



Crude alkaloid extract of *Rhazya stricta* inhibits cell growth and sensitizes human lung cancer cells to cisplatin through induction of apoptosis

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Abstract

There is an urgent need to improve the clinical management of non-small cell lung cancer (NSCLC), one of the most frequent causes of cancer-related deaths in men and women worldwide. *Rhazya stricta*, an important medicinal plant used in traditional Oriental medicine, possesses anti-oxidant, anti-carcinogenic and free radical scavenging properties. This study was done to explore the potential anticancer activity of a crude alkaloid extract of *R. stricta* (CAERS) against the NSCLC line A549. CAERS markedly suppressed the growth of A549 cells and considerably enhanced the anti-proliferative potential of cisplatin. CAERS-mediated inhibition of A549 cell growth correlated with the induction of apoptosis that was accompanied by numerous morphological changes, DNA fragmentation, an increase in the Bax/Bcl-2 ratio, the release of mitochondrial cytochrome *c*, activation of caspases 3 and 9 and cleavage of poly(ADP-ribose)-polymerase. CAERS reduced the constitutive expression of anti-apoptotic proteins (Bcl-2, Bcl-X_L, Mcl-1 and Survivin) and cell cycle regulating proteins (cyclin D1 and c-Myc), but enhanced expression of the proapoptotic proteins Noxa and BAD. These observations indicate that CAERS induced apoptosis and sensitized NSCLC to cisplatin via a mitochondria-mediated apoptotic pathway. These data provide a rationale for using a combination of CAERS and CDDP to treat NSCLC and other CDDP-resistant tumors.

Keywords: apoptosis, lung cancer, medicinal plant, RT-PCR, western blot.

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Introduction

Lung cancer is one of the most common cancers in many countries and accounts for 28% of all cancer-related deaths (Bilello *et al.*, 2002; Jemal *et al.*, 2008). Clinically, lung cancer is classified into two groups, namely, small cell and non-small cell lung cancer (NSCLC). The latter is more prevalent, accounting for almost 85% of lung cancers (Jemal *et al.*, 2004). Although early detection and treatment can improve the prognosis, only 15% of patients with NSCLC are diagnosed at an early stage since most lung cancer begins to grow silently without any symptoms until the cancer is in an advanced stage (Gargiullo *et al.*, 2002; Beadsmoore and Sreaton, 2003; Jemal *et al.*, 2004), at which point the treatment options are generally limited to surgery or radiation. Currently, the most active chemotherapeutic agent for the treatment of NSCLC is cisplatin (cis-diamminedichloroplatinum(II); CDDP) and CDDP-based combinations have remained the standard first-line chemotherapy for advanced NSCLC for more than two decades (Johnson, 2000; Wang *et al.*, 2004). Unfortunately, despite its great efficacy, CDDP is associated with several

major problems, including a number of side effects and the development of resistance that tend to limit the therapeutic potential of CDDP (Rom *et al.*, 2000; Gridelli *et al.*, 2003; Wang *et al.*, 2004). Consequently, new approaches such as novel effective drugs with low toxicities or excellent combination regimens are needed to overcome CDDP-resistance in NSCLC therapy.

During the past decade, various studies have shown that current cancer therapies (for example, chemotherapy, γ -irradiation, immunotherapy or suicide gene therapy) exert their antitumor effect primarily by triggering apoptosis in cancer cells (Makin and Dive, 2001). Consistent with this evidence, most tumor cells, including NSCLC, are naturally resistant to apoptotic-related cell death and to non-apoptotic cell death such as necrosis, autophagy, senescence, mitotic catastrophe and paraptosis (Okada and Mak, 2004; Debatin and Krammer, 2004). To date, three apoptotic pathways have been identified, *i.e.*, mitochondrial-, death receptor- and endoplasmic reticulum stress-mediated apoptosis (reviewed in Wong, 2011). Of these, the mitochondrial pathway initiates apoptosis in most physiological and pathological situations. A variety of stimuli such as stress and drugs induce disruption of the mitochondrial membrane and the release of mitochondrial proteins such as cytochrome *c* (cyt *c*).

The release of cyt *c* from mitochondria is tightly regulated by Bcl-2 family proteins that include anti-apoptotic (*e.g.*, Bcl-2) and pro-apoptotic (*e.g.*, Bax) members (Reed, 2000; Wong, 2011). Bcl-2 can stabilize mitochondrial permeability to prevent the release of cyt *c* whereas Bax increases the membrane permeability leading to the release of cyt *c* from mitochondria (Reed, 2000; Wong, 2011). Upon release from mitochondria into the cytosol, cyt *c*, together with Apaf-1, activates caspase-9 and the latter then activates caspases 3 and 7 (Cruchten and Den Broeck, 2002; Wong, 2011). Subsequently, active caspase-3 cleaves downstream substrates such as poly-(ADPribose) polymerase (PARP), which are responsible for the morphological and biochemical changes that are the hallmarks of apoptosis (Cruchten and Den Broeck, 2002; Wong, 2011). However, tumor cells do not undergo apoptosis easily because of defects in their ability to activate the death signaling pathway. Consequently, one effective strategy for the treatment/prevention of cancer is to search for agents that can activate apoptotic pathways in tumor cells (Call *et al.*, 2008; Liu *et al.*, 2011).

