

Virosecurinine induces apoptosis in human leukemia THP-1 cells and other underlying molecular mechanisms

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Abstract. Virosecurinine, a primary alkaloid from *Securinega suffruticosa* plant is known as a potent differentiation-inducing agent in acute leukemia cells. The present study aimed to investigate the effects and underlying mechanisms of virosecurinine on human leukemia THP-1 cells *in vitro*. The effects of virosecurinine on cell proliferation were assessed by CCK-8. The effects on apoptosis and cell cycle were assessed by staining with annexin V-fluorescein isothiocyanate and propidium iodide, respectively followed by flow cytometric analysis. The apoptotic cell bodies were observed using a transmission electron microscope, while the mRNA expression of phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), mechanistic target of rapamycin (mTOR) and phosphatase and tensin homolog (PTEN) in THP-1 was evaluated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Treatment with virosecurinine was able to decrease the viability of THP-1 cells in a dose- and time-dependent manner. The IC₅₀ values of virosecurinine at 24, 48, and 72 h post-treatment were 68.128, 23.615, and 13.423 μ mol/l, respectively. Cell cycle was arrested at the G1/S phase in virosecurinine-treated cells; however, not in untreated control cells. Numerous apoptotic bodies were observed in the THP-1 cells, which were treated with 12.5 μ mol/l virosecurinine for 48 h. RT-qPCR indicated that treatment with virosecurinine resulted in upregulated PTEN expression and downregulated expression of PI3K, AKT and mTOR in THP-1 cells. The present study demonstrated that treatment with virosecurinine was able to inhibit proliferation and induce apoptosis in THP-1 cells by exerting an inhibitory effect on the activation of PI3K/AKT/mTOR signaling pathways. Therefore, our data suggested that virosecurinine is a promising anti-tumor agent for the treatment of acute monocytic leukemia.

Introduction

Acute leukemia is characterized as a heterogeneous clonal disease with undifferentiated malignant growth. In total, ~140,000 people are diagnosed with acute leukemia worldwide annually (1). Acute myeloid leukemia (AML) is the most common type of acute leukemia in aging adults and is considered responsible for the highest annual mortality rate by leukemia globally (2). However, AML remains one of the most difficult malignant hematological diseases to treat, and has the lowest survival rate among all types of leukemia (3). Therefore, a deeper insight into the molecular etiology of AML will allow the development of an effective natural anti-tumor drug.

Several recent developments have been instigated for high efficacy and low toxicity antitumor drugs. Securinega alkaloids have been isolated from the *Euphorbiaceae* family of plants. Typically, securinine, an alkaloid from the leaves of *Securinega suffruticosa*, was initially isolated by a Russian scientist, Ia A, in 1956. Securinine has been structurally characterized into two optical isomers: L-securinine and virosecurinine, by Chinese scientists in 1963 (4). As a natural product, securinine was observed to exert several important roles in biological systems (5). Securinine is able to act as a γ -aminobutyric acid receptor antagonist (6) and exhibit antimalarial (7) and antibacterial activities (8). Li *et al* (9) have demonstrated that virosecurinine is able to exhibit apoptotic activity in human breast cancer MCF-7 cells, whereas Zhang *et al* (10) have demonstrated its apoptotic activity in human chronic myeloid leukemia K562 cells. Therefore, virosecurinine may be potentially used for cancer treatment.

Apoptosis is a physiological cell removal mechanism that is critical in the cancer cell cycle (11). Cancer cells have evolved multiple molecular mechanisms against the onset of apoptosis, and therefore the signaling pathways induced by natural products, including L-securinine, betulinic acid and resveratrol, may serve as key factors for antitumor activities (12). One such example is the phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling pathway, which is crucial for proliferation, development and cell death (13). Constitutive activation of the PI3K/AKT/mTOR signaling pathway is associated with the progression and pathogenesis of a broad spectrum of various types of human cancer, including acute leukemia (14-16). Therefore, investigating novel approaches for inhibiting this

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signaling pathway in order to develop targeted therapeutics while limiting the side effects is vital for increasing treatment efficacy and improving prognosis in patients (17).

