The Roles of Endoplasmic Reticulum Stress and Ca^{2+} on Rhein-induced apoptosis in A-549 Human Lung Cancer cells

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Abstract. *Although rhein has been shown to induce apoptosis in several cancer cell lines, the mechanism of action of rhein-induced cell cycle arrest and apoptosis at the molecular level is not well known. In this study, the mechanism of rhein action on A-549 human lung cancer cells was investigated. Rhein induced G_0/G_1 arrest through inhibition of cyclin D3, Cdk4 and Cdk6. The efficacious induction of apoptosis was observed at 50 μM for 12 h and up to 72 h as examined by a flow cytometric method. Flow cytometric analysis demonstrated that rhein increased the levels of GADD153 and GRP78, both hallmarks of endoplasmic reticulum stress, promoted ROS and Ca^{2+} production, induced the loss of mitochondrial membrane potential ($\Delta\Psi_m$), promoted cytochrome c release from mitochondria, promoted capase-3 activation and led to apoptosis. Rhein also increased the levels of p53, p21 and Bax but reduced the level of Bcl-2. The Ca^{2+} chelator BAPTA was added to the cells before rhein treatment, thus blocking the Ca^{2+} production and inhibiting rhein-induced apoptosis in A-549 cells. Our data demonstrate that rhein induces apoptosis in A-549 cells via a Ca^{2+}-dependent mitochondrial pathway.*

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Key Words: Rhein, apoptosis, caspase-3, ROS, mitochondria membrane potential, cytochrome *c*.

Antitumor research was based on the ability of agents to induce cellular lesions through cytotoxic mechanisms for many past years, however, evidence suggested that some antitumor agents can induce apoptosis, which leads to the rapid elimination of tumor cells (1-3). Apoptosis is a well-regulated and organized death process and is a general mechanism for removal of unwanted cells in the development and homeostasis of multicellular organisms (4). The characters of apoptosis include membrane blebbing, nuclear and cytoplasmic shrinkage, DNA fragmentation, translocation of phosphatidylserine of the plasma membrane from the inner to the outer leaflet, and activation of a family of caspases (5, 6). It is well documented that caspase activation is considered to be a key hallmark of apoptosis. Apoptosis is also accompanied by a loss of mitochondrial membrane potential ($\Delta\Psi_m$), before induction of cytochrome *c* release and activation of caspase-3 (7-9).

Rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), an anthraquinone found especially in the root of rhubarb (*Rheum palmatum* L. or *R. tanguticum* Maxim), was used to treat chronic liver disease in China. Many studies *in vivo* showed that rhein inhibits the growth of tumor cells in rat liver (10), human glioma (11) and Ehrlich ascites tumor (12). Rhein inhibits superoxide anion production, chemotaxis and phagocytic activity of neutrophils, and macrophage migration and phagocytosis, and these effects are dose dependent (13). Rhein reduced the concentration of Ca^{2+} and inhibition of protein kinase C that led to blocking of *interleukin-12* mRNA transcription (14). Rhein generated nitric oxide *in vitro* and led to induced apoptosis in human

colonic adenocarcinoma cell monolayers (15) and the production of ROS that led to apoptosis in human promyelocytic leukemia cells (HL-60) (16). Our previous studies also showed that rhein induced apoptosis through the mitochondria-dependent pathway before leading to cytochrome *c* release and activation of caspase-3 in human cervical cancer Ca Ski cells (17).

Although rhein was demonstrated to induce apoptosis in human cancer cell lines, there is no available information to address rhein affecting A-549 human lung cancer cells and also no information to address the role of ER stress and Ca^{2+} on rhein-induced apoptosis. Therefore, in this study we focused on the molecular mechanism and the role of endoplasmic reticulum stress and Ca^{2+} on the induction of apoptosis by rhein in A-549 cells.

Materials and Methods

Chemicals and reagents. Rhein, RNase, trypan blue, propidium iodide (PI), Tris-HCl and Triton® X-100 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). BAPTA, potassium phosphates and dimethyl sulfoxide (DMSO) were obtained from Merck Co. (Darmstadt, Germany). RPMI-1640, penicillin-streptomycin, trypsin-EDTA, fetal bovine serum (FBS) and glutamine were obtained from Gibco BRL (Grand Island, NY, USA). Caspase-3 activity assay kit was bought from OncoImmunin, Inc (Gaithersburg, MD, USA).

Human lung carcinoma cell line (A-549). A-549 cell line was obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan, ROC). The cells were placed into 75 cm^3 tissue culture flasks and grown at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere in F12K medium supplemented with 10% FBS, 1% penicillin-streptomycin (10 ng/ml penicillin and 10 U/ml streptomycin) and 1% L-glutamine.

Cell viability of A-549 cells treated with or without rhein were examined and determined by flow cytometry. The A-549 cells were plated in 12-well plates at a density of 2×10^5 cells/well and grown for 24 hours. The rhein (0, 25, 50 and 100 μM) was added to the cells while only adding DMSO (solvent) for the control regimen and they were grown at 37˚C, in 5% CO_2 and 95% air for different periods of time. For determining cell viability, a flow cytometric assay was used as described elsewhere (18, 19).

Flow cytometric analysis of DNA content for cell cycle distribution and apoptosis of A-549 cells treated with different concentrations of rhein. Approximately 2×10^5 cells/well of A-549 cells in 12-well plates at concentrations of 0, 25, 50 and 100 μM of rhein were incubated in an incubator for different time periods. Cells were harvested by centrifugation, fixed gently (drop by drop) by adding 70% ethanol (in PBS) in 4˚C overnight, before being re-suspended in PBS containing 40 μg/ml PI and 0.1 mg/ml RNase and 0.1% Triton® X-100 in a dark room. After 30 minutes at 37˚C, the cells were analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) with an argon ion laser at 488 nm. The cell cycle distribution was determined and analyzed (18-20).

