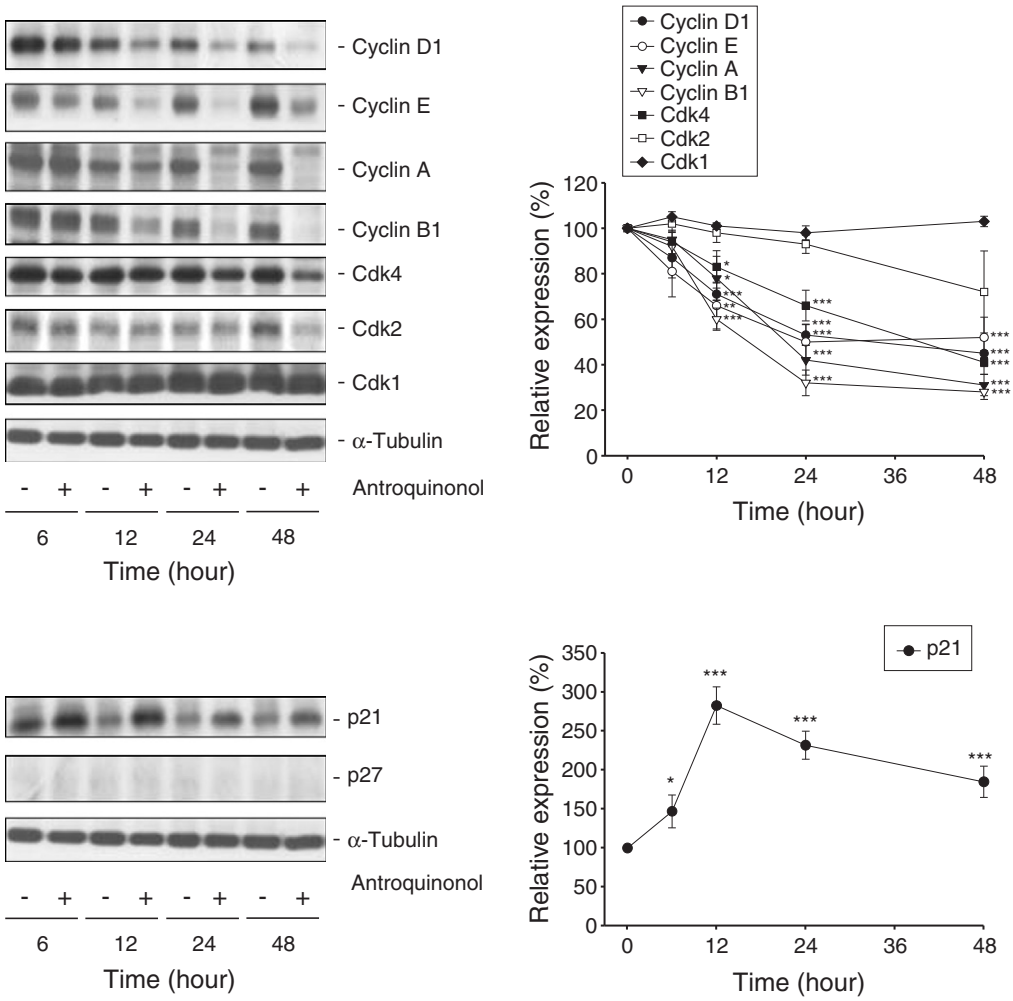

Fig. 4. Effect of antroquinonol on the expressions of several cell cycle regulators. AsPC-1 cells were incubated in the absence or presence of antroquinonol (30 μM) for the indicated times. Cells were harvested and lysed for the detection of the protein expression by Western blot. The expression was quantified using the computerized image analysis system ImageQuant (Amersham Biosciences). The data are expressed as mean±S.E.M. of three to five independent experiments. *P<.05, **P<.01 and ***P<.001 compared with the respective control.

critical residues within these effectors, including p70S6K at Thr^{389} and Thr^{421}/Ser^{424}, eIF4E at Ser^{209} and 4E-BP1 at $Thr^{37/46}$ and Thr^{70} (Fig. 3B). It revealed that mTOR/p70S6K/4E-BP1 signaling pathway was blocked by antroquinonol in AsPC-1 cells.

