

Growth inhibition and apoptosis-inducing effect on human cancer cells by RCE-4, a spirostanol saponin derivative from natural medicines

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Abstract. *Reineckia carnea* has been used to treat several diseases in folk remedies. RCE-4 has been isolated from several plants of the family Liliaceae, but its biological activity has not yet been reported. In the present study, we found that RCE-4 exhibited potent cytotoxicity to the tested human cancer cell lines, and the CaSki cell line was the most sensitive with an IC₅₀ of 3.37 μ M. Thus, we presented the apoptosis-inducing effect of RCE-4 on CaSki cervical cancer cells and investigated the relevant mechanisms. Based on observations using transmission electron microscopy, RCE-4-treated cells manifested nuclear shrinkage, condensation and fragmentation. Annexin V/PI dual staining flow cytometry assay further confirmed that RCE-4 caused a dose-dependent early apoptotic effect. Prior to these events, RCE-4 triggered a rapid decrease of the mitochondrial membrane potential and caused the release of cytochrome *c* from the mitochondria into the cytoplasm. RCE-4 increased the expression of Bax and decreased the expression of Bcl-2, thus augmenting the Bax/Bcl-2 ratio. These findings suggest that RCE-4 induces mitochondrial-mediated apoptosis in CaSki cells and has the potential to be developed as an anticancer agent.

Introduction

The constant increase in cancer incidence and the failure of conventional chemotherapy to protect against advanced cancer

warrants the development of novel agents to treat and prevent the malignancy. The search for successful anticancer agents began decades ago and is ongoing (1). For many years, the cytotoxic actions of the chemotherapeutic drugs were ascribed solely to their ability to induce genotoxic death. There is evidence that insufficient apoptosis has been associated with the development and progression of tumors (2). There is also accumulating evidence that many agents exert their cytotoxic effects mainly by inducing apoptosis in tumor cells (3,4). Currently, induction of apoptosis has become a useful marker for screening compounds for subsequent development as possible anticancer agents (5-7).

Natural medicine provides a rich pool of novel and efficacious agents for cancer prevention and treatment; previous research has resulted in the identification of several bioactive components from natural products such as resveratrol, curcumin, isothiocyanates, quercetin and polyphenols that selectively inhibit the growth of malignant cells *in vitro* by inducing apoptosis (8,9), and which have been used as cancer chemopreventive agents (10). Therefore, intensive efforts have been made to identify new bioactive compounds from natural products, through isolation of apoptosis-inducing agents and elucidation of the apoptosis mechanisms.

Reineckia carnea, also known as 'guanyin cao', one of the most popular traditional herbs in China, has been used to prevent cough, eliminate phlegm, as well as to treat rheumatism disease and hepatitis, for at least one thousand years (11,12). Previous studies showed that many bioactivity components including spirostanol sapogenin and spirostanol saponins were found in *Reineckia carnea* (13-15). It remains unclear whether *Reineckia carnea* contains any active chemical components with cytotoxic effects on cancer cells. RCE-4, a spirostanol saponin, was first isolated from *Reineckia carnea* ethyl acetate fraction, although this compound was firstly isolated from *Rohdea japonica* by Miyahara (16). However, it is still uncertain whether this kind of saponin has cytotoxic effects on cancer cells. Therefore, in this study we evaluated the cytotoxicity of RCE-4 on different cancer cell lines and further elucidated one of the possible mechanisms underlying its cytotoxic action.

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Materials and methods

Reagent and antibodies. RCE-4 (Fig. 1A) was isolated from the whole plant of *Reineckia carnea* and purified at the Hubei Key Laboratory of Natural Products Research and Development (China Three Gorges University); it was dissolved in DMSO at a stock concentration of 10 mM, and diluted to the indicated concentration with RPMI-1640 medium. Antibodies against Bax, Bcl-2, cytochrome *c* and HRP-labeled secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Annexin V-FITC/PI apoptosis assay kit, Cell Cycle assay kit and JC-1 mitochondrial membrane potential ($\Delta\psi_m$) assay kit were obtained from KeyGen Biotech Co., Ltd. (Nanjing, China). The NE-PER nuclear and cytoplasmic extraction kit were obtained from Thermo Scientific Pierce.