Members of the plant family Apocynaceae have been used in folk medicine for centuries and many of their constituents have been isolated and are now in clinical use, *e.g.*, vinblastine, vincristine and reserpine (Neuss, 1970). *Rhazya stricta* (harmal) is an important medicinal species of the Apocynaceae used in indigenous medicinal herbal drugs to cure various diseases in southern Asia (Pakistan, India and Afghanistan) and the Middle East (Saudi Arabia, Qatar, United Arab Emirates, Iran and Iraq) (Gilani *et al.*, 2007). Indeed, extracts of *R. stricta* leaves are prescribed in folklore medicine for the treatment of various disorders such as diabetes, sore throat, helminthiasis, inflammatory conditions and rheumatism (Ali *et al.*, 2000, 1995, 1998). Some of the chemical constituents of *R. stricta* and their pharmacological activities have been reviewed (Ali *et al.*, 2000). The plant extract contains many alkaloids, glycosides, flavonoids, tannins and triterpenes (Ahmed *et al.*, 1983; Al-Yahya *et al.*, 1990). The effective uses of *R. stricta* described in traditional medicine have been attributed to the presence of indole alkaloids. Indeed, activity-guided phytochemical analysis of *R. stricta* extract has shown that the alkaloidal fraction has the highest biological activity (Tanira *et al.*, 2000). In addition, two indole alkaloids, 16-*epi-Z*-isositsirikine and didemethoxycarbonyl-tetrahydrosecamine, isolated from *R. stricta* have antineoplastic activity (Mukhopadhyay *et al.*, 1981, 1983; Attaur-Rahman and Zaman, 1986).

Previously work has shown that an aqueous extract of *R. stricta*, acting as a single agent, inhibits cell proliferation in the breast cancer cell lines MCF-7 and MDA MB-231 (Baeshen *et al.*, 2012). In contrast, no detailed studies have addressed the effect of a crude alkaloid extract of *R. stricta* (CAERS) on cancers. The present study was undertaken to assess the impact of CAERS on the growth of NSCLC

A549 cells and to examine the mechanism of action. The results described here clearly show that CAERS suppressed the growth of A564 cells and increased the sensitivity to and cytotoxicity of CDDP. CAERS sensitized A549 cells to CDDP through a mitochondria-dependent apoptotic pathway. These data provide a basis for using a combination of CAERS and CDDP to treat lung carcinoma and other tumors.

Materials and Methods

Preparation of crude alkaloid extract from *R. stricta*

A crude alkaloid extract of *R. stricta* leaves was prepared essentially as described elsewhere (Tanira *et al.*, 2000), with some modifications. Briefly, air-dried leaves of *R. stricta* (350 g) were soaked in 80% methanol (1 L) at ambient temperature for seven days after which the methanolic extract was evaporated in a rotatory evaporator and the remaining residue was suspended in water and filtered. The aqueous extract was then acidified with 10% glacial acetic acid and extracted with chloroform. This chloroform fraction contained weakly basic alkaloids and neutral compounds. The remaining aqueous solution was alkalized using NaOH and the pH was adjusted to 11. The alkaline aqueous layer was extracted with chloroform to yield a chloroform fraction enriched in strongly basic alkaloids (Tanira *et al.*, 2000). The chloroform layer was evaporated to dryness and the resulting residue was re-dissolved in DMSO to the desired concentrations.

Cell culture

The human NSCLC cell line A549 and non-malignant human foreskin fibroblasts (HF-5 cells) were obtained from King Fahd Center for Medical Research, King Abdulaziz University, Saudi Arabia. The A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Promega) supplemented with 10% of FBS (Promega) and 1% penicillin-streptomycin antibiotics (Promega) and were grown at 37 °C in a humidified atmosphere with 5% CO₂.

Cell growth assay

Cell viability and the effects of drugs on the growth of NSCLC cells were assessed with the trypan blue dye exclusion assay. Briefly, A549 cells were seeded onto 24-well plates (50 x 10³ cells/well) and grown overnight. The cells were then treated with increasing concentrations of CAERS (0, 3, 6, 15, 30, 45 and 60 µg/mL) or CDDP (0, 5, 10, 25, 50, 75 and 100 µg/mL) and incubated for 24, 48 or 72 h. At the end of each incubation, the floating and adherent cells were collected (with care being taken that none of the floating cells were lost during washes) and pelleted by centrifugation (700 g, 5 min). The cells were re-suspended in 25 µL of phosphate-buffered saline (PBS), mixed with 5 µL of 0.4% trypan blue solution and counted using a hemocytometer under an inverted microscope. Cell growth

rates were determined by counting the number of viable cells in each CAERS-/CDDP-treated well and expressing this as a percentage of the total number of viable cells in the control well (no drugs added).

Clonogenic assay

The clonogenic assay was done by seeding 1000 cells/well onto 6-well plates. After incubation for 24 h, the cells were treated with increasing concentrations of CAERS (0, 3, 6, 15, 30, 45 and 60 $\mu\text{g}/\text{mL}$) for 72 h and then washed twice with PBS to remove any remaining CAERS before adding fresh medium. The cells were subsequently incubated for another 12 days after which the colonies were stained the 0.4% trypan blue solution. Colonies with > 50 cells were scored as surviving and the percentage survival was determined using the equation: percentage of survival = (colonies with drug treatment/colonies without drug treatment, *i.e.*, control) x 100.

Assessment of cell morphological changes

Cells were seeded ($50 \times 10^3/\text{well}$) onto 24-well plates and treated for 24 h with increasing concentrations (0, 3, 6, 15, 30, 45 and 60 $\mu\text{g}/\text{mL}$) of CAERS after which they were washed in PBS and fixed and permeabilized in 3.7% paraformaldehyde (Sigma) in PBS for 10 min at room temperature. Fixed cells were washed with PBS and stained with ethidium bromide solution (20 $\mu\text{g}/\text{mL}$) for 20 min at room temperature. The cells were washed twice more with PBS and analyzed. Representative images were captured with an inverted fluorescence microscope (Carl Zeiss, Germany) using the magnification indicated in the figures.

DNA fragmentation assay

Cells were seeded ($20 \times 10^4/\text{well}$) onto 6-well plates and treated for 24 h with different concentrations (0, 3, 6, 15 and 30 $\mu\text{g}/\text{mL}$) of CAERS after which the cells were harvested, washed in PBS and purified using a DNA purification kit (DNeasy Blood and Tissue kit, QIAGEN), according to the manufacturer's recommendations. The concentration of DNA was determined spectrophotometrically and DNA was electrophoresed on a 1% agarose gel at 100 V for 2 h and analyzed. The gel was stained with ethidium bromide (0.5 $\mu\text{g}/\text{mL}$) and DNA was visualized with a UV trans-illuminator (Bio-Rad).

