Fundamental Toxicological Sciences (Fund. Toxicol. Sci.)
Vol.2, No.1, 49-54, 2015

Letter

Comparison of cytotoxicity among pectenotoxin-2 and other oxidized pectenotoxins in a rat myoblast cell line (L6) and a human rhabdomyosarcoma cell line (RD)

Ryoji Matsushima[1], Saori Kikutsugi[1], Ryuichi Watanabe[1], Hajime Uchida[1,2], Takeshi Yasumoto[1], Hiroshi Nagai[2], Masaki Kaneniwa[1] and Toshiyuki Suzuki[1]

[1]National Research Institute of Fisheries Science, Fisheries Research Agency, 2-12-4 Fukuura, Kanazawa, Yokohama, Kanagawa 236-8648, Japan
[2]Tokyo University of Marine Science and Technology, 4-5-7 Konan, Minato-ku, Tokyo 108-8477, Japan

(Received January 6, 2015; Accepted January 13, 2015)

ABSTRACT — Lipophilic toxin pectenotoxin-2 (PTX2) is oxidatively metabolized to pectenotoxin-1 (PTX1), pectenotoxin-3 (PTX3), and pectenotoxin-6 (PTX6) in the Japanese scallop *Patinopecten yessoensis*. This particular metabolism has been observed only in Japanese scallops, in which PTX6 is the most dominant lipophilic toxin. We investigated the cytotoxicity of PTX2 and its metabolites PTX1,3,6 in a rat cell line (L6) and a human cell line (RD). RD showed an approximately three-fold greater sensitivity than L6 upon exposure to PTXs. The cytotoxicity of PTXs decreased with degree of oxidation in the order PTX2 > PTX1 > PTX3 > PTX6. The calculated half maximal inhibitory concentration (IC_{50}) values of PTX2 obtained for the L6 and RD cell lines were 60 and 23 ng/mL, respectively, while those obtained for PTX6 for both cell lines were over 2,000 ng/mL. These results demonstrate that PTX6 has extremely low cytotoxicity or is non-toxic and that the oxidative metabolism of PTX2 in *P. yessoensis* is a detoxification process.

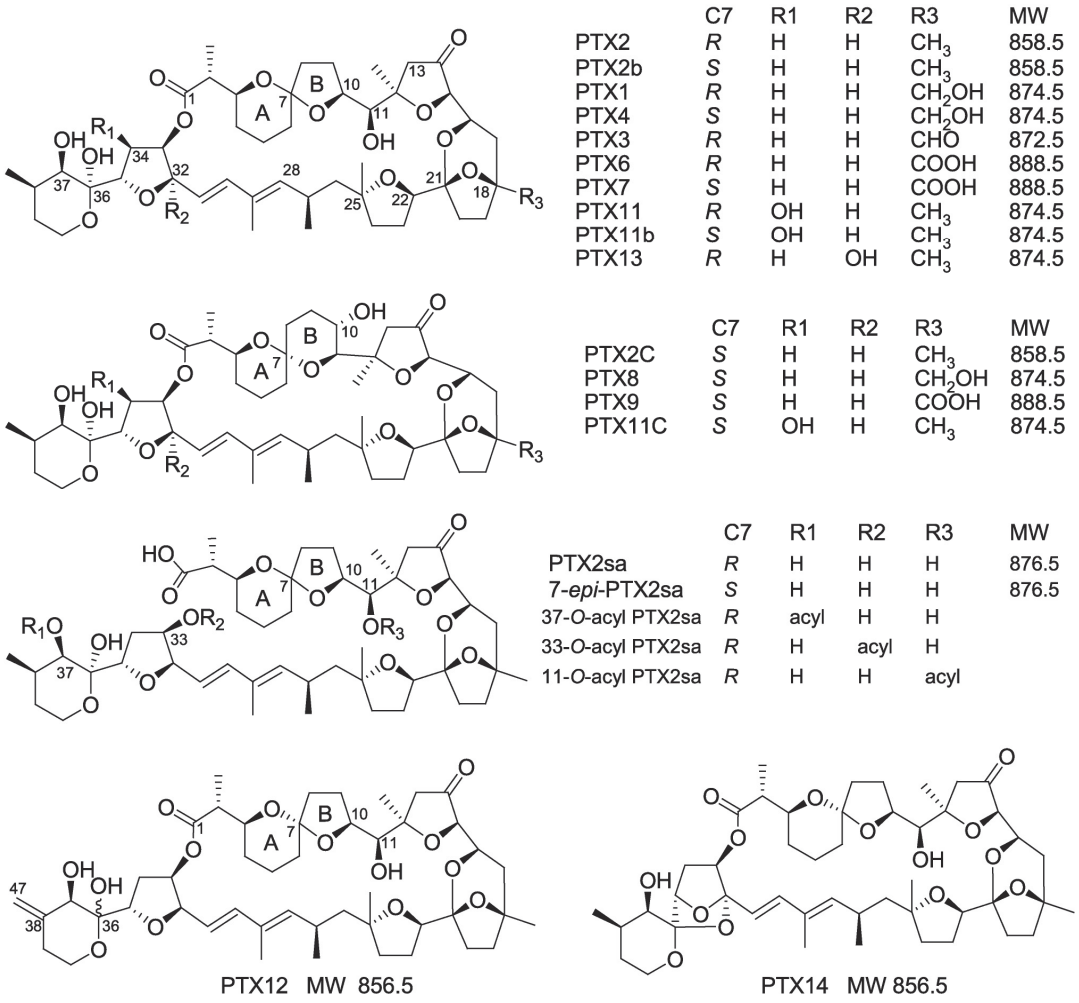
Key words: Diarrhetic shellfish poisoning, Lipophilic toxin, Pectenotoxin-2, Oxidized pectenotoxins, Apoptosis, Cytotoxicity assay

