Ovatoxin-a, A Palytoxin Analogue Isolated from *Ostreopsis* cf. *ovata* Fukuyo: Cytotoxic Activity and ELISA Detection

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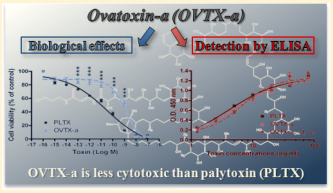
Supporting Information

ABSTRACT: This study provides the first evaluation of the cytotoxic effects of the recently identified palytoxin (PLTX) analog, ovatoxin-a (OVTX-a), the major toxin produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea. Its increasing detection during *Ostreopsis* blooms and in seafood highlights the need to characterize its toxic effects and to set up appropriate detection methods. OVTX-a is about 100 fold less potent than PLTX in reducing HaCaT cells viability (EC_{50} = 1.1 × 10^{-9} M vs 1.8 × 10^{-11} M, MTT test) in agreement with a reduced binding affinity (K_d = 1.2 × 10^{-9} vs 2.7 × 10^{-11} M, saturation experiments on intact cells). Similarly, OVTX-a hemolytic effect is lower than that of the reference PLTX compound. Ost-D shows the lowest cytotoxicity toward HaCaT keratinocytes, suggesting the lack of a hydroxyl group at C44 as a critical feature for PLTXs cytotoxic effects. A sandwich ELISA developed for PLTX detects also OVTX-a in a sensitive (LOD = 4.2 and LOQ = 5.6 ng/mL) and accurate manner (Bias = 0.3%), also in *O.* cf. *ovata* extracts and contaminated mussels. Although in vitro OVTX-a appears less toxic than PLTX, its cytotoxicity at nanomolar concentrations after short exposure time rises some concern for human health. The sandwich ELISA can be a viable screening method for OVTXs detection in monitoring program.



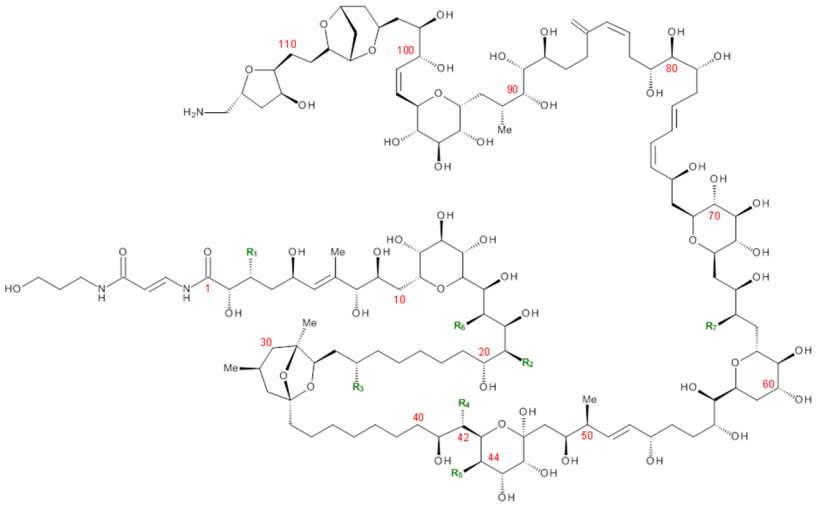
INTRODUCTION

Palytoxin (PLTX) is a highly toxic marine compound originally isolated in 1971 from *Palythoa toxica* corals[1,2] and subsequently identified in *Ostreopsis* dinoflagellates[3,4] and *Trichodesmium* marine cyanobacteria.[5]

A series of PLTX analogues have been identified, so far. Among them, four analogues have been chemically and/or biologically characterized: (i) 42*S*-OH-50*S*-PLTX, isolated from *Palythoa toxica*,[6] which in acute oral toxicity studies in mice is comparable in potency to PLTX;[7] (ii) its stereoisomer due to a conformational inversion on C50 (42*S*-OH-50*R*-PLTX) extracted from *Palythoa tuberculosa*, whose cytotoxicity is 100 times lower than that of PLTX;[8] (iii) ostreocin-D (Ost-D), isolated from *Ostreopsis siamensis*, which appears less toxic than PLTX in vivo;[9] (iv) ovatoxin-a (OVTX-a), produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea (Figure 1).[10] Intriguingly, the Mediterranean *Ostreopsis* cf. *ovata* strain was found to produce OVTX-a as the major toxin, in addition to other OVTXs (OVTX-b to -h), and only low amounts of a putative PLTX[10,11] recently renamed isobaric PLTX.[12]