3.4. Effect of antroquinonol on the expressions of several cell cycle regulators

Cell cycle progression is regulated by periodic activation of various Cdk/cyclin complexes. Since antroquinonol induced G1 arrest in AsPC-1 cells, the expressions of several critical cyclins were determined. As demonstrated in Fig. 4, the protein levels of all the examined cyclins, including cyclin D1, E, A and B1, and Cdk4 were decreased by antroquinonol in a time-dependent fashion, which correlated with the inhibition of mTOR-mediated pathways. In contrast to Cdk/cyclin complex, several Cdk inhibitors are important regulators to negatively control the cell cycle through the formation of heterotrimetric complexes with Cdks and cyclins. The study showed that $p21^{WAF1/CIP1}$ but not $p27^{KIP1}$ was the predominant Cdk inhibitor in AsPC-1 cells. Under antroquinonol-mediated stress, the expression of $p21^{WAF1/CIP1}$ was dramatically augmented in a p53-independent manner since AsPC-1 is a p53-negative human pancreatic cancer cell line (Fig. 4).

3.5. Effect of antroquinonol on protein expressions of Bcl-2 and Ras families

The flow cytometric analysis showed that antroquinonol induced a significant apoptosis after a 24-h treatment in AsPC-1 cells (Fig. 2). A large body of evidence suggests that mitochondria play a central role in the control of cell death [23]. Bcl-2 family proteins that regulate the integrity of mitochondrial membrane were examined. The Western blot revealed that antroquinonol induced a significant decrease of Bcl-xL expression in a 24-h treatment and the down-regulation of both Bcl-xL and Mcl-1 after a 48-h exposure (Fig. 5A). In a parallel experiment, the flow cytometric analysis showed that antroquinonol caused a time-dependent loss of $\Delta\Psi_m$ (Fig. 5B). The data correlated closely with the resulting apoptotic cell death (Fig. 2) and suggested the crucial role of mitochondria.

Ras family, including H-ras, N-ras and K-ras, regulates a wide variety of biological effects, including cell proliferation, survival and transformation [24]. However, it may also induce growth arrest, apoptosis, senescence and autophagy [25,26]. The data demonstrated that K-ras was a predominant member in the Ras family of AsPC-1 cells. Antroquinonol induced a significant increase of K-ras expression and phosphorylation as demonstrated on the band shift (Fig. 5A). Ras has been reported to display proapoptotic activity through a direct interaction with Bcl-2 or related family members [27]. We examined

