

Apoptosis induced by yessotoxins in HeLa human cervical cancer cells *in vitro*

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Abstract. To investigate the apoptotic effects of an extract of red-tide algae, *Protoceratium reticulatum*, on HeLa cells, we used liquid chromatography-tandem mass spectrometry, optical microscopy, Hoechst 33342/propidium iodide staining, and DNA gel electrophoresis to analyze its constituents and toxicity, as well as rhodamine 123 staining to investigate changes in mitochondrial membrane potential. Analysis showed that the *P. reticulatum* extract contained the yessotoxins (YTXs) homo-YTX, 45-OH-YTX and 45-OH-homo-YTX. The results indicated that *P. reticulatum* extract negatively influences HeLa cells and induces their apoptosis.

Introduction

Yessotoxins (YTXs) are sulfated polyether compounds (1) produced by the dinoflagellates *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum* (2,3). Analogue compounds of YTXs are a concern in seafood quality monitoring and the shellfish industries. YTXs were

first isolated and identified from the scallop *Patinopecten yessoensis* at Mutsu Bay in Japan in 1987 (1). Over the past two years, YTXs have been detected in shellfish around the world, including the coastal cities of China (4-9).

YTXs were initially classified as a diarrhetic shellfish poisoning (DSP) toxin since they were usually extracted with DSP toxins such as okadaic acid (OA) and dinophysins (DTXs). However, subsequent research found that, unlike OA and DTXs, YTXs do not induce diarrhea (1,10), nor do they inhibit protein phosphatase 2A activity (10). The structure of YTXs was shown to be similar to that of brevetoxins and ciguatoxins (11). In 2002, the European Union Commission formally delisted YTXs as a DSP toxin (12). However, YTXs may accumulate in shellfish and are a health concern for human consumption (13).

YTXs are mainly produced by the dinoflagellates *P. reticulatum*, *G. spinifera* and *L. polyedrum* (2,3). *P. reticulatum* is found worldwide and was the first biological source reported to produce YTXs (2). *L. polyedrum* and *G. spinifera* have been identified in several European countries and in the US (7,14-16). In China, *P. reticulatum*, *G. spinifera* and *L. polyedrum* have been found in the silt of the Yellow Sea, and *G. spinifera* has been found in the offshore areas of Shandong and Zhejiang provinces.

The toxic effects of YTXs have been studied in different contexts. YTXs administered through ingestion or intraperitoneal injection induce cardiotoxic effects in mice (10,17-19). At the molecular level, YTXs have been found to induce apoptosis *in vitro* (20,21). Apoptosis, sometimes called 'cell suicide', is a complex process involving a series of morphological and biochemical changes in cells, including DNA fragmentation, chromatin condensation, apoptotic body formation, membrane blebbing and cell shrinkage (22-24). The analysis of apoptosis can be used to determine the cytotoxic activity of biotoxins.

In this report, YTX-induced apoptosis as well as morphological and biochemical changes were investigated in a HeLa cell line. The study was undertaken to elucidate the apoptotic activity of *P. reticulatum* extract and to provide an analysis of *P. reticulatum* production. It is the first study in China to focus on the cytotoxic activity of YTXs in *P. reticulatum* extract (PRE).

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Abbreviations: DMSO, dimethyl sulphoxide; DSP, diarrhetic shellfish poisoning; DTX, dinophysins toxins; FCS, fetal calf serum; *G. spinifera*, *Gonyaulax spinifera*; HPLC, high-performance liquid chromatography; *L. polyedrum*, *Lingulodinium polyedrum*; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MTP, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRC, National Research Council; OA, okadaic acid; *P. reticulatum*, *Protoceratium reticulatum*; PBS, phosphate-buffered saline; PI, propidium iodide; PRE, *P. reticulatum* extract; Rh123, rhodamine 123; RNase A, ribonuclease A; YTX, yessotoxin

Key words: yessotoxins, apoptosis, HeLa cells

Materials and methods

Sources of toxins

Toxin standards. OA and YTX standards for the cytotoxic experiments were purchased from the NRC (National Research Council, Canada) and dissolved in RPMI-1640 medium to a final concentration of 2 ng/ μ l.

Cell culture. A YTX-producing strain of *P. reticulatum* was purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (USA). The strain was adapted in a silicate-free L1 medium prepared from filtered seawater (25). All cultures were inoculated with 500 cells/ml in 2,000 ml borosilicate glass bottles containing 1,800 ml of the same medium as stock. The inocula were maintained in thermostated chambers at 20°C in Erlenmeyer flasks. A 12:12 h light/dark cycle was used with an average irradiance on the surface of the culture. Lighting was provided by cool white fluorescent lamps (30 W).