PLTX has been detected in different edible marine organisms, including fish, crustaceans, mollusks, and echinoderms. Consumption of PLTX-contaminated fish and crabs has been associated with human poisonings, with some fatal outcomes in tropical and subtropical areas.[4] On the contrary, in temperate areas such as the Mediterranean Sea, human adverse effects ascribed to PLTXs have been associated mainly with cutaneous and inhalational exposure to aerosolized seawater during *Ostreopsis* blooms and with handling zoanthid corals in home aquaria. These problems, characterized mainly by dermatitis, respiratory distress, and fever, are far more frequent, but likely underestimated.[4,13] Intriguingly, OVTX-a seems to be the major toxin identified in *O.* cf. *ovata* in the Mediterranean area, so far. However, no human intoxications ascribed to these toxins have been documented in this area, despite the presence of high amounts of OVTX-a have been frequently detected in seafood.[11,14,15] This suggests a lower OVTX-a toxicity than that of the PLTX parent compound, although the knowledge on the toxic potential of OVTX-a is very scarce: the only available information derived from in vitro studies on *O. ovata* extracts containing OVTXs mixtures. In particular, the hemolytic activity of semipurified *O.* cf. *ovata* extracts was evaluated using sheep erythrocytes,[11,16] while Crinelli et al. showed an increased expression of genes encoding for inflammation-related proteins in human macrophages exposed to a semipurified extract.[17] Furthermore, uncharacterized *O.* cf. *ovata* extracts were shown to induce cytoskeletal disorganization, apoptosis, and dysregulation of gene expression in HeLa cells.[18] In addition, few studies highlighted a series of

Accepted: December 29, 2015



	R1	R2	R3	R4	R5	R6	R7
PLTX	Me	OH	Me	H	OH	OH	OH
42-OH-PLTX	Me	OH	Me	OH	OH	OH	OH
Ostreocin-D	H	H	H	OH	H	OH	OH
Ovatoxin-a	Me	OH	Me	OH	H	H	H

Figure 1. Molecular structure of PLTX and some analogues.

toxic effects for *O.* cf. *ovata* extracts on marine organisms, such as mussels,[19] crustaceans and fish,[20] and sea urchins.[21] Nevertheless, a complete characterization of OVTX-a adverse effects using predictive models of toxicity for humans is not available, so far.

Given the growing cases of adverse effects attributed to OVTXs detected in both *Ostreopsis* and marine aerosols in the Mediterranean Sea,[22−26] it is very important to define the toxicological profile of OVTX-a, and to develop a rapid detection method suitable for monitoring programs. Due to the limited availability of purified OVTX-a, an in vitro approach was used to investigate its toxic effects on skin HaCaT keratinocytes, whereas an immunoenzymatic assay set up for PLTX was evaluated for its ability to detect also OVTX-a.

EXPERIMENTAL SECTION

Toxins. Palytoxin, isolated from *Palythoa tuberculosa*, was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan; lot number WKL7151, purity >90%). A semipurified sample of OVTX-a at purity grade of 51% (Table S1) and a mixture of OVTXs, containing OVTX-a, -d, and -e, were isolated from cultures of Mediterranean strains of *O.* cf. *ovata* as previously described[10] and used for cytotoxicity assays.

Briefly, cell pellets (300×10^6 cells with a toxin content of 19 pg/cell) were extracted with MeOH/H_2O (1:1, v/v) and partitioned with CH_2Cl_2. A crude extract containing 5.6 mg of OVTX-a was obtained, evaporated to dryness, and loaded onto a 360-g Combiflash C-18 column. The column was eluted with H_2O:PrOH on a linear gradient from 60:40 to 10:90 over 50 min. Flow was 5 mL/min. A 75 mL fraction containing OVTXs was collected after 13 min, evaporated to dryness, and further separated on a Gemini 10 μm high performance liquid chromatography (HPLC) column, 10 × 200 mm (Phenomenex, Torrance, CA, USA) with a linear gradient of H_2O−CH_3CN−AcOH from 80:20:01 to 0:100:0.1 over 30 min. Final purification of OVTX-a was achieved on a Kinetex 2.6 μm HPLC column, 4.6 × 100 mm (Phenomenex, Torrance, CA, USA). This last purification was carried out by using a 20 min gradient with the same mobile phases as above. In preparative HPLC, high resolution mass spectrometry (HRMS) detection was used splitting the flow before the ion source. OVTX-a (700 μg, purity 51%) was isolated and the recovery of the entire isolation procedure was estimated to be 12.5%. Sample purity (%) was measured by liquid chromatography high-resolution mass spectrometry (LC-HRMS) in full MS positive ion mode using extracted ion chromatogram (XIC) areas.

