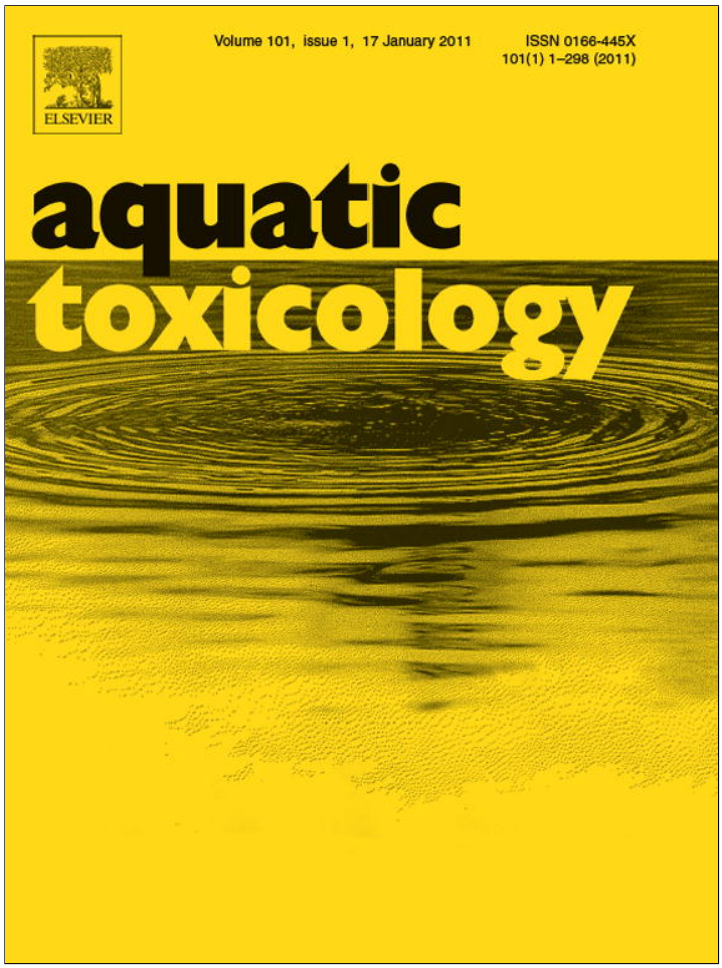

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Proteomic modification in gills and brains of medaka fish (*Oryzias melastigma*) after exposure to a sodium channel activator neurotoxin, brevetoxin-1

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ABSTRACT

Although brevetoxins (PbTxs) produced by the marine dinoflagellate *Karenia brevis* are known to be absorbed across gill membranes and exert their acute toxic effects through an ion-channel mediated pathway in neural tissue, the exact biochemical mechanism concerning PbTxs neurotoxicity in neural tissue and gas-exchange organs has not been well elucidated. In this study, we calculated the LC_{50} value of PbTx-1 using the medaka fish model, and presented the molecular responses of sub-acute exposure to PbTx-1 with proteomic method. By adopting two-dimensional electrophoresis, the abundances of 14 and 24 proteins were found to be remarkably altered in the gills and brains, respectively, in response to toxin exposure. Thirteen gill and twenty brain proteins were identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry analysis. These proteins could be categorized into diverse functional classes such as cell structure, macromolecule metabolism, signal transduction and neurotransmitter release. These findings can help to elucidate the possible pathways by which aquatic toxins affect marine organisms within target organs.

1. Introduction

The marine dinoflagellate *Karenia brevis* produces lipid-soluble polyether toxins (brevetoxins, PbTxs) that are accumulated by shellfish during coastal blooms of this organism. *K. brevis* blooms are implicated in massive fish kills, bird deaths, and marine mammal mortalities (Bossart et al., 1998; Flewelling et al., 2005). Studies indicate that the massive fish kills are probably due to the depletion of oxygen during biomass decomposition, the accumulation of PbTxs via direct ingestion by *K. brevis* cells or via contaminated prey, as well as the absorption of toxins across the gill membranes (Pierce and Henry, 2008). In humans, the consumption of PbTxs-contaminated shellfish causes food poisoning, known as neurotoxic shellfish poisoning.

Collectively, PbTxs are a family of ten naturally occurring derivatives, which could be differentiated into two distinct backbone structures, PbTx-1 (Type-A), and PbTx-2 and PbTx-3 (Type-B), with the latter being the predominant form (Pierce and Henry, 2008; Poli et al., 1986). Most efforts are devoted to exploring the action of PbTx-2 and -3 *in vivo* and *in vitro* (Choich et al., 2004; Plakas et al., 2002; Yan et al., 2006), and little is known concerning the toxicity of PbTx-1, albeit it is Type-A which presents the most potency among PbTxs (Kimm-Brinson and Ramsdell, 2001; Landsberg, 2002; Wang et al., 2005). PbTxs are known to exert their acute toxic effects in neural tissues through an ion-channel mediated pathway. Their primary mode of action is to act as sodium channel activators which interact with site 5 of the α-subunit of the voltage-gated sodium channel (VGSC), and augment Na^+ influx by increasing the mean opening time of the channel (Jeglitsch et al., 1998). The resultant VGSC activation promotes depolarization of neurons and, thus, can account for the neurotoxicity of PbTxs *in vitro*.

PbTxs are known to accumulate in the central nervous system (CNS) at concentrations sufficient to affect CNS functioning in animals (Cattet and Geraci, 1993). Lu and Tomchik (2002) describe a significant loss in auditory sensitivity in fish resulting from PbTx exposure and state that PbTx-3 could affect the CNS of fishes, as well as the peripheral auditory system. A recent study shows that *in vivo* sublethal PbTx exposure in bluegill *Lepomis macrochirus* increases neurological stimulation, as well as neurological alteration in

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calcium (Choich et al., 2004). The brain, as the major component of the CNS, is the primary target of PbTxs (Poli et al., 1986). Thus, investigation of the brain could help to elucidate the cellular consequences of PbTx exposure on the CNS. Additionally, studies on the mechanisms of freshwater toxins suggest that the massive fish kills involved may result from the loss of ion homeostatic processes produced by their inhibitory action on the ion pumps of gill (Gaete et al., 1994). Studies on fish kills caused by microbial toxins also involve observations of the pathological changes in the fish gills (Lewis et al., 2009). According to Montoya et al. (1996), South American kills of mackerel, *Scomber japonicus*, in part, can be attributed to direct exposure to *Alexandrium tamarense* blooms, and the accumulation of toxins in the gills. As a sophisticated organ, the gill performs the function of gas exchange, ion regulation, acid–base balance and nitrogen excretion (Evans et al., 2005). It also receives the entire cardiac output, and functions as a major site of circulatory or vasomotor control (Olson, 2002; Jonz and Zaccone, 2009). After being affected by PbTxs both from the outer aquatic environment and from the inner blood circulation during *K. brevis* blooms, the gills might play a vital role in fish mortality. Thus, investigation of the gills will help elucidate the mechanism of marine biotoxins in relation to massive fish kills.