RNA extraction and reverse transcriptase-PCR

Cells were seeded ($20 \times 10^4/\text{well}$) onto 6-well plates and treated with different concentrations (0, 3, 6, 15 and 30 $\mu\text{g}/\text{mL}$) of CAERS for 24 h. After this period, floating and adherent cells were collected (with care being taken that none of the floating cells were lost during washes) and pelleted by centrifugation (700 g, 5 min). RNA extraction and reverse transcriptase-PCR were done as previously described (Elkady, 2012). Briefly, total RNA was extracted,

reverse transcribed and amplified by PCR using QIAamp RNA Blood mini kits (QIAGEN) according to the manufacturer's instructions. The polymerase chain reaction (PCR) was done using gene-specific primers. The primer sequences for Bcl-2, Bcl-x_L, Mcl-1, survivin (BIRC5), Bad, Noxa, c-Myc, cyclin D1 and HPRT1 were described earlier (El-Kady *et al.*, 2011). Amplification products obtained by PCR were separated electrophoretically on 1% agarose gels and visualized by ethidium bromide (0.5 $\mu\text{g}/\text{mL}$) staining (Elkady, 2012).

Preparation of mitochondrial and cytosolic extracts

To detect cyt *c* release by western immunoblotting, mitochondrial and cytosolic extracts were obtained as described previously (Elkady, 2012). Briefly, cells were seeded ($20 \times 10^4/\text{well}$) onto 6-well plates, treated with the indicated concentrations of CAERS and CDDP and incubated for 24 h. After this incubation, the cells were collected by centrifugation, washed twice with cold PBS, re-suspended in 500 μL of ice-cold cytosol extraction buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA and 1 mM EGTA) containing a protease inhibitor cocktail (1 mM PMSF, 1% aprotinin, 1 mM leupeptin and 1 μg of pepstatin A/mL). After a 30 min incubation on ice, the cells were homogenized in the same buffer using a dounce homogenizer (30 strokes) and centrifuged (1000 x g, 10 min, 4 °C). The supernatant was collected and centrifuged again (14,000 x g, 30 min) to collect the mitochondria-rich (pellet) and cytosolic (supernatant) fractions. The supernatant was used as cytosolic lysate while the pellet was suspended in lysis buffer (137 mM NaCl, 20 mM Tris, pH 7.9, 10 mM NaF, 5 mM EDTA, 1 mM EGTA, 10% (v/v) glycerol and 1% Triton X-100) supplemented with a protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem) before being centrifuged to obtain the mitochondrial lysate. Proteins concentrations were determined with a BCA protein assay kit (Pierce) and equal amounts of protein fractions were subjected for further analyses as described below.

Western blot analysis

After treatment with CAERS and CDDP, the cells were harvested, washed three times with PBS and lysed in cold lysis buffer containing 0.05 mM Tris-HCl, 0.15 mM NaCl, 1 M EGTA, 1 M EDTA, 20 mM NaF, 100 mM Na₃VO₄, 0.5% NP40, 1% Triton X-100 and 0.5 mM phenylmethylsulfonyl fluoride, pH 7.4, with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem). The amount of protein was quantified using a BCA protein assay kit (Pierce). Western immunoblotting was done essentially as described elsewhere (Elkady, 2012). Briefly, equal amounts (20 μg per treatment) of protein from control and CAERS/CDDP-treated cells were boiled for 5 min in Laemmli buffer and separated by SDS-PAGE on 10% polyacrylamide gels. The proteins were sub-

sequently transferred to PVDF membranes that were then blocked and probed with primary antibodies (Spring Bioscience) against the desired proteins followed by incubation with secondary HRP-conjugated antibodies (Spring Bioscience). In all experiments, the blots were stripped with stripping buffer (62.5 mM Tris, pH 6.7, 2% SDS and 90 mM 2-mercaptoethanol) and reprobed with anti- β -actin (Spring Bioscience) antibody as a control for protein loading. Signals were detected with an enhanced chemiluminescence detection kit (Amersham).

Statistical analysis

All assays were done at least three times and the results were expressed as the mean \pm SD unless otherwise stated. Statistical comparisons were done by using Student's *t*-test. Probability values of $p < 0.05$ indicated statistical significance.

Results

CAERS suppresses cell growth and colony formation and sensitizes A549 cells to CDDP

Initially, the effect of CAERS on the death of A549 cells was evaluated by trypan blue dye exclusion, with the viability being expressed as a percentage of that of untreated (control) cells (assumed to be 100%). Figure 1 shows that continuous exposure to increasing concentrations of CAERS (0, 3, 6, 15, 30, 45 and 60 $\mu\text{g}/\text{mL}$) for 24, 48 and 72 h resulted in a concentration- and time-dependent decrease in cell viability relative to control cultures. The IC_{50} (inhibitory concentration 50%) for CAERS-induced cell death was ~ 28 , 15 and 8 μg at 24, 48 and 72 h, respectively. To assess whether CAERS-dependent growth inhibition was selective for cancer cells, the effect of CAERS on the viability of non-malignant human foreskin fibroblasts (HF-5 cells) was also assessed. Figure 1B shows that HF-5 cells were significantly more resistant to growth inhibition by CAERS than were A549 cells. The strongest effect was seen at the highest concentration of CAERS, where the survival of HF-5 cells was inhibited by only $\sim 15\%$ after 72 h, indicating that CAERS is not toxic to normal, non-transformed cells. These results suggest that CAERS may selectively target A549 cancer cells but spare normal fibroblasts, which is a highly desirable property of potential anticancer agents.