Caspase-3 activity determination of A-549 cells treated with or without rhein. Approximately 2×10^5 cells/well of A-549 cells in 12-well plates at concentrations of 0, 25, 50 and 100 μM of rhein were incubated in an incubator for 24 hours. Cells were harvested by centrifugation and the medium was removed. A 50 μl of 10 μM PhiPhilux substrate solution (a unique class of substrates for caspase-3) was then added to the cell pellet (1×10^5 cells per sample) without vortexing the cells. Cells were then incubated at 37˚C for 60 minutes. Cells were the washed once by adding 1 ml of ice-cold PBS and re-suspended in a fresh 1 ml. Cells were analyzed with flow cytometry (Becton-Dickinson) with an argon ion laser at 488 nm. Finally, the caspase-3 activity was determined and analyzed (21).

Inhibition of rhein-induced apoptosis by the caspase-3 inhibitor z-DEVD-fmk in A-549 cells. In order to examine whether or not caspase-3 activation was involved in apoptosis triggered by rhein, A-549 cells were pretreated with the caspase-3 inhibitor z-DEVD-fmk (20 μM) 3 h prior to treatment with 50 μM rhein. Apoptosis and caspase-3 activity were then determined by flow cytometry as described above (21).

Detection of reactive oxygen species (ROS) in A-549 cells after treatment with rhein by flow cytometry. The level of ROS of the A-549 cells was examined by flow cytometry (Becton Dickinson FACS Calibur), using 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma). Approximately 2×10^5 cells/well of A-549 cells in 12-well plates were incubated for 0, 15, 30, 60, 120, 240 and 480 minutes with 50 μM rhein to detect the changes in ROS. The cells were harvested and washed twice, re-suspended in 500 μl of DCFH-DA (10 μM) and incubated at 37˚C for 30 min before being analyzed by flow cytometry (20).

Detection of mitochondrial membrane potential ($\Delta\Psi_m$) in A-549 cells after treatment with rhein by flow cytometry. The level of cell $\Delta\Psi_m$ in A-549 was determined by flow cytometry (Becton Dickinson FACS Calibur) using $DiOC_6$ (4 μmol/l). Approximately 2×10^5 cells/well of A-549 cells in 12-well plates with 0, 25, 50 and 100 μM of rhein were incubated for 24 hours to detect the changes in $\Delta\Psi_m$. The cells were harvested and washed twice, re-suspended in 500 μl of $DiOC_6$ (4 μmol/l) and incubated at 37˚C for 30 min before being analyzed by flow cytometry (17).

Detection of Ca^{2+} concentrations in A-549 cells after treatment with rhein by flow cytometry. The level of Ca^{2+} of the A-549 cells was determined by flow cytometry (Becton Dickinson FACS Calibur), using Indo 1/AM (Calbiochem; La Jolla, CA, USA). Approximately 2×10^5 cells/well of A-549 cells in 12-well plates with 0, 25, 50 and 100 μM of rhein were incubated for 24 hours to detect the changes in Ca^{2+} concentration. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) and incubated at 37˚C for 30 min before being analyzed by flow cytometry (22).

Detection of Ca^{2+} concentrations and $\Delta\Psi_m$ levels in A-549 cells after pre-treatment with BAPTA. The level of Ca^{2+} and $\Delta\Psi_m$ of the A-549 cells were determined by flow cytometry (Becton Dickinson FACS Calibur) using Indo 1/AM (Calbiochem; La Jolla, CA). Approximately 2×10^5 cells/well of A-549 cells in 12-well plate were pre-treated with BAPTA, a Ca^{2+} chelator (10 μM), before adding 50 μM of rhein for incubation for 24 hours to

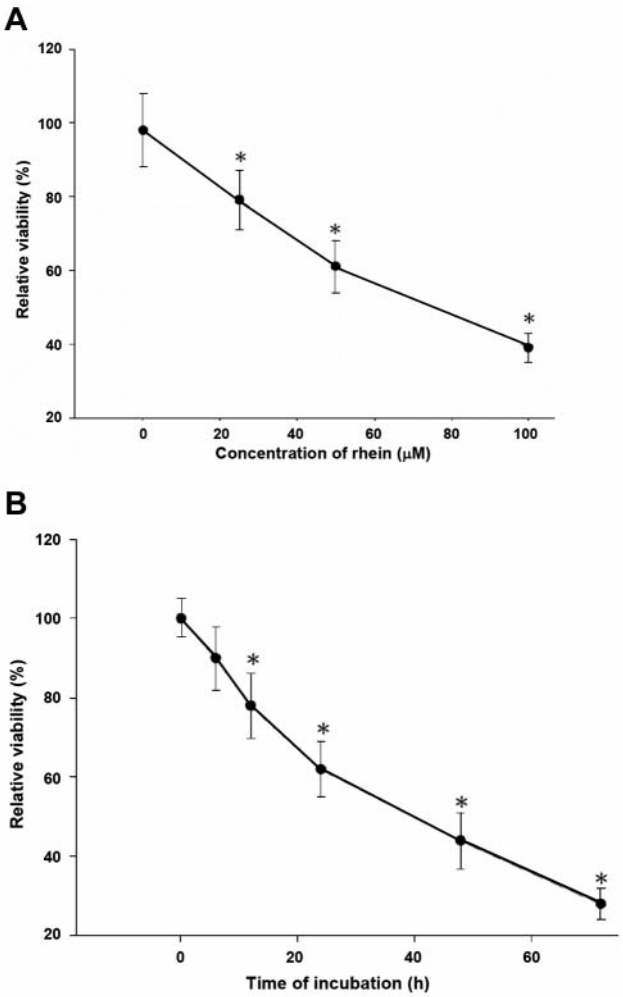

Figure 1. *The percentage of viable A-549 cells after rhein treatment. The A-549 cells (2×10^5 cells/well; 12 well plates) were plated in F12K + 10% FBS with different concentrations of rhein for 48 hours (A) or 50 μM rhein for 6, 12, 24, 48 and 72 hours (B). The cells were collected by centrifugation and the viable cells were determined by trypan blue exclusion and flow cytometry as described in Materials and Methods. Each point is mean±S.D. of three experiments. Significantly different from the control at *p<0.05.*

