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Virosecurinine induces apoptosis by affecting Bcl-2 and Bax expression in human colon cancer SW480 cells

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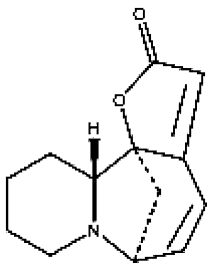
Virosecurinine, the major alkaloid isolated from *Securinega suffruticosa* Pall Rehd was found to exhibit growth inhibition and cytotoxicity against huaman colon cancer SW480 cells via the microculture tetrazolium (MTT) assay. Due to its greater cytotoxic potency and selectivity towards SW480 cells, flow cytometry was used to analyze the cell cycle distribution of control and treated SW480 cells whereas Annexin V-FITC/PI flow cytometry analysis was carried out to confirm apoptosis induced by virosecurinine in SW480 cells. Apoptotic regulatory genes were determined by RT-PCR analysis. Virosecurinine was found to induce G1/S cell cycle arrest which led to predominantly apoptotic mode of cell death. Mechanistically, virosecurinine was found to up-regulated the Bax gene expression and down-regulated the Bcl-2 expression in SW480, The ratio of Bcl-2 to Bax was significantly decreased. Hence, we suggest that virosecurinine induced apoptosis in SW480 cells by affecting the expression of bcl-2 and bax.

1. Introduction

Colon cancer is one of the most common malignancies worldwide, particularly in China. Surgical therapy, chemotherapy, and radiation have been used for the treatment of colon cancer. However, colon cancer remains one of the more difficult cancers to treat. Chemotherapy is a common therapeutic strategy after surgery, but, chemotherapy drugs usually cause problems with adverse reactions and drug resistance. Therefore, it is very important to develop an effective natural anti-tumor drug with low toxicity. Securinine is a major alkaloid of the plant Securinega suffruticosa. There are two optical isomers, l-securinine and virosecurinine. It has been reported to be a useful agent for treatment of poliomyelitis, aplastic anemia, and diseases related to the central nervous system (CNS) by acting as a GABA receptor antagonist.



Virosecurinine

Securinine has also been found to be a potent differentiation inducing agent in cancer cells with increased efficacy and low toxicity. Hence, it has potential for clinical use in the treatment of cancer (Buravtseva 1958; Copperman et al. 1973; Lubick et al. 2007; Dong et al. 1999). Our somatic cells are born by mitosis and almost all will die by apoptosis, a physiological process of cellular suicide. Cancer occurs when this balance is disturbed, either by an increase in cell proliferation or a decrease in cell death. The goal of cancer therapy is to promote the death of cancer cells without causing too much damage to normal cells (Gerl and Vaux 2005; Lowe and Lin 2000). Hence, apoptosis induction a very important way to cancer treatment. Our present study aimed at investigating the effect of virosecurinine on human colon cancer cell line SW480, and further to elucidate its mechanism.

Table 1: Cytotoxicity of virosecurinine on SW480 cells assessed by MTT assay ($\bar{x} \pm \mathbf{s}$, $\mathbf{n} = 5\%$)

Dose (μmol/L)	24 ± h	36 h	48 h
6.25	1.34 ± 1.0^{a}	8.74 ± 1.1^{b}	25.32 ± 6.7^{c}
12.5	7.15 ± 1.3^{a}	18.17 ± 5.8^{a}	34.57 ± 5.3^{b}
25	24.45 ± 3.6^{a}	39.63 ± 6.0^{a}	50.58 ± 8.1^{c}
50	38.48 ± 7.1^{a}	49.68 ± 7.5^{b}	65.35 ± 4.5^{c}
100	40.24 ± 7.9^{a}	60.21 ± 7.5^{b}	74.07 ± 4.5^{c}

*Between two differ letter $P < 0.05$, between the same letter $P > 0.05$.