INTRODUCTION

The presence of pectenotoxins (PTXs) in shellfish was discovered in a mouse bioassay (MBA) using intraperitoneal (i.p.) injection (Yasumoto *et al*., 1985; Yasumoto and Murata, 1993). About twenty analogues of PTXs (Fig. 1) have been reported. Among the PTX analogues, almost all PTXs, except for pectenotoxin-2 (PTX2) and pectenotoxin-11 (PTX11) are metabolites in bivalves (Suzuki *et al*., 1998, 2001a, 2001b, 2006). Animal studies indicate that PTXs are much less toxic via the oral route and that they do not induce diarrhea (Miles *et al*., 2004; Ito *et al*., 2008). Since PTXs do not fit the clinical case definition of diarrhetic shellfish poisoning (DSP) toxins, they have recently come to be considered a separate lipophilic toxin group. PTXs show high hepatotoxicity and nephrotoxicity by i.p. or intravenous (i.v.) injection to mice (Ito *et al*., 2008). It is also noteworthy that the induction of apoptosis by depolymerization of actin is the primary mechanism in cells exposed to PTXs (Ares *et al*., 2007; Espiña *et al*., 2008, 2010; Shin *et al*., 2011).

Many bivalve species such as the mussels *Perna canaliculus* and *Mytilus galloprovincialis*, and the oyster *Crassostrea gigas* (Suzuki *et al*., 2001a, 2001b; Miles *et al*., 2004; Amzil *et al*., 2007) hydrolyze PTX2 to pectenotoxin-2 seco acid (PTX2sa), which has been found to be nontoxic in a MBA and a cell cytotoxicity assay (Daiguji *et al*., 1998). On the other hand, the Japanese scallop *Patinopecten yessoensis* has a particularly unique PTX2 metabolism in which PTX2 is oxidatively metabolized to PTX1, PTX3, and PTX6 (Suzuki *et al*., 1998). Because PTX6 is the most dominant toxin in DSP and other lipophilic toxins in Japanese scallops (Suzuki *et al*., 1998), detailed investigations on the toxicity of PTX6 have been conducted. Assessment of the toxicity of PTX6 is also interesting in terms of detoxification of PTX2 in this bivalve species. The i.p. lethal level of PTX6 in mice is 500 μg/kg, thus its toxicity is approximately half of the

Correspondence: Toshiyuki Suzuki (E-mail: tsuzuki@affrc.go.jp)