CDDP is one of the most effective chemotherapeutic drugs for treating NSCLC but it has a narrow therapeutic window between efficacy and unacceptable toxicity (Rom *et al.*, 2000; Gridelli *et al.*, 2003). Since CAERS effectively inhibited the growth of A549 cells and had little effect on the growth of normal HF-5 fibroblasts, the ability of CAERS to enhance the cell killing effect of CDDP was examined. For this, the effect of CDDP alone or in combination with CAERS on the growth of A549 cells was assessed. Figure 1C shows the effect of increasing concen-

trations (0, 5, 10, 25, 50, 75 and 100 $\mu\text{g}/\text{mL}$) of CDDP alone on A549 cells; the IC_{50} values were 70, 60 and 40 $\mu\text{g}/\text{mL}$ at 24, 48 and 72 h, respectively. On the other hand, combined treatment with CAERS (6 $\mu\text{g}/\text{mL}$) and increasing concentrations of CDDP markedly increased the inhibition of A549 cell growth by CDDP. In this case, there was a decrease in the IC_{50} values of CDDP to 15, 8 and 4 $\mu\text{g}/\text{mL}$ at 24, 48 and 72 h, respectively, in the presence of CAERS (Figure 1D). Pair-wise comparisons between the IC_{50} values shown in panels C and D showed that, in general, CDDP alone had an approximately half-maximal inhibitory effect on cell growth at all concentrations over 24, 48 and 72 h. These results indicate that CAERS strongly enhances the inhibition of cell proliferation by CDDP in the human NSCLC A549 cell line.

Subsequently, a clonogenic assay was done to confirm the potential of CAERS to suppress the growth of A549 cells. As shown in Figure 1E, the clonogenic survival of A549 cells was significantly affected by exposure to CAERS for 72 h, with CAERS causing a concentration-dependent decrease in the number and size of growing colonies when compared to the untreated controls (100%). These results demonstrate that CAERS had a cytostatic effect on long-term colony formation by A549 cells.

CAERS induces apoptosis in A549 cells

The major goal of cancer chemotherapy is to commit tumor cells to apoptosis following exposure to anticancer agents, and it is generally believed that the induction of apoptosis is the primary cytotoxic mechanism of phytochemicals (Khan *et al.*, 2007). To determine whether CAERS inhibited the cell growth of A549 cells by inducing apoptosis and whether CAERS-induced apoptosis accounts for its ability to enhance CDDP-induced inhibition of cell growth and proliferation, cells were treated with increasing concentrations of CAERS for 24 h and frequency of apoptotic cell death was assessed by phase microscopy. As seen under an inverted phase microscope (Figure 2A), untreated A549 cells grew well to form confluent monolayers with a homogenous morphology containing lightly and evenly stained nuclei. In contrast, CAERS-treated cells showed marked morphological changes compared to untreated cells. Most of the cells treated with CAERS concentrations ≥ 30 $\mu\text{g}/\text{mL}$ had densely stained nuclear granular bodies containing highly condensed chromatin ('apoptotic bodies'). The nuclear changes and apoptotic body formation characteristic of apoptosis were visualized by incubating A549 cells with CAERS for 24 h followed by staining with ethidium bromide (Figure 2B).

DNA fragmentation to yield DNA ladders is a characteristic feature of apoptosis (Nagata, 2000). To examine whether CAERS might provoke such fragmentation in A549 cells, genomic DNA from A549 cells treated with CAERS was extracted and separated by agarose gel electrophoresis. Figure 2C shows that there were clear DNA