The medaka fish (*Oryzias melastigma*), as a marine counterpart of the freshwater Japanese medaka (*Oryzias latipes*), is a preferred model fish in toxicological studies (Padilla et al., 2009). In the current study, the medaka fish is used as an ideal model to simulate the fish in the natural marine environment. Meanwhile, global techniques, such as proteomics, provide effective strategies and tools for toxicological studies, and are regarded as a powerful tool in the investigation of the cellular responses to toxicants (Dowling and Sheehan, 2006). In this study, the medaka fish were exposed to a PbTx-1 concentration of 6 μg/L for 2 days (d) and the protein profiles of the gill and brain were analyzed using the proteomic approach, and then identified using matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry (MALDI-TOF/TOF MS) analysis. Our results showed that the expression of proteins involved in cell structure, macromolecule metabolism and signal transduction were significantly disrupted, which may help explain the damage mechanisms of aquatic toxins in fish.

2. Materials and methods

2.1. Medaka fish maintenance and LC_{50} calculation

Both sexes of medaka fish (weighing 0.5 ± 0.05 g) were acclimatized in aerated seawater tanks for 3 days prior to the toxicity assay at a water temperature of 25 °C with a 14 h:10 h light–dark cycle and fed twice a day (9:00 a.m. and 3:00 p.m.) with commercial dried artemia bait. Seven groups of fish, each consisting of eight individuals, were randomly placed into beakers, and then exposed to different concentrations (0, 6, 8, 10, 12, 16 and 18 μg/L) of PbTx-1, which was dissolved in dimethyl sulfoxide (DMSO). During the exposure period, fish were not fed and dead fish were removed. The acute exposure period lasted for 24 h, and fish mortality was assessed at the end of the exposure period. Two replicate experiments were performed, and the LC_{50} value ($p < 0.05$) was calculated with SPSS Statistics 17.0 using the Probit Analysis Statistical Method (Finney, 1971).

2.2. Medaka fish exposure experiment

Both sexes of medaka fish were acclimatized for 15 days under the conditions as described above, except a 12 h:12 h light–dark cycle was adopted to prevent fish entering the reproductive phase. They were then randomly assigned into two groups, the exposure group and the control group, each containing 16 individuals. Based on the 24 h LC_{50} value, the PbTx-1 exposure concentration was selected to be 6 μg/L. Exposure was carried out in glass tanks (25 cm × 20 cm × 20 cm) containing filtered water under acclimation conditions for 2 days. Each day, half of the aged water was renewed with fresh water containing 6 μg/L PbTx-1. The control group received the same volume of DMSO as that added into the dilution of the dosing concentration. No mortality occurred in the control group, but 10% mortality was observed in the exposure group. At the end of the experiment, the live medaka fish were anaesthetized and dissected on ice. Gills and brains were collected and stored at −80 °C for proteomic analysis. Two parallel replicates for each group were performed, and tissues were pooled.

2.3. Protein extraction and 2D electrophoresis

Frozen fish gills and brains were homogenized in 0.6 mL of 20% trichloroethane/acetone (w/v) with 20 mM dithiothreitol (DTT) using an ultrasonic disrupter (Branson, US). Protein pellet was collected by centrifugation at 17,000 × *g* for 30 min at 4 °C, washed twice with 80% acetone (v/v) and twice with ice-cold acetone containing 20 mM DTT, then dried with SpeedVac for 5 min, and finally dissolved in 100 μL rehydration buffer (8 M urea, 2% CHAPS, 2.8 mg/mL DTT, 0.5% immobilized pH gradient (IPG) buffer, a trace of bromophenol blue). Undissolved material was discarded by centrifugation at 20,000 × *g* for 30 min at 15 °C. The protein concentration was quantified using the 2-D Quant kit (GE Healthcare, US) in SmartSpec 3000 (Bio-Rad, US).

For the first dimension, 500 μg protein were loaded onto IPG strips of linear pH gradient 4–7 (GE Healthcare). Rehydration and subsequent isoelectric focusing were conducted using the Ettan IPGphor III Isoelectric Focusing System (GE Healthcare, USA) as follows: 40 V for 6 h, 100 V for 6 h, 500 V for 1 h, 1000 V for 1 h, 8000 V for 2 h and 8000 V for 50,000 V h. Before the second dimension, each strip was equilibrated with 10 mL of equilibration buffer I (50 mM Tris (pH 8.8), 6 M urea, 30% glycerol, 2% SDS, 1% DTT and a trace of bromophenol blue) for 17 min, and then with 10 mL of equilibration buffer II (equilibration buffer I containing 2.5% iodoacetamide instead of 1% DTT). The second dimension was performed on 11.5% SDS-PAGE at 10 mA/gel for 15 min and then 200 V until the bromophenol blue front reached the edge of the gels. Triplicates for both the exposure and control were performed.

2.4. Image capture and processing

Gels were visualized with the colloidal Coomassie staining following an improved blue silver method described by Candiano et al. (2004). Briefly, the gel was fixed for 1 h initially in a fixation solution containing 40% (v/v) methanol and 10% (v/v) acetic acid, followed by four Milli-Q water washes (15 min each time). Then the gel was stained overnight in the working colloidal "blue silver" solution with 0.12% Coomassie Brilliant Blue G-250, 10% ammonium sulfate, 10% phosphoric acid, and 20% methanol. Subsequently, the gels were destained for 2 h via six Milli-Q water washes (20 min each time).