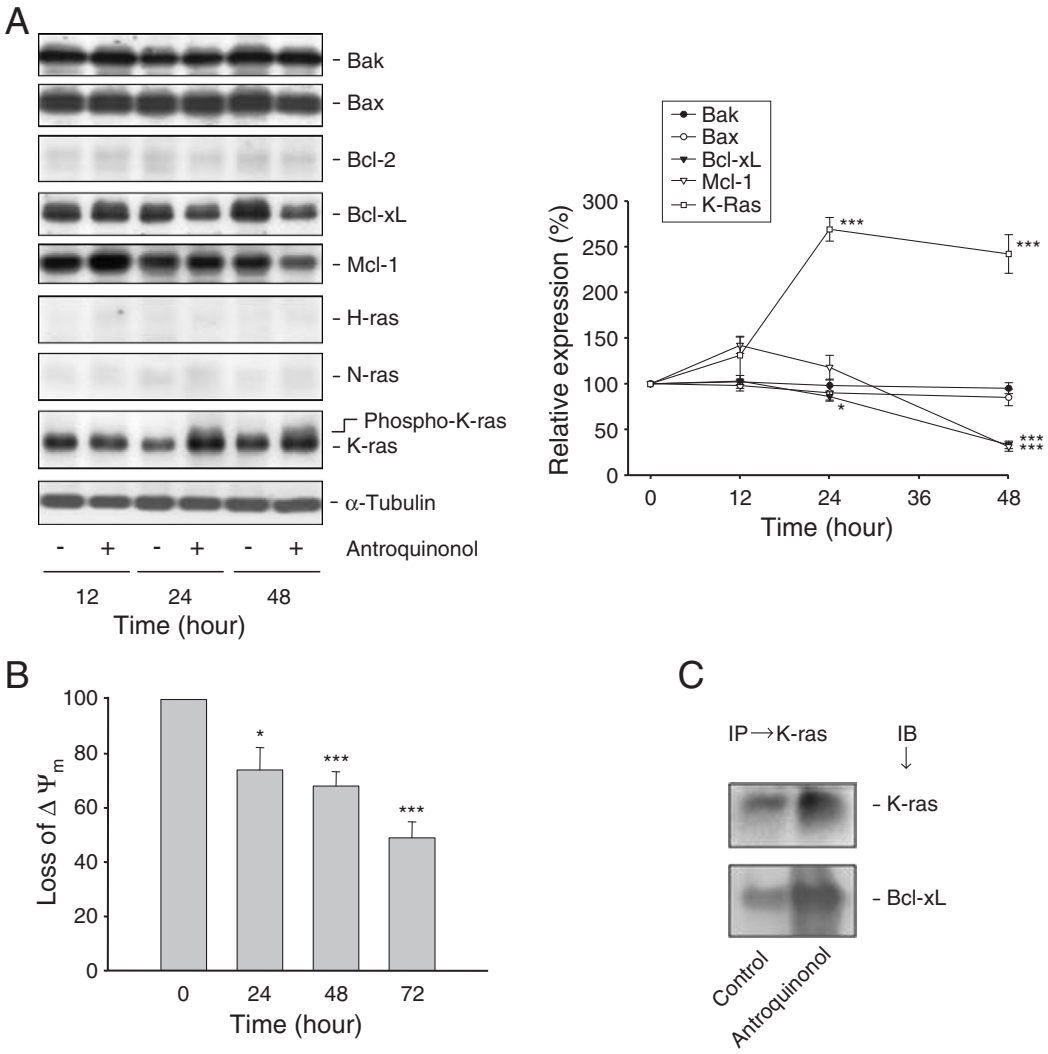

Fig. 5. Effect of antroquinonol on several protein expressions and $\Delta\Psi_m$. AsPC-1 cells were treated with antroquinonol (30 μM) for the indicated times. Cells were harvested and lysed for the detection of the protein expression by Western blot (A), or cells were incubated with rhodamine 123 for the detection of $\Delta\Psi_m$ using FACScan flow cytometric analysis (B). Data are expressed as mean±S.E.M. of three independent determinations. *P<.05 and ***P<.001 compared with the respective control. (C) AsPC-1 cells were treated without or with antroquinonol (30 μM) for 24 h. Cells were harvested for the immunoprecipitation assay. The data are representative of two independent experiments.

by coimmunoprecipitation the association between K-ras and Bcl-xL. The data showed that the capacity of the association was proportional to the level of K-ras expression (Fig. 5C).

3.6. Effect of antroquinonol on autophagy and senescence

To determine the autophagic activation, the generation of LC3 II, a protein that associates with autophagosome membranes, was detected [28]. After a long-term treatment (72 h), antroquinonol caused an increase of LC3 II formation (Fig. 6A). Furthermore, the activity of β-galactosidase was measured for the determination of senescence. As a result, a 72-h treatment of antroquinonol induced a small population (3%) of cellular senescence (Fig. 6B).