	C7	R1	R2	R3	MW
PTX2	*R*	H	H	CH_3	858.5
PTX2b	*S*	H	H	CH_3	858.5
PTX1	*R*	H	H	CH_2OH	874.5
PTX4	*S*	H	H	CH_2OH	874.5
PTX3	*R*	H	H	CHO	872.5
PTX6	*R*	H	H	COOH	888.5
PTX7	*S*	H	H	COOH	888.5
PTX11	*R*	OH	H	CH_3	874.5
PTX11b	*S*	OH	H	CH_3	874.5
PTX13	*R*	H	OH	CH_3	874.5

	C7	R1	R2	R3	MW
PTX2C	*S*	H	H	CH_3	858.5
PTX8	*S*	H	H	CH_2OH	874.5
PTX9	*S*	H	H	COOH	888.5
PTX11C	*S*	OH	H	CH_3	874.5

	C7	R1	R2	R3	MW
PTX2sa	*R*	H	H	H	876.5
7-*epi*-PTX2sa	*S*	H	H	H	876.5
37-*O*-acyl PTX2sa	*R*	acyl	H	H	
33-*O*-acyl PTX2sa	*R*	H	acyl	H	
11-*O*-acyl PTX2sa	*R*	H	H	acyl	

PTX12 MW 856.5

PTX14 MW 856.5

Fig. 1. Chemical structure of pectenotoxins.

lethal level of PTX1, PTX2 or PTX11 (Yasumoto *et al*., 1989; Miles *et al*., 2004; Suzuki *et al*., 2006). Given the toxicities of PTXs determined by i.p. or i.v. MBA, cultured cell assays have been reported with various kinds of cells derived from humans, fish, rats, *etc*., for basic and application purposes (Fladmark *et al*., 1998; Chae *et al*., 2005; Kim *et al*., 2008; Shin *et al*., 2008).

In the present study, we investigated the cytotoxicities of PTXs in a newly selected rat skeletal muscle myoblast cell line (L6) and in a human rhabdomyosarcoma cell line (RD). Because there have been few reports comparing cytotoxicities among a series of oxidative metabolites of PTXs (PTX2,1,3,6) using the same cell lines under the same experimental conditions and cancer cell lines considered more sensitive than normal ones (Kim *et al*., 2008, 2011). We also evaluated quantitative cytotoxicity among PTX2,1,3,6 with PTXs-induced reduction of cell viability and apoptosis (Fladmark *et al*., 1998; Korsnes *et al*., 2006; Kobayashi *et al*., 2007).

MATERIALS AND METHODS

Chemicals

PTX2, PTX1, PTX3 and PTX6 were isolated from the hepatopancreas of bivalves following the method previously described (Yasumoto *et al*., 1989). Isolated PTXs were identified by nuclear magnetic resonance (NMR) spectrometry and mass spectrometry (MS). The purity of the PTXs isolated in the present study was confirmed by positive and negative full-scan liquid chromatography (LC)/MS analysis (Suzuki *et al*., 2006) and LC-diode array detection (DAD) with slight modifications of

the conditions previously described (Suzuki *et al*., 2006). PTXs were dissolved in MeOH at a concentration of 20 μg/mL (w/v).

Cell culture

The L6 and RD cell lines were purchased from the Health Science Research Resources Bank (Osaka, Japan). Both cell lines were maintained in plastic culture flasks (TPP, St. Louis, MO, USA). Medium and reagents were purchased from Wako Pure Chemical (Osaka, Japan). The L6 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin at 37°C under 5% CO_2. Eagle's minimal essential medium including 2 × amino acids and 2 × vitamins with 10% fetal bovine serum were employed for the RD cells. The cells were observed by microscopy with a 4 × objective and a Moticam 2300 (Shimadzu, Tokyo, Japan).

WST-8 assay

Cytotoxicity was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Highly water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activities in cells to yield a yellow formazan dye, which is soluble in tissue culture media. For the WST-8 assay, L6 and RD cells were seeded at a density of 5×10^3 and 1×10^4 cells/50-μL well, respectively, on 96-well microtiter plates. Twenty-four hours following cell attachment, 50-μL aliquots of PTXs with various concentrations were added. Control cells were incubated in the same conditions with the toxin vehicle, methanol. After 48 hr of incubation, 10 μL of CCK-8 was added and the cells were incubated for another 2 hr. The developed colors were measured to determine the optical density (OD) value at 450 nm using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). Absorbance measured in WST-8 assays was expressed as a percent of the control (defined as 100%).