Gels were scanned using a Gel-documentation system on a GS-670 Imaging Densitometer (Bio-Rad, US). Gels were analyzed with the ImageMaster 2D Elite. Spot intensities were normalized with total valid spot volume in order to minimize the non-expression related variations in spot intensity and hence accurately provide semiquantitative information across different gels. Differences of ≥1.5 in expression (ratio %V) between matched spots were considered significant when a spot group passed statistical analysis (an independent-samples *t*-test, $p < 0.05$).

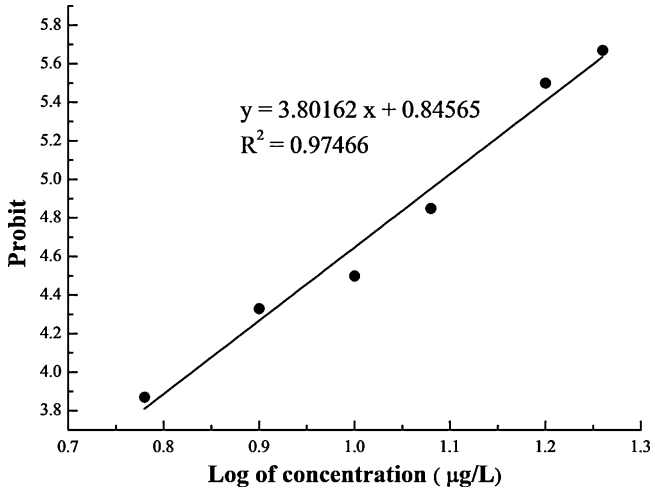

Fig. 1. The relationship between PbTx-1 concentration and mortality probit of the medaka fish during the 24 h acute toxicity test.

2.5. MALDI-TOF mass spectrometry

Differentially expressed protein spots were manually excised from 2-DE gels, and washed sequentially with water and 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate, until they were completely destained. After dehydration with 100% ACN, the dry gel pieces were digested by adding 10.0 ng/μL trypsin (Promega, Madison, WI) in 25 mM ammonium bicarbonate overnight at 37 °C.

For MALDI-TOF/TOF MS analysis, 2 μL of the digest peptide was mixed with 1 μL of matrix (100 mM α-cyano-4-hydroxy-cinnamic acid in 50% ACN and 0.1% trifluoroacetic acid) on the target plate. MALDI-TOF MS and TOF-TOF tandem MS were performed on a 4800 Proteomics Analyzer (Applied Biosystems, Foster City, CA), and data were acquired in the positive MS reflector mode with a scan range of 900–4000 Da, and five monoisotopic precursors (S/N > 200) were selected for MS/MS analysis. For interpretation of the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm. One missed cleavage site was allowed for trypsin digestion; cysteine carbamidomethylation was assumed as a fixed modification; and methionine was assumed to be partially oxidized. Results with CI% (Confidence Interval %) values greater than 95% were considered to be a positive identification. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (http://www.geneontology.org/).

3. Results

3.1. The 24 h LC_{50} value for medaka fish exposed to PbTx-1

According to the method of Probit analysis (Finney, 1971), Probit value 5.0 corresponds to 50% mortality. With the Probit Analysis Statistical Method and

SPSS Statistics 17.0, the linear relationship between PbTx-1 concentration and the mortality Probit of the medaka fish was calculated (Fig. 1). Based on this linear relationship, the 24 h LC_{50} value was 12.38 μg/L with a 95% confidence limit of 10.43–14.69 μg/L.

3.2. Identification of proteins in medaka fish

Representative 2-DE gels of gill and brain proteins of PbTx-1 exposed and non-exposed medaka fish are shown in Figs. 2 and 3, and quantitative spot comparisons were made with ImageMaster Platinum software version 5.0. On average, more than 800 protein spots were detected in each gel using the Coomassie Blue Staining method.

3.2.1. Changes in protein profiles in medaka fish gill exposed to PbTx-1

Compared with the 2-DE gels of the non-exposed medaka fish gills, a total of 14 protein spots from the PbTx-exposed medaka fish gills were found to be significantly altered in abundance (percentage volume ≥1.5, $p < 0.05$). Among these altered proteins, one protein spot disappeared in the PbTx-exposed treatment, eight protein spots were significantly downregulated, and five protein spots were noticeably upregulated (Fig. 2). Thirteen protein spots were successfully identified (Table 1) and all the matched proteins came from the NCBI database for fish species. Of these, six protein spots (1, 2, 5, 6, 7 and 8) were involved in cell structure; two (spots 9 and 12) were concerned with metabolism, one participating in lipid binding and the other in carbohydrate metabolism; and five proteins (spots 3, 4, 10, 11 and 13) were involved in signal transduction, mostly in calcium ion binding.

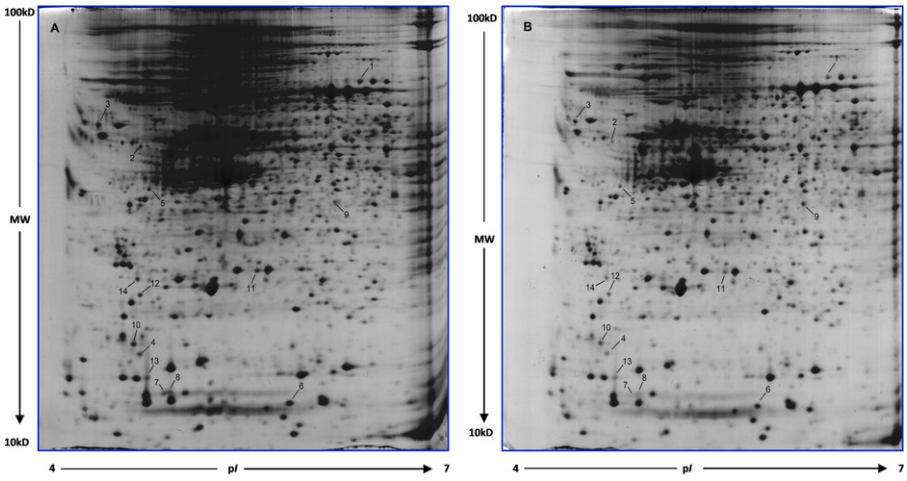

Fig. 2. Representative 2-DE gels of gill proteins in the medaka fish after 2 d exposure to PbTx-1. (A) Control and (B) 6 μg/L. The soluble proteins from medaka fish gills were separated using 2-DE and visualized with colloidal Coomassie G-250 staining. The protein spots altered by PbTx-1 exposure are labeled with numbers. The molecular weights (MW) and p*I* scales are indicated. Each gel is representative of three independent replicates.