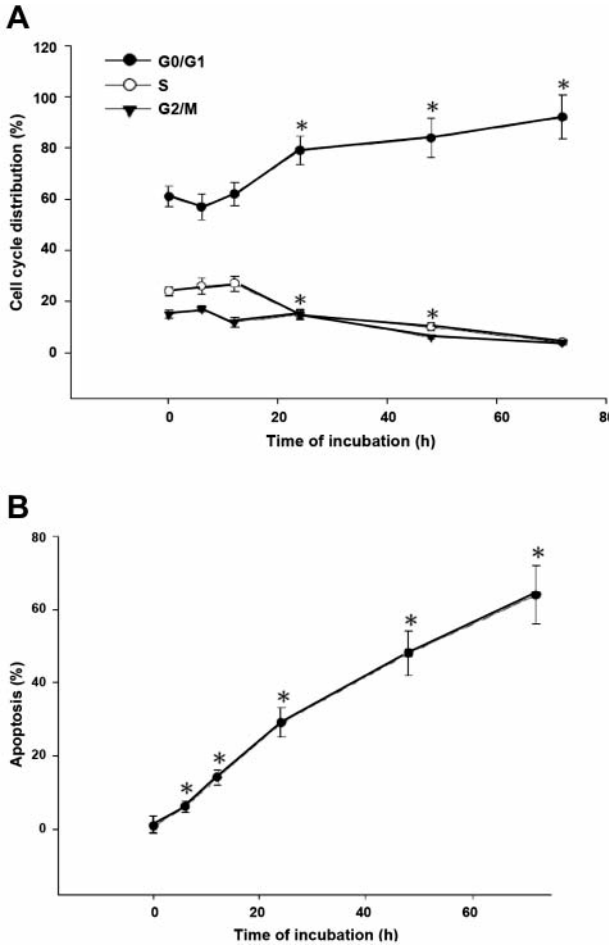

Figure 2. *Flow cytometric analysis of the effects of rhein on the A-549 cell cycle and apoptosis. The A-549 cells were exposure to different concentrations of rhein for 48 hours or 50 μM rhein for 6, 12, 24, 48 and 72 hours, and the cells were harvested and analyzed for cell cycle (A: the percent of cells in phase) and sub-G1 group (B: the percentage of cells in apoptosis) were analyzed by flow cytometry as described in Materials and Methods. Data represent mean±S.D. of three experiments. Significantly different from the control at *p<0.05.*

detect the changes in Ca^{2+} concentration and the levels of $\Delta\Psi_m$. The cells were harvested and washed twice, once for apoptosis analysis and the other for re-suspension in Indo 1/AM (3 μg/ml) and incubated at 37˚C for 40 min before being analyzed by flow cytometry (22).

Western blotting for examining the effect of rhein on Cdk4 and 6, cyclin E and Cdk2, p53, p21, Bax, Bcl-2 and cytochrome c of A-549 cells. The total proteins were collected from A-549 cells treated with or without various concentrations of rhein for 48 hours before p53 p21, Bax, Bcl-2, Cdk1, Cdk2, cyclin B1, D3 and cytochrome *c* (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were measured by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot as described elsewhere (18-20).

Confocal laser microscopy. A-549 cells (5×10^4 cells/well) plated on 4-well chamber slides were treated with or without 30 μM rhein for 24 hours then the cells were fixed in 4% formaldehyde in PBS for 15 min, permeabilized with 0.3% Triton®-X 100 in PBS for 1 h with blocking of non-specific binding sites using 2% boven serum albumin. Fixed cells were then incubated with antihuman GADD153 and GRP78 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) (1:100 dilution) overnight and then exposed to the secondary antibody (FITC-conjugated goat anti-mouse IgG at 1:100 dilution) (Santa Cruz Biotechnology Inc.), followed by DNA staining with PI. Photomicrographs were obtained using a Leica TCS SP2 Confocal Spectral Microscope (23)

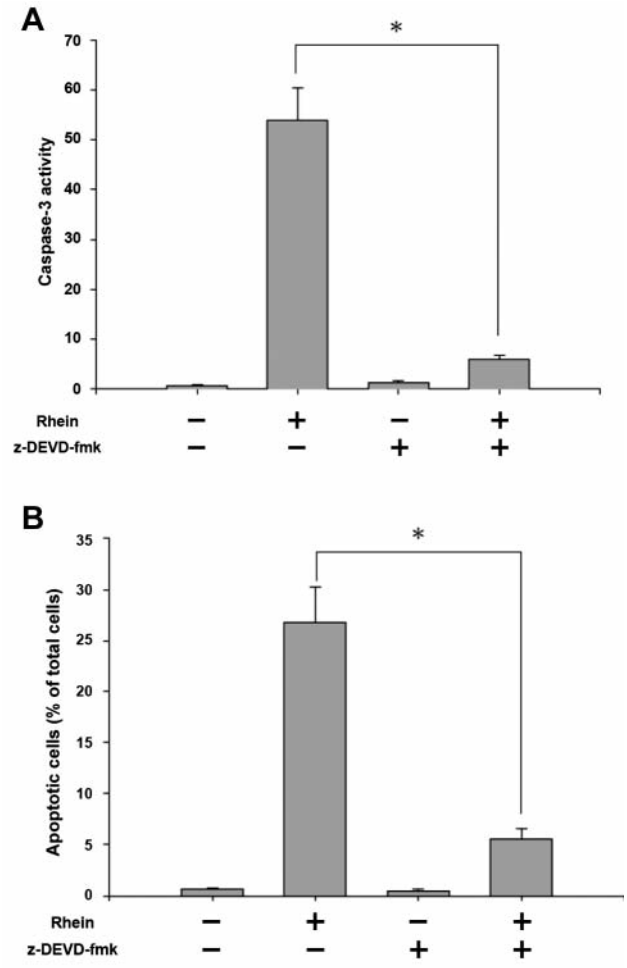

Figure 3. *Flow cytometric analysis of the effects of rhein on caspase-3 activity and apoptosis in A-549 cells. The A-549 cells were incubated with 50 μM rhein and/or with or without z-DEVD-fmk treatment for caspase-3 activity (A) and apoptosis determination (B) as described in Materials and Methods. Data represent mean±S.D. of three experiments. *p<0.05. Significantly different from the control at *p<0.05.*

Statistical analysis. Student's *t*-test was used to analyze the differences between the rhein treated and control groups.