Cell lines. The cancer cell lines CaSki, HT-29, CNE-2 and the normal cell lines Marc-145 and MDCK, were obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Science, Shanghai, China. All cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 25 mM HEPES buffer, 2 mmol/l L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cultures were incubated in a humidified atmosphere of 5% CO₂ at 37°C.

Cell viability assay. Cells (1x10⁴/well) were seeded in supplemented culture medium (100 μ l/well) in a 96-well plate and incubated for 24 h. Then the medium was replaced with a drug-containing medium, and the cells were further incubated for 48 h. All experiments were run in parallel with controls (0.1% DMSO) and the cell viabilities were evaluated by MTT assays. The absorbance of formazan formed was measured at 570 nm by a microplate reader. Each experiment was repeated at least 3 times.

Transmission electron microscopy. CaSki cells treated with DMSO or RCE-4 were collected by trypsinization, washed twice with PBS, and then fixed with 0.5 ml of ice-cold glutaraldehyde (2.5% in 0.1 cacodylate buffer, pH 7.4) at 4°C overnight. The subsequent steps were performed according to standard procedures, including fixing, incubation, rinsing, gradient dehydration, embedding and ultrathin sections. Ultrathin sections were doubly stained with uranyl acetate and lead citrate and analyzed by transmission electron microscopy (Hitachi H-7500).

Annexin V-FITC/PI cytometric analysis. Early apoptosis was assessed by detecting surface exposure of phosphatidylserine (PS) in cells using an Annexin V-FITC/PI kit. Briefly, CaSki cells (2x10⁵) were seeded into a 100-ml culture flask and incubated for 12 h. Then, cells were treated with RCE-4 of 2.5, 5 and 10 μ M for 24 h. The cells (both adherent and floating cells) were collected and treated according to the manufacturer's instructions. Finally, the cells were analyzed with FITC/PI double-staining using a flow cytometer (Beckman Coulter, USA) with the single beam at 488 nm excitation.

Cell cycle analysis. To investigate the effect of RCE-4 on the cell cycle distribution, CaSki cells (2x10⁶) were subcultured into culture flasks and treated with 2.5, 5 and 10 μ M of RCE-4 for 24 h. The cells were resuspended in 2 ml of 70% ice-cold

ethanol solution and fixed at 4°C overnight. After washing with PBS, the pellets were resuspended in 100 mg/ml PI solution containing 100 mg/ml RNase, and then incubated at 37°C for at least 30 min. The cellular DNA content was then detected by flow cytometer.

Analysis of mitochondrial membrane potential (JC-1 staining). The change in mitochondrial membrane potential ($\Delta\psi_m$) was measured using a JC-1 fluorescent probe assay kit, according to the kit's instructions. Briefly, following RCE-4 treatment of 2.5, 5 and 10 μ M for 24 h, the cells were washed with PBS and incubated for 30 min with JC-1 at 37°C. After washing in PBS twice, the cells were subjected to two-color analysis by a flow cytometer.

Real-time quantitative PCR (qPCR). To examine the role of Bcl-2 family members in RCE-4-induced apoptosis, we measured the gene expression of Bax and Bcl-2 using qPCR. CaSki cells were treated with 10 μ M of RCE-4 for 0, 6, 12 and 24 h in 6-well plates. Total cellular RNA was isolated using the TRIzol Reagent. The qPCR reaction was carried out on the LightCycler 2.0 instrument (software v4.0; Roche Applied Science) using the double-stranded DNA dye SYBR-Green I strategy. The oligonucleotide sequences of the PCR primers used herein were designed based on the human mRNA encoding the respective genes. Quantification was based on threshold cycle (Ct) difference performed according to the $\Delta\Delta C_t$ method, using the following equation: expression ratio = $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_t \text{ target} - C_t \text{ reference}) \text{ time } x - (C_t \text{ target} - C_t \text{ reference}) \text{ time } 0$. The expression level of each target gene was normalized to that of glyceraldehyde-3 phosphate dehydrogenase (GAPDH), which fulfils the requirements for validation of reference genes.