The present study investigated the effects and underlying mechanisms of virosecurinine on apoptosis in human AML THP-1 cells. Furthermore, the present study also searched for natural anti-tumor drugs that exhibit a high efficacy and low toxicity.

Materials and methods

Chemicals. Virosecurinine (Fig. 1) was provided by the Institute of Traditional Chinese Medicine and Natural Products, Jinan University (Guangzhou, China). Cell Counting Kit-8 (CCK-8) was purchased from Nanjing KeyGen Biotech Co., Ltd., (catalog no. KGA317; Nanjing, China). The cell culture media (RPMI-1640) and solutions were bought from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Cell line and culture. Human AML THP-1 cell lines were obtained from the Key Gen Serving Science Company and were grown in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% fetal bovine serum (FBS; Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China), 100 IU/ml penicillin, and 100 µg/ml streptomycin. The cells were grown and maintained at 37°C in a humidified incubator with 5% CO₂.

Cell viability analysis. Cell Counting Kit-8 assay (CCK-8; catalog no. KGA317; Nanjing KeyGen Biotech Co., Ltd.) was used to measure cell viability. THP-1 cells in the exponential phase were plated in 100 µl into 96-well plates (Corning Incorporated, Corning, NY, USA) at a density of 5x10³ cells/well. After 24 h at 37°C, THP-1 cells were replenished with RPMI-1640 medium containing 10% FBS and treated with virosecurinine (200 µl/well) at concentrations ranging from 0 to 200 µmol/l. Then, the plates were incubated in a humidified incubator for 24, 48, and 72 h at 37°C. A total of 10 µl CCK-8 solution was added to each well three hours prior to measurement of absorbance. The optical density was measured at 450 nm with a microplate reader (EL-x 800; BioTek Instruments, Inc., Winooski, VT, USA). Relative cell proliferation inhibition rate (IR) was calculated using the following formula: IR=(absorbance of the control group-absorbance of the experimental group)/(absorbance of the control group-absorbance of the blank control group)x100%. All experiments were performed four times.

Transmission electron microscopy. THP-1 cells were seeded at a density of 5x10⁵ cells/well in 6-well plates (Corning Incorporated) with or without 12.5 µmol/l virosecurinine for 48 h at 37°C. Subsequently, the cells were harvested and fixed for 2 h at 4°C in 2.5% chilled glutaraldehyde followed by three washes in 0.1 mol/l PBS (Nanjing KeyGen Biotech Co., Ltd.). The cells were then post-fixed at 4°C in 1% osmium tetroxide for 2 h, dehydrated sequentially in 50, 70, 90 and 100% ethanol for 15 min each (three times in 100% ethanol), and embedded in epoxy resin. Consequently, the embedded cells were sliced into 50-60 nm sections and stained with uranyl acetate for 30 min at room temperature and lead citrate for 30 min at room

temperature, and subsequently observed under a transmission electron microscope (TEM-1011; JEOL, Ltd., Tokyo, Japan).

Apoptosis analysis. For apoptosis analysis, THP-1 cells were cultured in 6-well plates at a density of 3.0x10⁵ cells/well for 24 h, and then treated with various concentrations of virosecurinine, 6.25, 12.5 and 25 µmol/l, respectively, for 48 h. The cells were washed twice with cold PBS and gently resuspended in 500 µl binding buffer. Thereafter, 5 µl annexin V-fluorescein isothiocyanate (FITC) (KGA105; Nanjing KeyGen Biotech Co., Ltd.) and propidium iodide (KGA511; Nanjing KeyGen Biotech Co., Ltd.) were added. After 15 min incubation at room temperature in the dark, the cells were subjected to flow cytometric analysis. The fluorescence was measured using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA) equipped with an argon laser (488 nm). The percentage of apoptotic cells was calculated using the FACSscan software (version 6.0; BD Biosciences).