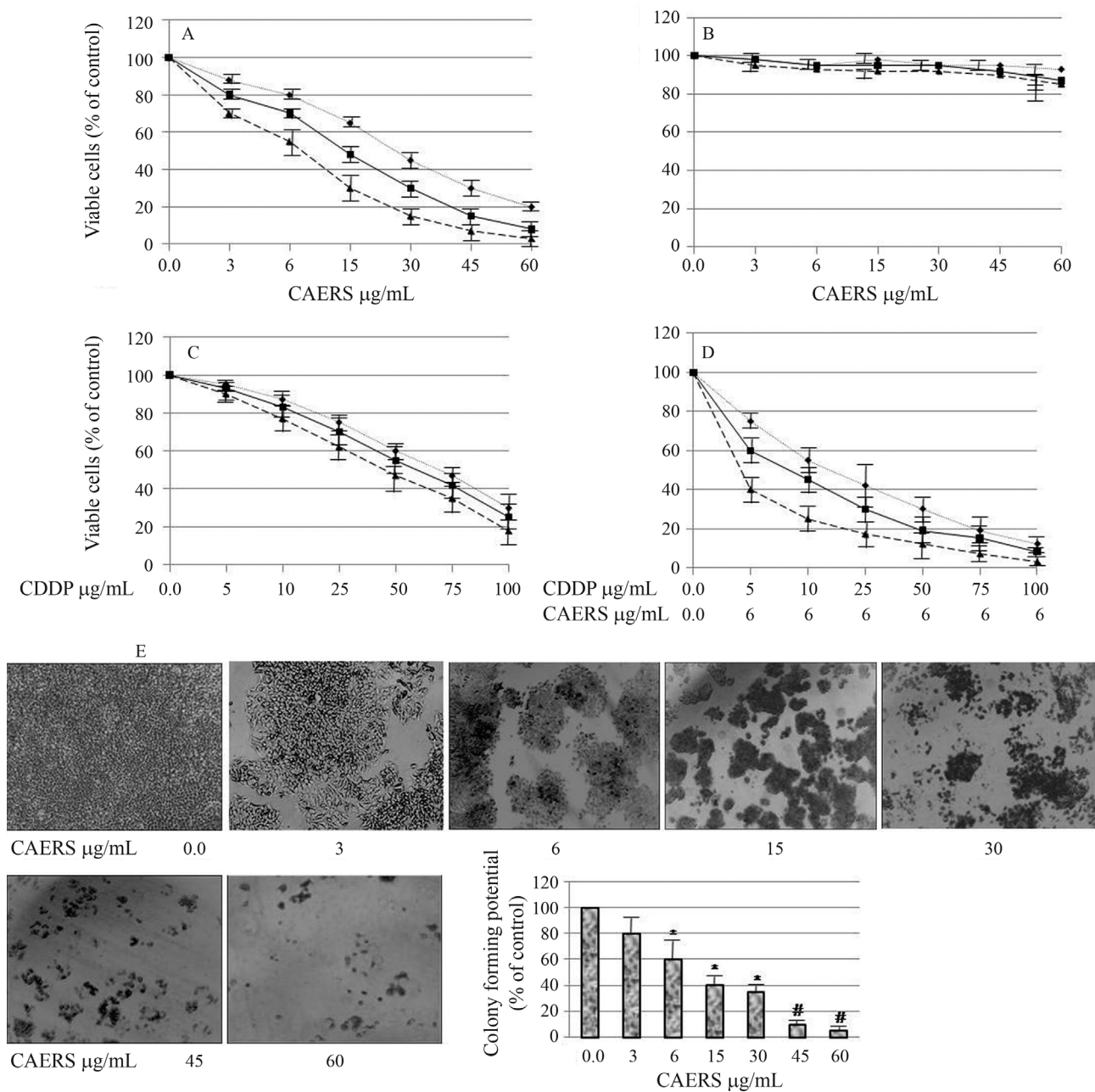


Figure 1 - CAERS suppresses cell growth and colony formation and sensitizes A549 cells to CDDP. A549 (A) and HF-5 (B) cells (50×10^3 cells/well) were seeded onto 24-well plates and treated with the indicated concentrations of CAERS alone for 24 h (dotted line), 48 h (solid line) and 72 h (dashed line). (B and C) A549 cells (50×10^3 cells/well) were seeded onto 24-well plates and treated with the indicated concentrations of CDDP alone (C) or CDDP + CAERS (D) for 24, 48 and 72 h. The inhibition of cell proliferation was assessed by the trypan blue dye exclusion assay. Cell viability was expressed as a percentage of control (no drug treatment) cell viability. The points are the mean \pm SD of at least three independent experiments. (E) Effects of CAERS on clonogenicity of A549 cells. Cells were seeded onto a 6-well plate at 1000 cells/well and treated with the indicated concentrations of CAERS as detailed in Materials and Methods. The colonies were counted under a dissection microscope. A survival of 100% corresponded to the number of colonies obtained with cells that were not treated with CAERS. The experiment was repeated three times and the colony forming potential of the cells at each concentration of CAERS is expressed as a percent of the control and is reported as the mean \pm SD. * $p < 0.05$ and # $p < 0.001$ compared to control cells (100%).

fragmentation ladders in samples from cells treated with all concentrations of CAERS. The comet assay was used to confirm DNA fragmentation after incubation with CAERS. This assay is a sensitive method for monitoring single strand (ss) DNA breaks at the single cell level (reviewed in

Tice *et al.*, 2000). When A549 cells were treated with 3, 6 and 15 μg of CAERS/mL for 24 h there was significant ssDNA damage, as indicated by the increased tail length in the CAERS-treated cells compared with the controls (data not shown). Thus, these two independent methods of as-

