Yessotoxin induces apoptosis in HL7702 human liver cells

MIN PANG[1], PEI QU[2], CHUN-LEI GAO[1] and ZONG-LING WANG[1]

[1]The First Institute of Oceanography, State Oceanic Administration, Qingdao 266061;
[2]College of Environmental Science and Engineering, Ocean University of China, Qingdao 266100, P.R. China

Received June 23, 2011; Accepted September 21, 2011

DOI: 10.3892/mmr.2011.606

Abstract. The marine toxin yessotoxin (YTX) is found in numerous aquatic environments and poses a potential threat to the shellfish industry and to public health. We analyzed the toxicity of YTX on HL7702 human liver cells using optical microscopy, Hoechst 33342 chromatin staining, DNA gel electrophoresis, rhodamine 123 staining and calcium-sensitive laser scanning confocal microscopy. The results demonstrated that YTX induced the usual hallmarks of apoptosis, including chromatin condensation, DNA laddering, activity of caspase-3 deregulation and loss of mitochondrial membrane potential. Furthermore, YTX caused cytosolic calcium levels to increase in HL7702 cells. YTX may cause liver damage through hepatocyte apoptosis.

Introduction

Yessotoxin (YTX) is a disulfated polyether lipophilic marine toxin that was first isolated from the digestive organs of scallops in Japan (1). Since YTX and its analogs were always detected together with diarrhetic shellfish poisoning (DSP) toxins, such as okadaic acid (OA), they were initially classified in the DSP toxin group. However, YTX does not induce diarrhea, nor does it inhibit protein phosphatase 2A, such as OA or certain dinophysistoxins (DTXs) (2). In 2002, YTX and its analogs were removed from the DSP toxin group by the European Union Commission and classified as an independent group (3).

Correspondence to: Dr Min Pang, The First Institute of Oceanography, State Oceanic Administration, Qingdao 266061, P.R. China
E-mail: pm_cat@hotmail.com

Abbreviations: AM, acetoxymethyl ester; DMSO, dimethyl sulphoxide; DSP, diarrhetic shellfish poisoning; DTXs, dinophysistoxins; EB, ethidium bromide; FACS, fluorescent-activated cell sorting; FCS, fetal calf serum; HBSS, Hank's balanced salt solution; LSCM, laser scanning confocal microscopy; MTP, mitochondrial transmembrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRC, National Research Council; OA, okadaic acid; pNA, p-nitroanilide; Rh123, rhodamine 123; RNase A, ribonuclease A; SMACs, second mitochondria-derived activator of caspases; YTX, yessotoxin

Key words: yessotoxin, apoptosis, HL7702 cells, mitochondrial transmembrane potential, calcium level

YTX and its analogs are mainly produced by the algal dinoflagellates *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum* (4-6). *Protoceratium reticulatum* was the first reported producer of YTX and it has been identified in numerous countries around the world (4). The species *Gonyaulax spinifera* and *Lingulodinium polyedrum*, which may also produce YTX, have been identified in certain European and American countries (4,7-11). Recently, the cysts of *Protoceratium reticulatum*, *Gonyaulax spinifera* and *Lingulodinium polyedrum* have been identified in the silt of the Yellow Sea (12). The species *Gonyaulax spinifera* has also been found in offshore areas of Shandong and Zhejiang Province. In 2010, Gao *et al* detected YTX and its analogs in shellfish samples collected from Chinese coastal areas (13). Thus, YTX and its analogs are of major concern to the shellfish industry in China.

Although to date there are no reports on toxic episodes caused by YTX-contaminated seafood consumption, high toxicity has been revealed following intraperitoneal injection in mice in a number of studies (14-17). The maximum acceptable YTX contents (1 mg/kg shellfish flesh) have been established by quality monitoring agencies (3). However, YTX and its analogs do not induce diarrhea and no lethality was found in mice following oral exposure to high doses of YTX (1-54 mg/kg) or repeated oral administration (1 and 2 mg/kg/day, for 1 week; 1-5 mg/kg, 7 times during 3 weeks) (17-19). Numerous *in vitro* studies have been carried out at the single-cell level to investigate the mechanisms of YTX toxicity. The chemical structure of YTX is similar to that of the neurotoxic brevetoxins, and interactions between YTX and transmembrane ion channels have been studied (20,21). Furthermore, YTX has been identified as an inducer of apoptosis in the BE (2)-M17 neuroblastoma cell line (22), in primary neuronal cultures (21), in myoblast cell lines from rat and mouse (23,24) and in the HeLa human cervical cancer cell line (25). However, the exact molecular mechanisms remain unclear.