Cell cycle analysis. For cell cycle analysis, THP-1 cells were cultured in 6-well plates and treated with virosecurinine as described above. The cells were then washed, harvested and fixed with 70% ethanol at 4°C overnight. Subsequently, the cells were treated with Tris-HCl buffer (pH 7.4) containing 1% RNase A (KGA511; Nanjing KeyGen Biotech Co., Ltd.) and stained with propidium iodide (PI, 5 mg/ml; Nanjing KeyGen Biotech Co., Ltd.). The distribution of cells with different DNA contents was determined by flow cytometry.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR assays were performed on THP-1 cells treated with or without virosecurinine in order to evaluate the expression of PI3K, AKT, mTOR and phosphatase and tensin homolog (PTEN). Total RNA was isolated from THP-1 cells with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A two-step reverse transcription PCR was performed. First-strand cDNA synthesis was performed using the ProSTARt First Strand RT-PCR kit (catalog no. PC0002; Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers. CK8 and GAPDH genes were quantified by RT-PCR for mRNA level and as an endogenous control, respectively. Subsequently, 20 µl reaction mixture was used for qPCR, using a qPCR kit (catalog no. DA7600; OriGene Technologies, Inc., Rockville, MD, USA), which consisted of 40 cycles: Denaturation (15 sec at 94°C), annealing (20 sec at 60°C) and extension (40 sec at 72°C). The reaction mixture (catalog no. EP0702; Fermentas; Thermo Fisher Scientific, Inc.) contained 10 µM of each primer, 2 µl of 2x QuantiTect SYBR Green RT-PCR Master Mix, 10 µl QuantiTect reverse transcriptase mix, and 8 µl nuclease-free water. The relative quantification was analyzed by the 2^{-ΔΔCq} method (18) with GAPDH as the housekeeping gene and the control cells as the baseline. The results were expressed as fold changes. Each experiment was repeated three times. The sequences of the primers used are as follows: PI3K forward, 5'-GGGGATGATTTACGGCAAGATA-3' and reverse, 5'-CACCACCTCAATAAGTCCCA-3'; AKT forward, 5'-GCAGCACGTGTACGAGAAGA-3' and reverse,

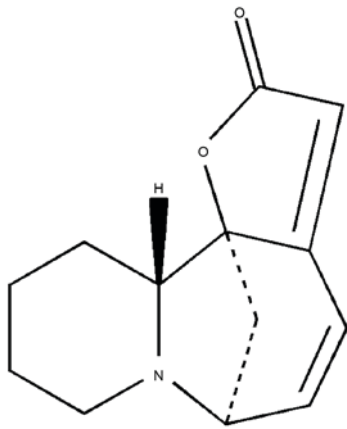


Figure 1. Structure of virosecurinine.

5'-GGTGTCACTCTCCGACGTG-3'; mTOR forward, 5'-ATT TGATCAGGTGTGCCAGT-3', and reverse, 5'-GCTTAGGAC ATGGTTCATGG-3'; PTEN forward, 5'-CAAGATGATGTT TGAAACTATTCCAATG-3', and reverse, 5'-CCTTTAGCT GGCAGACCACAA-3'; GAPDH forward, 5'-TGTTGCCAT CAATGACCCCTT-3', and reverse, 5'-CTCCACGACGTA CTCAGCG-3'.

Statistical analysis. All data are represented as the mean \pm standard deviation. SPSS (version 18.0; SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistically significant differences between the groups were analyzed by Student's t-test, and multiple comparisons were performed using one-way analysis of variance followed by Tukey's test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Virosecurinine treatment inhibits growth THP-1 cells in vitro. In order to determine the mechanistic effects of virosecurinine-induced apoptosis, CCK-8 assay was employed to investigate the proliferation of THP-1 cells. The inhibitory effect on proliferation was determined by treating cells with a range of virosecurinine concentrations ranging from 0 to 200 $\mu\text{mol/l}$ for 24, 48 and 72 h, respectively. The assay revealed that treatment with virosecurinine was able to significantly inhibit the proliferation of THP-1 cells in a dose- and time- dependent manner (Fig. 2). The IC_{50} values at 24, 48 and 72 h following treatment were 68.128, 23.615 and 13.423 $\mu\text{mol/l}$, respectively.

Ultrastructure of THP-1 cells. The ultra-structural analysis of virosecurinine-induced apoptosis in THP-1 cells was carried out by electron microscopy (Fig. 3). The appearance of apoptotic bodies in THP-1 cells that were treated with 12.5 $\mu\text{mol/l}$ virosecurinine for 48 h (Fig. 3B) confirmed that apoptosis was taken place in these treated cells. Similar results were not observed in untreated cells (Fig. 3A).