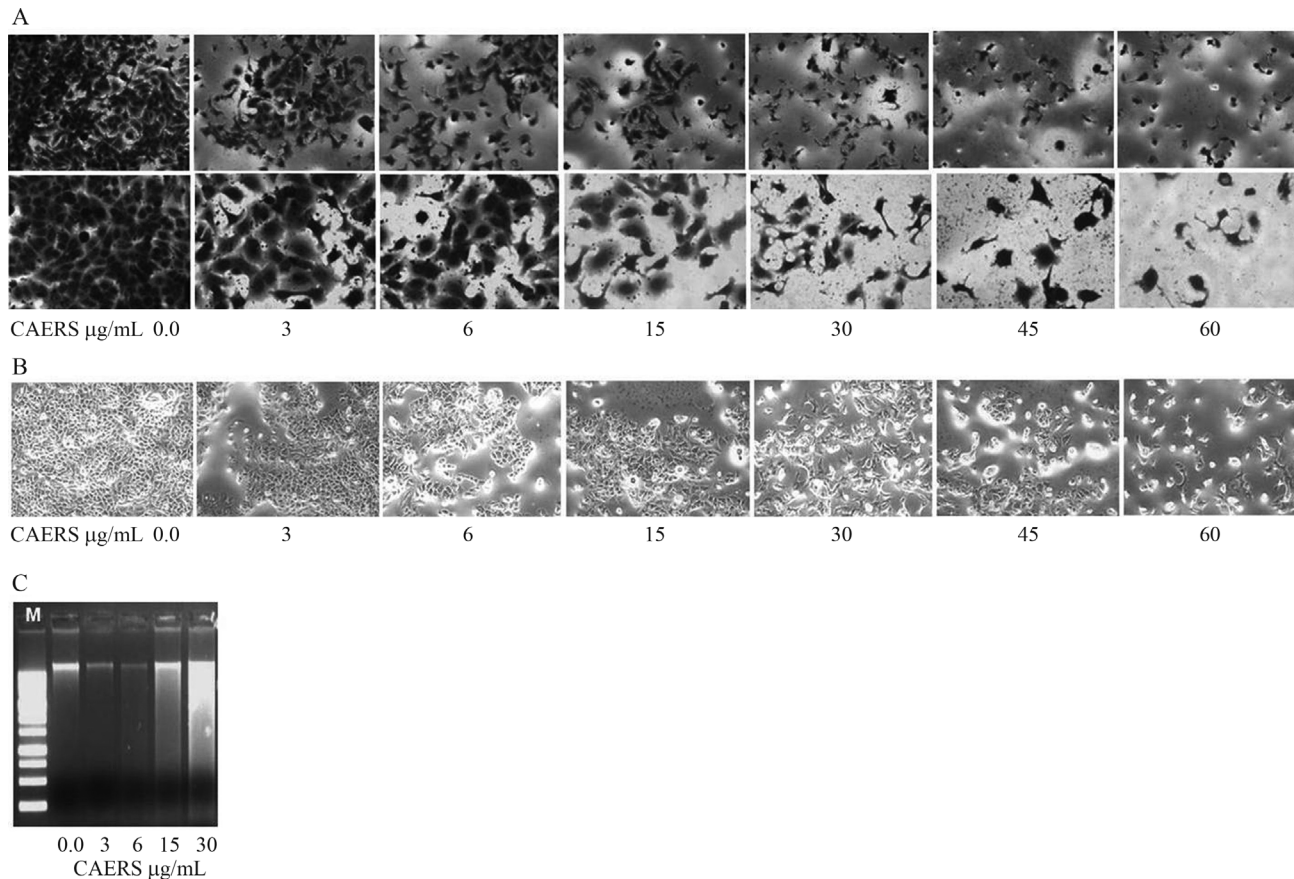


Figure 2 - CAERS induces apoptosis in A549 cells. (A) Morphological changes in A549 cells after incubation with CAERS. (A) The cells were treated with the indicated concentrations of CAERS for 24 h after which they were examined using an inverted microscope (first row: magnification - 200x; second row: magnification - 400x). (B) The cells were stained with ethidium bromide for 20 min and photographed with a fluorescence microscope using a red filter (magnification: 200x). (C) Fragmentation of DNA isolated from A549 cells incubated with CAERS. The cells were incubated with the indicated concentrations of CAERS for 24 h after which DNA fragmentation was assessed as described in Materials and Methods. Lane 'M' indicates the DNA marker ladder. The data are representative of three independent experiments with similar results.

sessing apoptosis provided similar results, suggesting that the anti-proliferative potential of CAERS was linked to its ability to induce apoptosis in A549 cells.

CAERS modulates the expression of apoptotic regulatory proteins and their activation in A549 cells

To elucidate how CAERS induced A549 cell apoptosis, changes in the expression of several key apoptosis-related proteins, including Bcl-2, Bax, *cyt c*, caspases 9 and 3, and poly(ADP-ribose) polymerase (PARP) were examined. Bcl-2 and Bax play critical roles in the regulation of *cyt c* release from the mitochondria into the cytosol; the released *cyt c* initiates caspase activation and apoptotic cell death. PARP is an early marker of chemotherapy-induced apoptosis (Reed, 2000; Cruchten and Den Broeck, 2002; Wong, 2011). A549 cells were treated with increasing concentrations of CAERS for 24 h after which the levels of Bcl-2, Bax, *cyt c*, caspases 9 and 3, and cleaved PARP were analyzed by western blotting. Figure 3 shows that incubation with CAERS significantly reduced the level of Bcl-2 and increased those of Bax (A) and cytosolic *cyt c* (B), as

well as the activation of caspases 9 and 3 and cleavage of PARP (C). These results demonstrate that CAERS induced A549 cell apoptosis at the molecular level, possibly by activating an intrinsic apoptotic pathway.

CAERS modulates the expression of antiapoptotic and cell cycle-regulating genes in A549 cells

To assess the significance of the expression patterns of antiapoptotic and cell cycle regulating genes in response to CAERS, A549 cells were treated with CAERS for 24 h and then possible alterations in the mRNA expression levels of various apoptosis-/cell cycle-related genes were analyzed by RT-PCR using gene-specific primers. The proteins examined included the anti-apoptotic proteins Bcl-2, Bcl-X_L and Mcl-1, a member of the IAP family of proteins, Survivin (Reed, 2000) and the cell cycle-regulating proteins cyclin D1 and c-Myc (Liao *et al.*, 2007). In addition, the expression levels of the proapoptotic BH3-only protein, Noxa (Reed, 2000; Wong, 2011), and a positive regulator of cell death, Bad (Danial, 2009), were also examined. Figure 4 shows that incubation with CAERS consistently mod-

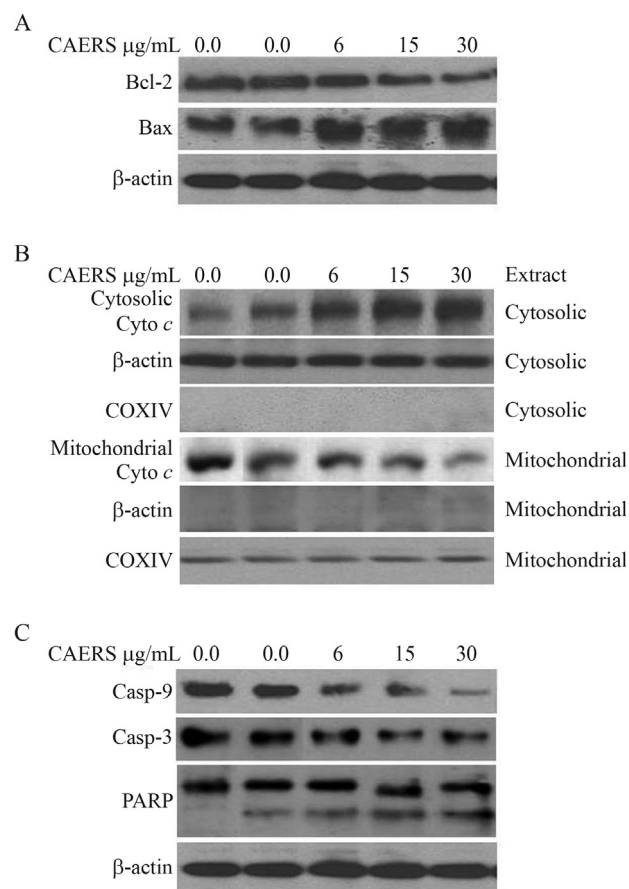


Figure 3 - CAERS modulates expression of apoptosis regulatory proteins and their activation in A549 cells. A549 cells (20×10^4 cells/well) were seeded onto 6-well plates and treated with the indicated concentrations of CAERS for 24 h. Subsequently, 20 μg of cell extract protein isolated from CAERS-treated A549 cells was subjected to SDS-PAGE in 10% polyacrylamide gels, transferred to PVDF membranes and immunoblotted with antibodies against the proteins of interest. Treatment with CAERS increased the Bax/Bcl-2 ratio (A), promoted mitochondrial cyto *c* release (B) and activated caspases 9 and 3 and PARP cleavage (C). The immunoblots of cyto *c* (B) were stripped and re-probed with anti-cytochrome oxidase IV (COXIV) to confirm the purity of the cytoplasmic fraction and equal loading of the mitochondrial fraction. In all experiments, the membranes were stripped and re-probed with anti- β -actin antibody as a loading control. The data are representative of three separate experiments.

ulated the expression of this panel of genes in a concentration-dependent manner. Thus, CAERS down-regulated the expression of Bcl-2, Bcl-X_L, Mcl-1, Survivin, c-Myc and cyclin D1, but enhanced that of Bad and Noxa. These data suggest that CAERS induced apoptosis in A549 cells by selectively inhibiting anti-apoptotic gene expression and up-regulating Bad and Noxa expression.