Western blot analysis. CaSki cells (6x10⁶) were treated with 10 μ M RCE-4 for 0, 6, 12 and 24 h. The cells were harvested and washed with cold PBS twice. Cell pellets were lysed in 40 μ l lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail) for 20 min on ice. The lysis solution was centrifuged at 25,000 x g for 10 min at 4°C and protein contents in the supernatant were measured using a Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA). The protein expression of Bax, Bcl-2, and cytochrome *c* were detected by western blot analysis. Briefly, equal amounts of protein were electrophoresed on 12% SDS acrylamide. Following electrophoresis, the proteins were transferred from the gel to a PVDF membrane. Non-specific binding was blocked with 5% skim milk in TBST buffer (5 mM Tris-HCl, pH 7.6, 136 mM NaCl, 0.05% Tween-20) at 4°C overnight. Blots were incubated at 37°C for 4 h each with primary and secondary antibody conjugated with peroxidase (HRP)-labeled anti-rabbit/mouse IgG. Blots were developed with ECL western blotting detection reagents (Multi Sciences). Experimental values were normalized to β -actin reactivity.

Statistical analysis. Statistical analyses were performed using Prism software (GraphPad, San Diego, CA, USA). Data are presented as the means \pm SEM. Data were analyzed by one-way ANOVA for multiple comparisons. $P < 0.05$ was considered to indicate statistically significant differences.

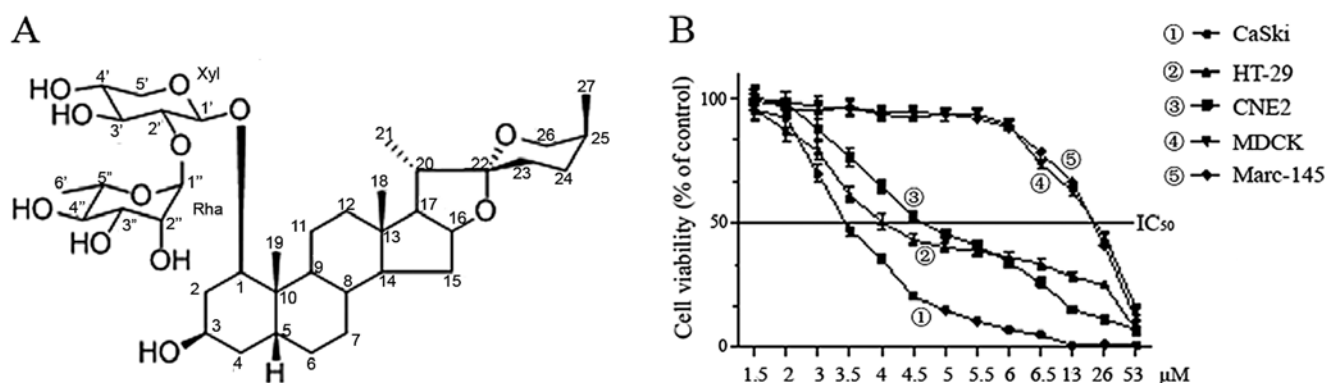


Figure 1. Structure of RCE-4 and its effect on cancer cell lines. (A) The structure of RCE-4, which consists of hydrophobic (spirostanol aglycone) and hydrophilic (disaccharide) moieties. (B) RCE-4 exhibits cytotoxic effects against various cancer cell lines whereas it exhibits relatively weaker cytotoxic effects to normal cells, as determined by MTT assay. Values are the means \pm SEM (n=3). The results shown are from three independent experiments.