2. Investigations and results

2.1. Growth inhibition in SW480 cells exposed to virosecurinine

We used the MTT assay to determine the effects of virosecurinine on the proliferation of SW480 cells. Using virosecurinine at concentrations of 6.25, 12.5, 25, 50, and 100 μM, we observed an anti-proliferative effect on SW480 cells that was time- and dose-dependent (Table 1). The IC_{50} of virosecurinine to

SW480 cells 24, 36 and 48 h post-treatment were 102.26, 92.96, 19.10 μM, respectively.

2.2. *Apoptosis occurred in virosecurinine-treated cells*

To determine whether virosecurinine-treated SW480 cells undergo apoptosis, we stained cells using annexin V-FITC/PI. We observed a significant increase in the number of cells that exhibited nuclear condensation when treated with virosecurinine for 48 h. Our data also showed that the apoptotic index of SW480 cells increased in a dose-dependent manner. The proportion of apoptotic cells significantly elevated in virosecurinine (2.5, 5, 10 μM)-treated cells from 5.6% in untreated cells to 10.3–56.8% (Fig. 1).

2.3. *Inhibition of cell cycle progression*

Since virosecurinine displayed greater cytotoxic selectivity towards SW480 cells, flow cytometric analysis was used to measure the DNA contents of control and virosecurinine-treated SW480 cells to determine the cell cycle perturbations induced by the compound. Any changes in the cell cycle progression will be reflected in Fig. 2. Following an exposure time of 48 h, virosecurinine induced a G1 phase cell cycle arrest in SW480 cells. Treatment with 2.5, 5 and 10 μM concentrations for 48 h caused accumulation of cells in G1 phase with a concomitant decrease of both S and G2 phase in SW480 cells. It is noteworthy that increasing the concentration of the compound also promoted appearance of a sub-G1 population indicative of apoptotic cells.

2.4. *Gene expression effects*

Bcl-2 is an antiapoptotic protein, whereas Bax is a proapoptotic protein belonging to the Bcl-2 gene family. Together, the levels of these anti-and proapoptotic proteins determine a cell's fate for survival or cell death (Hasnan et al. 2010; Coultas and Strasser 2003). The mRNA levels of Bcl-2 and Bax were measured in virosecurinine-deprived and control SW480 cells using semi-quantitative RT-PCR analysis. The results showed a decrease in the level of Bcl-2 with a concomitant increase in the level of Bax mRNA in virosecurinine deprived cells. Compared with control cells,the ratio of Bcl-2 to Bax significantly declined (Fig. 3).

3. Discussion

Although securinine, a major alkaloid of the plant *Securinega suffruticosa*, has been known to inhibit the growth of several types of tumor cells, the mechanism is not yet clear (Buravtseva 1958; Copperman et al. 1973; Lubick et al. 2007; Dong et al. 1999). In this study, we explored the efficacy of virosecurinine for its antiproliferative activity against human colon cancer cell line SW480 and found that virosecurinine significantly inhibited proliferation of SW480 cells at low concentrations. The IC_{50} of virosecurinine to SW480 cells 48 h after treatment was 19.1 μM. In addition, the study found that virosecurinine could induce cell apoptosis we observed a significant increase in the number of cells that exhibited nuclear condensation when treated with virosecurinine for 48 h. Our data also showed that the apoptotic index of SW480 cells increased in a dose-dependent manner treated by virosecurinine. The presence of apoptotic cells can also be demonstrated in DNA histograms by the appearance of a sub-G1 peak, which is an established indicator of apoptosis. Through the cell cycle analysis by flow cytometry (Packham 1998; Bryson et al. 1994), in cells exposed to virosecurinine, there were progressively fewer cells in G2/S phase and more cells in G1-phase. This may represent an increase in cellular proliferation or an arrest of the cell cycle in G1-phase, which prevented cells from progressing towards mitosis. Apoptosis plays an essential role as a protective mechanism against neoplastic development by eliminating genetically damaged cells or excess cells. Inducing cancer cell apoptosis can inhibit their proliferation and then is a potential anti-tumor method (Gerl an Vaux 2005; Lowe and Lin 2000; Hasnan et al. 2010; Rautureau et al. 2010). All these results indicate that the inhibitory effect of virosecurinine on human colon cancer cell line SW480 growth may be in part due to the induction of apoptosis.