Ost-D, used for structure−activity relationship analysis, was kindly provided by Prof. Yasumoto (Japan Food Research Laboratories) and extracted from *O. siamensis* collected along Aka Island (Okinawa, Japan) coasts as previously described.[27]

Extraction of Naturally Contaminated *Ostreopsis* cf. *ovata* Cells and Mussels. *O.* cf. *ovata* OOAN0816 strain was isolated by the capillary pipet method[28] from samples collected in 2008 in the Northern Adriatic Sea (Marche region, Italy) and deposited in the culture collection of University of Bologna (Ravenna campus, Italy). Cultures were maintained in natural seawater, at salinity of 36 ppt in modified *f*/2 medium containing 5-fold diluted macronutrients and selenium, and kept in a thermostatic chamber at 20 °C, 16:8 h light−dark cycle and irradiance of 100−110 μmol/m^2/s from cool white lamps. Cells were then separated from culture media by gravity filtration and extracted three times with MeOH/H_2O 1:1 obtaining an extract at a final concentration of 0.1 g/mL. Cell media were extracted by three repartitions with BuOH (*Ostreopsis* culture media) as previously described.[29]

Mussels (*Mytilus galloprovincialis*) edible parts were extracted three times with MeOH/H_2O 8:2 obtaining an extract at a final concentration of 0.1 g/mL as previously described.[29]

These extracts were analyzed by the sandwich ELISA after 1:10 or 1:100 dilution in assay buffer to overcome matrix effect, and the results confirmed by LC-HRMS.

Liquid Chromatography High-Resolution Mass Spectrometry Analyses. LC-HRMS analyses were performed on a hybrid linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer (FTMS) with an ESI ION MAX source (Thermo-Fisher, USA) combined with an Agilent 1100 LC

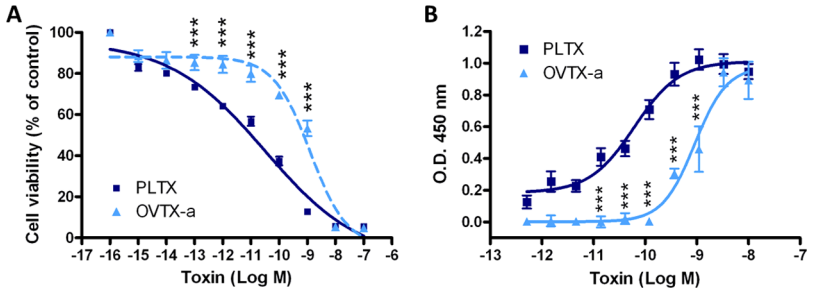


Figure 2. Effects of OVTX-a on HaCaT cells. (A) Cells were exposed to OVTX-a and PLTX (10^{-16}–10^{-7} M) for 4 h and cell viability evaluated by MTT assay. Results are reported as % of control (untreated cells). (B) Specific binding of OVTX-a compared to that of PLTX; nonspecific bindings were obtained in the presence of 1.0×10^{-3} M OUA. Results are the means ± SE of five different experiments performed in triplicate. Statistical differences: ***, $p < 0.001$ (two-way ANOVA and Bonferroni's post test).

binary system (USA) according to Ciminiello et al.[15] A Kinetex C18, 2.6 μm, 2.10 × 100 mm (Phenomenex, USA) column was used. Mobile phase was A = H_2O, 30 mM acetic acid, and B = MeCN/H_2O (95:5), 30 mM acetic acid. Flow rate was 0.2 mL/min and injection volume 5 μL. The following gradient elution was used: 25−30% B in 15 min; 30−100% B in 1 min; 100% B for 5 min. PLTX standard was used to generate a five-level calibration curve (25, 12.5, 6.25, 3.13, and 1.6 ng/mL). Calibration points were the result of triplicate injections, and peak areas were used for plotting. The curve was used to quantify positive samples assuming that OVTXs present the same molar response as PLTX. Under the described instrumental conditions, measured limit of detection (LOD) and quantitation (LOQ) for the PLTX standard was 1.6 ng/mL and 3.13 ng/mL.