Apoptosis is a normal event in the development and stress response of multicellular organisms. To adequately study apoptosis, it is necessary to combine experimental techniques to assess the temporal progression of multiple apoptotic processes, including DNA fragmentation, calcium deregulation, activation of caspases and decreased mitochondrial transmembrane potential. Bianci *et al* assessed mitochondrial depolarization in hepatoma cells isolated from the livers of male Wistar rats following YTX treatment (26), but the mechanism of action of YTX in liver cells has not been investigated in

detail. In terms of solubility, YTX is described as a lipophilic polyether. Since lipophilic drugs are typically metabolized in the liver, we chose a human liver cell line (HL7702) to evaluate the cytotoxicity and pro-apoptotic responses induced by YTX.

Materials and methods

Chemicals and solutions. YTX was purchased from the National Research Council (NRC; Canada), and it was stocked in methanol. An apoptosis and necrosis assay kit, including a fluorescence DNA probe Hoechst 33342, was purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rhodamine 123 (Rh 123) and Fluo-3 acetoxymethyl ester (AM) solution (5 mM) were also purchased from Beyotime.

The vital stain 3-(4,5)-dimethylthiazol-2-y1)-3,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St. Louis, MO, USA). Nifedipine was from Sigma and dissolved in DMSO. Other chemicals were of analytical reagent grade or higher purity. Hank's balanced salt solution (HBSS) for cell washing consisted of: NaCl 137 mM; KCl 5.6 mM; $CaCl_2$ 1.26 mM; $MgSO_4$ 0.81 mM; Na_2HPO_4 0.38 mM; KH_2PO_4 0.44 mM and $NaHCO_3$ 4.2 mM. pH was adjusted to 7.4 with NaOH and HCl.

Cell culture. The HL7702 human liver cell line was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were seeded in 25-cm^2 flasks and cultured in RPMI-1640 (Gibco, USA) with 300 mg/l L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, plus 15% fetal calf serum (FCS; TBD, China). Cells were maintained at 37˚C in a humidified 5% CO_2 atmosphere until 80-90% confluence was reached.

Measurement of anti-proliferative activity. Prior to microplate assays, the cells in culture flasks were trypsinized after reaching optimum confluence and the concentration of the cell suspension was adjusted to $1x10^4$ cells/ml. Following an additional incubation of 20 h at 37˚C, cells were grown in 96-well microplates. YTX was added in serial concentrations (50-350 ng/ml). HBSS alone was added as the control and all groups were re-incubated for 20 h in drug or vehicle. Then, 20 μl of 2 mg/ml MTT solution was added to the medium and the incubations continued for 4 h at 37˚C. The medium containing MTT was removed and 150 μl DMSO was added to each well to dissolve the formazan. Absorbance at 490 nm was read by a microplate reader (Rayto, China). The inhibition ratio (%) of toxins was calculated by the equation: Inhibition ratio (%) = $(1 - A_{treatment}/A_{control}) \times 100\%$ (1).

Morphological observation. Following incubation with 50 ng/ml of YTX for 48 h, the morphological change in HL7702 cells was observed by an optical microscope.

In addition, cell nuclei were visualized using the fluorescent dye Hoechst 33342 following 24 h of drug treatment. Briefly, after removing the culture medium, the treated cells were re-suspended in staining buffer containing Hoechst 33342 for 30 min at 4˚C. Following washing with PBS, fluorescent signals from 350 nm excitation were measured at 461 nm emission under a fluorescent microscope (Nikon, Japan).

Determination of endonuclease-dependent genomic DNA fragmentation by agarose gel electrophoresis. Liver cells were incubated with medium containing 50 ng/ml YTX for 48 h. Cells in medium were collected, washed with HBSS and resuspended in 50 μl lysis buffer. Following treatment with 10 μl RNase A (1 mg/ml) at 37˚C for 1 h, the samples were treated with 10 μl proteinase K (20 mg/ml) and incubated at 55˚C for at least 3 h. The lysates were added to 10X loading buffer and electrophoresis of extracted DNA was performed on 2.0% agarose gels. A 200-bp DNA marker was used to compare the sizes of DNA fragments. Bands were visualized by ethidium bromide (EB) staining (Molecular Probes Inc.).

Measurement of caspase-3 activity. The activation of caspase-3 was determined using a commercial caspase-3 assay kit (Beyotime Institute of Biotechnology). Following cleavage from the labeled substrate DEVD-pNA, the chromophore p-nitroanilide (pNA) may be quantified by 405 nm absorbance using an ELISA plate reader. Comparison of the absorbance of pNA from an apoptotic sample to an untreated control allows for determination of the increase in caspase-3 activity.

Analysis of mitochondrial transmembrane potential. The mitochondrial transmembrane potential (MTP) was measured using flow cytometry (BD FACScanto, USA) with the fluorescent probe Rh 123. Prior to treatment, Rh 123 was diluted in PBS to a final concentration of 2 μM. Approximately 10^6 cells were collected following trypsinization and incubated with 50 ng/ml YTX for 2, 4, 6, 8, 10 or 12 h. Treated cells were washed twice with HBSS and incubated in the dark with Rh 123 for 30 min at 37˚C. Samples were then analyzed by flow cytometry.