Results

Effects of cell viability by rhein on A-549 cells. The results from PI staining experiments indicated increasing the dose of rhein and/or increasing the time of incubation with 50 μm rhein led to a significant decrease in the percentage of viable cells. These effects of rhein were dose- and time-dependent (Figure 1A and B).

Induction of cell cycle arrest and apoptosis by rhein on A-549 cells. We investigated the occurrence of cell cycle arrest and apoptosis of A-549 cells by treatment with rhein.

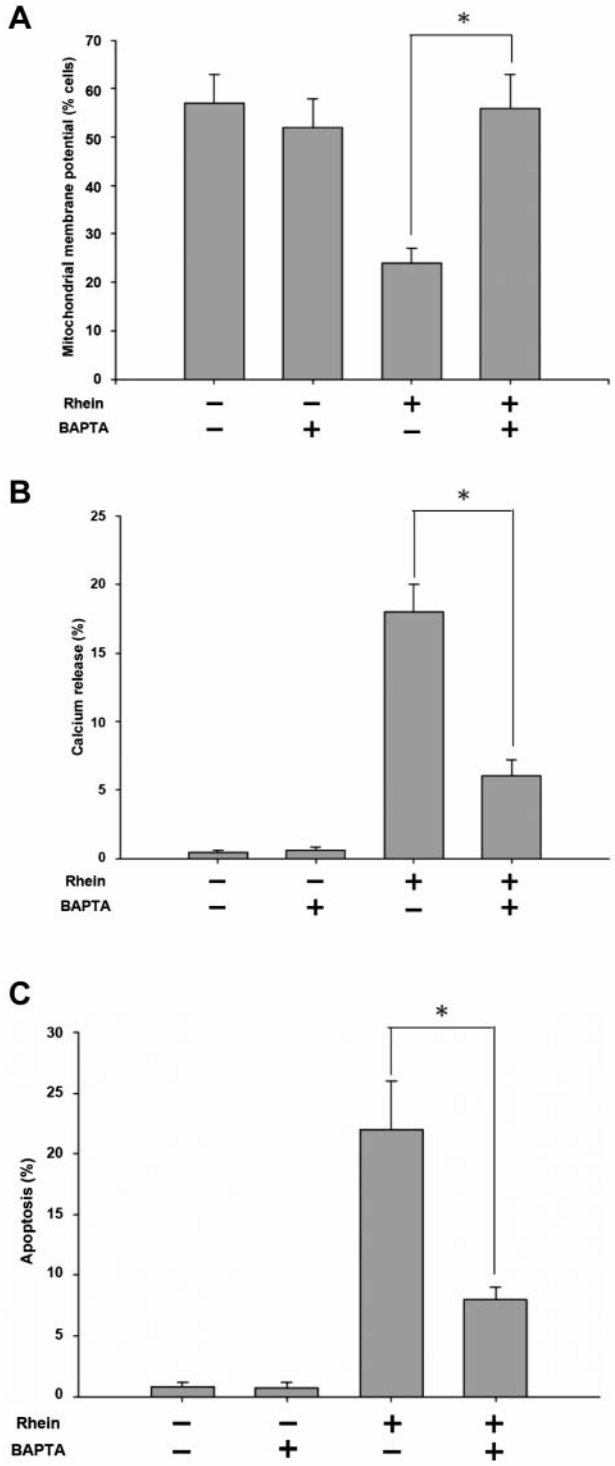

Figure 4. *Effects of calcium antagonist BAPTA (Ca^{2+} chelator) on the rhein effects on the levels of $\Delta\Psi_m$, Ca^{2+} and apoptosis in A-549 cells. The A-549 cells were pre-treated with BAPTA for 3 hours then treated with rhein and cells were harvested for $\Delta\Psi_m$ (A), Ca^{2+} (B) and apoptosis (C) determinations as described in Materials and Methods. Data represent mean±S.D. of three experiments. Significantly different from the control at *p<0.05.*

As shown in Figure 2A and B, rhein induced G_0/G_1 arrest in a concentration- and induction of apoptosis in a time-dependent manner. The percentage of cells in the G_0/G_1 phase and apoptosis increased significantly in A-549 cells by treatment with rhein. The percentage of apoptosis reached 58% with 50 μM rhein treatment for 48 hours.

Inhibition of rhein-induced caspase-3 activity and apoptosis by the caspase-3 inhibitor z-DEVD-fmk on A-549 cells. This was conducted in order to examine whether or not caspase-3 activation is involved in the apoptosis of cells triggered by rhein. The results indicate that rhein increased caspase-3 activity and apoptosis (Figure 3A and B). The A-549 cells were pretreated with the cell permeable broad-spectrum caspase inhibitor (z-DEVD-fmk) 3 hours prior to the treatment with rhein. After treatment with rhein and z-DEVD-fmk, inhibition of rhein-mediated caspase-3 activation in A-549 cells was accompanied by the marked attenuation of rhein-induced apoptotic cell death (Figure 3B).

