Antitumor activity of nervosine VII, and the crosstalk between apoptosis and autophagy in HCT116 human colorectal cancer cells

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[ABSTRACT] Nervosine VII is one of the known saturated pyrrolizidine alkaloids isolated from the plant of Liparis nervosa. This is the first study to investigate the antitumor activity of nervosine VII in vitro, and the results indicated that nervosine VII induced autophagy and apoptosis in HCT116 human colorectal cancer cells. Mechanistic studies showed that nervosine VII-induced apoptosis was associated with the intrinsic pathway by the activation of caspase-9, -3 and -7. Autophagy induced by nervosine VII was characteristic with the regulation of autophagic markers including the increase of LC3-II and beclin 1 proteins, and the decrease of p62 protein. Nervosine VII simultaneously induced autophagy and apoptosis by activated MAPKs signaling pathway including JNK, ERK1/2 and p38, suppressing the p53 signaling pathway.

(KEYWORDS) Liparis nervosa; Pyrrolizidine alkaloids; Nervosine VII; Autophagy; Apoptosis; HCT116 cells


Introduction

Since ancient times, natural products are always to be the main source of traditional medicine. Up to now, numbers of natural compounds have been discovery to have growth inhibitory effects on tumor cells. Pyrrolizidine alkaloids (PAs) are a fascinating group of secondary metabolites which most likely evolved as plant defensive chemicals under the selection pressure of herbivory. It is well known that the majority of PAs cause serious diseases in domestic animals and humans through liver bioactivation[1]. However, the saturated PAs are not hepatotoxic and must be distinguished from the 1, 2-unsaturated PAs[1-2]. More evidence presented that PAs are biologically active molecules, and have anti-tumor, antibiosis, antifeedant activities[3-4]. Liparis nervosa(Thunb.) Lindl belonging to the Orchidaceae family, was used as a medical plant widely distributed in China. L. nervosa exhibits various biological effects on inflammation, detoxication and hemostatic[5-7]. Previous investigations on chemical constituents of L. nervosa in our group have reported several pyrrolizidine alkaloids and nervogenic acid derivatives[8-12]. Nervosine VII (Fig. 1A), as a known saturated PA, was isolated from the whole plant of L. nervosa[11]. However, the antitumor potential and its underlying mechanism of action of Nervosine VII against HCT116 human cancer cells have not been elucidated yet.

Extensive evidence has indicated that the regulation of programmed cell death (PCD) is an important target in cancer chemotherapy[13]. Apoptosis, type I PCD, which is a major cytotoxic mechanism of antitumor agents, is known to be driven by two major pathways: the cell death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic)[13]. Both of those pathways are dependent upon the activation of caspas[14]. Autophagy, type II PCD, is involved in the formation of autophagosomes, and further fused with lysosomes to form acidic vesicular organelles including autolysosomes[15]. Autophagy has pro-death and pro-survival functions depending on the circumstances, and modulate autophagy to be useful for anticancer treatment. The cross-talk between autophagy and apoptosis is common in mammalian cells, and response machineries are complex, controversial, and difficult to distinguish for they can occur simultaneously, sequentially, or exclusively[16].

In the present study, the cytotoxicity of Nervosine VII on HCT116 human colon cancer cells, and its underlying molecular mechanism of action with the modulation of crosstalk between autophagy and apoptosis were investigated.

Materials and Methods

General experimental procedures

Spectra were recorded on a Bruker AV 600 NMR spec-
trometer with TMS. HR-ESI-MS were carried out on a Q-TOF micro mass spectrometer (Waters, USA). Silica gel (Qingdao Haiyang Chemical Co., Ltd., China, 200–300 mesh) were used for column chromatography (CC). The TLC plates were precoated with silica gel GF\textsubscript{254} (Qingdao Haiyang Chemical Co., Ltd., China). Bafilomycin A1 (BaF), 3-Methyl-adenine (3-MA) and other chemicals were purchased from Sigma-Aldrich (St, Louis, MO, USA). The antibodies for Bcl-2, Bax, p-ERK1/2, ERK1/2, p-p38, p38, JNK, p-JNK, LC3-II, p62, Beclin 1, and the secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

Plant material

The whole plants of \textit{L. nervosa} were collected in Zunyi, Guizhou Province (N28°14′35.48″E107°0′55.40″), China in July 2014. A voucher specimen (No. ZN361520140801) is deposited in School of Life Science and Engineering, Southwest Jiaotong University, Sichuan, China.