Analysis of intracellular calcium levels. HL7702 cells were transplanted in a 24-well microplate and cultured at 37˚C until 90% confluence was reached. After washing twice with HBSS, the cells were loaded with Fluo-3 AM at a final concentration of 5 μM for 1 h at 37˚C in the dark. Then, the dye loading solution was removed and the adherent cells were washed twice using HBSS.

Fluorescence in labeled cells was analyzed by Olympus FluoView FV1000 laser scanning confocal microscopy (LSCM). An Argon laser was used in LSCM. The excitation wavelength was set at 488 nm and the emission wavelength was set at 525 nm for Fluo-3 fluorescence reading. Special software was used to measure fluorescence and for initial analysis of results. Calcium concentration values of all cells observed in each experiment were averaged.

Statistical analysis. All experiments were carried out at least three times in duplicate. Results were analyzed using the Student's t-test for unpaired data. A probability level of 0.01 was used for statistical significance. Results were expressed as the means ± SEM.

Results

Cell proliferation and toxicity during treatment with YTX. In order to verify whether the cell proliferation was inhibited by YTX, an MTT assay was performed. MTT, a yellow

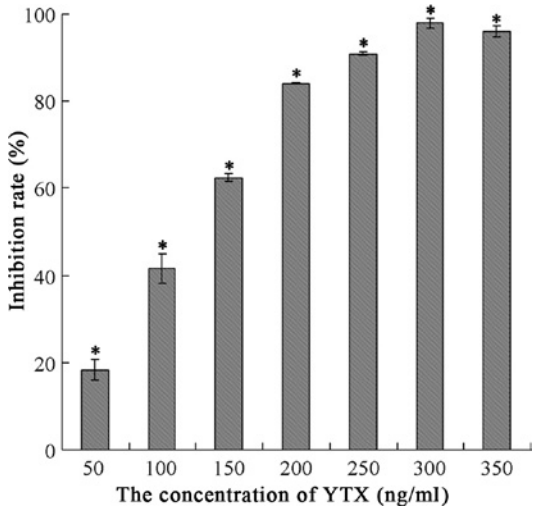

Figure 1. Effect of YTX on HL7702 cell proliferation. As the concentration of YTX increased, the growth of HL7702 cells was markedly inhibited. *P<0.01 compared to the control cells.

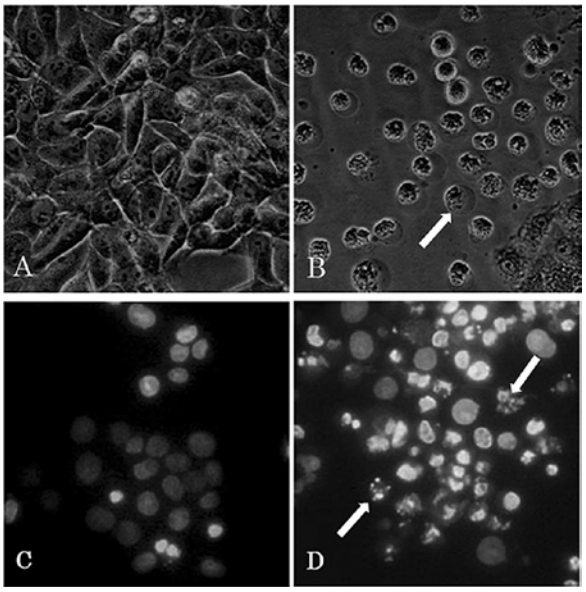

Figure 2. Morphological changes in HL7702 cells treated with 50 ng/ml YTX (magnification, x200). (A and B) Cells observed by optical microscope. Cells under optical microscope showed marked alterations in shape associated with apoptosis following treatment with YTX for 48 h. These demarcated cells eventually detached from the culture plate. (C and D) Hoechst 33342 staining of nuclei in untreated control cells and toxin-treated cells. As indicated by arrows, cells incubated with YTX demonstrated chromatin condensation and nuclear fragmentation.

water-soluble compound, may be converted to a purple insoluble formazan crystal based on the activity of the mitochondrial dehydrogenases in viable cells (27). In our pre-experiments, serial concentrations of YTX were used and the toxin-treated cells were observed every 2 h using an optical microscope. Few morphological changes were found in cells treated with lower concentrations of YTX (10 and 20 ng/ml) in 24 h (data not shown). Thus, increasing concentrations from 50 to 350 ng/ml were used in the MTT-test. As shown in Fig. 1, a significant reduction in HL7702 cell viability was found following 24 h of exposure to YTX, and the cytotoxicity of the toxin was dose-dependent.