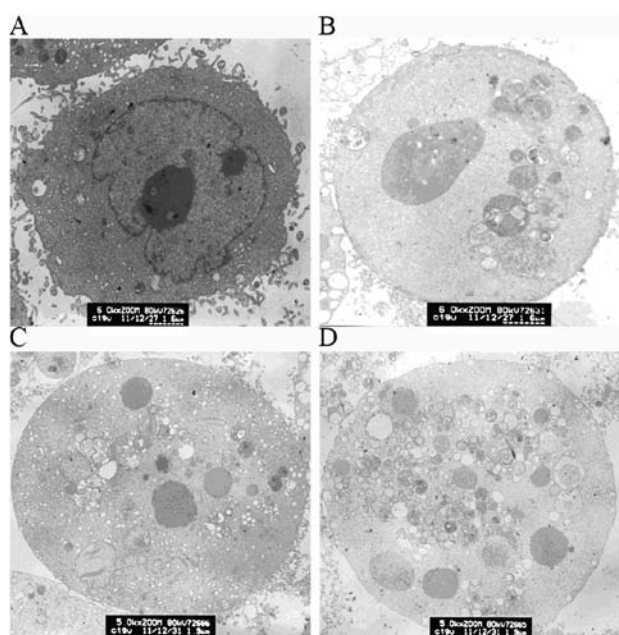


Figure 2. Apoptotic morphological changes in CaSki cells induced by RCE-4. Representative transmission electron microscopy images depicting ultrastructures of cells treated with (A) 0 or (B-D) 2.5, 5 and 10 μ M RCE-4 for 24 h. The characteristic apoptotic morphological changes including nuclear fragmentation, chromatin condensation, nucleoli absence, the cilia of the cell surface disappear and a large number of vacuoles is present in the cytoplasm.

Results

Chemical structure of RCE-4. The structure of RCE-4 was identified as (1 β ,3 β ,5 β ,25 S)-spirostan-1,3-diol-1-[α -L-rhamnopyranosyl-(1 \rightarrow 2)- β -D-xylopyranoside] (Fig. 1A).

Effect of RCE-4 on tumor cell viability. MTT assays were performed to investigate the effects of RCE-4 on the proliferation of tumor and normal cells. After treatment for 48 h with different concentrations, RCE-4 exhibited the greatest growth inhibitory effect against CaSki cells, followed by HT-29, CNE2 cells and much less cytotoxicity to Marc 145 and MDCK normal cells (~6-fold higher IC_{50}) (Fig. 1B). The IC_{50} values were 3.37, 4.31, 4.81, 18.73 and 19.75 μ M for CaSki, HT-29, CNE2,

Marc-145 and MDCK, respectively. These results indicated that RCE-4 had a good growth-inhibitory effect on CaSki cells in a dose-dependent manner and relatively high selectivity.

Apoptotic morphological changes in CaSki cells induced by RCE-4. To evaluate cellular ultrastructure for indications of the mode of death, we compared RCE-4-treated and untreated CaSki cells using transmission electron microscopy. Inspection of the ultrastructural details revealed the presence of RCE-4-induced apoptosis. CaSki control cells (Fig. 2A) showed a clear nucleus, nuclear membrane and nucleoli, irregular cell surface with cells very densely packed together. Following treatment with different concentrations of RCE-4 (2.5, 5 and 10 μ M), cells exhibited pronounced morphological changes and typical apoptosis features (Fig. 2B-D), including cell shrinkage, nuclear fragmentation, and chromatin condensation. The nucleus appeared to have broken down and their volume decreased. The nucleoli were also absent and no subcellular organelles were observed. Furthermore, irregular cell surface disappeared and a large number of vacuoles in the cytoplasm was present.

Effect of RCE-4 on apoptosis in CaSki cells. To further differentiate between apoptosis or necrosis, the cytotoxic effects of RCE-4 on CaSki cells were evaluated using the early marker of apoptosis Annexin V, and the dead cell marker PI. Numerous studies have reported that advanced nuclear fragmentation is preceded by alteration in the plasma membrane, such as PS externalization (17). Hence, cells treated with RCE-4 of 2.5, 5 and 10 μ M for 24 h were double stained with Annexin V-FITC/PI and analyzed by flow cytometry. Apoptotic cells were determined by counting the percentage of early apoptosis in the upper left quadrant (Annexin V⁺/PI⁻), and late apoptosis in the upper right quadrant (Annexin V⁺/PI⁺). Treatment with different doses of RCE-4 (2.5, 5 and 10 μ M) for 24 h resulted in separately 9.5, 23.3 and 26.3% early apoptosis compared with the control (0.8%) (Fig. 3A). These results suggest that RCE-4 can effectively induce apoptosis, and, particularly, early apoptosis. The necrotic cell population (Annexin V⁻/PI⁺) did not change apparently following exposure to different concentrations of RCE-4, indicating that apoptosis is the preferential cell death induced by RCE-4 in CaSki cells.