Virosecurinine treatment inhibits cell cycle progression in THP-1 cells. To understand the mechanisms of virosecurinine-induced THP-1 cell apoptosis, flow cytometric cell cycle analysis of THP-1 cells following treatment with

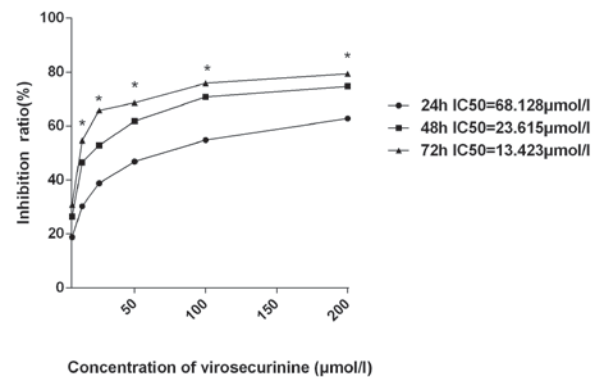


Figure 2. Dosage and time dependent inhibition of THP-1 cells by virosecurinine. $P < 0.05$ vs. untreated control.

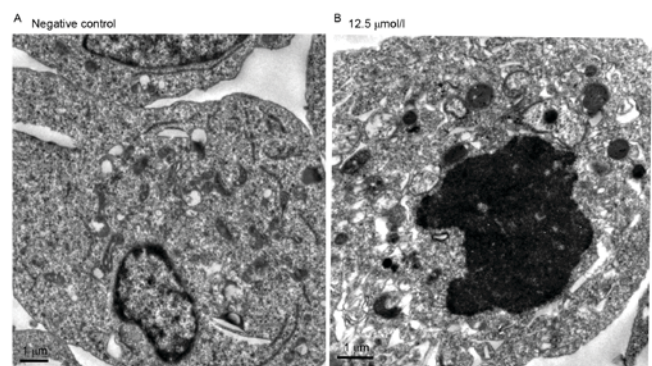


Figure 3. Electron micrographs demonstrating the ultrastructure of THP-1 cells, which were treated with 12.5 $\mu\text{mol/l}$ virosecurinine for 48 h. (A) A small number of apoptotic bodies were observed in THP-1 cells that were treated without virosecurinine for 48 h (control). (B) Numerous apoptotic bodies were observed in virosecurinine treated-THP-1 cells.

0, 6.25, 12.5 and 25 $\mu\text{mol/l}$ virosecurinine, respectively, for 48 h was performed. The results demonstrated that treatment with virosecurinine resulted in cell-cycle arrest in the G1 phase. (Fig. 4). Notably, a sub-G1 peak was observed, which represented an apoptotic population as a response to virosecurinine treatment.

Virosecurinine treatment induces apoptosis in THP-1 cells in vitro. Apoptotic rate in THP-1 cells treated with virosecurinine was determined by FITC-annexin V and PI double staining followed by flow cytometric analysis. The quantification of cells in each quadrant in Fig. 5 are representative of necrosis (C1), late apoptosis (C2), live cells (C3) and early apoptosis (C4). This indicated that virosecurinine concentration was directly proportional to the rate of apoptosis in THP-1 cells. The proportion of apoptotic cells treated with 6.25, 12.5 and 25 $\mu\text{mol/l}$ of virosecurinine for 48 h was 25.47, 38.29 and 64.31%, respectively (Fig. 5).

Virosecurinine treatment affects the expression of genes in the PI3K/AKT/mTOR signaling pathway in THP-1 cells. To further elucidate the mechanism underlying virosecurinine-induced apoptosis in THP-1 cells, the expression of PI3K, AKT, mTOR and PTEN in treated and control THP-1 cells was evaluated.