4. Discussion

An increasing body of evidence shows that numerous common foods make up the anticancer diet, such as garlic, soy beans, turmeric, green tea and red wine [29,30]. *Antrodia camphorata*, a camphor tree mushroom, is a health food and has been produced in agricultural manufacturing scales in Taiwan. Antroquinonol, an active component from *A. camphorate*, showed anticancer activity against human pancreatic cancers. It is of particular importance because pancreatic cancer has a very poor prognosis and is highly resistant to current cancer chemotherapeutic drugs. Antroquinonol induced an arrest of cell cycle at G1 phase and subsequent apoptosis in pancreatic cancer AsPC-1 cells through mTOR-mediated translational pathways. It has been well identified that mTOR is directly phosphorylated by Akt at Ser^{2448}, leading to an increase of mTOR activity [21]. More importantly, Akt can phosphorylate and inactivate tuberous sclerosis complex (TRC) 2, a tumor suppressor and an inhibitor of mTOR, leading to dissociation of TSC1/TSC2 complex and a subsequent increase of mTOR activity [31]. Antroquinonol inhibited Akt activity that in turn caused the suppression of mTOR activity as substantiated by the inhibition of phosphorylation at Ser^{2448}. The suppression of mTOR activity by antroquinonol also impeded protein synthesis by inactivating p70S6K activity and stimulating the translation repressor 4E-BP1. 4E-BP1 inhibited cap-dependent translational pathways through the association with the translation initiation factor eIF4E. The hyperphosphorylation of 4E-BP1 disrupted the association, leading to the activation of cap-dependent translation [31,32]. Antroquinonol inhibited 4E-BP1 phosphorylation at $Thr^{37/46}$ and

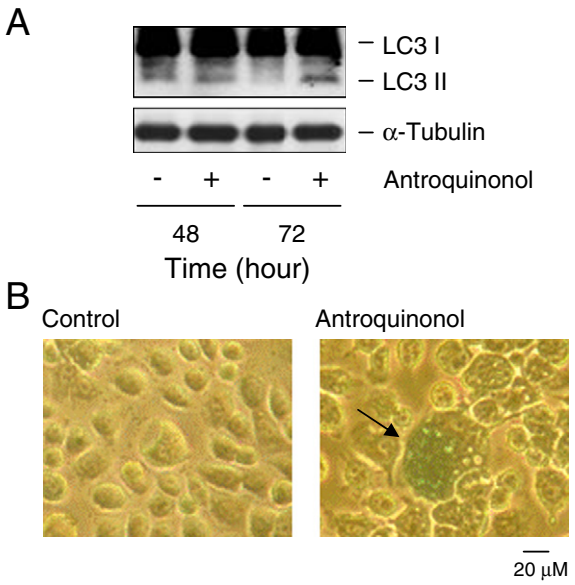

Fig. 6. Effect of antroquinonol on LC3 expression and senescence in AsPC-1 cells. (A) Cells were incubated without or with antroquinonol (30 μM) for 48 and 72 h. Cells were harvested and lysed for the detection of LC3 expression by Western blot. The data are representative of two independent experiments. (B) Cells were incubated without or with antroquinonol (30 μM) for 72 h. Cells were fixed and stained for the detection of β-galactosidase activity as described in the Materials and Methods section. The data are representative of four independent experiments. Arrow, senescent cell.

Thr^{70} and recovered the interaction between 4E-BP1 and eIF4E, resulting in the inhibition of cap-dependent translation. However, mTOR activity can be regulated through Akt-independent mechanism. 5′Adenosine-monophosphate-activated protein kinase, a serine/threonine protein kinase, may serve as a cellular energy sensor and regulate cellular adaption to environmental or nutritional stress [33]. Recent work has revealed that AMPK is able to block mTOR pathways through the phosphorylation of TSC2, leading to the association of TSC1/TSC2 complex and suppression of mTOR and p70S6K activity [34]. 5′Adenosine-monophosphate-activated protein kinase may also directly phosphorylate mTOR at Thr^{2446}, a phosphorylation site that negatively regulates mTOR activity [35]. However, antroquinonol did not induce AMPK activation in AsPC-1 cells. The data ruled out the involvement of AMPK in antroquinonol-mediated down-regulation of mTOR activity.