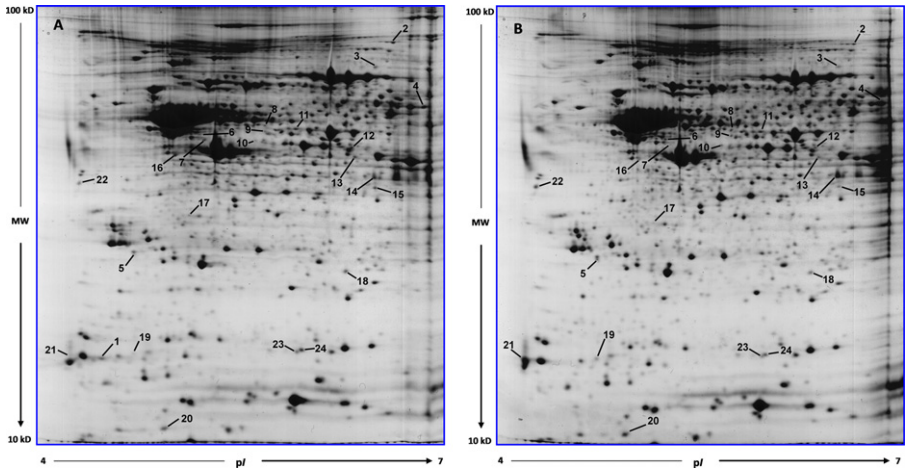

Fig. 3. Representative 2-DE gels of brain proteins in the medaka fish after 2 d exposure to PbTx-1. (A) Control and (B) 6 μg/L. The soluble proteins from medaka fish brains were separated using 2-DE and visualized with colloidal Coomassie G-250 staining. The protein spots altered by PbTx-1 exposure are labeled with numbers. The molecular weights (MW) and p*I* scales are indicated. Each gel is representative of three independent replicates.

3.2.2. Changes in protein profiles in medaka fish brain exposed to PbTx-1

Compared with the 2-DE gels of the non-exposed medaka fish brains, a total of 24 protein spots from the PbTx-exposed medaka fish brains were found to be significantly altered in abundance (percentage volume ≥1.5 fold, $p < 0.05$). Among these altered proteins, one protein spot disappeared in the PbTx-exposed treatment, 12 protein spots were significantly downregulated, and 11 protein spots were noticeably upregulated (Fig. 3). However, only 20 protein spots were successfully identified with CI% values greater than 95% (Table 2), and all the matched proteins came from the NCBI database for fish species. The 20 successfully identified protein spots were distinguished into 19 different proteins. Of these, eight protein spots (3, 6, 7, 8, 9, 16, 19 and 20) were involved in cell structure, and eight proteins (2, 4, 5, 11, 12, 13, 14 and 15) were concerned with metabolism, with most of them participating in carbohydrate metabolism. Two proteins were involved in signal transduction, corresponding to calmodulin and transforming protein RhoA. The other two proteins were characterized as Beta-synuclein and SH3-domain GRB2-like endophilin B2 (SH3GLB2). Beta-synuclein is related to neurotransmitter release; however the function of SH3GLB2 is unknown (Fig. 4).

4. Discussion

In this study, the 24 h LC_{50} value of PbTx-1 in medaka fish (*O. melastigma*) was found to be 12.38 μg/L. When exposed to PbTx-1 at half of the concentration of LC_{50} (6.0 μg/L) for 48 h, medaka fish may have metabolized and accumulated the toxin within the body, thus leading to a significant proteomic response. The brain, which is the major component of the central nervous system, is the main target of the neurotoxin, PbTx-1 (Poli et al., 1986). Additionally, previous studies emphasized that the loss of normal gill functions led to fish kills (Gaete et al., 1994; Lewis et al., 2009). Therefore,

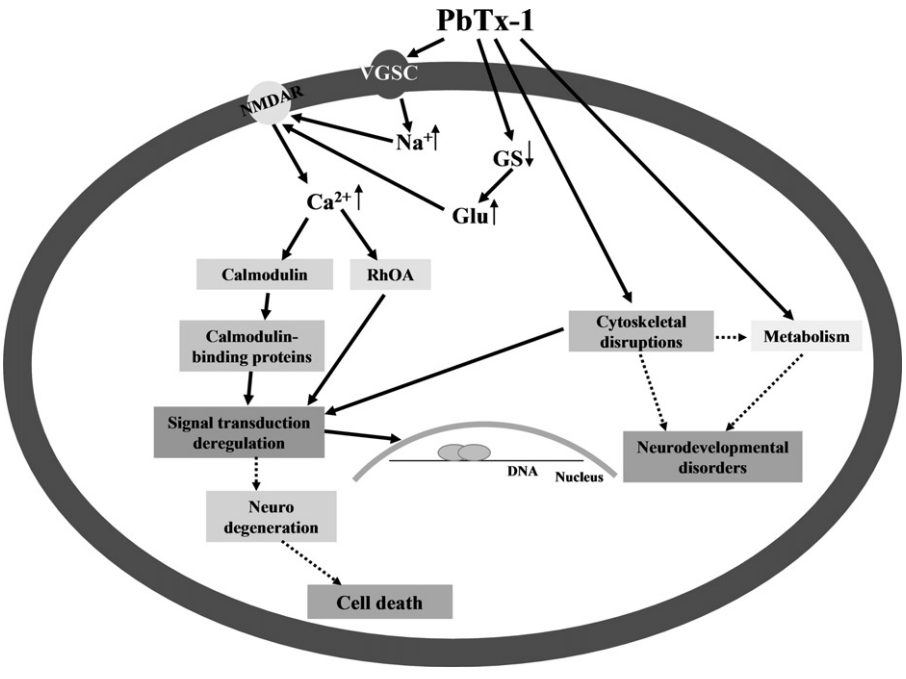

Fig. 4. The proposed scheme illustrates the cellular events in medaka fish brains following PbTx-1 treatment. Glu: glutamate; GS: glutamine synthetase; NMDAR: N-methyl-D-aspartate receptor; VGSC: voltage-gated sodium channel.

Table 1
A detailed list of protein spots identified using MALDI-TOF/TOF MS from the gill of medaka fish (*Oryzias melastigma*) following PbTx-1 exposure.