Isolation and identification of nervosine VII

The whole plants of \textit{L. nervosa} (14 kg) were extracted with 95\% ethanol at room temperature to obtain the ethanol extract (750 g). The extract was then suspended in H\textsubscript{2}O and extracted with petroleum ether (60–90 °C), EtOAc, and n-butanol to obtain the petroleum ether extract (260 g), EtOAc extract (160 g) and n-butanol extract (180 g) successively. The EtOAc extract (120 g) was subjected to a silica gel column eluted in a step gradient manner with CH\textsubscript{2}Cl\textsubscript{2}–CH\textsubscript{3}OH (50 : 1–0 : 1) to afford fractions (A–F) based on TLC analysis. Fraction E (7 g) was subjected to silica gel column, eluted with petroleum ether–Me\textsubscript{2}CO–Et\textsubscript{2}N (20 : 1 : 1) to yield nervosine VII (50 mg). The structure of nervosine VII was identified by comparing their spectroscopic data (\textit{\textsuperscript{1}H NMR, \textit{\textsuperscript{13}C NMR, and HR-ESI-MS}) with those reported in the literature\cite{11}.

Cell culture

\textit{HCT116} human colon cancer cell line were obtained from ATCC. These cells were maintained at subconfluence in a 95\% air and 5\% CO\textsubscript{2} humidified atmosphere at 37 °C. McCoy’s 5A medium supplemented with 5\% fetal bovine serum, 100 U·mL\textsuperscript{−1} penicillin, and 100 μg·mL\textsuperscript{−1} streptomycin was used for routine culturing.

Cytotoxicity assay

The cytotoxicity of nervosine VII against \textit{HCT116} cells were evaluated by the MTT method as described in our previous paper\cite{8}. Cells were dispensed in 96-well plates at a dens-
ity of $5 \times 10^3$ cells/mL. After 24 h incubation, cells were treated with different concentrations of nervosine VII for the indicated time periods. Cell cytotoxicity was measured by the MTT assay. Cells treated with DMSO (0.1%, V/V) were used as negative controls, whereas paclitaxel (Sigma Chemical Co., Ltd., Shanghai, China) was used as the positive control.

**Hoechst 33258 staining**

After incubation with nervosine VII for 24 h, the cells were stained with Hoechst 33258 at 37 °C for 30 min, and then the morphology was observed by fluorescence microscopy (Olympus, Tokyo, Japan)\(^{[8]}\).

**Annexin-V/propidium iodide (PI) dual staining assay**

Annexin V/PI dual staining assay was employed to determine the involvement of apoptosis in nervosine VII-induced cell death, using Annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer’s instructions\(^{[17]}\).

**CATO-ID® Autophagy detection kit staining assay**

CATO-ID® Autophagy detection kit staining assay was employed to determine the involvement of autophagy in nervosine VII-induced cell death, using CATO-ID® Autophagy Detection Kit (Roche) according to the manufacturer’s instructions.

**Western blot analysis**

The HCT116 cells were collected and lysed in lysis buffer (20 mmol L\(^{-1}\) HEPES, 350 mmol L\(^{-1}\) NaCl, 20% glycerol, 1% Nonidet P-40, 1 mmol L\(^{-1}\) MgCl\(_2\), 0.5 mmol L\(^{-1}\) EDTA, 0.1 mmol L\(^{-1}\) EGTA, 1 mmol L\(^{-1}\) DTT, 1 mmol L\(^{-1}\) PMSF, protective case inhibitor cocktail, and phosphatase inhibitor cocktail). A 20 μg aliquot of total protein was separated by 10% SDS-PAGE gel, transferred to nitrocellulose membranes, blocked with 5% BSA, and probed with a primary antibody (1 : 1000) followed by the corresponding secondary antibody (1 : 5000). The signal was detected with Amersham Imager 600 (GE Healthcare, England)\(^{[18]}\).

**Results**

**Nervosine VII exhibits antiproliferative activity against several human cancer cell lines**

To evaluate the effects of nervosine VII on the growth of human cancer cells, the growth inhibitory potential of Nervosine VII for 24 h was determined in a panel of cancer cell lines (Table 1, Figs. 1B and 1C). As shown in Table 1, Nervosine VII inhibited cancer cells growth with IC\(_{50}\) values ranging from 11.27 to 33.8 μmol·L\(^{-1}\). In addition, based on the sensitivity of the HCT116 human colon cancer cells to nervosine VII, the plausible mechanisms of action of nervosine VII were further elucidated using HCT116 cells. The HCT116 cells were treated with different concentrations of nervosine VII (6.25, 12.5, 25, 50, and 100 μmol·L\(^{-1}\) ) for 24, 48, and 72 h and cell viability was determined by MTT assay (Fig. 1D). Our results showed that nervosine VII inhibited HCT116 cells growth in a time- and concentration-dependent manner with an IC\(_{50}\) value (at 24 h) of 11.72 μmol·L\(^{-1}\).