Extraction of yessotoxins. The *P. reticulatum* cultures were filtered using a 0.45- μ m cellulose acetate membrane and immersed in the extraction solvent (80% methanol, v/v). The sample was then vortexed for 1 min, homogenized in an ultrasonic bath for 10 min, soaked for 30 min and washed again in an ultrasonic bath for 10 min. The sample was then centrifuged for 10 min at 3,000 rpm and filtered with single-use syringe filters (0.45- μ m, polypropylene). For the HeLa cell experiments, the extract was further purified using a solid phase extraction column.

Cell culture and treatment. Human cervical cancer HeLa cells were donated by the Institute of Oceanology, CAS (Chinese Academy of Sciences, Qingdao, China) and seeded in 25 cm² flasks. HeLa cells were cultured in RPMI-1640 medium (Gibco, USA) with 300 mg/l L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Fetal calf serum (FCS) (TBD, China) (15%) was added to the medium. Cells were maintained at 37°C until 80-90% confluence was reached. For microplate assays, the split cells were trypsinized after reaching optimum confluence, and the concentration of the culture was adjusted to 1x10⁴ cells/ml. Following an additional incubation of 48 h at 37°C, cells grown in microplates were used for the microplate assays.

Cell proliferation assay. The inhibition of cell proliferation by PRE was detected by 3-(4,5)-dimethylthiazol-2-yl)-3,5-diphenyltetrazolium bromide (MTT). Cells were placed in microplates at an initial density of 1x10⁴ cells/well. Various concentrations of PRE were added to each well, then the cells were incubated at 37°C for 44 h. The control group was treated simultaneously with solvent only. Following the initial incubation, MTT solution was added, and samples were further incubated for 4 h at 37°C. The absorbance at 490 nm was read by an ELISA reader following the addition of dimethyl sulphoxide (DMSO, 150 μ l/well) as a stop solution. The inhibition ratio (%) was calculated using the equation: inhibition ratio (%) = (1 - A_{treatment}/A_{control}) x 100%.

Morphological changes. Morphological changes in the HeLa cell groups treated with 50 ng/ml of the standards and various concentrations of PRE were monitored under an inverted light

microscope. After 24 h of treatment, cells were stained with Hoechst 33342 and PI for 20 min, then washed with phosphate-buffered saline (PBS). Cell nuclei were then visualized using a UV fluorescence microscope (Nikon, Japan) to detect morphological evidence of apoptosis.

DNA fragmentation. The presence of a 'ladder-like' DNA fragmentation pattern typical of apoptotic cells was evaluated after 48 h of exposure to medium containing 50 ng/ml of the toxin standards or extract at a volume of 3, 6 or 9% (6, 12 or 18 μ l in 200 μ l). There was also a blank group with no additive and a positive control using H₂O₂. To differentiate the effect of the solvent, an 80% (v/v) methanol group was added using the same concentration as the maximum dose level of the extract.

Both floating and attached cells in medium were collected, washed in PBS and resuspended in 50 μ l lysis buffer, 20 mM EDTA, 100 mM Tris/HCl (pH 8.0) and 0.8% (w/v) sodium dodecyl sulfate. After treatment with RNase A (1 mg/ml, 10 μ l) at 37°C for 1 h, DNA samples were added to 10 μ l proteinase K (20 mg/ml) and incubated at 55°C for at least 3 h. Electrophoresis of the extracted DNA, to which a 10X loading buffer had already been added, was carried out on 2.0% agarose gels; a 200-bp DNA marker was used to compare the size of the DNA fragments. DNA bands were visualized after ethidium bromide staining (Molecular Probes Inc.).

Mitochondrial membrane potential. Mitochondrial transmembrane potential (MTP) was measured using flow cytometry with the fluorescent probe rhodamine 123 (Rh 123). Rh 123 accumulates in normal mitochondrial due to its high negative charge; loss of MTP leads to the release of Rh 123 and a reduction in its fluorescence intensity. Before treatment, Rh 123 was diluted in PBS to a final concentration of 10 mg/l. Treated cells were incubated for 8, 16, 24, 32, 40 or 48 h. After treatment, ~10⁶ cells were collected by trypsinization, washed twice in PBS and incubated with Rh 123 for 30 min at 37°C in the dark. The samples were analyzed by flow cytometry.