Morphological changes related to YTX treatment. Morphological observations revealed that untreated control cells grew well and attached firmly onto the culture flask (Fig. 2A). By contrast, cells treated with 50 ng/ml YTX for 48 h demonstrated loss of native morphology (cell rounding) and marginalization of the nuclei (Fig. 2B). Control cells had round nuclei and uniform chromatin revealed by Hoechst 33342 staining; however, cells treated with YTX for 24 h demonstrated the typical morphological features of early-stage apoptosis, chromatin condensation and apoptotic bodies (Fig. 2D).

Effects of YTX on chromatin DNA. Following drug incubation for 48 h, possible DNA damage was evaluated by 2.0% agarose gel electrophoresis. The DNA isolated from vehicle-treated control cells remained completely intact and devoid of any fragmentation, while DNA fragmentation ('laddering') was clearly apparent in the toxin-treated cells (Fig. 3).

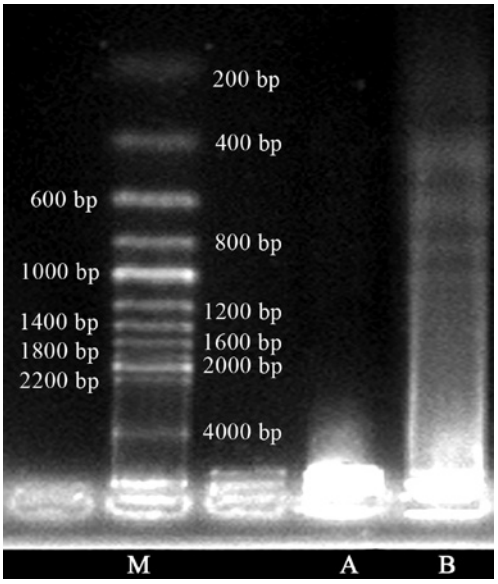

Figure 3. Electrophoretic analysis of purified DNA from YTX-treated HL7702 cells. Sample identities for each lane are as follows: M, 200-bp DNA ladder markers; A, untreated control cells; B, cells treated with 50 ng/ml YTX. Electrophoresis was carried out on 2.0% agarose gels.

Effects of YTX on caspase-3 activity and mitochondrial transmembrane potential. Caspases are critical mediators of cell apoptosis. The pro-apoptotic caspase-3 was detected in cells following incubation with 50 ng/ml YTX for at least 3 h (Fig. 4).

Disruption of the MTP is an early intracellular event in cell apoptosis. When HL7702 cells were treated with YTX for various periods at 37˚C, a decrease in Rh 123 fluorescence was observed, indicating a loss of MTP (Fig. 5). The MTP began to depolarize following 2 h of incubation with YTX and continued to decrease over the next 9 h.

Effects of YTX on the intracellular calcium levels. To investigate whether Ca^{2+} concentrations were changed in toxin-treated apoptotic human liver cells, the average fluorescence intensity

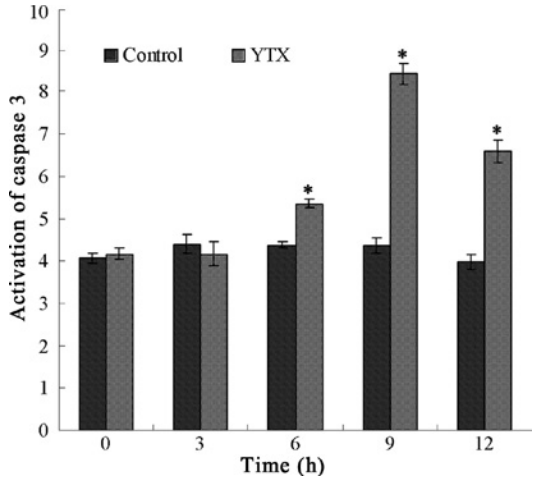

Figure 4. The activity of caspase-3 was elevated in HL7702 cells treated with YTX or OA for 0-12 h compared to untreated control cells. *P<0.01.

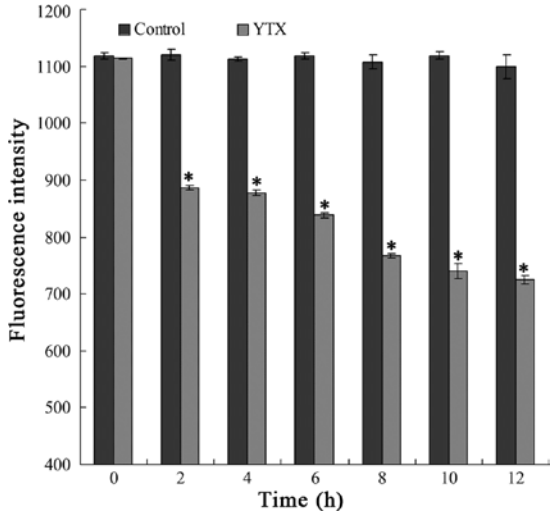

Figure 5. The MTP of HL7702 cells treated with OA or YTX decreased over time as revealed by Rh 123 staining. *P<0.01 relative to untreated control cells.