Spot ID	Protein identity	Accession number	Protein score	Expectation value	MW/p*I*	Fold change	Organism	Function category
Cell structure								
2	Histone-binding protein RBBP4	gi| 88930443	138	2.4e−9	47.65/4.74	−1.62	*Danio rerio*	Core histone-binding subunit
1	Gelsolin	gi|9800189	127	3.1e−8	80.07/6.95	−1.90	*Danio rerio*	Actin binding
5	Krt4 protein	gi|161612220	258	2.4e−21	54.01/5.34	−1.52	*Danio rerio*	Keratin filament
6	Hemoglobin beta chain	gi|13876583	132	9.6e−9	16.54/6.95	3.33	*Epinephelus coioides*	Hemoglobin complex; oxygen binding
7	Histone H3	gi|148372467	85	4.7e−4	12.30/10.6	D[a]	*Sillago chondropus*	Chromosomal protein
8	Histone H3	gi|30140280	107	3.1e−6	12.35/10.8	−4.70	*Nectonemertes mirabilis*	Chromosomal protein
Metabolism								
12	apoA-IV4	gi|74096419	127	3.1e−8	28.49/4.78	−1.68	*Takifugu rubripes*	Lipid binding
9	Aldose reductase	gi|209733154	83	7.7e−4	35.40/6.76	1.84	*Salmo salar*	Oxidoreductase activity
Signal transduction								
4	Putative transient receptor protein 2	gi|37779086	126	3.8e−8	19.57/4.67	2.23	*Pagrus major*	Calcium ion binding; receptor activity
10	Myosin regulatory light chain 2	gi|225706388	231	1.2e−18	19.83/4.68	1.87	*Osmerus mordax*	Calcium ion binding
11	Grancalcin	gi|213514306	66	4.1e−2	24.54/5.02	−1.59	*Salmo salar*	Calcium ion binding
13	Myosin light chain 2	gi|222087969	68	2.3e−2	18.16/4.48	2.40	*Epinephelus coioides*	Calcium ion binding
3	Calreticulin, like 2	gi|41054373	182	9.6e−14	48.95/4.40	−1.65	*Danio rerio*	Calcium ion binding

Table 2
A detailed list of protein spots identified using MALDI-TOF/TOF MS from the brain of medaka fish (*Oryzias melastigma*) following PbTx-1 exposure.

Spot ID	Protein identity	Accession number	Protein score	Expectation value	MW/p*I*	Fold change	Organism	Function category
Cell structure								
3	Gelsolin	gi|9800189	74	6.4e−3	80.07/6.95	−1.75	*Danio rerio*	Non-motor actin binding protein
6	Glial fibrillary acidic protein	gi|20977259	66	3.8e−2	42.15/5.03	3.52	*Danio rerio*	Intermediate filament
7	Keratin 15	gi| 47087241	78	2.4e−3	49.15/5.13	−5.00	*Danio rerio*	Intermediate filament
8	Zgc:65851	gi|41054736	71	1.3e−2	64.83/4.79	1.76	*Danio rerio*	Intermediate filament
9	Zgc:65851	gi|41054736	183	7.7e−14	64.83/4.79	1.91	*Danio rerio*	Intermediate filament
16	Type I cytokeratin, enveloping layer	gi|41388915	69	1.8e−2	46.53/5.13	−6.67	*Danio rerio*	Intermediate filament
19	Myosin light chain 2	gi|16117353	554	6.1e−51	19.12/4.67	1.71	*Pseudocaranx dentex*	Calcium ion binding
20	Tropomyosin alpha-3 chain	gi|223647762	68	2.7e−2	28.44/4.71	1.64	*Salmo salar*	Actin binding motor protein
Metabolism								
2	Pyruvate carboxylase	gi|27884125	89	2.1e−4	79.73/5.99	−2.04	*Danio rerio*	Carbohydrate metabolism
4	Dpysl5a protein	gi|66392186	124	6.1e−8	61.71/6.36	2.64	*Danio rerio*	Pyrimidine metabolism
5	Triosephosphate isomerase	gi|77417615	491	1.2e−44	27.38/4.77	2.14	*Oryzias latipes*	Carbohydrate metabolism
11	Enolase	gi|226441951	408	2.4e−36	43.91/5.16	−1.92	*Gillichthys mirabilis*	Carbohydrate metabolism
12	Glutamine synthetase	gi|185135730	116	3.8e−7	42.52/5.93	−3.70	*Oncorhynchus mykiss*	Amino acid metabolism
13	Isovaleryl coenzyme A dehydrogenase	gi|26788031	146	3.8e−10	46.39/7.96	−1.89	*Danio rerio*	Acyl-CoA metabolism
14	Glyceraldehyde 3-phosphate dehydrogenase	gi|51895785	437	3.1e−39	36.17/6.40	1.61	*Haplochromis burtoni*	Carbohydrate metabolism
15	Aldose reductase	gi|209733154	99	1.8e−5	35.67/6.76	−1.61	*Salmo salar*	Carbohydrate metabolism
Signal transduction								
18	Transforming protein RhoA	gi|209734062	401	1.2e−35	22.19/5.83	−3.57	*Salmo salar*	Small GTPase
21	Calmodulin	gi|78099193	389	1.9e−34	16.84/4.05	8.90	*Oreochromis mossambicus*	Calcium ion binding
Other function related								
1	Beta-synuclein	gi|41055752	70	1.4e−2	13.33/4.37	D[a]	*Danio rerio*	Neurotransmitter release
10	SH3-domain GRB2-like endophilin B2	gi|123232871	169	1.9e−12	45.00/5.59	1.80	*Danio rerio*	Unknown

MW: molecular weight. p*I*: isoelectric point.
Note: Variations were calculated as treated/control spot volume ratio and if the result was below 1, it is reported as − control/treated ratio.
D[a] indicates a protein which disappeared in the treated group.

both the brain and the gills were selected as the two target tissues for the proteomic analysis. In this study, the expression levels of myosin like proteins, aldose reductase, gelsolin and keratin were found to be altered in both tissues, which may be the common cellular targets of PbTx-1.