**Nervosine VII-induced apoptotic cell death in HCT116 cells**

To determine the features of HCT116 cells death, morphological changes were observed. Compared with the control group, treatment with Nervosine VII caused significant morphological changes, including the appearance of membrane blebbing and granular apoptotic bodies (Fig. 2A). To further confirm the occurrence of apoptosis, Hoechst 33258 staining was performed, turn out the cells in the control group showed uniform dispersion of low-density fluorescence, but Nervosine VII treated cells showed condensed, bright fluorescence and nuclear fragmentation. The number of bright spots increases as the concentration of drug increases (Fig. 2B). Moreover, flow cytometric analysis with Annexin V/PI demonstrated that nervosine VII significantly induced apoptosis (Fig. 2C). As shown in Fig. 2D, the pro-apoptotic protein, Bax, was up-regulated, and the anti-apoptotic protein, Bcl-2, was down-regulated by nervosine VII. In summary, these findings indicate nervosine VII induces apoptosis in HCT116 cells.

**Induction of apoptosis by nervosine VII is associated with caspase-mediated pathway**

As we know, the modulation of programmed cell death (PCD) is an important target in cancer chemotherapy. Apoptosis (type I PCD), is highly regulated signaling pathway characterized by cell shrinkage, membrane blebbing, DNA fragmentation, and apoptotic body formation, induced by two known pathways leading to caspase activation: the extrinsic (death receptor) pathway with the activation of caspase-8 and the intrinsic (mitochondrial) pathway with the activation of caspase-3 and -9.

To further explore the molecular mechanism involved in the nervosine VII-mediated apoptosis of HCT116 cells, the effect of nervosine VII on the activation of caspases was determined by western blot analysis. The active cleavage forms of caspase-3, -8, and -9 were observed after treatment with nervosine VII. Dose-dependent activation of caspase-3 and PARP-1 cleavage were detected, both of which are key mediators of apoptosis (Fig. 3). Caspase-9 and -7 activation were observed, while the procaspase-8 and 6 protein level was not changed and no caspase-8 and 6 active form were detected. The cytosolic levels of cytochrome c were also increased in a concentration-dependent manner. These results demonstrate that nervosine VII induced intrinsic pathway-mediated apoptosis.

<table>
<thead>
<tr>
<th>cell line</th>
<th>classification</th>
<th>IC(_{50})(μmol·L(^{-1}) )</th>
<th>Nervosine VII</th>
<th>Paclitaxel*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116 colon cancer cells</td>
<td>11.72 ± 0.61</td>
<td>11.72 ± 0.61</td>
<td>0.82 ± 0.22</td>
<td>3.26 ± 0.18</td>
</tr>
<tr>
<td>786-O renal cancer cells</td>
<td>29.66 ± 2.18</td>
<td>6.3 ± 0.25</td>
<td>1.60 ± 0.33</td>
<td>2.34 ± 0.18</td>
</tr>
<tr>
<td>A549 lung cancer cells</td>
<td>33.8 ± 4.52</td>
<td>5.26 ± 0.18</td>
<td>6.3 ± 0.25</td>
<td>3.26 ± 0.18</td>
</tr>
<tr>
<td>MCF-7 breast cancer cells</td>
<td>19.8 ± 0.47</td>
<td>0.82 ± 0.32</td>
<td>1.60 ± 0.33</td>
<td>2.34 ± 0.18</td>
</tr>
</tbody>
</table>

*Paclitaxel was used as a positive control.
Nervosine VII induces autophagy in HCT116 cells.

Cyto-ID flow cytometry analysis showed that Nervosine VII significantly induced autophagy, and autophagy increased with the increase of drug concentration (Fig. 4A). To confirm nervosine VII-induced autophagy, western blot analysis was used to detect autophagy biomarkers. Accumulation of microtubule-associated protein light chain 3 (LC3)-II and beclin 1 was detected in HCT116 cells after 24 h treatment with nervosine VII in a concentration-dependent manner. Treatment with nervosine VII also down-regulated the expression of p62 in HCT116 cells, indicating the presence of autophagy induced by nervosine VII (Fig. 4B). In addition, LC3-II accumulation was further enhanced by cotreatment with nervosine VII and bafilomycin A1 (BaF), indicating that nervosine VII induced autophagosome accumulation by increasing autophagic flux, rather than by blocking autophagic degradation (Fig. 4C). Taken together, these findings suggested that treatment with nervosine VII for 24 h induces autophagy in HCT116 cells.