Detection of reactive oxygen species (ROS) in A-549 cells after treatment with different concentrations of rhein by flow cytometry. The percentage of ROS was higher in the rhein-treated groups than in the control. The ROS production was maximal at 15 minutes after treatment with rhein before decreasing for up to 6 hours (Table I).

Effects of rhein on the levels of Ca^{2+} and mitochondrial membrane potential ($\Delta\Psi_m$) of A-549 cells. The percentage of Ca^{2+} production was significantly lower in the rhein-treated groups than in the control. Increasing the time of rhein treatment led to an increase in the Ca^{2+} production in A-549 cells (Table II). The $\Delta\Psi_m$ was significantly lower in the rhein-treated groups than in the control. Increasing the dose of rhein led to greater reduction of the $\Delta\Psi_m$ in A-549 cells (Table II). The effects of rhein on the levels of Ca^{2+} and $\Delta\Psi$m were dose dependent.

Effects of BAPTA on rhein-induced production of $\Delta\Psi_m$, Ca^{2+} and apoptosis of A-549 cells. The $\Delta\Psi_m$, Ca^{2+} and apoptosis production levels were significantly different between rhein and/or pretreated with BAPTA treated and control groups. Pre-treatment with BAPTA mitigated the $\Delta\Psi_m$ induced by rhein (Figure 4A) and reduced Ca^{2+} levels (Figure 4B) and apoptosis (Figure 4C) in A-549 cells.

Western blotting for examining the effect of rhein on Cdk1, Cdk4, Cdk6, Cdk2, cyclins D3 and E, p53, p21, Fas, FasL, bax, Bcl-2 and cytochrome c of A-549 cells. The results are shown in Figure 5A. Western blot indicated that the levels of Cdk4, Cdk6, Cyclin D3 and cyclin E were reduced, which may have led to G_2/M arrest. Rhein increased the expressions of p53, p21, Bax and cytochrome *c* but reduced the expression of Bcl-2, which may have led to apoptosis in these cells.

Table I. *Flow cytometric analysis of reactive oxygen species in A-549 human lung cancer cells with or without rhein treatment*.

Time of incubation (min)	Percentage of cells stained by DCFH-DA (% control)
0	2.4±0.6
15	23.4±3.8*
30	18.2±2.1*
60	14.6±1.6*
120	11.1±1.2*
240	5.9±1.0*
480	3.7±0.9*

Values are mean±S.D. n=3. The A-549 cells (5×10^5 cells/ml) were treated with 50 μM rhein. The zero concentration was defined as control. The percentage of cells that were stained by DCFH-DA dye were determined by flow cytometry as described in the Materials and Methods section. *Difference between rhein and control at $p<0.05$.

Table II. *Flow cytometric analysis of Ca^{2+} concentration and mitochondrial membrane potential in A-549 human lung cancer cells with or without rhein treatment*.

Rhein (μM)	Percentage of cells stained by	
	Indo 1/AM (% control)	$DiOC_6$
0	1.2±0.3	86.8±8.2
25	19.6±1.2*	61.8±6.1*
50	44.8±2.8*	48.1±2.8*
100	63.6±4.2*	16.4± 2.4*

Values are mean±S.D., n=3. The A-549 cells (5×10^5 cells/ml) were treated with various concentrations of rhein. The zero concentration was defined as control. The percentage of cells that were stained by Indo 1/AM and/or $DiOC_6$ dye were determined by flow cytometry as described in the Materials and Methods section. *Difference between rhein and control at $p<0.05$.

Confocal laser microscopy for examining the levels of GADD153 and GRP78. As illustrated in Figure 6A, rhein-treated cells reacted with GADD153 antibodies and PI staining results showed that rhein treatment for 24 h increased the levels of GADD153, which was translocated to nuclei. The results from Figure 6B also showed that rhein treatment for 24 h increased the levels of GRP78, which was translocated to nuclei.

Discussion

Many anthraquinones have been reported to possess tumor cell inhibitory effects through a variety of mechanisms, including induction of apoptosis (24, 25), intercalation and binding with cellular DNA (26), redox-cycling radical