Although there are multiple pathways leading to apoptosis, most of them are ultimately regulated by the Bcl-2 family of proteins.

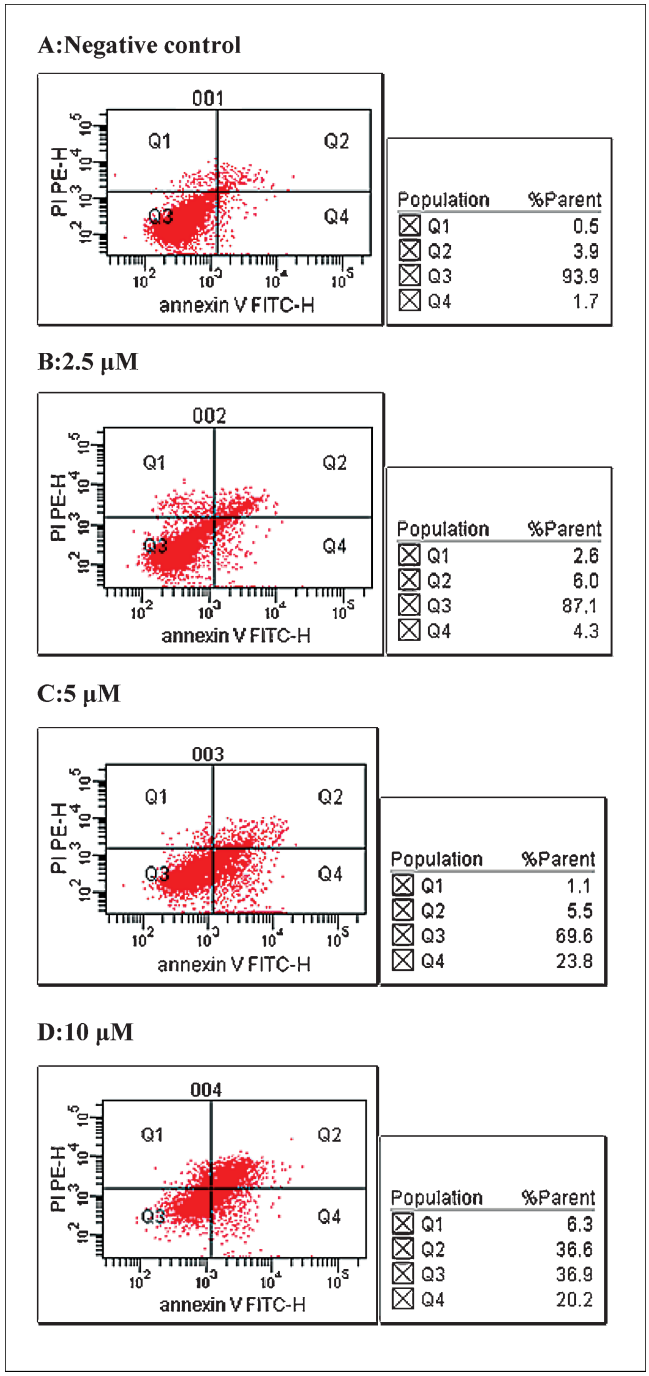


Fig. 1: Apoptosis induced by virosecurinine on SW480 cells. SW480 cells were treated for 0, 2.5, 5 and 10 μM of virosecurinine for 48 h. Lower left quadrants, viable cells. Lower right quadrants, necrotic cells. Upper left quadrants, early apoptotic cells. Upper right quadrants, nonviable late apoptotic cells