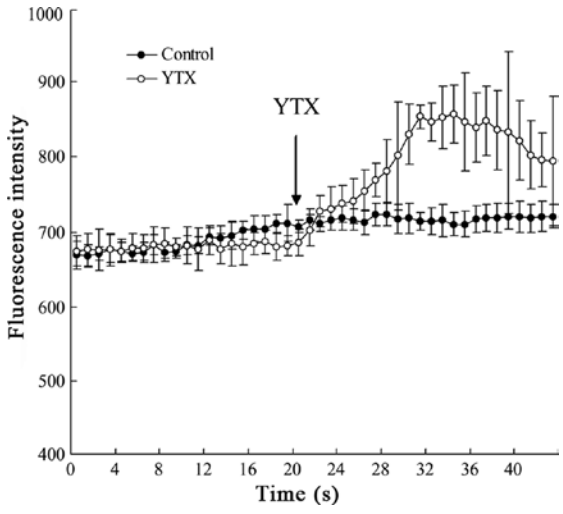

Figure 6. Effect of YTX on the calcium levels in a human liver cell line, compared to the control group. HL7702 cells, attached to a 24-well microplate and loaded with Fluo-3, were bathed in a balance salt solution containing 1 mM $CaCl_2$. As the arrow indicated, 100 ng YTX was added to the bathing medium.

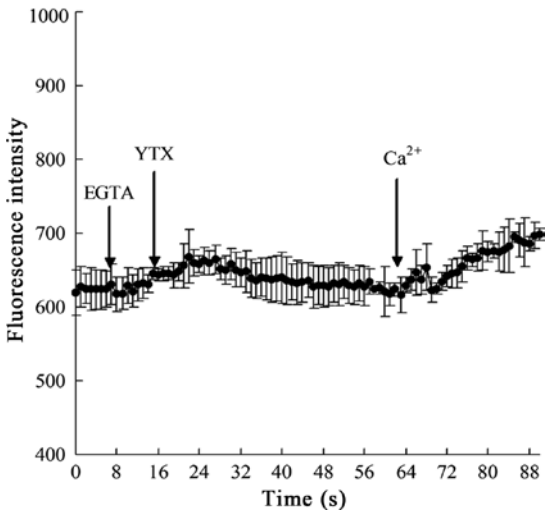

Figure 7. Effect of YTX in a Ca^{2+}-free solution. Cells attached to the microplate were loaded with Fluo-3, and the Ca^{2+} in the extracellular medium was removed using EGTA as indicated by the first arrow. Then, 100 ng YTX was added to the medium. Approximately 1 min later, $CaCl_2$ at a final concentration of 2 mM was added.

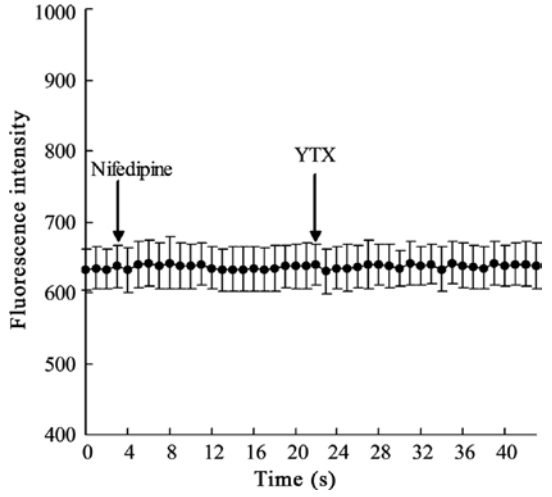

Figure 8. Effect of the calcium channel blocker nifedipine on the Ca^{2+} concentration rise induced by YTX. Following addition of nifedipine, YTX was added to the extracellular medium as indicated.

of intracellular Ca^{2+} concentration was detected using laser scanning confocal microscope (Olympus FluoView FV1000, Japan). As shown in Fig. 6, the intracellular calcium levels of HL7702 cells were markedly increased following the addition of YTX to the medium.

In order to determine whether YTX-evoked Ca^{2+} increase was caused by Ca^{2+} in the extracellular medium or released from cytoplasmic organoids, EGTA at a final concentration of 2 mM was added. In the Ca^{2+}-free solution, YTX could not evoke a Ca^{2+} concentration increase (Fig. 7). $CaCl_2$ was then added in the medium and a general Fluo-3 fluorescence increase was detected.

The possible involvement of the calcium channel was investigated in the calcium concentration increase induced by YTX. There are a number of drugs known to block various calcium conductive pathways. In this study, nifedipine was used at a final concentration of 1 μM to block the L-type Ca^{2+}

channels of HL7702 cells; the YTX-induced Ca^{2+} entry was inhibited absolutely by nifedipine (Fig. 8).