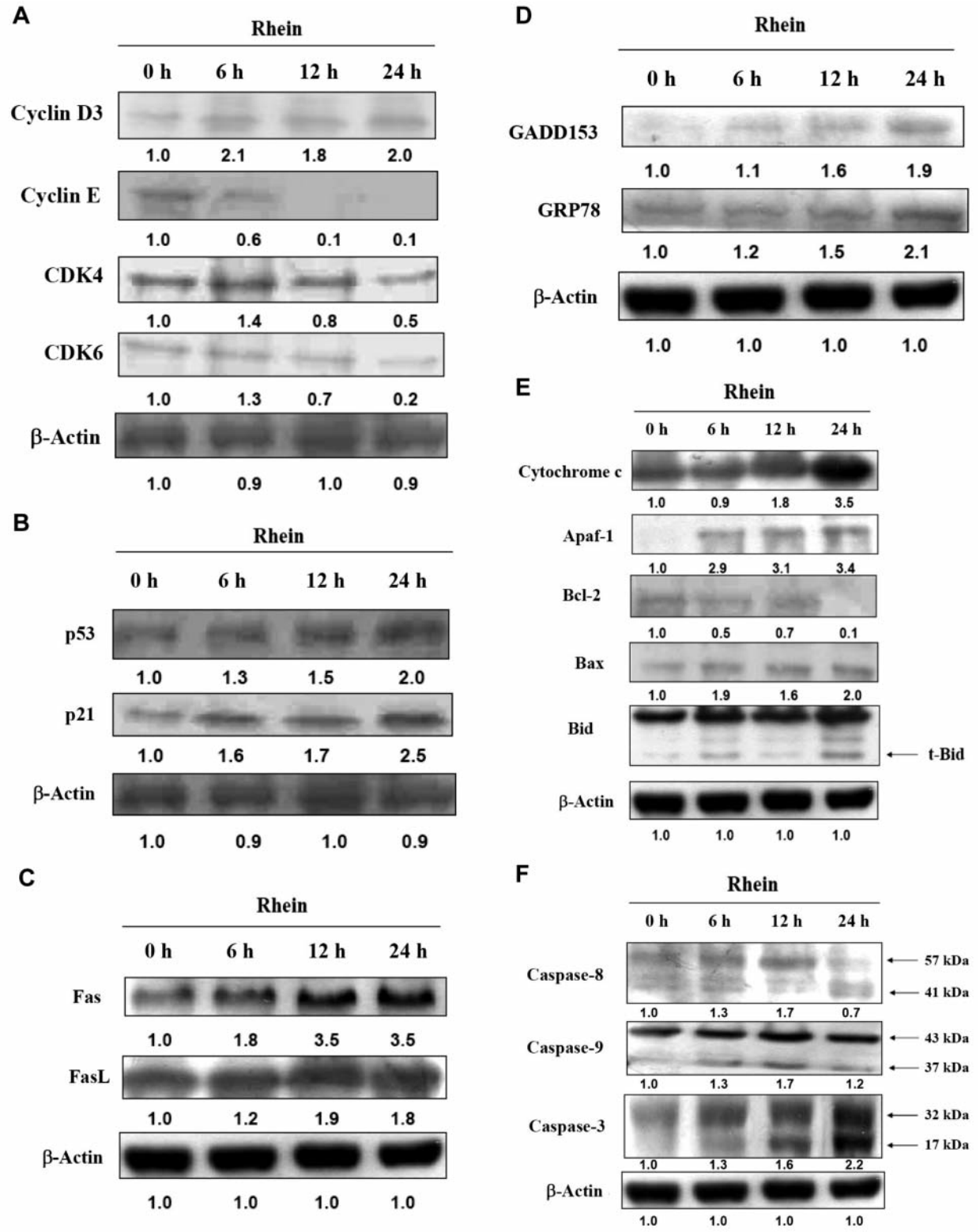

Figure 5. *Representative Western blots showing changes in the levels of cyclin D3 and E, and Cdk4 and 6 (A), p53 and p21 (B), Fas and FasL (C), GADD153 and GRP78 (D), cytochrome c, Apaf-1, Bcl-2, Bax and Bid (E) and caspases 8, 9 and 3 (F) in A-549 cells after treatment with rhein. The A-549 cells (5×10^6/ml) were treated with 50 μM rhein for 6, 12, and 24, then cytosolic fraction and total protein were prepared as described in Materials and Methods followed by evaluation of the levels of associated proteins by Western blotting as described in Materials and Methods.*