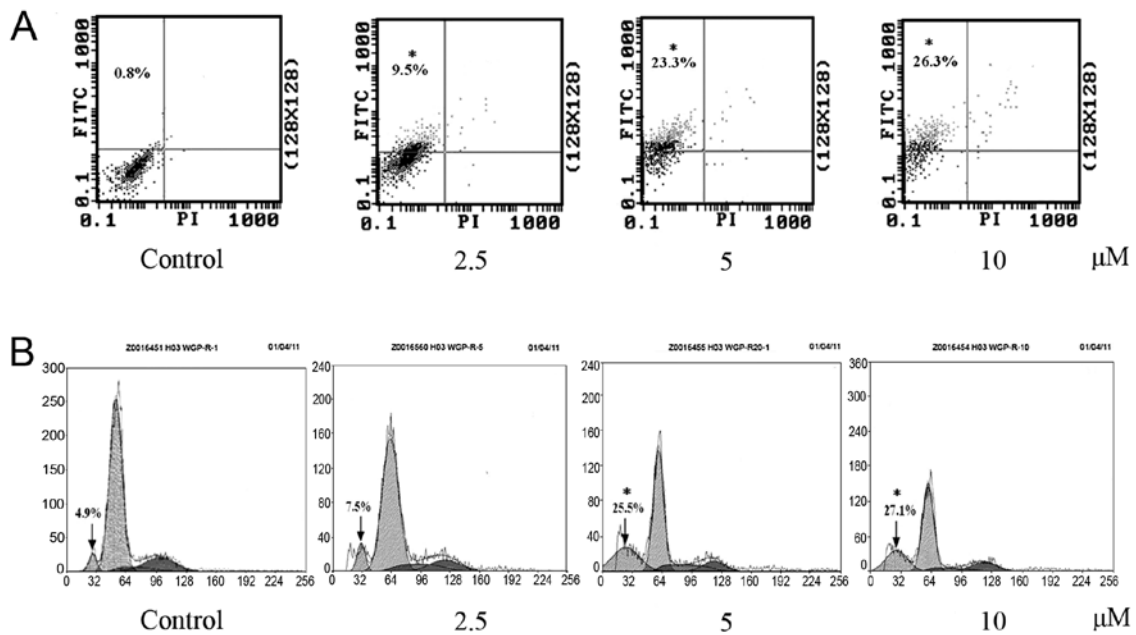


Figure 3. RCE-4 induces apoptosis and causes cell cycle arrest in CaSki cells. (A) Compared to control, the apoptosis percentage of CaSki cells treated with RCE-4 (2.5, 5 and 10 μ M) increased in a dose-dependent manner. Early apoptosis was reflected in the upper left quadrant (Annexin V⁺/PI⁻). The results shown are from three independent experiments; * P <0.05. (B) Flow cytometry analysis was used to determine the DNA content of CaSki cells treated with RCE-4 (2.5, 5 and 10 μ M) for 24 h. The number of cells in the sub-G1 phase (arrows) and S phase increased in a dose-dependent manner. The results shown are from three independent experiments; * P <0.05.