Progression of cell cycle is regulated by periodic activation of various Cdk/cyclin complexes. G1 phase is delicately regulated by cyclin D1 and its catalytic partner Cdk4. p21 and p27 belong to the family of Cdk inhibitors. The ability of the family member to enforce G1 arrest is through the inhibitory association with Cdk/cyclin complexes. However, G1 phase is prone to arrest when translational process is disrupted since protein synthesis is highly demanded at this phase. Accordingly, suppression of Akt and mTOR activity may efficiently arrest the progression of cell cycle at G1 phase [15,20,21]. As expected, antroquinonol inhibited mTOR activity, leading to the translational inhibition of cyclin D1, Cdk4 and several related cell cycle regulators. The G1 arrest was subsequently induced in AsPC-1 cells. Mitochondrial function is critical to cell viability. Numerous cellular stresses may eventually result in the damage and loss of mitochondrial function. Bcl-2 family proteins play a critical role in the control of mitochondrial integrity. Bcl-xL and Mcl-1, but not Bcl-2, were major antiapoptotic Bcl-2 family members in AsPC-1 cells. The significant decrease of Bcl-xL and Mcl-1 expression correlated well with antroquinonol-induced loss of $\Delta\Psi_m$ and apoptotic cell death, indicating the central role of mitochondria in cell survival.

More than 90% of pancreatic adenocarcinomas harbor a mutation within codon 12 of the K-ras gene, leading to a constitutive activation of K-ras and cell proliferation [36]. Moreover, raf activation and the resulting increase of p42/44 mitogen-activated protein kinase (MAPK) activity are required for K-ras-mediated cancer cell growth [37]. On the other hand, K-ras may also inhibit cell growth and induce apoptosis [25–27]. It has been reported that phosphorylation and internalization of K-ras induced apoptotic cell death in a Bcl-xL-dependent pathway [27]. In our work, antroquinonol induced up-regulation and phosphorylation of K-ras, although the phosphorylation site has yet to be identified. However, antroquinonol had no effect on p42/44 MAPK activity (data not shown), indicating that K-ras did not serve as a stimulator of cell proliferation. Of note, the coimmunoprecipitation assay demonstrated the association between K-ras and Bcl-xL to antroquinonol action. Bivona and colleagues reported that the phosphorylated K-ras on outer mitochondrial membrane may interact with and convert Bcl-xL to a proapoptotic protein [27]. However, we did not observe that mitochondria were specific organelles where the phosphorylated K-ras associated with Bcl-xL by confocal immunomicroscopic analysis (data not shown). We did not speculate that Bcl-xL was converted to be proapoptotic. Nevertheless, it is plausible that phosphorylated K-ras promoted apoptosis by sequestering Bcl-xL and triggering its down-regulation.

Autophagy, a major intracellular degradation system, and senescence, a cellular program of terminal growth arrest, are closely linked and are induced by numerous forms of stress, including a wide variety of anticancer drugs and radiation [38,39]. Several key signals have been suggested to induce autophagic cell death and accelerated senescence, such as p53 and p21 [38,40]. The data demonstrated that antroquinonol induced not only apoptosis but also a certain degree of autophagy and senescence in p53-negative AsPC-1 cells. The dramatic increase of p21 expression may serve as a key event in the signaling pathway. Furthermore, Bihani and colleagues reported that forced expression of H-ras as well as K-ras induced a senescence-like permanent growth arrest [41]. The senescence caused by antroquinonol might be, at least partly, explained by K-ras up-regulation since antroquinonol stimulated a profound increase of K-ras protein level.

Taken together, the data suggest that antroquinonol induces anticancer activity in human pancreatic cancer AsPC-1 cells through a sequential signaling cascade. It induces an inhibitory effect on PI3-kinase/Akt activities that in turn block mTOR/p70S6K/4E-BP1 signaling pathways, leading to the down-regulation of cyclin proteins and Cdks. The translational inhibition results in G1 arrest of the cell cycle and an ultimate mitochondria-dependent apoptosis. The up-regulated K-ras may also contribute to apoptosis through the association with Bcl-xL. Moreover, autophagic cell death and accelerated senescence also explain, at least partly, the antroquinonol-mediated anticancer effect in AsPC-1 cells.

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