Discussion

Tubaro *et al* demonstrated that YTX may be partly eliminated in urine and the concentration of YTX in the blood of mice was approximately 6 ng/ml following repeated oral administration (1 mg/kg/day, for 7 days) (28). However, YTX absorption could be affected by the course of biological metabolism *in vivo* and the toxic effect of low concentrations may be inconspicuous. A number of studies *in vitro* used higher concentrations to investigate the mechanism of cytoxicity induced by YTX. For example, Pérez-Gómez *et al* used YTX concentrations up to 150 nM (21), and Korsnes *et al* used 100 nM in routine experiments (23,24). Dell'Ovo *et al* indicated that the IC_{50} in the primary culture of rat cardiomyocytes exposed to the toxin for 48 h was approximately 10 nM. However, they used 1 μM YTX in the subsequent study to analyze the possible effect of YTX on changes of membrane potential and Ca^{2+} transients in cells (29). Our results from the MTT test suggested that a concentration of approximately 100 ng/ml YTX was close to the IC_{50} in HL7702 cells exposed to toxin for 24 h. Cells cultured in lines seemed to have higher drug tolerance than those in primary culture. As concentrations starting from 200 ng/ml may damage cells markedly, we chose 50 ng/ml YTX to study the apoptotic processes in the following experiments.

Apoptosis is characterized by an ordered progression of morphological changes that include cell shrinkage, condensation of chromatin and externalization of membrane phosphatidylserine (30). Our study revealed that YTX triggered all the usual hallmarks of apoptosis: morphological changes, DNA fragmentation, increased capase-3 activity, calcium deregulation and the loss of MTP. Following treatment with YTX, human liver cells exhibited clear chromatin condensation as revealed by changes in the spatial profile of Hoechst 33342 fluorescence. The Hoechst stains are a family of fluorescent stains used for labeling DNA in live or fixed cells for fluorescence microscopy and fluorescent-activated cell sorting (FACS). However, since Hoechst 33342 binds to DNA, it disrupts DNA replication during cell division (31), and so may have decreased proliferation in the absence of apoptosis. Agarose gel electrophoresis confirmed that YTX induced the 'ladder' pattern of DNA fragmentation caused by endonuclease activation and intranucleosomal DNA breaks (32-34). Therefore, the inhibition of growth was likely related to the induction of apoptosis.

The caspase family of intracellular cysteine proteases constitutes a pivotal signaling cascade in apoptosis (35). YTX elevated caspase-3 activity in HL7702 cells, allowing this cysteine protease to degrade cytoskeletal and nuclear proteins. The caspase pathway is triggered by the release of mitochondrial proteins concomitant with an increase in membrane permeability induced by the pore transition complex. Once the pore transition complex forms, specific apoptotic activators, including a second mitochondria-derived activator of caspases and cytochrome C, are released and activate the caspase protease cascade. Indeed, loss of MTP is a final common pathway in the early events of apoptosis (36). The disruption of MTP precedes nuclear DNA fragmentation in the majority of models of apoptosis (37-40), indicating that mitochondrial factors and caspase activation trigger nuclease activity. The disruption of MTP is also associated with increased generation of reactive oxygen species and elevation of the Ca^{2+} concentration (41).

The intracellular Ca^{2+} concentration is also a major regulator of programmed cell death; Ca^{2+} overload or deregulation over-stimulates proteases, lipases and endonucleases leading to cellular damage and eventually cell death (42). The activation of endonucleases that cleave DNA during apoptosis is Ca^{2+} and Mg^{2+}-dependent (43). Calcium deregulation may also lead to caspase-independent apoptosis (44). It has been reported that YTXs induce accumulation of Ca^{2+} in numerous cell types (20,21). In our study, YTX also induced Ca^{2+} entry in human liver cells. The mechanism for this calcium increase remains unknown, as is the role of calcium mobilization from various sources in apoptotic death. In this study, nifedipine was used to block the L-type calcium channels and the YTX-induced Ca^{2+} increase was inhibited. It appears that YTX may act on the L-type calcium channels of HL7702 cells. Indeed, while YTX appears to activate L-type calcium channels, channel blockers did not rescue primary neurons from apoptosis (21). In other studies, calcium was shown to play a more permissive effect in that calcium elevations were necessary for YTX-induced mitochrondrial depolarization, but alone did not induce MTP change. Aside from activating calcium-dependent lipases, proteins and nucleases, this Ca^{2+} deregulation and mitochrondrial uptake has been shown to hasten PTP formation and loss of MTP. Additional studies are required to reveal the exact mechanisms of apoptosis induced by YTX.