RESULTS

Effect of PTX2, -1, -3, or -6 on cell viability

The effects of PTXs on L6 and RD cell viability for 48 hr are shown in Fig. 2. PTX2 showed the highest inhibition activity for both cell lines in a concentration-dependent manner (Figs. 2A, E), and PTX1 showed the second highest inhibition activity (Figs. 2B, F). While PTX3 exhibited inhibition activity for RD, it barely affected the growth activity of L6 (Figs. 2C, G). PTX6 showed clearly lower inhibition activities than the other PTXs for both L6 and RD cells although slight inhibition activity was observed on the RD cells (Figs. 2D, H).

The half maximal inhibitory concentration (IC_{50}) values of PTXs calculated from the dose-response curves of L6 and RD are listed in Table 1. The IC_{50} values obtained for RD were lower than those obtained for L6. This ten-

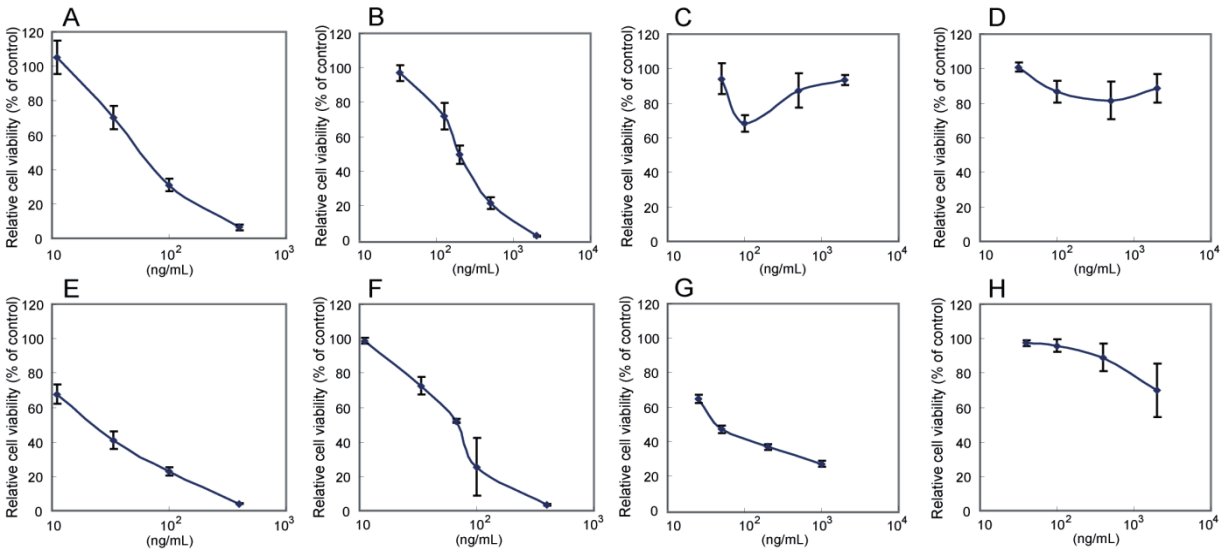


Fig. 2. Dose effect for L6 and RD cells treated with PTX2, -1, -3, or -6. Cell viability was measured by WST-8 assay. L6 and RD cells were exposed to various concentrations of PTXs for 48 hr. Each point represents the mean with S.D. of 3-6 determinations. (A-D) Values acquired from L6 cells; (E-H) values acquired from RD cells; (A and E) measured value of PTX2; (B and F) measured value of PTX1; (C and G) measured value of PTX3; and (D and H) measured value of PTX6.

Table 1. IC_{50} values of PTXs in L6 and RD.