Analysis of the extract by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using an Agilent 1200 high-performance liquid chromatography (HPLC) system with a G1311A binary pumping system and a G1329A automated injector coupled to an API4000 mass spectrometer with Turbo Spray interface. The column (100 x 4.6 mm) was packed with 3.5- μ m Agilent Zorbax SB-C18 and maintained at 20°C. The extract was analyzed in the negative mode.

The YTX standard was used as the calibration standard (200 ng/ml), and was prepared by appropriate dilution with methanol and stored at -80°C.

The mobile phase consisted of acetonitrile and water arranged in different proportions as solvent A (10:90, v/v) and solvent B (90:10, v/v), both containing 53 mM formic acid and 5 mM ammonium formate. Gradient elution was carried out according to the following program: min 0-5, 100% A; min 5-10, 40% A/60% B; min 10-20, 100% B; min 20-45, 100% A. The flow rate was 0.3 ml/min, and 20 μ l of the sample was injected into the column at room temperature. The exact mass spectrometer conditions of the YTXs are listed in Table I.

Table I. Mass spectrometer conditions of the yessotoxins.

Toxin	Mode	MRM transition ($m/z > m/z$)	DP	FP	CE
YTX	Negative	1141.5>1061.5	-12	-180	-45
Homo-YTX	Negative	1155.5>1075.5	-12	-180	-45
45-OH-YTX	Negative	1157.5>1077.5	-12	-180	-45
45-OH-homo-YTX	Negative	1171.5>1091.5	-12	-180	-45

CE, collision energy; DP, declustering potential; FP, focusing potential; MRM, multiple reaction monitoring; YTX, yessotoxin.

Results

Antiproliferative activity of the extract. To investigate the inhibitory effect of PRE on HeLa cells, an MTT assay was conducted. HeLa cells were treated with PRE dissolved in 80% methanol and the same volume of solvent as a control. As shown in Fig. 1, PRE inhibited the growth of HeLa cells in a concentration-dependent manner compared to the controls. In order to reduce the effect of 80% methanol, the extract used in the subsequent experiment did not exceed 10%.

Morphological changes. Morphological changes were observed under a microscope. The cells of the untreated control group and the group treated with 80% methanol underwent rapid growth, and their nuclei were round and clear. Cells in the positive control group treated with 0.3% H₂O₂ were irregularly shaped; some cells were broken with no content, while the nuclei were marginalized in other cells. Morphological changes characteristic of apoptosis were noted in cells treated with 50 ng/ml of the toxin standards and with the different PRE concentrations for 48 h (Fig. 2).

Hoechst 33342-positive cells were defined as being in early-stage apoptosis, while PI-positive cells were determined to be in late-stage apoptosis or even necrosis. Upon fluorescent microscopy, the HeLa cells treated with the standards and the indicated concentrations of the extract for 24 h presented the typical features of early-stage apoptosis. Bright nuclear condensation was noted by Hoechst 33342 staining, and apoptotic bodies were observed as masses in the cells (Fig. 3). The apoptotic and total cells in each field of view were counted, and the ratio of apoptotic cells was calculated (Fig. 4). The numbers of broken and necrotic cells increased in conjunction with increasing concentrations of PRE.

DNA fragmentation. Nuclear DNA fragmentation was examined using agarose gel electrophoresis. As shown in Fig. 5, DNA laddering was noted 48 h after the HeLa cells were exposed to various concentrations of PRE and the toxin standards.

Assessment of changes in mitochondrial membrane potential. As shown in Fig. 6, the MTP of the treated cells decreased with increasing time. When the cells were incubated with the YTX and OA standards or with 3% PRE for 8 h, the MTP began to decrease; 32 h after incubation, the decrease in MTP had not progressed further, indicating that the standards and PRE affected MTP within the first 32 h.