In conclusion, YTX has now been shown to exert cytotoxic effects on a variety of mammalian cell types at nanogram per ml concentrations. Ingestion of contaminated shellfish could, therefore, result in neural, cardiac or hepatic dysfunction. Whether the apoptotic effect of YTX differs for normal liver and liver cancer cells would be determined in the next step of research into potential cancer therapeutics. Aside from the potential effects on human health, marine toxins, such as YTX, are invaluable probes to elucidate cellular signaling pathways under normal physiological and pathological conditions.

Acknowledgements

This study was supported by the International Cooperation Program of the Ministry of Science and Technology of the People's Republic of China (no. 2007DFA30710) and the scientific research fund of the First Institute of Oceanography, SOA (2011T07).

References

1. Murata M, Masanori K, Lee JS and Yasumoto T: Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. Tetrahedron Lett 28: 5869-5872, 1987.
2. Yasumoto T and Takizawa A: Fluorometric measurement of yessotoxins in shellfish by high-pressure liquid chromatography. Biosci Biotechnol Biochem 61: 1775-1777, 1997.
3. EC: Commission Decision 2002/225/EC of 15 March 2002 laying down detailed rules for the implementation of Council Directive 91/492/EEC as regards the maximum levels and the methods of analysis of certain marine biotoxins in bivalve mollusks, echinoderms, tunicates and marine gastropods. Official Journal of the European Communities, p62, 2002.