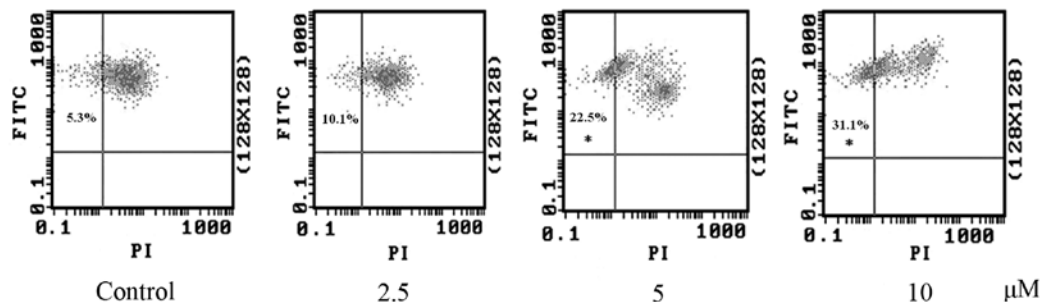


Figure 4. Effects of RCE-4 on $\Delta\psi_m$ disruptions in CaSki cells. The upper/right region represents the percentage of disrupted mitochondrial membrane. Quantitative analysis of the disruption of $\Delta\psi_m$ was calculated with the flow cytometer simultaneously. Compared to control (5.3%), the percentage of disrupted mitochondrial membrane increased from 10.1 to 31.1% respectively when treated with different concentrations of RCE-4. The results shown are from three independent experiments; * P <0.05.

Effect of RCE-4 on cell cycle distribution. An experiment was performed to evaluate the effect on the cell cycle phase distribution of CaSki cells after treatment with 2.5, 5 and 10 μ M of RCE-4. Results shown in Fig. 3B indicate that there was a significant increase in the Sub-G1 DNA fraction (4.9, 7.5, 25.5 and 27.1%) at a concentration of 0-10 μ M in a dose-dependent manner, which responded to apoptotic cells. Results of this experiment demonstrated that RCE-4 arrested the cell cycle progression of CaSki cells at S phase. Compared with the control (5.8%), RCE-4 (2.5, 5 and 10 μ M) led to an S phase increase of 8.4, 11.3 and 12.5%, respectively.

RCE-4 decreases the $\Delta\psi_m$ in CaSki cells. Apart from PS externalization, dissipation of $\Delta\psi_m$ has also been reported to be an early apoptosis event in several different systems. It is commonly used to detect mitochondrial depolarization that occurs in early apoptosis (18). To signal the loss of $\Delta\psi_m$,

JC-1 probe was applied to test the occurrence of apoptosis. As shown in Fig. 4, the majority of the untreated cells were identified in the upper right quadrant (FITC⁺/PI⁺). This corresponded to mitochondria with a polarized $\Delta\psi_m$. However, following treatment with different concentrations of RCE-4 for 24 h, cells moved towards the upper/left region (FITC⁺/PI⁻) and $\Delta\psi_m$ began to decrease, suggesting disruption of mitochondrial function. As can be seen in Fig. 4, RCE-4 treatment significantly decreased the $\Delta\psi_m$ in CaSki cells, compared with the control. These results were dose-dependent. These findings demonstrated that the RCE-4-induced apoptosis in CaSki cells involved mitochondria dysfunction associated with dissipation of the $\Delta\psi_m$.

mRNA expression of Bax and Bcl-2. The Bcl-2 family plays an important regulatory role in apoptosis, either as an activator (Bax) or inhibitor (Bcl-2). Since RCE-4 showed the ability to