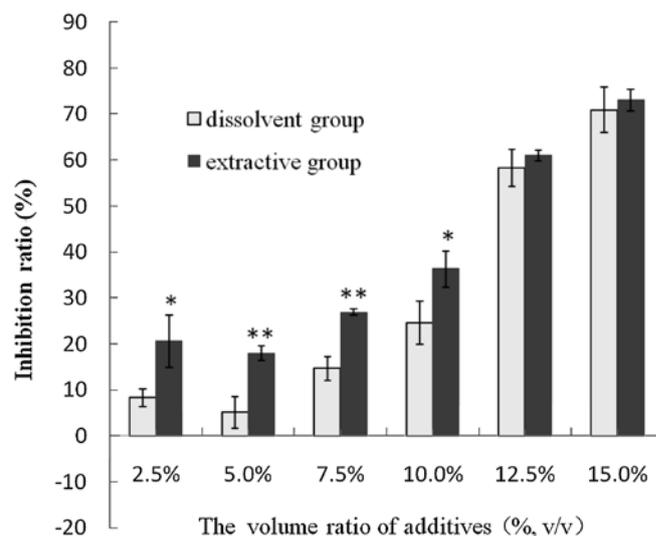


Figure 1. Inhibitory effect of the extract on the viability of HeLa cells in comparison with the dissolvent group. *P<0.01.

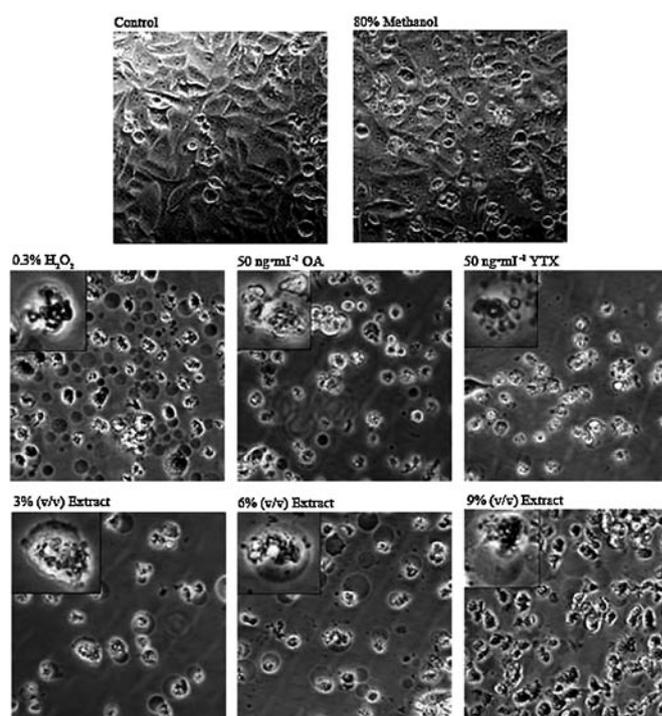


Figure 2. Morphological changes in HeLa cells treated with the standards and different concentrations of extract for 48 h observed with an inverted microscope (x200).

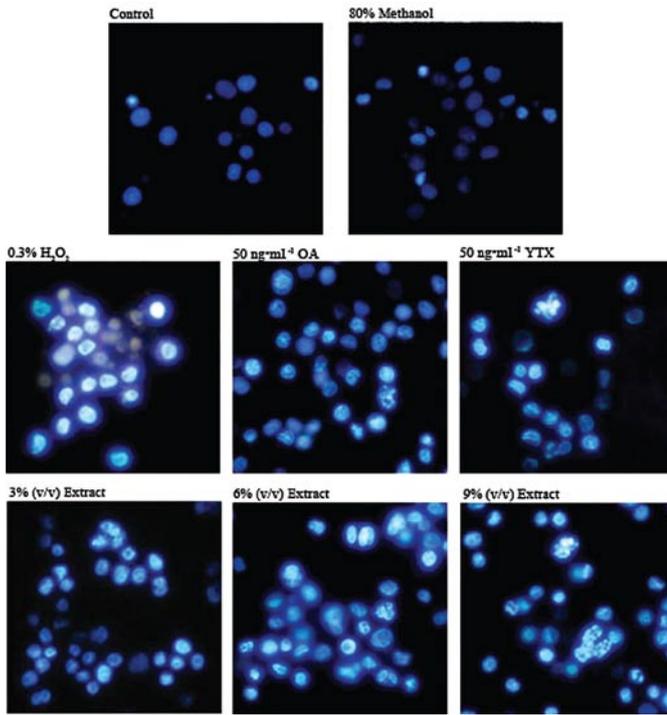


Figure 3. Fluorescent staining (Hoechst 33342 and PI) of nuclei in toxin-treated or untreated cells (x200).

Analysis of extract by LC-MS/MS. The extract was quantified by comparison with an authentic standard of YTX. The assumption was made that closely related analogues of homo-YTX and derivatives such as 45-OH-YTX, 45-OH-homo-YTX would give the same responses as the parent toxins. Calibration curves from the same quantitative standard were therefore applied to estimate the concentration of the respective related compounds.