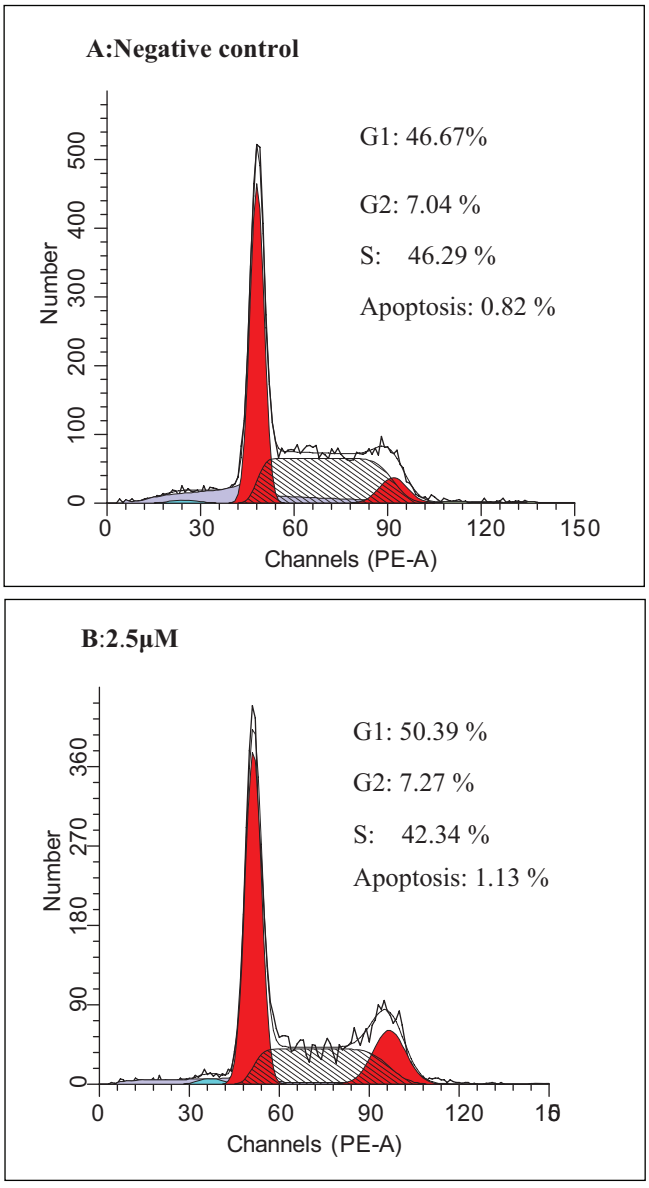


Fig. 2: Flow cytometry analysis of cells cycle treated of Virosecurinine. A: normal SW480cells; B,C and D cells treated with 2.5, 5, and 10 μM of virosecurinine for 48 h, respectively. The number in the top right quadrant represents the percentage of autophagic cells