PTX	IC_{50} (ng/mL)	
	L6	RD
PTX2	60.82	23.02
PTX1	198.34	68.85
PTX3	> 2,000	44.32
PTX6	> 2,000	> 2,000

dency was also observed for statins (lovastatin, simvastatin, and mevastatin), which are known to be reagents inducing cell apoptosis (Kobayashi *et al*., 2008), in the WST-8 assay (data not shown). These results suggested that L6 cell line had high tolerance property against agents than RD cell line. The IC_{50} values for PTX2 were the lowest among the compared PTXs, and the values for PTX1 were approximately three times higher than those obtained for PTX2 for both L6 and RD. Although the IC_{50} value obtained for PTX3 was lower than that obtained for PTX1 for RD cells, no significant inhibitory action was observed in L6 cells. It is noteworthy that the IC_{50} values for PTX6 were more than 2,000 ng/mL in both L6 and RD cells. The value was considerably higher than those obtained for PTX1,2,3.

Observation of cell morphology under light phase-contrast microscopy

Fig. 3 shows morphological observation of L6 and RD cell lines exposed to PTXs. Both L6 and RD cells exposed to PTX1,2,3 became round, indicating induced compound-dependent apoptosis, as described in previous studies (Fladmark *et al*., 1998; Kim *et al*., 2011). Similar results were also observed in cells exposed to statins (lovastatin, simvastatin, and mevastatin) (data not shown). PTX3 slightly inhibited the growth of L6 cells at a concentration of 100 ng/mL (Fig. 2C), however, the morphology of L6 cells was clearly affected by PTX3 in a concentration-dependent manner (Figs. 3A-D). Figs. 3F-N show morphological observation of the RD cell lines exposed to PTX1 and PTX3. The ratio of degenerative cells increased with increasing concentrations of PTX1 and PTX3. In agreement with growth curve results, no morphological changes were observed in either L6 cells (data not shown) or RD cells (Fig. 3O) exposed to PTX6 at a concentration of 2,000 ng/mL. The results of this cytotoxicity assay thus indicate the extremely low toxicity or non-toxicity of PTX6.

DISCUSSION

The cytotoxicity of PTXs has been reported in several previous studies, and the primary mechanisms of action have been elucidated with apoptosis by depolymerization of actin (Ares *et al*., 2007; Espiña *et al*., 2008, 2010; Shin *et al*., 2011). On the other hand, to the best of our knowledge, no quantitative analyses of the inhibitory activity of oxidative metabolites of PTXs (PTX2,1,3,6) have been reported in previous studies. Therefore, quantitative analyses of the inhibitory activity of PTXs were carried out using tumor-derived cell lines (L6 and RD). It was unexpected that the IC_{50} value of PTX1 for RD was higher than that of PTX3 while the response curves (Figs. 2F, G), but morphological defects (Figs. 3F-N) of RD for PTX1 and PTX3 indicated a higher toxicity of PTX1 than of PTX3. The full cytoxcity curves of PTX1 versus PTX3 on RD cells clearly show a dose response in which PTX1 is significantly more toxic than PTX3 at the higher doses. The IC_{50} values differences between RD and L6 cells are small and it may be an anomaly. Furthermore, PTX3 decreased L6 cell viability at 100 ng/mL (Fig. 2C) and this phenomenon had good reproducibility. But the morphology of L6 did not exhibit specific toxicity at 100 ng/mL of PTX3 (Figs. 3A-E). In any event the overall toxicity of PTX1 is clearly more potent when the entire dosing curves are considered and are consistent with the dosing response and morphological changes. The evaluation of toxicity requires prudent observation that takes into account the response curve and morphological observation.

Our present results that the cytotoxicity of PTXs decreased in accordance with the oxidation of PTX2 to PTX6 and PTX6 shows extremely low cytotoxicity is robust answer to prove PTX6 is nearly non-toxic. This tendency was the same as that obtained in a previous i.p. MBA (Yasumoto *et al*., 1989). These results are also consistent with the stronger actin-disassembling activity of PTX2 in comparison with that of PTX1 (Ares *et al*., 2007). Although the Japanese scallop *Patinopecten yessoensis* lacks the ability to detoxify PTX2 by hydrolyzing it to PTX2sa (Suzuki *et al*., 1998), PTX2 could be detoxified by an alternative conversion of PTX2 upon the oxidization of the 43-methyl group to the less cytotoxic PTX6 in this species. Our previous study found that the injection of *P. yessoensis* with PTX6 caused non-toxic symptoms (Suzuki *et al*., 2005). Thus, the present evaluation of the cytotoxicity of PTX6 is consistent with the results of our previous *in vivo* experiment on the scallops. PTX6 could be a rather harmless compound in comparison with PTX2 for *P. yessoensis*. It is believed that the enzyme(s)