Discussion

Lung cancer is the most frequent cause of cancer-related deaths in men and women worldwide, with NSCLC accounting for 75-80% of lung cancers (Bilello *et al.*, 2002; Jemal *et al.*, 2008). CDDP-based combination treatments

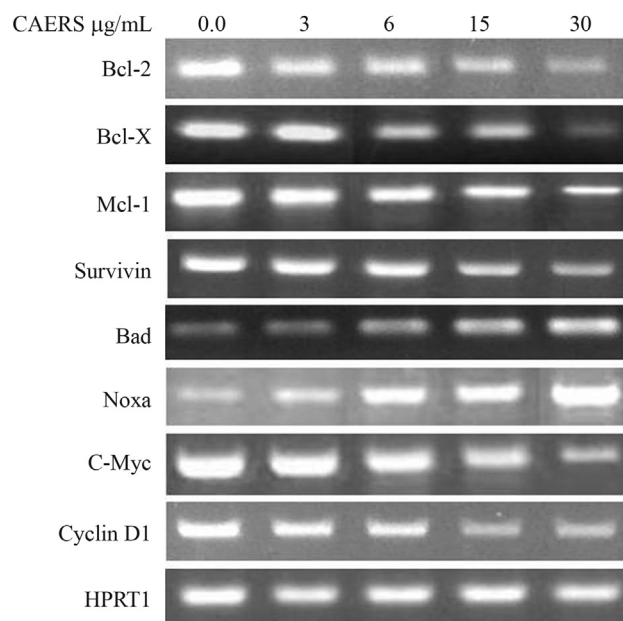


Figure 4 - CAERS modulates the expression of antiapoptotic and cell cycle-regulated gene products in A549 cells. A549 cells (20×10^4 cells/well) were seeded onto 6-well plates and treated with the indicated concentrations of CAERS for 24 h. Total RNA was then isolated, reverse transcribed and subjected to PCR with gene-specific primers. The PCR products of the genes were then subjected to electrophoresis in 1% agarose gels and visualized by staining with ethidium bromide. HPRT1 was used as the internal control. A typical result from three independent experiments is shown.

are the first-line chemotherapy for NSCLC (Johnson, 2000; Wang *et al.*, 2004). However, since current CDDP-based chemotherapy has a narrow therapeutic window between efficacy and unacceptable toxicity (Johnson, 2000; Ramalingam and Belani, 2004), there is an urgent need to develop low toxicity chemotherapeutics for NSCLC to reduce treatment failure. Recent work has shown that an aqueous extract of *R. stricta* successfully inhibited the proliferation and induced apoptotic cell death in breast cancer cell lines (Baeshen *et al.*, 2012). The present study was done to investigate whether CAERS, an aqueous extract of *R. stricta*, could inhibit A549 cell growth and enhance the cytotoxicity of CDDP; the mechanism of CAERS action was also examined.

The results described here show that CAERS significantly suppressed the growth of human A549 cells in a time- and concentration-dependent manner. Furthermore, cytotoxicity assays indicated that CDDP and CAERS acted synergistically to suppress the growth of A549 cells. In contrast, CAERS had no significant effect on non-malignant human fibroblasts (HF-5 cells), which raises the possibility that CAERS may selectively target lung cancer cells. However, the experiments described here cannot exclude the possibility of tissue-specific differences between human fibroblasts and lung cells. Additional cytotoxicity studies using a normal lung cell line and different lung cancer cell lines, as well as studies *in vivo* in nude mice are nec-

essary to prove that CAERS can inhibit tumor growth without major side effects. Further proof of the growth-suppressing potential of CAERS was provided by the colony formation assay which showed a significant reduction in the number and size of colonies in CAERS-treated cells compared with untreated control cells. Collectively, these data indicate that CAERS could be a promising candidate for restricting the growth of lung cancer cells.

The ability of CAERS to induce cell death in A549 cells was examined in detail in order to elucidate the mechanism by which CAERS markedly enhanced the CDDP-induced inhibition of NSCLC cell growth. The inhibitory activity of CAERS on cell growth was related to the induction of apoptosis since CAERS-treated A549 cells exhibited the typical morphological features of apoptosis (Cruchten and Den Broeck, 2002), such as a loss of cell viability, cell shrinkage, irregularity in cell shape, and cell detachment. In addition, staining with ethidium bromide revealed nuclear condensation, DNA fragmentation and perinuclear apoptotic bodies in CAERS-treated cells. Furthermore, DNA laddering and the comet assay (data not shown) demonstrated that CAERS induced apoptosis in A549 cells and augmented the apoptotic potential of CDDP in A549 cells. Since CDDP can trigger cancer cell death by inducing apoptosis (Sorenson *et al.*, 1990; Wang *et al.*, 2000; Del Bello *et al.*, 2001) it is possible that CAERS enhances the effect of CDDP on A549 cell proliferation partly by augmenting the ability of CDDP to initiate apoptosis.

Most anticancer agents induce DNA damage by interacting with DNA either directly or indirectly; the latter route may involve secondary stress-response signaling pathways that trigger apoptosis by activating the intrinsic (mitochondrial) apoptotic pathway and, in some cases, simultaneously activating the extrinsic (death receptor) pathway (Green, 2000). A variety of stimuli such as stress and drugs induce perturbation of the mitochondrial membrane and the release of mitochondrial proteins, such as *cyt c*, leading to the initiation of apoptotic cascades (Green, 2000). However, the mitochondria-dependent pathway for apoptosis is governed by Bcl-2 family proteins; the proteins Bax and Bcl-2 have opposing roles in initiating mitochondrial apoptotic events and modulating apoptosis (Wong, 2011). Bcl-2 is associated with the outer mitochondrial membrane where it plays a pivotal and overriding protective role by preserving mitochondrial structure and function, thereby preventing the onset of a mitochondrial permeability transition and inhibiting the release of *cyt c* to the cytosol. In contrast, Bax, a dominant-negative inhibitor of Bcl-2, induces a mitochondrial permeability transition and promotes apoptosis (Wong, 2011). Thus, when the level of Bax increases, the mitochondrial membrane becomes permeable to *cyt c*.