HaCaT Cells Culture. HaCaT cell line was purchased from Cell Line Service (DKFZ, Eppelheim, Germany) and cultured in DMEM containing 10% fetal bovine serum (FBS), 1.0×10^{-2} M glutamine, 1.0×10^{-4} g/mL penicillin, and 1.0×10^{-4} g/mL streptomycin, at 37 °C in a humidified 95% air/5% CO_2 atmosphere. Cell passage was performed 2 days postconfluence, once per week. All of the experiments were performed between passage 50 and 65.

MTT Assay. Cells (3×10^3 cells/well) were seeded in 96-wells plates and, after 72 h, exposed to the toxins for 4 h. Cells were then washed, and fresh culture medium containing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) was added. After 4 h, the insoluble crystals were solubilized in 200 μL of DMSO/well, and the absorbance was measured by an Automated Microplate Reader EL 311s (Bio-Tek Instruments, Winooski, VT, USA) at 540/630 nm.

Binding Assay. Saturation experiments were performed on HaCaT cells (1×10^4/well) as previously described.[30] Briefly, after 10 min exposure to PLTX or OVTX-a, cells were washed with Dulbecco's phosphate buffered saline (D-PBS), fixed with 4% paraformaldehyde (PFA) and blocked in TBB buffer (50 mM Tris-HCl, 0.15 M NaCl, 2% BSA, and 0.2% Tween 20, pH 7.5) containing 10% horse serum. Nonspecific binding was determined in the presence of 1×10^{-3} M ouabain (OUA). Toxin binding was detected by exposing the cells to 2 μg/mL mouse anti-PLTX 73D3 monoclonal antibody (mAb) and 1:3000 HRP conjugated antimouse IgG (DakoCytomation; Milan, Italy) for 1 h at 37 °C each. After washing, the colorimetric reaction was started by adding 60 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate and stopped after 20 min with 30 μL of 1 M H_2SO_4. The absorbance was read at 450 nm by a Spectra photometer (Tecan Italia; Milan, Italy).

Mouse anti-PLTX 73D3 mAb was produced and purified from a hybridoma cell culture as previously described.[29]

Hemolytic Assay. In 96 multiwell plates, 125 μL of toxin was added to wells containing 125 μL of human red blood cells (10^8 cells/ml in K^+-free D-PBS) for 5 h at 37 °C. For negative controls, 125 μL of red blood suspension were added to 125 μL of K^+-free D-PBS without toxin. The 100% of hemolysis (positive control) was given by 125 μL of 0.1% Tween 20 (v/v). After 5 h, samples were centrifuged at 300*g* for 5 min to collect the supernatants. The absorbance of each supernatant was subsequently measured at 405/540 nm using a Spectra photometer (Tecan Italia; Milan, Italy).

Indirect Sandwich ELISA. The ELISA assay was carried out as previously described.[29] Briefly, multiwell strips coated with 100 μL/well of mouse anti-PLTX 73D3 mAb (20 μg/mL in PBS) and blocked with 200 μL of 2% skimmed milk (w/v) dissolved in D-PBS containing 0.1% Tween 20 (PBS-Tw) were filled with toxins (100 μL) diluted in PBS-Tw. After 2 h at room temperature (RT), 100 μL of rabbit anti-PLTX polyclonal antibody (pAb) (0.17 μg/mL) followed by 100 μL of 1:2000 HRP-conjugated goat antirabbit pAb were added. After washes, 60 μL of TMB were added to each well and the reaction stopped after 30 min with 30 μL of H_2SO_4 1M. The absorbance was read at 450 nm (Spectra photometer; Tecan Italia; Milan, Italy).