Brevetoxins are known to activate the voltage-gated sodium channel, which will induce the subsequent increase of intracellular Ca^{2+} levels (George et al., 2009; Liberona et al., 2008). In this study, several proteins related to calcium ion binding were found to be altered. Myosin like proteins, which are involved in calcium uptake and transduction, and associated with muscle contraction (Kendrick-Jones and Jakes, 1976), were found up-regulated in both the gills and the brains after exposure to PbTx-1. In addition, it had been previously demonstrated that the symptom of smooth muscles contraction was induced after PbTxs treatment (Gallagher and Shinnick-Gallagher, 1985; Ishida and Shibata, 1985). Similarly, calmodulin, as one major Ca^{2+}-binding protein found mainly in the brain, displayed an increase in expression, which was implicated in the calcium signaling process and this process was known to be affected by PbTx-2 (George et al., 2009). However, there was a decrease in the amount of calreticulin in the gills, which is one calcium modulator in endoplasmic reticulum (Kales et al., 2007), and this was speculated to be partially caused by the increase of Ca^{2+} flux outside the cells. This is the first study that directly relates the changed expressions of calcium ion binding proteins to the PbTx-1 exposure.

Aldose reductase, which was found up-regulated in the gills of the PbTx-1 exposed fish, is an NADPH-dependent oxidoreductase responsible for the conversion of glucose to sorbitol. A study on diabetes indicated that the production of sorbitol was associated with the inactivation of nodal Na^{+}-channels (Cherian et al., 1996). Furthermore, aldose reductase was suggested to be a primary extrahepatic detoxification enzyme for the protection against xenobiotic and endogenous aldehydes (Vander Jagt et al., 1995). Therefore, the increased expression of aldose reductase might play a multi-functional role against the sodium channel activator PbTx-1, and which requires further study. However, in the brain, aldose reductase showed down-regulation, together with another gluconeogenesis enzyme pyruvate carboxylase, while the glycolytic enzymes triosephosphate isomerase (Tpi) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) exhibited up-regulation. Taken together, the down-regulation of aldose reductase and pyruvate carboxylase, and the simultaneous up-regulation of Tpi and GAPDH suggested a metabolic shift from gluconeogenesis to glycolysis, and thus an increased energy supply. However, enolase, another glycolytic enzyme, was down-regulated, whereas according to recent studies, enolase possesses a variety of regulating properties besides glycolytic function in the brain (Pancholi, 2001), and its function here remains unknown.

Gelsolin is an actin regulatory protein, which plays roles in the organization of the cytoskeleton, cell motility, cell growth and apoptosis (Koya et al., 2000). The significant decrease of gesolin expression under toxin treatment highlighted the increasing cell damage (e.g. cell death) in the brains and gills due to PbTx-1 attack.

Additionally, the proteins keratin4 (Krt4), keratin 15 (Krt15) and type I cytokeratin, which belong to cytokeratins, were significantly depressed under PbTx-1 treatment. Krt15 and cytokeratin I are noted in fish glial cells (Giordano et al., 1990; Merrick et al., 1995). Their primary function is to protect cells from stress damage which may result in cell death and to maintain normal signal transduction (Looi et al., 2009; Schaffeld and Schultess, 2006). Likewise, Krt4 in the gills is known to be involved in the maintenance of cellular architecture and to provide mechanical resistance against stress (Kirfel et al., 2002), the down-regulation of which suggests an effect on tissue integrity (Bohne-Kjersem et al., 2010).

Since gills receive the entire cardiac output to complete the blood gas exchange (Olson, 2002), hemoglobin, the characteristic protein in blood, was observed in the 2-D gels from the gills. Up-regulation of blood characteristic protein hemoglobin might suggest hyperemia after PbTx-1 exposure, which was previously proposed to be related to chronic exposure of brevetoxins from diet (Jacobson et al., 2006). However, a histological study needs to be involved as validation in future work. Lipid is the major energy source for fish and lipid metabolism is important for fish homeostasis maintenance. Apolipoprotein IV4 (apoA-IV4), a member of the apolipoprotein family, involved in the functions of lipid transportation and branchial epithelial integrity maintenance (Smith et al., 2005), showed down-regulation in gills of exposed fish. The down-regulation of apoA-IV4 might be a sign of the reduced energy supply in the gill and decreased levels of apoA is also thought to be a response to inflammation (Tietge et al., 2002; He et al., 2003). Moreover, histone, the chromosomal protein, also exhibited decreased levels, and in particular, the histone H3 spot disappeared in the treated fish gills. Histones are the chief protein components of chromatin, acting as spools around which DNA winds, thus enabling the compaction necessary to fit the large genomes of eukaryotes inside cell nuclei. Histones also act in diverse biological processes, such as gene regulation, DNA repair and chromosome condensation.

In brain, an important signal transduction protein, RhoA, was found to be down-regulated. RhoA has been reported to prevent apopotosis through Mek/ErK pathway during zebrafish embryogenesis (Zhu et al., 2008). We proposed that when PBTx enters the brain, the neurotoxin may cause increased cell death by inhibition of RhoA. Additionally, beta-synuclein, an inhibitor of alpha-synuclein aggregation (Hashimoto et al., 2001), was found to be inhibited in the brain by PbTx-1 treatment. Alpha-synuclein is an abnormal protein inclusion associated with several neurodegenerative disorders (e.g., Parkinson's disease) (Goedert et al., 1998). Therefore, the inhibition of beta-synuclein may depress its protective role in the central nervous system. Taken together, these proteins may be the preliminary targets of PbTx-1 in brain.

Proteins involved in amino acid metabolism were also altered in the brains of medaka fish exposed to PbTx-1. Isovaleryl coenzyme A dehydrogenase (IVD), a nucleus-encoded mitochondrial flavoprotein involved in the leucine catabolism pathway (Volchenboum and Vockley, 2000), showed down-regulate after exposure. Glutamine synthetase (GS) catalyzes the synthesis of glutamine from glutamate and ammonia. Down-regulation of GS in PbTx-1 treated brain suggested an increased level of glutamate, which is responsible for mediating both synaptic and extraynaptic neurotransmission. High glutamate concentrations can even cause excitotoxicity, the uncontrolled influx of calcium into the cells and consequent dysfunction of calcium-dependent homeostatic mechanisms (Johnston, 2005). Previous studies reported the same excitotoxicity associated with high glutamate concentration and elevated intracellular Ca^{2+} after brevetoxin exposure (Berman and Murray, 2000; Cao et al., 2007).