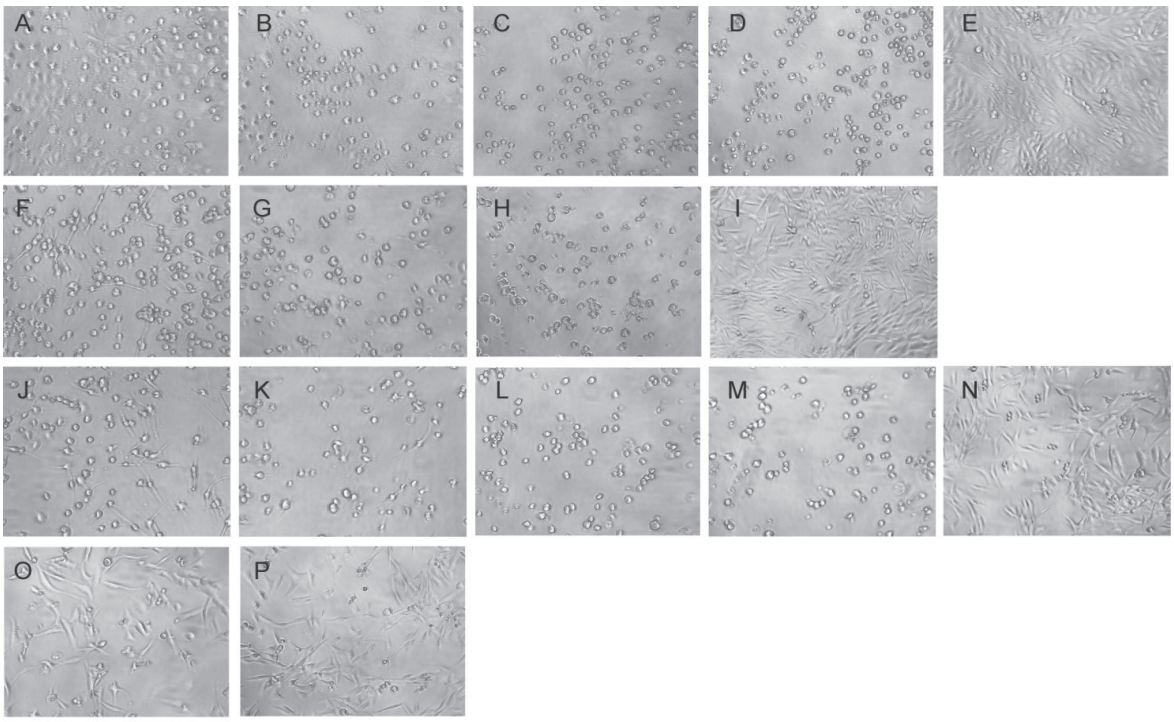


Fig. 3. Morphological observation of L6 and RD cells treated with PTXs. Both cell lines were exposed to PTXs for 48 hr, and the cells were observed by microscopy with a 4 × objective and a Moticam 2300. L6 cells: (A to E) treated with PTX3 at concentrations of 50 ng/mL, 100 ng/mL, 500 ng/mL, 2,000 ng/mL, and 2,000 ng/mL vehicle control, respectively. RD cells: (F to I) treated with PTX1 at concentrations of 33 ng/mL, 66 ng/mL, 100 ng/mL, and 100 ng/mL vehicle control, respectively; (J to N) treated with PTX3 at concentrations of 25 ng/mL, 50 ng/mL, 200 ng/mL, 1,000 ng/mL, and 1,000 ng/mL vehicle control, respectively; (O and P) treated with PTX6 at a concentration of 2,000 ng/mL and vehicle control, respectively.

responsible for the oxidation of PTX2 should be present in *P. yessoensis* as well as in the Greenshell mussel *Perna canaliculus*, in which such an enzyme hydrolyzes PTX2 to PTX2sa (MacKenzie *et al*., 2012).