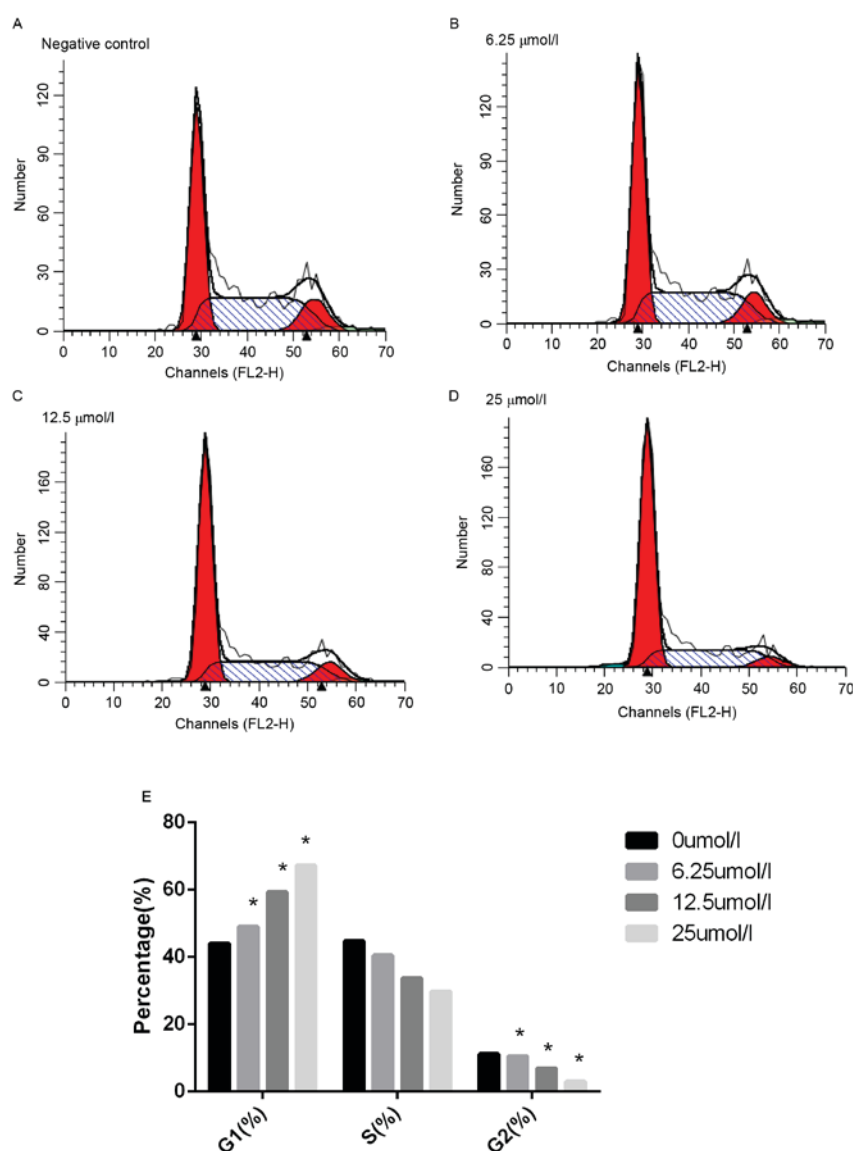


Figure 4. Flow cytometric analysis of cell cycle of cells treated with virosecurinine. (A) Flow cytometric analysis of normal THP-1 cells and cells treated with (B) 6.25, (C) 12.5 and (D) 25 $\mu\text{mol/l}$ virosecurinine for 48 h. (E) Cell cycle of THP-1 cells treated with virosecurinine at different concentrations for 48 h and detected using flow cytometry. The results demonstrated that the cell cycle was arrested at the G0/G1 phase when treated with virosecurinine. * $P < 0.05$ vs. control.

RT-qPCR analysis revealed that treatment with virosecurinine was able to downregulate the level of PI3K, AKT and mTOR expression and upregulate the expression of PTEN ($P < 0.05$; Fig. 6). These results suggested that virosecurinine-induced apoptotic cell death was associated with the activation of PI3K, AKT, mTOR and PTEN.