Nervosine VII induces the switch from autophagy to apoptosis in HCT116 cells

The time-dependent expression of autophagy- and apoptosis-specific proteins after treatment with 10 μmol·L⁻¹ nervosine VII was determined to confirm whether nervosine VII induces autophagy and apoptosis sequentially. As a result, the induction of autophagy by nervosine VII, as indicated...
Fig. 3 Effect of nervosine VII on the expression of apoptosis-related proteins in HCT116 cells. HCT116 cells were cultured with 10, 20 and 30 μmol·L⁻¹ of nervosine VII for 24 h. The activation of caspase-8, caspase-9 and caspase-3, caspase-6, caspase-7 and PARP, and cytochrome c was determined by western blot analysis. GAPDH was used as an internal standard. The data are presented as the mean ± SD of the results from three independent experiments.

Fig. 4 (A) The cells were treated with Nervosine VII (10, 20 and 30 μmol·L⁻¹) for 24 h, then revealed by CYTO-ID® Autophagy detection kit staining using flow cytometry analysis. (B) HCT116 cells were cultured with the indicated concentrations of nervosine VII for 24 h. The protein expression levels of autophagy markers were determined by western blot analysis. GAPDH was used as an internal standard. (C) Cells were treated with 10 μmol·L⁻¹ nervosine VII or 2.5 nmol·L⁻¹ BaF for 24 h. The protein expression levels of LC3-II were detected by western blot analysis. GAPDH was used as an internal standard. The data are presented as the mean ± SD of the results from three independent experiments.
by the expression of LC3-II, and beclin 1, was gradually increased and reached a peak at 24 h, but the activation of apoptosis by nervosine VII, as indicated by the levels of cleaved caspase-9, continued up to 48 h simultaneously (Fig. 5A). These data suggest that the induction of autophagy and apoptosis by nervosine VII is not associated with a successive process but a simultaneous approach in an early stage, and the prolonged treatment with nervosine VII induces the switch from autophagy to apoptotic cell death. To confirm the role of autophagy in nervosine VII-induced cell death, 3-methyladenine (3-MA), a specific autophagy inhibitor, was used to block acidic vacuole formation. HCT116 cells were pretreated with 2 mmol·L\(^{-1}\) 3-MA for 1 h followed by a 24 h treatment with 10 μmol·L\(^{-1}\) nervosine VII. Although the nervosine VII-induced LC3-II accumulation was suppressed by 3-MA, the levels of cleaved PARP were increased in cells co-treated with nervosine VII alone (Fig. 5B). These findings suggested that inhibition of autophagy by 3-MA potentiates Nervosine VII-mediated apoptotic cell death in human colorectal cancer cells.

**Effect of the MAPK signaling pathway on nervosine VII-induced apoptosis and autophagy**

MAPKs signaling pathway consisting of ERK1/2, p38 MAPK, and JNK have also been considered as chemotherapeutic targets for sensitizing cancer cells to autophagy and apoptosis. To determine whether MAPK signaling pathway was closely related to nervosine VII-induced autophagy and apoptosis, we treated HCT116 with nervosine VII and then detected the levels of p-ERK1/2, p-JNK and p-p38. As shown in Fig. 6A, Nervosine VII activated the phosphorylation of ERK1/2, p38 and JNK in a dose-dependent manner.

**Effect of p53 on nervosine VII induced apoptosis and autophagy**

P53 is linked to autophagy via inhibition of mTOR by AMPK, TSC1, and TSC2, and plays an important role in the transcriptional regulation of autophagy-related target genes. The effect of nervosine VII on the expression levels of p53

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**Fig. 5** Effect of Nervosine VII on the modulation of crosstalk between autophagy and apoptosis. (A) HCT116 cells were cultured with 10 μmol·L\(^{-1}\) of Nervosine VII for 0, 6, 12, 24, 36 and 48 h, and the protein expression levels of autophagy marker (LC3-II and beclin 1) and apoptosis-related proteins (cleaved caspase 9) were determined by western blot analysis. GAPDH was used as an internal standard. (B) Cells were treated with 2 mmol·L\(^{-1}\) 3-MA for 1 h and then co-incubated with nervosine VII for an additional 24 h. Western blot analysis was performed for LC3-II and cleaved PARP. GAPDH was used as an internal standard. The data are presented as the mean ± SD of the results from three independent experiments.
was further examined (Fig. 6B). Nervosine VII downregulated the expression levels of p53 in a dose-dependent manner.