ACKNOWLEDGMENT

This work was supported in part by a grant-in-aid (Development of evaluation and management methods for the supply of safe, reliable and functional food and farm produce) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

Conflict of Interest---- We declare that there is no conflict of interest including financial or personal relationship with other people or organizations that could inappropriately influence in our present research.

REFERENCES

Amzil, Z., Sibat, M., Royer, F., Masson, N. and Abadie, E. (2007): Report on the first detection of pectenotoxin-2, spirolide-A and their derivatives in French shellfish. Mar. Drugs, **5**, 168-179.

Ares, I.R., Louzao, M.C., Espiña, B., Vieytes, M.R., Miles, C.O., Yasumoto, T. and Botana, L.M. (2007): Lactone ring of pectenotoxins: a key factor for their activity on cytoskeletal dynamics. Cell. Physiol. Biochem., **19**, 283-292.

Chae, H.D., Choi, T.S., Kim, B.M., Jung, J.H., Bang, Y.J. and Shin, D.Y. (2005): Oocyte-based screening of cytokinesis inhibitors and identification of pectenotoxin-2 that induces Bim/Bax-mediated apoptosis in p53-deficient tumors. Oncogene, **24**, 4813-4819.

Daiguji, M., Satake, M., James, K.J., Bishop, A., Mackenzie, L., Naoki, H. and Yasumoto, T. (1998): Structures of new pectenotoxin analogs, pectenotoxin-2 seco acid and 7-*epi*-pectenotoxin-2 seco acid, isolated from a dinoflagellate and Greenshell mussels. Chem. Lett., **7**, 653-654.

Espiña, B., Louzao, M.C., Ares, I.R., Cagide, E., Vieytes, M.R., Vega, F.V., Rubiolo, J.A., Miles, C.O., Suzuki, T., Yasumoto, T. and Botana, L.M. (2008): Cytoskeletal toxicity of pectenotoxins in hepatic cells. Br. J. Pharmacol., **155**, 934-944.

Espiña, B., Louzao, M.C., Ares, I.R., Fonfría, E.S., Vilariño, N., Vieytes, M.R., Yasumoto, T. and Botana, L.M. (2010): Impact of the pectenotoxin C-43 oxidation degree on its cytotoxic effect on rat hepatocytes. Chem. Res. Toxicol., **23**, 504-515.

Fladmark, K.E., Serres, M.H., Larsen, N.L., Yasumoto, T., Aune, T. and Døskeland, S.O. (1998): Sensitive detection of apoptogenic toxins in suspension cultures of rat and salmon hepatocytes. Toxicon, **36**, 1101-1114.

Ito, E., Suzuki, T., Oshima, Y. and Yasumoto, T. (2008): Studies of diarrhetic activity on pectenotoxin-6 in the mouse and rat. Toxicon, **51**, 707-716.

Kim, M.O., Moon, D.O., Kang, S.H., Heo, M.S., Choi, Y.H., Jung, J.H., Lee, J.D. and Kim, G.Y. (2008): Pectenotoxin-2 represses telomerase activity in human leukemia cells through suppression of hTERT gene expression and Akt-dependent hTERT phosphorylation. FEBS Lett., **582**, 3263-3269.

Kim, G.Y., Kim, W.J. and Choi, Y.H. (2011): Pectenotoxin-2 from marine sponges: a potential anti-cancer agent-a review. Mar. Drugs, **9**, 2176-2187.

Kobayashi, M., Kagawa, T., Takano, R., Itagaki, S., Hirano, T. and Iseki, K. (2007): Effect of medium pH on the cytotoxicity of hydrophilic statins. J. Pharm. Pharmaceut. Sci., **10**, 332-339.

Kobayashi, M., Kagawa, T., Narumi, K., Itagaki, S., Hirano, T. and Iseki, K. (2008): Bicarbonate supplementation as a preventive way in statins-induced muscle damage. J. Pharm. Pharmaceut. Sci., **11**, 1-8.