In conclusion, our study provided a new insight into PbTx-1 neurotoxicity in medaka gills and brains at the proteomic level. To the best of our knowledge, this is the first study that has established a direct relationship between Ca^{2+}-binding proteins and the sodium channel activator, PbTx-1. Moreover, the down-regulation of several proteins involved in cell protection, such as cytokeratins, glutamine synthetase, RhoA, beta-synuclein, may lead to the dysfunction of cytoskeleton assembly, lipid, carbohydrate and amino acid metabolism. With the assistance of the proteomic approach combined with other methods, we hope to elucidate the toxicological mechanism of aquatic toxins in marine animals in the future.

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References

Berman, F.W., Murray, T.F., 2000. Brevetoxin-induced autocrine excitotoxicity is associated with manifold routes of Ca^{2+} influx. J. Neurochem. 74, 1443–1451.

Bohne-Kjersem, A., Bache, N., Meier, S., Nyhammer, G., Roepstorff, P., Sæle, Ø., Goksøyr, A., Grøsvik, B.E., 2010. Biomarker candidate discovery in Atlantic cod (*Gadus morhua*) continuously exposed to North Sea produced water from egg to fry. Aquat. Toxicol. 96, 280–289.

Bossart, G.D., Baden, D.G., Ewing, R.Y., Roberts, B., Wright, S.D., 1998. Brevitoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 epizootic: gross, histologic and immunohistochemical features. Toxicol. Pathol. 26, 276–282.

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G.M., Carnemolla, B., Orecchia, P., Zardi, L., Righetti, P.G., 2004. Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteomic analysis. Electrophoresis 25, 1327–1333.

Cao, Z., George, J., Baden, D.G., Murray, T.F., 2007. Brevetoxin-induced phosphorylation of Pyk2 and Src in murine neocortical neurons involves distinct signaling pathways. Brain Res. 1184, 17–27.

Cattet, M., Geraci, J.R., 1993. Distribution and elimination of ingested brevetoxin (PbTx-3) in rats. Toxicon 31, 1483–1486.

Cherian, P.V., Kamijo, M., Angelides, K.J., Sima, A.A., 1996. Nodal Na^+-channel displacement is associated with nerve-conduction slowing in the chronically diabetic BB/W rat: Prevention by aldose reductase inhibition. J. Diabetes Complicat. 10, 192–200.

Choich, J., Salierno, J.D., Silbergeld, E.K., Kane, A.S., 2004. Altered brain activity in brevetoxin-exposed bluegill, *Lepomis macrochirus*, visualized using in vivo ^{14}C 2-deoxyglucose labeling. Environ. Res. 94, 192–197.

Dowling, V.A., Sheehan, D., 2006. Proteomics as a route to identification of toxicity targets in environmental toxicology. Proteomics 6 (20), 5597–5604.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Finney, D.J., 1971. Statistical Analysis. Cambridge University Press.

Flewelling, L.J., Naar, J.P., Abbott, J.P., Baden, D.G., Barros, N.B., Bossart, G.D., Bottein, M.-Y.D., Hammond, D.G., Haubold, E.M., Hei, L.C.A., Henry, M.S., Jacocks, H.M., Leighfield, T.A., Pierce, R.H., Pitchford, T.D., Rommel, S.A., Scott, P.S., Steidinger, K.A., Truby, E.W., Van Dolah, F.M., Landsberg, J.H., 2005. Brevitoxicosis: red tides and marine mammal mortalities. Nature 435, 755–756.

Gaete, V., Canelo, E., Lagos, N., Zambrano, F., 1994. Inhibitory effects of *Microcystis aeruginosa* toxin on ion pumps of the gill of freshwater fish. Toxicon 32, 121–127.

Gallagher, J.P., Shinnick-Gallagher, P., 1985. Effects of crude brevetoxin on membrane potential and spontaneous or evoked end-plate potentials in rat hemidiaphragm. Toxicon 23 (3), 489–496.

George, J., Dravid, S.M., Prakash, A., Xie, J., Peterson, J.H., Jabba, S.V., Baden, D., Murray, T.F., 2009. Sodium channel activation augments NMDA receptor function and promotes neurite outgrowth in immature cerebrocortical neurons. J. Neurosci. 29, 3288–3301.

Giordano, S., Hall, C., Quitschke, W., Glasgow, E., Schechter, N., 1990. Keratin 8 of simple epithelia is expressed in glia of the goldfish nervous system. Differentiation 44 (3), 163–172.

Goedert, M., Spillantini, M.G., Davies, S.W., 1998. Filamentous nerve cell inclusions in neurodegenerative diseases. Curr. Opin. Neurobiol. 8, 619–632.

Hashimoto, M., Rockenstein, E., Mante, M., Mallory, M., Masliah, E., 2001. β-Synuclein inhibits α-synuclein aggregation: a possible role as an anti-parkinsonian factor. Neuron 32, 213–223.

He, Q.Y., Lau, G.K.K., Zhou, Y., Yuen, S.T., Lin, M.C., Kung, H.F., Chiu, J.F., 2003. Serum biomarkers of hepatitis B virus infected liver inflammation: a proteomic study. Proteomics 3, 666–674.

Ishida, Y., Shibata, S., 1985. Brevetoxin-B of *Gymnodinium breve* toxin-induced contractions of smooth muscles due to the transmitter release from nerves. Pharmacology 31 (4), 237–240.

Jacobson, E.R., Homer, B.L., Stacy, B.A., Greiner, E.C., Szabo, N.J., Chrisman, C.L., Origgi, F., Coberley, S., Foley, A.M., Landsberg, J.H., Flewelling, L., Ewing, R.Y., Moretti, R., Schaf, S., Rose, C., Mader, D.R., Harman, G.R., Manire, C.A., Mettee, N.S., Mizisin, A.P., Shelton, G.D., 2006. Neurological disease in wild loggerhead sea turtles *Caretta caretta*. Dis. Aquat. Org. 70 (1–2), 139–154.

Jeglitsch, G., Rein, K., Baden, D.G., Adams, D.J., 1998. Brevetoxin-3 (PbTx-3) and its derivatives modulate single tetrodotoxin-sensitive sodium channels in rat sensory neurons. J. Pharmacol. Exp. Ther. 284, 516–525.

Johnston, M.V., 2005. Excitotoxicity in perinatal brain injury. Brain Pathol. 15, 234–240.

Jonz, M.G., Zaccone, G., 2009. Nervous control of the gills. Acta Histochem. 111 (3), 207–216.