Upon its release into cytosol, *cyt c*, together with Apaf-1, activates caspase-9 that then activates caspase-3 (Reed, 2000; Wong, 2011). Active caspase-3 cleaves the

116 kDa PARP protein to an 89 kDa fragment, which is a characteristic marker of the execution of apoptosis (Reed, 2000; Cruchten and Den Broeck, 2002). The findings described here fit the above scenarios perfectly since CAERS significantly enhanced cytosolic *cyt c* that was associated with a concomitant decrease in the level of mitochondrial *cyt c*, suggesting that this treatment resulted in the release of *cyt c* from the mitochondrion into the cytosol. In addition, CAERS enhanced the activation of caspase-9, implying that CAERS mediated A549 cell growth via the caspase-9 apoptotic pathway, in agreement with previous studies showing that the caspase-9-dependent mitochondria-mediated apoptotic pathway is the primary pathway for antitumor agent-induced apoptosis (Khan *et al.*, 2007). Furthermore, western blotting demonstrated down-regulation of Bcl-2 expression and up-regulation of Bax expression.

Based on these observations, it is tempting to speculate that treatment with CAERS resulted in *cyt c* release and the activation of caspase-9 and caspase-3 partly via the disruption of a balance between Bax and Bcl-2. Subsequently, active caspase-3 mediated the cleavage of PARP to account for DNA fragmentation as well as other morphological and biochemical changes during apoptosis (Cruchten and Den Broeck, 2002). CAERS therefore appears to exert its anti-proliferative potentiality on A549 cell growth via the intrinsic apoptosis pathway involving Bcl-2 family proteins and caspase-9 activation. The down-regulation of Bcl-2 and up-regulation of Bax by CAERS corroborates published reports indicating that the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax protein at least partly determines the susceptibility of the cell to a death signal (Vander Heiden and Thompson, 1999) and can be used as a predictive marker for therapeutic response to therapy (Mackey *et al.*, 1998). The down-regulation of Bcl-2 by CAERS highlights the potential usefulness of CAERS as an ideal therapeutic agent for treating NSCLC.

RT-PCR showed that CAERS down-regulated the expression of genes known to have a pro-survival role, such as Bcl_{x_L}, Mcl-1 and Survivin (Reed, 2000). For example, Bcl_{x_L} and Mcl-1 suppress the release of *cyt c* from mitochondria and prevent apoptosis (Reed, 2000; Liu *et al.*, 2001) and overexpression of Mcl-1 delayed apoptosis induced by growth factor withdrawal and other cytotoxic agents (Liu *et al.*, 2001). Survivin is selectively expressed in most human neoplasms, including NSCLC, and is associated with a poor prognosis in patients with NSCLC (Zhang *et al.*, 2012); Survivin also confers tumor cell resistance to anticancer agents and ionizing radiation (Pennati *et al.*, 2007). Recent findings suggest that Survivin and Bcl-2 are up-regulated in NSCLC tissues and may act synergistically in the development, invasion and metastasis of NSCLC (Gao *et al.*, 2012). In addition, CAERS down-regulated the mRNA levels of c-Myc and cyclin D1. The c-Myc oncoprotein promotes cell cycling progression,

plays a vital role in most human cancers (Dang *et al.*, 2006) and cooperates with Mcl-1 in the development of NSCLC (Allen *et al.*, 2011). Similarly, cyclin D1 is a central player in the cell cycle and plays an essential role in the development of NSCLC (Ai *et al.*, 2012). Consequently, the down-regulation of Bcl-2, Bcl_{xL}, Mcl-1, Survivin, c-Myc and cyclin D1 by CAERS imply that this extract could effectively target the survival advantage provided by these effectors. However, it remains unclear whether the down-regulation of mRNA expression by CAERS was due to transcriptional down-regulation, mRNA stabilization or both. On the other hand, CAERS up-regulated the expression of genes involved in counteracting cell survival, such as Bad and Noxa. Down- and up-regulation of Mcl-1 and Noxa expression, respectively, by CAERS is important since studies of molecular targeted therapies have shown that ABT-737, a Bad mimetic, binds with high affinity to BCL-X_L, BCL-2 and BCL-w but not to Mcl-1 (Labi *et al.*, 2008). Hence, therapy with ABT-737 would be ineffective in cells expressing significant amounts of Mcl-1 (van Delft *et al.*, 2006; Huang and Sinicrope, 2008). This would be critical, particularly in tumor cells such as NSCLC that have a high expression of Mcl-1 (Luo *et al.*, 2011) since only Noxa, but not other BH3-only family members, appears to be crucial in fine-tuning cell death decisions by targeting the Mcl-1 for proteasomal degradation (Ploner *et al.*, 2009). Consequently, the down-regulation of Mcl-1 and up-regulation of Noxa by CAERS may tip the balance of events towards apoptosis in A549 cells.

In summary, the results described above have shown that a natural plant-derived antitumor agent (CAERS) inhibits growth and potentiates the anticancer activity of the cytotoxic drug CDDP in human A549 cells. CAERS inhibited A549 cell growth via apoptotic mechanisms. The induction of apoptosis by CAERS was mediated by regulation of the levels and activities of apoptosis regulatory proteins. Together, these findings provide a scientific basis for using a combination of CAERS and CDDP as a potentially effective chemotherapy regimen for treating patients with resistant lung cancer, although further preclinical investigations are warranted. The ability of CAERS to improve the antitumor effects of CDDP in A549 orthotopic xenografts is currently being studied.

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