Korsnes, M.S., Hetland, D.L., Espenes, A., Tranulis, M.A. and Aune, T. (2006): Apoptotic events induced by yessotoxin in myoblast cell lines from rat and mouse. Toxicol. In Vitro, **20**, 1077-1087.

MacKenzie, L.A., Selwood, A.I. and Marshall, C. (2012): Isolation and characterization of an enzyme from the Greenshell™ mussel *Perna canaliculus* that hydrolyses pectenotoxins and esters of okadaic acid. Toxicon, **60**, 406-419.

Miles, C.O., Wilkins, A.L., Munday, R., Dines, M.H., Hawkes, A.D., Briggs, L.R., Sandvik, M., Jensen, D.J., Cooney, J.M., Holland, P.T., Quilliam, M.A., MacKenzie, A.L., Beuzenberg, V. and Towers, N.R. (2004): Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. Toxicon, **43**, 1-9.

Shin, D.Y., Kim, G.Y., Kim, N.D., Jung, J.H., Kim, S.K., Kang, H.S. and Choi, Y.H. (2008): Induction of apoptosis by pectenotoxin-2 is mediated with the induction of DR4/DR5, Egr-1 and NAG-1, activation of caspases and modulation of the Bcl-2 family in p53-deficient Hep3B hepatocellular carcinoma cells. Oncol. Rep., **19**, 517-526.

Shin, I.J., Ahn, Y.T., Kim, Y., Kim, J.M. and An, W.G. (2011): Actin disruption agents induce phosphorylation of histone H2AX in human breast adenocarcinoma MCF-7 cells. Oncol. Rep., **25**, 1313-1319.

Suzuki, T., Mitsuya, T., Matsubara, H. and Yamasaki, M. (1998): Determination of pectenotoxin-2 after solid-phase extraction from seawater and from the dinoflagellate *Dinophysis fortii* by liquid chromatography with electrospray mass spectrometry and ultraviolet detection: Evidence of oxidation of pectenotoxin-2 to pectenotoxin-6 in scallops. J. Chromatogr. A, **815**, 155-160.

Suzuki, T., Mackenzie, L., Stirling, D. and Adamson, J. (2001a): Pectenotoxin-2 seco acid; a toxin converted from pectenotoxin-2 by New Zealand Greenshell mussel, *Perna canaliculus*. Toxicon, **39**, 507-514.

Suzuki, T., Mackenzie, L., Stirling, D. and Adamson, J. (2001b): Conversion of pectenotoxin-2 to pectenotoxin-2 seco acid in the New Zealand scallop, *Pecten novaezelandiae*. Fisheries Science, **67**, 506-510.

Suzuki, T., Igarashi, T., Ichimi, K., Watai, M., Suzuki, M., Ogiso, E. and Yasumoto, T. (2005): Kinetics of diarrhetic shellfish poisoning toxins, okadaic acid, dinophysistoxin-1, pectenotoxin-6 and yessotoxin in scallops *Patinopecten yessoensis*. Fisheries Science, **71**, 948-955.

Suzuki, T., Walter, J.A., LeBlanc, P., Mackinnon, S., Miles, C.O., Wilkins, A.L., Munday, R., Beuzenberg, V., Mackenzie, A.L., Jensen, D.J., Cooney, J.M. and Quilliam, M.A. (2006): Identification of pectenotoxin-11 as 34*S*-hydroxypectenotoxin-2, a new pectenotoxin analogue in the toxic dinoflagellate *Dinophysis acuta* from New Zealand. Chem. Res. Toxicol., **19**, 310-318.

Yasumoto, T., Murata, M., Oshima, Y., Sano, M., Matsumoto, G.K. and Clardy J. (1985): Diarrhetic shellfish toxins. Tetrahedron, **41**, 1019-1025.

Yasumoto, T., Murata, M., Lee, J.S. and Torigoe, K. (1989): Polyether toxins produced by dinoflagellates, in Proc. Mycotoxins and Phycotoxins '88 (Natori, S., Hashimoto, K., Ueno, Y., Eds.), pp.375-382, Elsevier, Amsterdam.

Yasumoto, T. and Murata, M. (1993): Marine toxins. Chem. Rev. **93**, 1897.