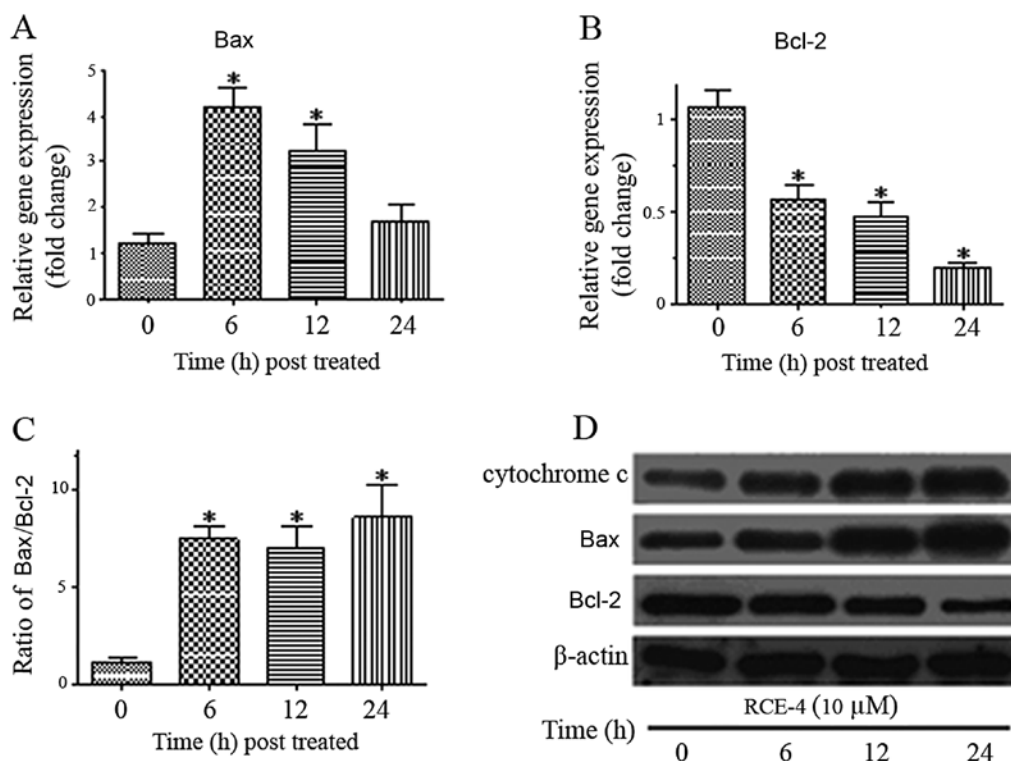


Figure 5. Kinetic effect of RCE-4 on the expression of apoptosis-related proteins in CaSki cells. (A and B) The effect of RCE-4 on Bax and Bcl-2 mRNA expression was determined at the indicated times in CaSki cells treated with RCE-4 (10 μ M); * P <0.05. (C) Bar charts show the ratio of Bax/Bcl-2, analyzed from the qPCR results. Values are the means \pm SEM (n=3); * P <0.05. (D) The protein expression of Bax and Bcl-2 was determined when CaSki cells were treated with RCE-4 (10 μ M) for the indicated times. The expression of cytochrome *c* was also increased in a time-dependent manner when treated with RCE-4.

interfere with the $\Delta\psi_m$, we hypothesized that Bax and Bcl-2 are involved in the RCE-4-induced apoptosis. Therefore, we investigated the gene expression level of Bax and Bcl-2 using qPCR. As depicted in Fig. 5A, Bax gene expression increased following treatment with 10 μ M RCE-4 and reached its peak at 6 h (~4.5-fold higher compared to the untreated). In addition, Bcl-2 gene expression was clearly suppressed as it was found to decrease 2-fold at 6 h and remained lower during the treatment hours (Fig. 5B). The increase in Bax and the decrease in Bcl-2 expression significantly elevated the Bax/Bcl-2 expression ratio (Fig. 5C).

Effects of RCE-4 on the expression of apoptosis-related proteins in CaSki cells. In order to further prove that the anti-proliferative effect of RCE-4 was due to mitochondria-mediated apoptosis, CaSki cells were treated with RCE-4 at 10 μ M for 6, 12 and 24 h and proteins implicated in apoptosis were evaluated using western blot analysis. The release of cytochrome *c* from mitochondria into cytosol induces the mitochondrial-dependent apoptotic pathway. Cytochrome *c* gradually increased in cytosol with a time-dependent increase as exposed to RCE-4 (Fig. 5D). We further investigated the involvement of Bcl-2 and Bax, the key regulatory factor, when cytochrome *c* was released into the cytosol during apoptosis induction. RCE-4 treatment resulted in the upregulation of Bax and downregulation of Bcl-2, leading to an increase in the Bax/Bcl-2 ratio (Fig. 5D). These results strongly indicate that apoptosis induced by RCE-4 in CaSki cells occurs via the mitochondria-dependent signal pathway.

Discussion

In the present study, we first demonstrated that RCE-4 possesses a sound antitumor activity against three cancer cell lines and limited toxicity against two normal cell lines. Furthermore, the involvement of antitumor mechanisms in this cytotoxic effect was clarified showing that apoptotic cell death of human cervical cancer CaSki cells was induced by the mitochondria-dependent activation of caspase cascade.