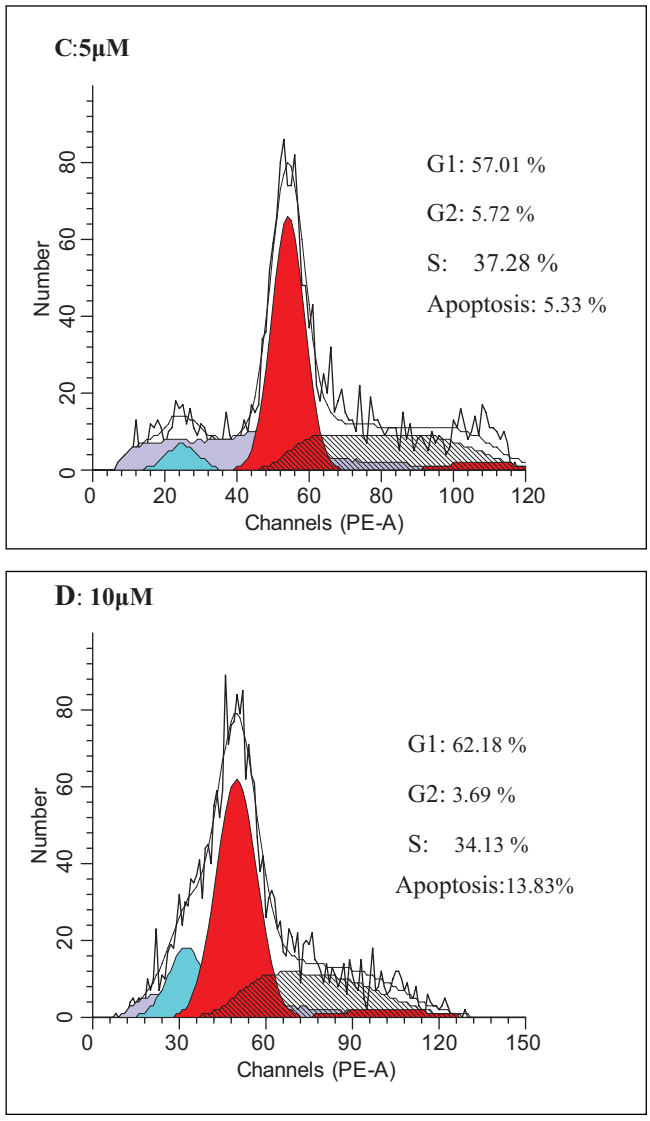


Fig. 2: *(Continued)*

The Bcl-2 proteins are regarded as important regulators of the apoptotic process, since they include at least 15 proteins that can be divided into two subclasses that either promote apoptosis (BAX, BCL-XS, Bad, Bik and Bak) or suppress it (Bcl-2, Bcl-xl and Mcl-1) (Adams and Cory 1998; Wei et al. 2001; Sedlak et al. 1995). Bax is a crucial mediator and as a tumor suppressor (Yamaguchi et al. 2003; Sedlak et al. 1995). These proteins can form heterodimers, and the ratio of apoptosis promoters to apoptosis suppressors is one determinant of cellular response. Therefore, the ratio between bcl-2/bax ratio is thought to be a decisive factor in activating cell death (Ghobrial et al. 2005). To further investigate its molecular mechanism, we measured the levels of two key apoptosis-linked gene bcl-2 and bax in virosecurinine-treated SW480 cells. Our data showed that virosecurinine up-regulated the Bax gene expression and down-regulated the Bcl-2 expression in SW480. The ratio of Bcl-2 to Bax was significantly decreased. Thus, we demonstrated for the first time that virosecurinine induces apoptosis in SW480 cells by changing the expression of bcl-2 and bax. Taken together, these results reveal that virosecurinine is an effective agent in suppressing human colon cancer cell proliferation and suggest that growth inhibition may be partly due to its induction of apoptosis through down-regulation of Bcl-2 and up-regulation of bax. From laboratory experiments, we suggest that virosecurinine may serve as potential lead for future drug development for prevention and treatment of colon cancer.

4. Experimental

4.1. Chemicals

Pure virosecurinine was extracted and isolated from *Securinega suffruticosa* (Pall) Rehd by ion exchange resin method. 3-(4,5-Dimethylthiazol-2-yl)-

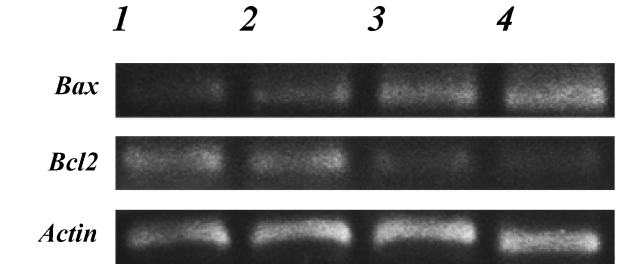


Fig. 3: Semiquantitative reverse transcription-PCR(RT-PCR) analysis of *Bcl-2 and Bax* mRNA levels in SW480 cells treated with virosecurinine.Lane 1: Negative control; Lane 2,3,4:cells treated with virosecurinine at.2.5 μM,5 μM, 10 μM for 48 h

Table 2: Gene stucture

Primer	Stucture		Length
β-Actin	Sense primer	5' ACAAAGTGGTCATTGAGGGC 3'	510bp
	Antisense primer	5' GCCGTCAGGCAGCTCGTAGC 3'	
Bcl-2	Sense primer	5' GTGGAGGAGCTCTTCAGGGA 3'	304bp
	Antisense primer	5' AGGCACCCAGGGTGATGCAA 3'	
Bax	Sense primer	5' GGCCCACCAGCTCTGAGCAGA 3'	477bp
	Antisense primer	5' GCCACGTGGGCGTCCCAAAGT 3'	

2,5-diphenyl-tetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Cell culture media and solutions were obtained from Gibco BRL (Karlsruhe, Germany).