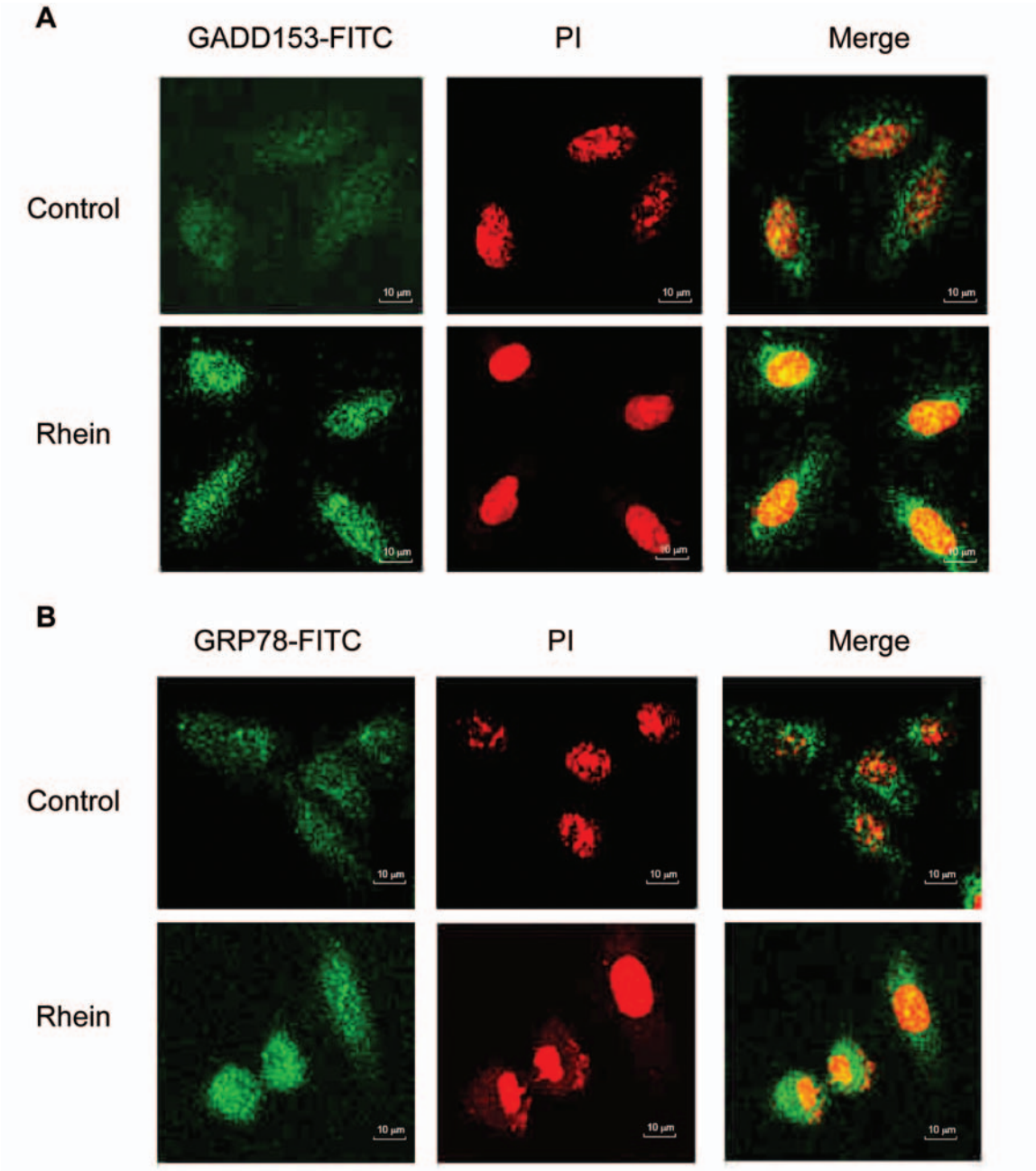

Figure 6. *Effects of rhein on GADD1153 and GRP78 nuclear translocation in A549 cells. A-549 cells (5×10^5 cells/well; 12-well plates) were incubated with or without rhein (50 μM) for 24 hours. The cells were fixed and stained with primary antibodies to GADD1153 (A) (Control: GADD153-FITC, MitoTracker and GADD153/MitoTracker; rhein: GADD153-FITC, MitoTracker and GADD153/MitoTracker) and GRP78 (B) (Control: GRP78-FITC, MitoTracker and GRP78/MitoTracker; rhein: GRP78-FITC, MitoTracker and GRP78/MitoTracker). FITC-labeled secondary antibodies were then used (green fluorescence) and the proteins were detected by a confocal laser microscopic system. The nuclei were stained by MitoTracker (red fluorescence). Areas of co-localization between GADD153 and/or GRP78 expressions and nuclei in the merged panels are yellow. Scale bar, 10 μm.*

formation (27, 28) and inhibition of topoisomerase (29). A comparison of the results obtained from viability assays suggest that the antitumor activity of rhein was through the induction of apoptosis in A-549 cells. Rhein is an anthraquinone existing in rhubarb which inhibits the growth of tumor cells, which may be associated with apoptotic cell death. In this study, we demonstrated that rhein reduced the percentage of viable cells and induced apoptosis in A-549 cells in dose- and time-dependent manners. Comparing the inhibition of viable cells (A-549) (IC_{50} is 45 μM) to other

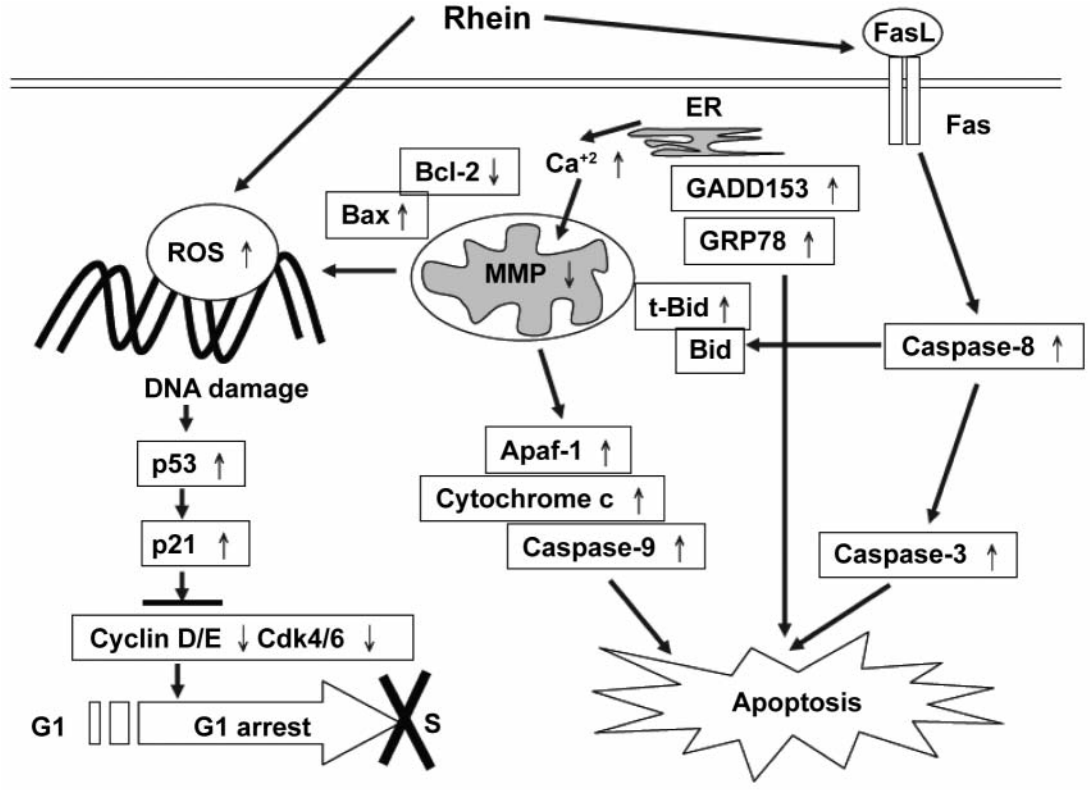

Figure 7. *Proposed model of rhein mechanism of action for G_0/G_1 arrest and apoptosis in A-549 cells. Rhein reduces Cdk4 and 6, cyclin E and D3, leading to G_0/G_1 arrest. Rhein induced p53 and p21 expression and increased the production of ROS and Ca^{2+} production and reduced $\Delta\Psi_m$ levels, leading to caspase-3 activity causing apoptosis in A-549 cells.*

cells from other investigators, IC_{50} values of rhein for KB, hepatoma BEL-7402 and mammary carcinoma MCF-7 cells were 11.5 μg/ml, 14.0 μg/ml and 18.4 μg/ml, respectively (30) and 25 μM for Ca Ski cells (17). Apparently, IC_{50} values of rhein are dependent on cell lines.