An important parameter in the evaluation of chemotherapeutic agents is their ability to inhibit cancer cell growth and induce cancer cell death. Apoptosis is an important means to maintain cellular homeostasis between cell division and cell death (19,20). Apoptosis and its related signaling pathways have a profound effect on the progression of cancer and so induction of apoptosis is a highly desirable goal of preventive strategies for cancer control (21). RCE-4 was found to induce a significant loss of cell viability in a time-dependent manner when CaSki cells were treated with RCE-4 for 48 h; a significant reduction of cell viability was induced with the IC_{50} value 3.37 μ M. In agreement with the cytotoxic effects of RCE-4 on CaSki cells, marked morphological changes indicative of cell apoptosis were clearly observed under transmission electron microscopy, including cell shrinkage, nuclear fragmentation, loss of cell-cell adhesion, membrane blebbing, and chromatin condensation, alterations commonly associated with apoptosis (22).

Furthermore, to confirm that RCE-4 induces apoptosis in CaSki cells, an Annexin V binding assay, which measures another feature of apoptosis, was conducted by flow cytometric

analysis. Consequently, the population of early apoptotic cells increased with increasing RCE-4 concentrations, but the late apoptotic cells and necrotic cells did not change notably. These results demonstrated that RCE-4 induces significant apoptosis instead of necrosis in a dose-dependent manner. At the same time, RCE-4-induced apoptosis was again identified by cell cycle analysis. The visible sub-G1 peak, which represents apoptotic cells, appeared to increase in a dose-dependent manner following RCE-4 treatment, suggesting that the cytotoxic effect of RCE-4 on CaSki cells is attributable to induced apoptotic cell death.

There are two main signaling pathways involved in apoptosis, the extrinsic and the intrinsic pathway (23). The extrinsic pathway is activated by ligand-bound death receptors of the tumor-necrosis factor receptor (TNFR) super-family (22). The intrinsic pathway, also called the mitochondria mediated apoptosis pathway, is a signal transduction pathway involving the mitochondria and the Bcl-2 family. In the present study, we examined whether RCE-4-induced apoptosis was mediated by the mitochondrial pathways. Since loss of mitochondrial membrane potential ($\Delta\psi_m$) is the primary target for the majority of intrinsic apoptotic signals, we investigated the integrity of mitochondrial membrane using JC-1 metachromatic dye. The results clearly demonstrated that RCE-4 drastically reduced the depolarization of the $\Delta\psi_m$ in CaSki cells and thus proceeded through apoptosis. In the mitochondria-mediated apoptosis pathway, an apoptotic stimulus is followed by the release of cytochrome *c* from mitochondria into the cytosol. Following the release, cytochrome *c* forms a complex in the cytoplasm with adenosine triphosphate (ATP) and others and then triggers the apoptosis (24). The present study found that RCE-4 promoted the release of cytochrome *c* into the cytosol with its protein expression upregulation in CaSki cells. These experimental results suggested that the intrinsic pathway was involved in RCE-4 induced apoptosis.

Of the Bcl-2 family members, the Bcl-2 and Bax protein ratio has been recognized as a key factor in regulation of the apoptotic process (25). Other studies have suggested that Bcl-2 maintains the mitochondrial integrity, while Bax destroys the mitochondrial integrity and causes loss of $\Delta\psi_m$ (26). Consequently, the ratio between Bcl-2 and Bax determines the susceptibility to apoptosis and thus dictates the life and death of a cell (27,28). In this study, RCE-4 treatment altered the gene expression level of Bcl-2 and Bax proteins, showing that Bcl-2 was decreased, while Bax was increased. Furthermore, western blotting showed that RCE-4 was able to inhibit the protein expression of Bcl-2 and stimulate the protein expression of Bax and so significantly increase the Bax/Bcl-2 ratio. These results are consistent with those from the gene expression studies.

In conclusion, the present study is the first to report a molecular pathway of apoptosis induced by RCE-4 from *Reineckia carnea*. This study also suggests that RCE-4 may be a natural potential apoptosis-inducing agent for human cervical cancer and possibly to treat other types of cancer.

Acknowledgements

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