4.2. Cell line and culture

The human colon cancer SW480 cell line was purchased from the KeyGen Serving Science Company (Nanjin, China). SW480 cells were cultured in RPMI-1640 medium supplemented with 10% heatin- activated fetal bovine serum and 100 U/ml of both penicillin and streptomycin. Cells were routinely cultured in a humidified incubator at 37 °C with 5% carbon dioxide.

4.3. MTT assay

MTT was used to measure the viability. Exponentially growing SW480 cells (5×10^3) were seeded into 96-well plates. After 24 h, tumor cells were fed with RPMI-1640 medium containing 0.1% fetal bovine serum and treated with virosecurinine (10 μl per well) at concentrations ranging from 6.25 to 100 μM and every kind cell was repeated in three holes. Then the plates were cultured for 24, 36 and 48 h, followed by incubation with 20 μl of stock solution 5 mg/ml of MTT for 4 h.The supernatant was removed after centrifugation. Finally, 100 μl of dimethyl sulphoxide (DMSO) was added and oscillated for 10 min. The optical density was measured by means of enzyme-labeling instrument (680 type, BIO-RAD) at 450 nm wavelength. Relative cell proliferation inhibition rate (IR) = (1-averageA450 of the experimental group/average A450 of the control group) × 100%.

4.4. Apoptosis analysis

The SW480 cells were cultured in 6-well plates (3.0×10^5 cells/well) and treated with different concentrations of virosecurinine (2.5, 5, 10 μM) at 37 °C in a humidified atmosphere with 5% CO_2 for 48 h. The cells were washed with clod PBS twice, then added 5 μl of annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI 5 mg/ml). After 15 min, incubation at room temperature in the dark, cells were used for flow cytometry analysis. Fluorescence was measured by FACScan flow cytometry (Becton Dickinson) equipped with an argon laser (488 nm). The percent age of apoptotic cells was calculated using the internal software system of the FACScan (Becton Dickinson).

4.5. Cell cycle analysis

The SW480 cells were cultured in 6-well plates (3.0×10^5 cells/well) and treated with different concentrations of virosecurinine (2.5, 5,10 μM) at 37 °C in a humidified atmosphere with 5% CO2 for 48 h. Cells were collected and fixed in 70% ethanol at 4°C overnight. Subsequently, cells were treated with Tris–HCl buffer (pH 7.4) containing 1% Rnase A and were stained with propidium iodide (PI, 5 mg/ml). The distribution of cells with different DNA contents was determined by flow cytometry (Facscalibur, Becton Dickinson, USA) and the data were analyzed by multicycle DNA content and cell cycle analysis software.

4.6. RT-PCR analysis

The mRNA levels of Bcl-2 and Bax were measured in virosecurinine-deprived and control SW480 cells using the semiquantitative RT-PCR analysis. The total RNA was extracted from the cell cultures with the use of the RNeasy kit (Qiagen Inc,Valencia, CA). First-strand cDNA was synthesized with total RNA (0.5 mg) using the ProSTARt First Strand RT-PCR Kit (Invitrogen,C arlsbad, CA, USA). A volume of
2 ml of cDNA reaction was amplified by PCR kit (Invitrogen) using primer β-actin (5' ACAAAGTGGTCATTGAGGGC 3'),Bcl-2(5'AGGCACCCAGGGTGATGCAA 3') and Bax (5' GCCACGTGG GCGTCCCAAAGT 3') (Table 2). The thermal cycle profile was 94 °C for 5 min, 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 32 s for 32 cycles. The amplified products were fractionated on a 2% agarose gel containing 0.5 mg/ml ethidium bromide, gels were photographed with BIO-RAD Gel DocTM XR(BIO-RAD corporation). The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers.

4.7. Statistical analysis

The data were expressed as means ± S.D. Analysis of variance was performed with SPSS software for windows 17.0 by using one way ANOVA and pairwise comparison with Student's t test. The criterion for statistical significance was taken as $P < 0.05$.

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