Discussion

AML treatment remains a major challenge due to poor efficacy of the current chemotherapeutics. Therefore, investigating natural plants as resources for antitumor agents is an increasingly important topic in cancer research. Herein, the effect of virosecurinine on the proliferation of human AML THP-1 cells was investigated. In the present study, it was demonstrated that virosecurinine was able to inhibit proliferation of THP-1 cells at low concentrations. The IC_{50} values were determined to be 68.128, 23.615, and

13.423 $\mu\text{mol/l}$, respectively at 24, 48 and 72 h post-treatment. The US National Cancer Institute Plant Screening Program demonstrated *in vitro* cytotoxicity of a crude extract, with a IC_{50} value of $< 20 \mu\text{g/ml}$ (919 $\mu\text{mol/l}$) following incubation between 48 and 72 h (19). Therefore, the present study exhibited the *in vitro* cytotoxic activity of virosecurinine in THP-1 cells. This result also illustrated that the proliferation of THP-1 cells was markedly inhibited by virosecurinine in a dose- and time-dependent manner. Moreover, apoptosis was also confirmed by the appearance of apoptotic bodies, and a sub-G1 peak was observed in THP-1 cells that were treated with 25 $\mu\text{mol/l}$ virosecurinine for 48 h.

Several studies demonstrated that apoptosis and autophagy are two predominant cell death routes in various types of cancer (20,21). Apoptosis or programmed cell death can be activated by anti-neoplastic drugs, which interfere with cell proliferation mediated by (22). In agreement with previous studies, the present study also demonstrated that treatment