Rabbit anti-PLTX pAb was produced after rabbit immunization with a bovine serum albumin-conjugated PLTX, as previously reported.[29]

Statistical Analysis. The effective concentration giving 50% of the maximal response (EC_{50}) was calculated by a four-parameter curve-fitting nonlinear regression and K_d constant by a one-site binding hyperbola nonlinear regression analysis using the GraphPad software, version 6.0 (Prism GraphPad, Inc.; San Diego, CA, USA); the statistical analysis was performed by *t* test (significant differences, $p < 0.05$).

Concentration-effect curves of the different toxins were analyzed by a two-way ANOVA analysis followed by Bonferroni's post-test (PrismGraphPad, Inc.; San Diego, CA, USA) and significant differences were considered at $p < 0.05$.

Limit of detection (LOD), limit of quantitation (LOQ), and accuracy (Bias %) were calculated according to the international principles as described by Eurachem Guide.[31] LOD and LOQ were expressed as the analyte concentration corresponding to the average of 10 blank values plus 3 or 10 times the standard deviations, respectively. Accuracy was measured as % Bias over 10 replica, calculated as % difference between PLTX concentration measured by the assay and the theoretical

concentration in the sample divided by the theoretical concentration.

RESULTS

OVTX-a Cytotoxicity Is Lower than That of PLTX. OVTX-a cytotoxicity was evaluated on HaCaT cells by MTT assay, after 4 h exposure to each toxin (10^{-16}–10^{-7} M). Figure 2A shows the % of cell viability compared to negative controls (untreated cells). The concentration–effect curve of OVTX-a was compared to that of PLTX obtained under the same experimental conditions. OVTX-a reduced cell viability from the concentration of 10^{-11} M (79.9 ± 3.9% cell viability) up to the concentration of 10^{-8} M (5.4 ± 1.3% cell viability), with an EC_{50} = 1.1 × 10^{-9} M (95% confidence interval, CI: 0.7–1.7 × 10^{-9} M).

Exposure to PLTX induced a reduction of cell viability from the concentration of 10^{-14} M (80.2 ± 1.5% cell viability) up to the concentration of 10^{-8} M (5.8 ± 0.7% cell viability). The PLTX EC_{50} value was 1.8 × 10^{-11} M (95% CI: 1.0–3.4 × 10^{-11} M), about 2 orders of magnitude lower than that of OVTX-a (p < 0.001) (Figure 2A).

OVTX-a Binding Affinity to HaCaT Cells Is Lower than That of PLTX. To characterize OVTX-a binding to HaCaT cells, saturation experiments were performed exposing the intact cells to the toxin (5.1 × 10^{-13}-1.0 × 10^{-8} M) for 10 min. Nonspecific binding was measured in the presence of 1.0 × 10^{-3} M OUA, added 10 min before OVTX-a. Figure 2B shows the saturation curves of the specific bindings of OVTX-a and PLTX obtained by subtracting the respective nonspecific bindings from the total ones. Regarding OVTX-a, a single binding site was found with a K_d value of 1.2 ± 0.4 × 10^{-9} M, about 2 orders of magnitude higher (p < 0.001) than that of PLTX (K_d = 2.7 ± 0.6 × 10^{-11} M).

OVTX-a Hemolytic Activity Is Lower than That of PLTX. The biological activity of OVTX-a was also evaluated using the human red blood cell hemolysis assay. As shown in Figure 3, hemolysis induced by OVTX-a was significantly lower

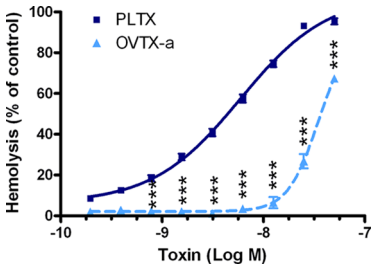


Figure 3. Hemolytic activity of OVTX-a. Human red blood cells were exposed to OVTX-a and PLTX (5.0 × 10^{-8}–1.9 × 10^{-10} M) for 5 h at 37 °C. Results are reported as % of hemolysis with respect to the positive control (0.1% Tween 20) and are the means ± SE of five different experiments performed in triplicate. Statistical differences: ***, p < 0.001 (two-way ANOVA and Bonferroni's post test).

than that induced by PLTX, with an EC_{50} value of 3.4 × 10^{-8} M (95% CI: 2.3–5.1 × 10^{-8} M), more than an order of magnitude higher (p < 0.001) than that of the parent compound (EC_{50} = 5.9 × 10^{-9} M; CI 95% = 4.9–7.2 × 10^{-9} M).