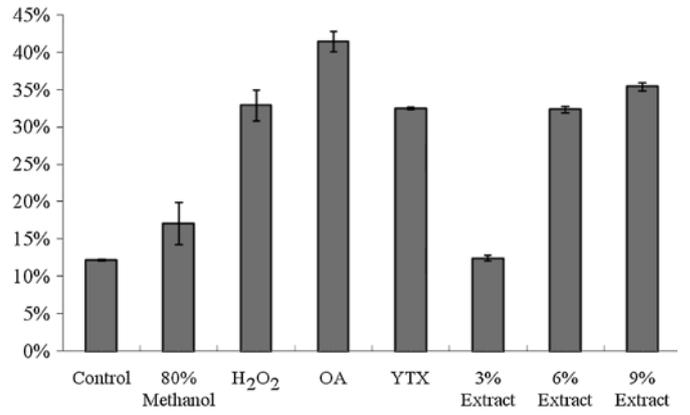


Figure 4. Ratio of apoptotic cells in the treatment groups.

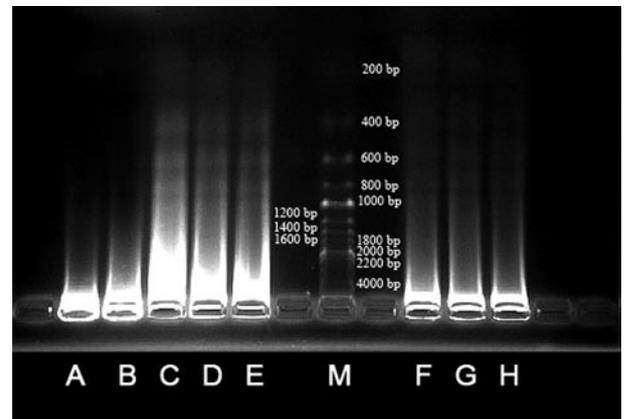


Figure 5. DNA fragmentation measured by ethidium bromide staining. (A) Control, (B) 80% methanol, (C) 0.3% H₂O₂, (D) 50 ng/ml OA, (E) 50 ng/ml YTX, (M) 200-bp DNA ladder marker, (F) 3% (v/v) extract, (G) 6% (v/v) extract, (H) 9% (v/v) extract.

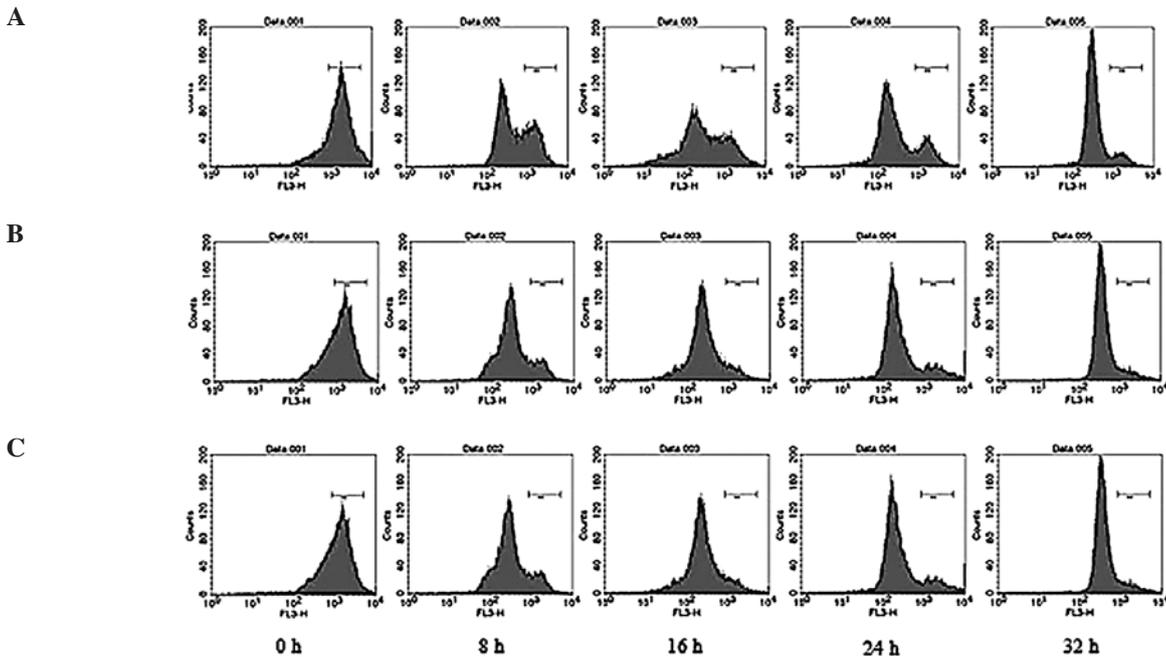


Figure 6. Changes in the mitochondrial membrane potential of HeLa cells treated with OA standard (A), YTX standard (B) or 3% extract (C) for different time periods.