Dietary antioxidants may play a role in potentiating the effects of certain antitumor agents including anthraquinone derivatives (31). Therefore, we also examined the ROS in A-549 cells after treatment with rhein and results showed that rhein quickly induced ROS production, but 8 hours later levels returned to normal levels. We also added vitamin C with rhein to the cells and saw a significant increase of cell viability (data not shown). This is in agreement with other reports that co-treatment of breast cancer cells with both anthraquinones and vitamins C and E generally resulted in a significant increase in cell viability. Similar effects have been found when cancer cells were treated with paclitaxel and carboplatin and an antioxidant vitamin mixture containing vitamins C and E and β-carotene (32). These data indicate that anthraquinones might play various redox reaction roles among different cancer cell lines.

Incubation of A-549 cells with rhein led to G_0/G_1 arrest and apoptosis (sub-G1 group), which appeared to be mediated by a mitochondria-dependent and caspase-3 activation pathway in a dose- and time-dependent fashion. Other investigators showed that rhein induced apoptosis in human colonic adenocarcinoma cell monolayer (15) and human promyelocytic leukemia cells (HL-60) (16) and our previous studies also had similar results in Ca Ski cells (17).

The root of rhubarb has been used at an average daily dosage of 2-20 g of powered root for stomach cancer, leukemia and liver cancer in the Chinese population (15, 33). Anthraquinone comprise 3-12% of rhubarb root, 60-80% of which include rhein, emodin, and aloe-emodin (equivalent of approximate 400 μM in blood concentration). Although many experiments demonstrated that emodin and aloe-emodin induced cell cycle arrest and apoptosis in human and animal cancer cell lines, there are no studies that show rhein affecting the A-549 human lung cancer cell line. In the present study, 50 μM rhein induced apoptosis at 12, 24, 48 and 72 hours in A-549 cells in a dose- and time-dependent manner. This concentration can be obtained from the daily therapeutic dosage of rhubarb root (25-100 μM). The induction of apoptosis and cell cycle arrest by rhein in animal model *in vivo* for lung cancer needs to be investigated.

Our data showed that rhein induced G_0/G_1 phase arrest in A-549 cells and this is in agreement with other investigators who demonstrated that rhein blocked cell cycle progression in the G1-phase of human hepatoblastoma G_2 (Hep G_2) cell line through the significantly increased expressions of p53 and p21/WAF1 protein (34). However, the further check point enzymes that may or may not be affected by rhein are not known. Our previous studies showed that rhein induced G_0/G_1 arrest in human cervical Ca Ski cell through a caspase-dependent and mitochondria-dependent pathway (17). Currently our data showed that rhein induced the expression of p53 and p21 and inhibited the expressions of Cdk2, 4 and 6 and cyclin E that led to G_0/G_1 arrest in A-549 cells.

It is well known that caspases play critical roles in the initiation of apoptosis. Two major apoptosis pathways, caspase-dependent and -independent, are well established. Caspases can be grouped into apoptotic initiators, such as caspase-8, and apoptotic effectors, such as caspase-3, based on their substrate specificities and target proteins (35). We showed rhein induced activation of caspase-3, promoted Bax expression and reduced Bcl-2 expression in A-549 cells. We also showed that adding the caspase-3 inhibitor (z-DEVD-fmk), thus inhibiting rhein-induced caspase-3 activity, led to a decrease of apoptosis in these cells.

The mitochondrion plays an important role in the regulation of apoptosis *via* mitochondria-dependent and -independent pathways (36, 37). The characters of mitochondrial dysfunctions are the loss of $\Delta\Psi_m$, permeability transition and release of cytochrome *c* from the mitochondrion into the cytosol, and these are associated with apoptosis (38). In these studies, rhein induced the rapid loss of $\Delta\Psi_m$, the release of cytochrome *c* and promoted capase-3 activity in A-549 cells. Western blot also showed that rhein increased Bax protein which is a pro-apoptotic protein associated with apoptosis. Our data showed that rhein also reduced Bcl-2 protein which is an antiapoptotic protein. Caspase-8 can be activated either by cytokines or chemicals that in turn induce Bid cleavage. Cleaved Bid causes cytochrome *c* efflux from mitochondria leading to caspase-3 activation and PARP cleavage, which in turn results in apoptosis (39, 40). Our data also showed that rhein induced Ca^{2+} production and pre-treatment with a Ca^{2+} chelator (BAPTA) led to a decrease in the levels of Ca^{+2} and $\Delta\Psi_m$ in A-549 cells. After pre-treating A-549 cells with BAPTA for 3 hours and adding rhein to the cells, the levels of $\Delta\Psi_m$ and caspase-3 activation decreased, and the percentage of apoptosis also decreased. Apparently, Ca^{2+} plays an important role in rhein-induced apoptosis in A-549 cells.

A number of chemopreventive agents had been shown to induce apoptosis in part with the generation of ROS and the disruption of redox homeostasis (38, 41). Our data showed that rhein induced ROS production up to 15 minutes treatment, before decreasing so that by six hours, there was only ~20% left. By 8 hours' treatment, the ROS level was close to that of the control group. The ROS production may lead to ER stress and DNA damage causing p53 levels to increase in A-549 cells. We used confocal microscopy to show rhein increased the levels of GADD153 and GRP78, a hallmark of ER stress. When the antioxidant catalase was added to the rhein-treated cells, the results showed lower ROS production and also lower rhein-induced apoptosis (data not shown).

In conclusion, this is the first report that rhein induced G_0/G_1-phase arrest and induced apoptosis in A549 human lung cancer cells. The proposed flow chart of rhein-induced G_0/G_1 arrest and apoptosis is shown in Figure 7. Rhein appears to induce G_0/G_1 arrest by the inhibition of Cdk2, 4 and 6 and cyclin E, but promotes the expressions of p53 and p21. It also induces apoptosis through increasing the level of Bax and decreasing the level of Bcl-2, before decreasing the levels of $\Delta\Psi_m$. Caspase-3 activation then occurs and ROS and Ca^{2+} levels are also involved in this process in these cells. These findings may offer some information regarding the probable therapeutic function of rhein in human lung cancer.

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