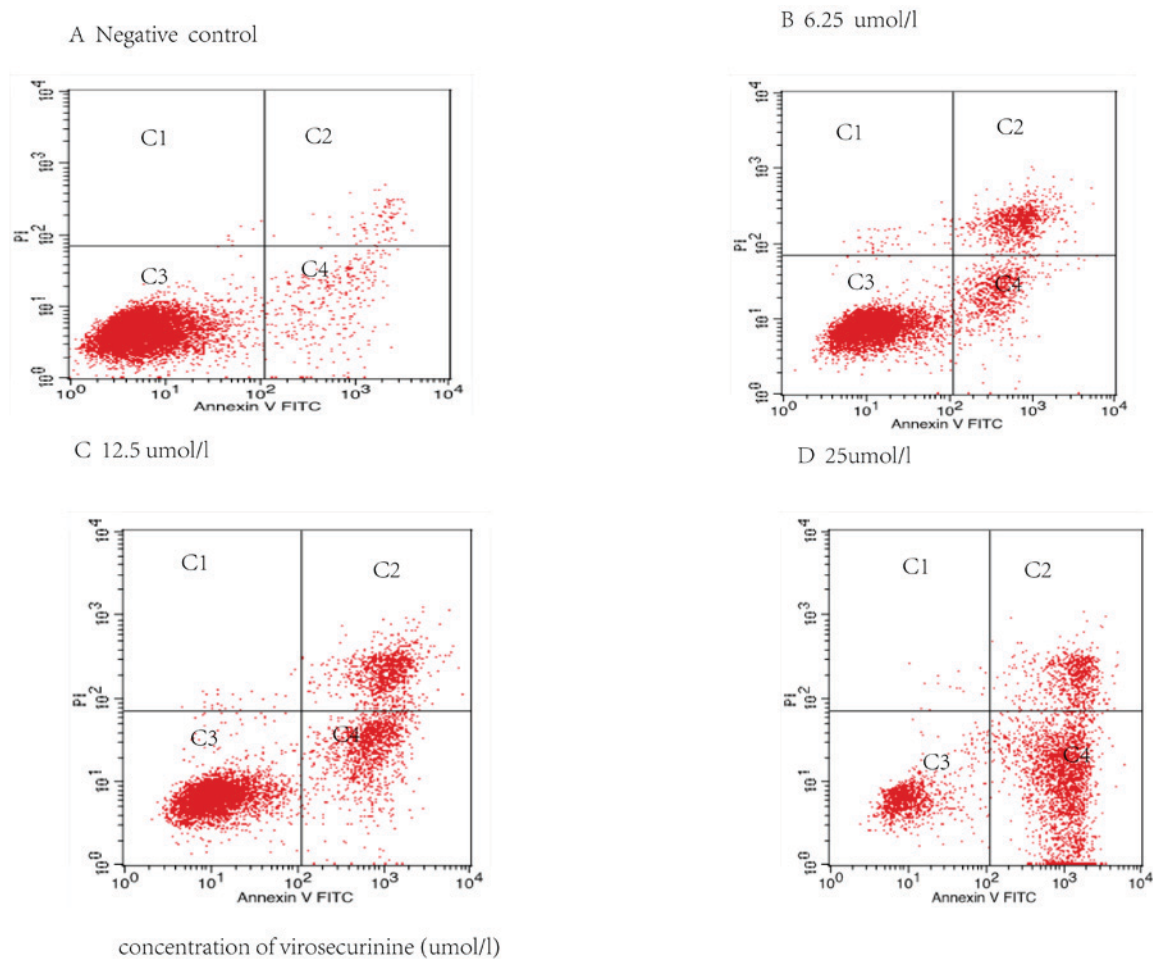


Figure 5. Flow cytometric analysis of apoptotic cells treated with virosecurinine. Apoptotic rates analyzed by fluorescence-activated cell sorting following treatment with virosecurinine. THP-1 cell. A, normal THP-1 cells; B, C and D demonstrate THP-1 cells treated with 6.25, 12.5 and 25 $\mu\text{mol/l}$ of virosecurinine for 48 h respectively.

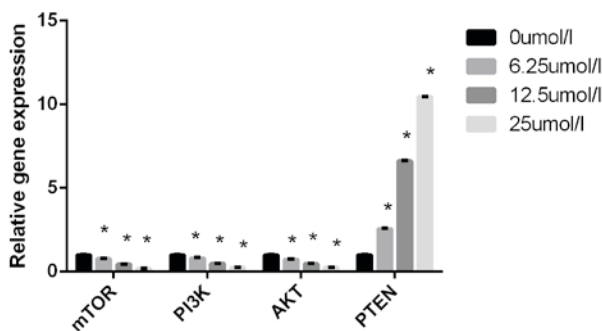


Figure 6. Reverse transcription-quantitative polymerase chain reaction analysis of gene expression of mTOR, PI3K, AKT and PTEN in THP-1 cells treated with 6.25, 12.5 and 25 $\mu\text{mol/l}$ virosecurinine for 48 h. mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homolog. * $P < 0.05$ vs. the control.

with virosecurinine was able to induce apoptosis in AML cell line THP-1 via an inhibitory effect on the cell apoptosis. The PI3K/AKT/mTOR signaling pathway is one of the major intracellular pathways, which are tightly regulated under normal physiological conditions. However, the PI3K/AKT/mTOR signaling pathway is frequently activated in human cancer types (23,24). Upon stimulation by receptor tyrosine kinases or G-protein coupled receptors, PI3K is translocated to the plasma

membrane, resulting in the phosphorylation of phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 3,4,5-triphosphate. In previous studies, dysregulation of PI3K/AKT/mTOR signaling has been observed to exhibit a vital role in the onset of various types of human cancer (25,26). This may be an innate characteristic of the signaling pathway or the consequence of mutations that are able to activate or regulate the PI3K/AKT/mTOR signaling pathway. Some of these mutations include activating mutations in Fms-like tyrosine kinase, N- or KRAS, and c-kit tyrosine kinase receptor (27-29). The phosphatase, PTEN, functions as a tumor suppressor through negative regulation of the PI3K/AKT/mTOR signaling pathway (30). Inactivation of PTEN leads to increased ATP-binding cassette transporter G2 expression, which inhibits the PI3K/AKT/mTOR signaling pathway, thereby designating it as a potential therapeutic target in the treatment of AML (31). The factors modulating PI3K and mTOR have been accentuated to function in a synergistic association with the current chemotherapeutic drugs in the treatment of AML (32,33).

To further investigate the molecular etiology, analysis of the four apoptosis-linked genes demonstrated that treatment with virosecurinine was able to downregulate PI3K, AKT and mTOR gene expression and upregulate the PTEN expression in THP-1 cells. Herein, to the best of our knowledge, it was demonstrated for the first time that virosecurinine is able to

induce apoptosis in THP-1 cells, which is regulated by altered expression of PI3K, AKT, mTOR and PTEN. These results suggest that virosecurinine is an effective agent for suppressing the proliferation of AML THP-1 cells and that this may be partially mediated by the downregulation of PI3K, AKT and mTOR and upregulation of PTEN.

In the present study, it was indicated that virosecurinine may be a potential therapeutic for the prevention and treatment of AML.

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