**Discussion**

Nervosine VII is a naturally occurring saturated PA from *L. Nervosa*, and has been reported that it has no significant cytotoxicity to A549 (human lung adenocarcinoma epithelial cell line), MCF-7 (human breast adenocarcinoma cell line) and 786-0 (renal clear cell adenocarcinoma cells). However, its antitumor potential and underlying mechanism against HCT116 cells have not been explored yet.

Apoptosis, is highly regulated by the family of caspases characterized by cell shrinkage, membrane blebbing, DNA fragmentation, and apoptotic body formation. An increasing number of studies have suggested that the activating the apoptosis in tumor cells is an effective method of chemotherapy. Autophagy, another programmed cell death process, has two effects on tumors, which can inhibit apoptosis by promoting cell survival, or cooperated with apoptosis to induce cell death. A multitude of antitumor agents has been shown to induce autophagy in different types of tumor cells, and induction of autophagy in tumor cells can be therapeutically useful to evade chemo-resistance.

Both apoptosis and autophagy are high important in cellular degradation pathways that are often induced or regulated by similar signal pathways. Up to now, natural products are a rich source for the novel natural anticancer agents. The present study indicated that nervosine VII exhibited efficient cytotoxic effects on HCT116 cells by the activation of both apoptosis and autophagy, which coexist and promote each other in HCT116 cells.

The present study demonstrated that nervosine VII has antitumor activities against human colorectal cancer in vitro, and the cell death induced by nervosine VII was due to apoptosis, which was associated with the modulation of the intrinsic pathway by the release of cytochrome c from the mitochondria to activate caspase-9, thereby promoting the activation of caspase-3 and -7.

Autophagy plays the different roles in cells, the function as a protective mechanism a survival pathway, or may induce autophagic cell death. Western blot analysis was used to detect autophagy biomarkers to confirm nervosine VII-induced autophagy. The results showed nervosine VII significantly upregulated the expression of both LC3-II and beclin 1 protein, and downregulated the levels of p62 protein, which...
demonstrated that nervosine VII induced autophagy in HCT116 cells.

The time-dependent expression of autophagy- and apoptosis-specific proteins suggest that the induction of autophagy and apoptosis by nervosine VII is not associated with a successive process but a simultaneous approach in an early stage, and the prolonged treatment with nervosine VII induces the switch from autophagy to apoptotic cell death. 3-Methyladenine (3-MA), a specific autophagic process, was used in autophagy studies. Our study found LC3-II accumulation was suppressed by 3-MA, the levels of cleaved PARP were upregulated in cells cotreated with nervosine VII and 3-MA compared to cells treated with nervosine VII alone, indicating that inhibition of autophagy with 3-MA enhanced cell death.

We further investigated the roles of PI3K/Akt, MAPKs, and p53 pathways on the interactions of the apoptosis and autophagy processes. The MAPKs signal, which includes three main family members p38, JNK and ERK, is involved in inducing both autophagy and apoptosis\[22\]. The results showed that nervosine VII upregulated the phosphorylation of ERK1/2, JNK and p38 in a dose-dependent manner. Accumulating evidence has suggested that the effects of JNK and p38 were consistent on both of apoptosis and autophagy. JNK and p38 regulate the expression level of Bcl-2 and Bax in apoptotic cells. JNK and p38 are able to initiate autophagy by targeting Bcl2/BclXL and abrogating their binding to beclin 1, and activated JNK and p38 are contribute to the accumulation of LC3-II. Numerous of studies\[??\] have suggested that ERK plays a role in modulating autophagy, and the activation of ERK1/2 pathways induces autophagy.

The tumor suppressor p53 plays an important role in cell cycle arrest and apoptosis, and p53 mutations are linked to the pathology of most cancer cells\[23\]. In theory, activation of p53 is beneficial in inhibiting tumor growth. However, in the current study, nervosine VII downregulated the expression levels of p53 in a dose-dependent manner. This may be due to the influence of other signaling pathways. The interpretation of this result requires further experimental confirmation in our future studies.

In summary, this study demonstrated that nervosine VII simultaneously induced apoptosis and autophagy by activation the ERK1/2, JNK and p38 pathway, and suppressing the p53 signaling pathway. Moreover, nervosine VII induced apoptosis by activating the intrinsic pathway by the release of cytochrome c from the mitochondria to activate caspase-9, thereby promoting the activation of caspase 3, and PARP cleavage. A schematic diagram of these observed effects of nervosine VII is shown in Fig. 7. Those finding indicate the nervosine VII may be a novel therapeutic method for the treatment of cancer.

**References**


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