4. Satake M, MacKenzie L and Yasumoto T: Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. Nat Toxins 5: 164-167, 1997.
5. Paz B, Daranas AH, Norte M, *et al*: Yessotoxins, a group of marine polyether toxins; an overview. Marine Drugs 6: 73-102, 2008.
6. Tubaro A, Sidari L, Della LR and Yasumoto T: Occurrence of homoyessotoxin in phytoplankton and mussels from Northern Adriatic Sea. In: Harmful Algae. Reguera B, Blanco J, Fernández ML and Wyatt T (eds). Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO, Grafisant, Santiago de Compostela, pp470-472, 1998.
7. Draisci R, Ferretti E, Palleschi L, *et al*: High levels of yessotoxin in mussels and presence of yessotoxin and homoyessotoxin in dinoflagellates of the Adriatic Sea. Toxicon 37: 1187-1193, 1999.
8. Stobo LA, Lewis J, Quilliam MA, *et al*: Detection of yessotoxin in UK and Canadian isolates of phytoplankton and optimization and validation of LC-MS methods. In: Proceedings of the Eighth Canadian Workshop on Harmful Marine Algae. Bates S (ed). Gulf Fisheries Centre, Moncton, New Brunswick, Canada, pp8-14, 2003.
9. Ciminiello P, Dell'Aversano C, Fattorusso E, *et al*: Complex yessotoxins profile in *Protoceratium reticulatum* from north-western Adriatic sea revealed by LC-MS analysis. Toxicon 42: 7-14, 2003.
10. Arévalo F, Pazos Y, Correa J, *et al*: First report of yessotoxins in mussels of Galician Rías during a bloom of *Lingulodinium polyedra* Stein (Dodge). In: Molluscan Shellfish Safety. Henshilwood K, Deegan B, McMahon T, *et al* (eds). Galway, Ireland, pp184-189, 2006.
11. Morton SL, Vershinin A, Leighfield TA, *et al*: Identification of yessotoxin in mussels from the Caucasian Black Sea Coast of the Russian Federation. Toxicon 50: 581-584, 2007.
12. Pan J, Li RX, Li Y and Sun P: Distribution of dinoflagellate cysts in surface sediments from the Southern Yellow Sea in autumn. Adv Marine Sci 28: 41-49, 2010.
13. Gao CL, Liu RY, Liang YB, *et al*: First report of the presence of yessotoxins (YTXs) in shellfish from China's coastal areas. Acta Oceanol Sin 3: 129-137, 2010.
14. Aune T, Sørby R, Yasumoto T, *et al*: Comparison of oral and intraperitoneal toxicity of yessotoxin towards mice. Toxicon 40: 77-82, 2002.
15. Ogino H, Kumagai M and Yasumoto T: Toxicologic evaluation of yessotoxin. Nat Toxins 5: 255-259, 1997.
16. Terao K, Ito E, Oarada M, *et al*: Histopathological studies on experimental marine toxin poisoning – 5. The effects in mice of yessotoxin isolated from *Patinopecten yessoensis* and of a desulfated derivative. Toxicon 28: 1095-1100, 1990.
17. Tubaro A, Sosa S, Carbonatto M, *et al*: Oral and intraperitoneal acute toxicity studies of yessotoxin and homoyessotoxins in mice. Toxicon 41: 783-792, 2003.
18. Tubaro A, Sosa S, Altinier G, *et al*: Short-term oral toxicity of homoyessotoxins, yessotoxin and okadaic acid in mice. Toxicon 43: 439-445, 2004.
19. Callegari F, Sosa S, Ferrari S, *et al*: Oral administration of yessotoxin stabilizes E-cadherin in mouse colon. Toxicology 227: 145-155, 2006.
20. De la Rosa LA, Alfonso A, Vilarino N, *et al*: Modulation of cytosolic calcium levels of human lymphocytes by yessotoxin, a novel marine phycotoxin. Biochem Pharmacol 61: 827-833, 2001.
21. Pérez-Gómez A, Fererro-Gutierrez A, Novelli A, *et al*: Potent neurotoxic action of the shellfish biotoxin yessotoxin on cultured cerebellar neurons. Toxicol Sci 90: 168-177, 2006.
22. Malaguti C, Ciminiello P, Fattorusso E and Rossini GP: Caspase activation and death induced by yessotoxin in HeLa cells. Toxicol In Vitro 16: 357-363, 2002.
23. Korsnes MS, Hetland DL, Espenes A and Aune T: Induction of apoptosis by YTX in myoblast cell lines via mitochondrial signalling transduction pathway. Toxicol In Vitro 20: 1419-1426, 2006.
24. Korsnes MS, Hetland DL, Espenes A, *et al*: Apoptotic events induced by yessotoxin in myoblast cell lines from rat and mouse. Toxicol In Vitro 20: 1077-1087, 2006.
25. Leira F, Alvarez C, Vieites JM, *et al*: Characterization of distinct apoptotic changes induced by okadaic acid and yessotoxin in the BE (2)-M17 neuroblastoma cell line. Toxicol In Vitro 16: 23-31, 2002.
26. Bianchi C, Fato R, Angelin A, *et al*: Yessotoxin, a shellfish biotoxin, is a potent inducer of the permeability transition in isolated mitochondria and intact cells. Biochim Biophys Acta 1656: 139-147, 2004.
27. Mosmann T: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63, 1983.
28. Tubaro A, Giangaspero A, Ardizzone M, *et al*: Ultrastructural damage to heart tissue from repeated oral exposure to yessotoxin resolves in 3 months. Toxicon 51: 1225-1235, 2008.
29. Dell'Ovo V, Bandi E, Coslovich T, *et al*: In vitro effects of yessotoxin on a primary culture of rat cardiomyocytes. Toxicol Sci 106: 392-399, 2008.
30. Martin SJ, Reutelingsperger CP, McGahon AJ, *et al*: Early redistribution of plasma membrane phosphatidylserines a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 182: 1545-1556, 1995.
31. Telford WG, Komoriya A and Packard BZ: Multiparametric analysis of apoptosis by flow and image cytometry. In: Flow Cytometry Protocols (Methods in Molecular Biology). Hawley TS and Hawley RG (eds.). Humana Press, USA, pp141-160, 2004.
32. Williams JR, Little JB and Shipley WU: Association of mammalian cell death with specific endonucleolytic degradation of DNA. Nature 252: 754-756, 1974.
33. Wyllie AH: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. Nature 284: 555-556, 1980.
34. Tilly JL: Use of the terminal transferase DNA labeling reaction for the biochemical and in situ analysis of apoptosis. In: Cell Biology: A Laboratory Handbook. Celis JE (ed.). Academic Press, San Diego, pp330-337, 1994.
35. Thornberry NA and Lazebnik Y: Caspases: enemies within. Science 281: 1312-1316, 1998.
36. Lemasters JJ, Nieminen AL, Qian T, *et al*: The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim Biophys Acta 1366: 177-196, 1998.
37. Cossarizza A, Franceschi C, Monti D, *et al*: Protective effect of N-acetylcysteine in tumor necrosis factor-alpha induced apoptosis in U937 cells: the role of mitochondria. Exp Cell Res 220: 232, 1995.
38. Petit PX, Lecoeur H, Zorn E, *et al*: Alterations of mitochondrial structure and function are early events of dexamethasone-induced thymocyte apoptosis. J Cell Biol 130: 157, 1995.
39. Zamzami N, Marchetti P, Castedo M, *et al*: Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. J Exp Med 182: 367, 1995.
40. Marchetti P, Susin SA, Decaudin D, *et al*: Apoptosis associated derangement of mitochondrial function in cells lacking mitochondrial DNA. Cancer Res 56: 2033, 1996.
41. Macho A, Hirsch T, Marzo I, *et al*: Glutathione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. J Immunol 158: 4612-4619, 1997.
42. Smaili SS, Hsu YT, Carvalho ACP, *et al*: Mitochondria, calcium and pro-apoptotic proteins as mediators in cell death signaling. Braz J Med Biol Res 36: 183-190, 2003.
43. McConkey DJ and Orrenius S: The role of calcium in the regulation of apoptosis. Biochem Bioph Res Commun 239: 357-366, 1997.
44. Pivovarova NB and Andrews SB: Calcium-dependent mitochondrial function and dysfunction in neurons. FEBS J 277: 3622-3636, 2010.