Kales, S.C., Bols, N.C., Dixon, B., 2007. Calreticulin in rainbow trout: a limited response to endoplasmic reticulum (ER) stress. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 147, 607–615.

Kendrick-Jones, J., Jakes, R., 1976. The regulatory function of the myosin light chains. Trends Biochem. Sci. 1, 281–284.

Kimm-Brinson, K.L., Ramsdell, J.S., 2001. The red tide toxin, brevetoxin, induces embryo toxicity and developmental abnormalities. Environ. Health Perspect. 109 (4), 377–381.

Kirfel, J., Peters, B., Grund, C., Reifenberg, K., Magin, T.M., 2002. Ectopic expression of desmin in the epidermis of transgenic mice permits development of a normal epidermis. Differentiation 70 (1), 56–68.

Koya, R.C., Fujita, H., Shimizu, S., Ohtsu, M., Takimoto, M., Tsujimoto, Y., Kuzumaki, N., 2000. Gelsolin inhibits apoptosis by blocking mitochondrial membrane potential loss and cytochrome c release. J. Biol. Chem. 275, 15343–15349.

Landsberg, J.H., 2002. The effects of harmful algal blooms on aquatic organisms. Rev. Fish. Sci. 10, 113–390.

Lewis, J.W., Morley, N.J., Drinkall, J., Jamieson, B.J., Wright, R., Parry, J.D., 2009. Toxic effects of *Streptomyces griseus* spores and exudate on gill pathology of freshwater fish. Ecotoxicol. Environ. Saf. 72, 173–181.

Liberona, J.L., Cárdenas, J.C., Reyes, R., Hidalgo, J., Molgó, J., Jaimovich, E., 2008. Sodium-dependent action potentials induced by brevetoxin-3 trigger both IP3 increase and intracellular Ca^{2+} release in rat skeletal myotubes. Cel. Calc. 44, 289–297.

Looi, M., Karsani, S., Rahman, M., Dali, A., Ali, S., Ngah, W., Yusof, Y., 2009. Plasma proteome analysis of cervical intraepithelial neoplasia and cervical squamous cell carcinoma. J. Biosci. 34, 917–925.

Lu, Z., Tomchik, S.M., 2002. Effects of red tide toxin on fish hearing. J. Comp. Physiol. A 188, 807–813.

Merrick, S.E., Pleasure, S.J., Lurie, D.I., Pijak, D.S., Selzer, M.E., Lee, V.M., 1995. Glial cells of the lamprey nervous system contain keratin-like proteins. J. Comp. Neurol. 1355, 199–210.

Montoya, N.G., Akselman, R., Franco, J., Carreto, J.I., 1996. Paralytic shellfish toxins and mackerel (*Scomber japonicus*) mortality in the Argentine Sea. In: Harmful and Toxic Algal Blooms, Intergovernmental Oceanographic Commission , UNESCO, pp. 417–420.

Olson, K.R., 2002. Gill circulation: regulation of perfusion distribution and metabolism of regulatory. J. Exp. Zool. 293 (3), 320–335.

Padilla, S., Cowden, J., Hinton, D.E., 2009. Use of medaka in toxicity testing. Methods in toxicity testing. Curr. Protoc. Toxicol. 39, 1.10.1–1.10.36.

Pancholi, V., 2001. Multifunctional α-enolase: its role in diseases. Cell. Mol. Life Sci. 58, 902–920.

Pierce, R., Henry, M., 2008. Harmful algal toxins of the Florida red tide (*Karenia brevis*): natural chemical stressors in South Florida coastal ecosystems. Ecotoxicology 17, 623–631.

Plakas, S.M., El-Said, K.R., Jester, E.L., Granade, H.R., Musser, S.M., Dickey, R.W., 2002. Confirmation of brevetoxin metabolism in the Eastern oyster (*Crassostrea virginica*) by controlled exposures to pure toxins and to *Karenia brevis* cultures. Toxicon 40, 721–729.

Poli, M.A., Mende, T.J., Baden, D.G., 1986. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. Mol. Pharmacol. 30, 129–135.

Schaffeld, M., Schultess, J., 2006. Genes coding for intermediate filament proteins closely related to the hagfish "thread keratins (TK)" alpha and gamma also exist in lamprey, teleosts and amphibians. Exp. Cell Res. 312 (9), 1447–1462.

Smith, R.W., Wood, C.M., Cash, P., Diao, L., Pärt, P., 2005. Apolipoprotein AI could be a significant determinant of epithelial integrity in rainbow trout gill cell cultures: a study in functional proteomics. Biochim. Biophys. Acta Proteins Proteomics 1749, 81–93.

Tietge, U.J., Maugeais, C., Lund-Katz, S., Grass, D., deBeer, F.C., Rader, D.J., 2002. Human secretory phospholipase A2 mediates decreased plasma levels of HDL cholesterol and apoA-1 in response to inflammation in human apoA-1 transgenic mice. Aterioscler. Thromb. Vasc. Biol. 22, 1213–1218.

Vander Jagt, D.L., Kolb, N.S., Vander Jagt, T.J., Chino, J., Martinez, F.J., Hunsaker, L.A., Royer, R.E., 1995. Substrate specificity of human aldose reductase: identification of 4-hydroxynonenal as an endogenous substrate. Biochim. Biophys. Acta Protein Struct. Mol. Enzymol. 1249, 117–126.

Volchenboum, S.L., Vockley, J., 2000. Mitochondrial import and processing of wild type and type III mutant isovaleryl-CoA dehydrogenase. J. Biol. Chem. 275, 7958–7963.

Wang, W., Hua, Y., Wang, G., Cole, R., 2005. Characterization of rat liver microsomal and hepatocytal metabolites of brevetoxins by liquid chromatography–electrospray tandem mass spectrometry. Anal. Bioanal. Chem. 383, 67–75.

Yan, X.Z., Benson, J.M., Gomez, A.P., Baden, D.G., Murray, T.F., 2006. Brevetoxin-induced neural insult in the retrosplenial cortex of mouse brain. Inhal. Toxicol. 18, pp. 1109–1116(8).

Zhu, S., Korzh, V., Gong, Z., Low, B.C., 2008. RhoA prevents apoptosis during zebrafish embryogenesis through activation of Mek/Erk pathway. Oncogene 27, 1580–1589.