Cultured *P. reticulatum* cells were extracted by sonicating with methanol. The crude extract was directly analyzed by LC-MS/MS. Although homo-YTX, 45-OH-YTX and 45-OH-homo-YTX were detected in the resulting PRE, the amount of YTX was too small to detect. In the study, 4.5×10^7 *P. reticulatum* cells were collected, compared with the YTX standard (200 ng/ml), and analyzed; the products of *P. reticulatum* included 2.88 pg/cell for homo-YTX, 0.842 pg/cell for 45-OH-YTX and 0.0681 pg/cell for 45-OH-homo-YTX.

Discussion

In this study, we showed that apoptotic events were induced by PRE in a cell-specific manner in HeLa human cervical cancer cells, and compared these effects with those of OA and YTX standards. After treatment with various PRE concentrations, the morphology of the cells showed typical characteristics of apoptosis, including cell shrinkage, nuclear fragmentation and chromatin condensation.

To determine the extent of apoptosis in HeLa cells, Hoechst 33342 and PI staining were used to observe morphological changes in HeLa cells treated or not with the toxins. Hoechst stains are fluorescent stains used for labeling DNA in fluorescence microscopy and for fluorescent-activated cell sorting (FACS), and can be used on live or fixed cells (26). Since Hoechst 33342 binds to DNA, it disrupts DNA replication during cell division. PI is an intercalating agent and fluorescent molecule that is used to stain DNA. It can be used to differentiate necrotic, apoptotic and normal cells. PI readily enters and stains non-viable cells, but cannot cross the membrane of viable cells. In the present study, in HeLa cells treated with the toxin standards or various concentrations of the extract for 48 h then stained with Hoechst 33342 and PI, morphological alterations characteristic of early-stage apoptosis were observed, including chromatin condensation and nuclear fragmentation. However, few cells were stained by PI, indicating that most of the treated cells were in the early stages of apoptosis.

Changes in mitochondrial membrane permeability transition are a common and crucial mechanism in cell death (27). It has been found that MTP is an early event in apoptosis (28-30). In most models of apoptosis, the disruption of MTP often precedes nuclear DNA degradation (31-34). In our study, the toxin standards and the extract decreased MTP, indicating the initiation of apoptosis in treated cells. It has been reported that YTXs induce the accumulation of Ca^{2+} . We previously investigated YTX toxicity in Purkinje cells from the cerebellum of ICR mice, and showed a more positive response to YTX by immunohistochemical staining of the calcium-binding protein S-100. Further investigation of the mechanisms behind the effect of YTX are planned by our group.

In the marine biotoxin field, LC-MS is becoming the prime technology for improved screening due to its high sensitivity and specificity (35-37). Tandem mass spectrometry (LC-MS/MS) has been developed to detect marine biotoxins in plankton and shellfish. In the present study, the retention time and mass spectra of each peak were compared with those of individual reference samples injected under the same experimental conditions. The YTX content of PRE was also determined by comparison with an individual standard solu-

tion of YTX injected under the same experimental conditions. As analyzed by LC-MS/MS, YTX levels in the PRE were too low to be detected. Although there were no appropriate standards for YTX analogues, the assumption was made that the closely related analogue, homo-YTX, and derivatives such as 45-OH-YTX and 45-OH-homo-YTX gave the same molar response as YTX. As a result, the YTX analogue content in the extract of *P. reticulatum* was calculated as 2.88 pg/cell for homo-YTX, 0.842 pg/cell for 45-OH-YTX and 0.0681 pg/cell for 45-OH-homo-YTX. Different strains of *P. reticulatum* have different characteristics and products. The strain used in the present study produced low levels of YTX but high levels of homo-YTX and 45-OH-YTX. Apoptosis was observed in HeLa cells after incubation with PRE. However, subsequent studies planned by our group of *P. reticulatum* in China are required to confirm the toxicity of these compounds.

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