OVTX-a Is Detected by the ELISA Assay. The ability of the sandwich ELISA recently developed for PLTX quantitation[29] to detect OVTX-a and a mixture of OVTXs (-a, -d, and -e) was evaluated. The toxins were analyzed at concentrations within the working range of the sandwich ELISA for PLTX (1.3–80.0 ng/mL), and the obtained curves were compared to that of PLTX.

As shown in Figure 4A, both OVTX-a and the OVTXs mixture were detected in a concentration-dependent manner, similarly to PLTX: the EC_{50} values for OVTX-a (4.8 ng/mL; 95% CI: 4.3–5.3), OVTXs mixture (6.6 ng/mL; 95% CI: 4.6–9.7), and PLTX (4.3 ng/mL; 95% CI: 4.0–4.7 ng/mL) were comparable, with overlapping confidence intervals (p > 0.05).

The calculated LOD and LOQ for OVTX-a were 4.2 and 5.6 ng/mL, respectively. The working range has been analyzed by linear regression, plotting the theoretical OVTX-a concentrations submitted to the analysis against toxin concentrations measured by the assay (Figure 4B), with a good correlation coefficient (r^2 = 0.9814; n = 6) and a good accuracy (Bias = 0.3%; range: −7.3 to 15.8%).

The ability of the sandwich ELISA to quantify OVTX-a in natural samples was then assessed. Eleven *Ostreopsis* cf. *ovata* extracts, nine *Ostreopsis* cf. *ovata* culture media and five mussel (*Mytilus galloprovincialis*) extracts containing OVTX-a at different concentrations (as assessed by LC-HRMS analysis) have been analyzed by the ELISA assay (Figure 4C,D,E). On the whole, the sandwich ELISA was able to quantify OVTX-a similarly to LC-HRMS (r^2 = 0.9540, 0.9849, and 0.9281 for *Ostreopsis* cells, *Ostreopsis* culture media, and mussels extracts, respectively).

Ost-D Cytotoxicity and Detection by the ELISA Assay. The cytotoxicity of Ost-D was evaluated for structure–activity relationship analysis. Ost-D cytotoxicity was evaluated on HaCaT cells by MTT assay after 4 h exposure to the toxin (10^{-16}–10^{-7} M) and compared to that of PLTX (Figure 5A). Ost-D induced a reduction of cell viability from the concentration of 10^{-14} M (83.2 ± 2.9% cell viability) up to the concentration of 10^{-6} M (8.7 ± 0.6% cell viability), with an EC_{50} = 2.3 × 10^{-8} M (95% CI: 1.2–4.4 × 10^{-8} M), about 3 orders of magnitude higher than that of PLTX (p < 0.001).

The sandwich ELISA was investigated also for Ost-D detection at concentrations within the working range for PLTX (1.3–80.0 ng/mL), and the obtained curve was compared to that of PLTX. As shown in Figure 5B, Ost-D was detected in a concentration-dependent manner by the sandwich ELISA, but considerably in lesser extent than PLTX. Indeed, the EC_{50} values for Ost-D and PLTX were equal to 40.6 ng/mL (95% CI: 38.0–43.4 ng/mL) and 4.3 ng/mL (95% CI: 4.0–4.7 ng/mL), respectively, being significantly different (p < 0.001).

DISCUSSION

Toxic benthic dinoflagellates of the genus *Ostreopsis* have been recognized for decades in tropical and subtropical regions. Only recently they have appeared in temperate areas, such as the Mediterranean Sea and NE Atlantic ocean.[32] In these areas cases of sanitary problems after skin contact and/or inhalational exposure to marine aerosol or direct contact with seawater during *Ostreopsis* cf. *ovata* blooms have been reported almost every year.[4] In the past few years, it has been proven that among PLTXs, OVTX-a is the major toxin produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea and high amounts of this toxin have been found in edible marine organisms all along the Mediterranean coasts.[10,11,23,26] In addition, *Ostreospsis* cf. *ovata* cells fragments were recently identified in marine aerosol along the Spanish coasts by qPCR assays[33] along with

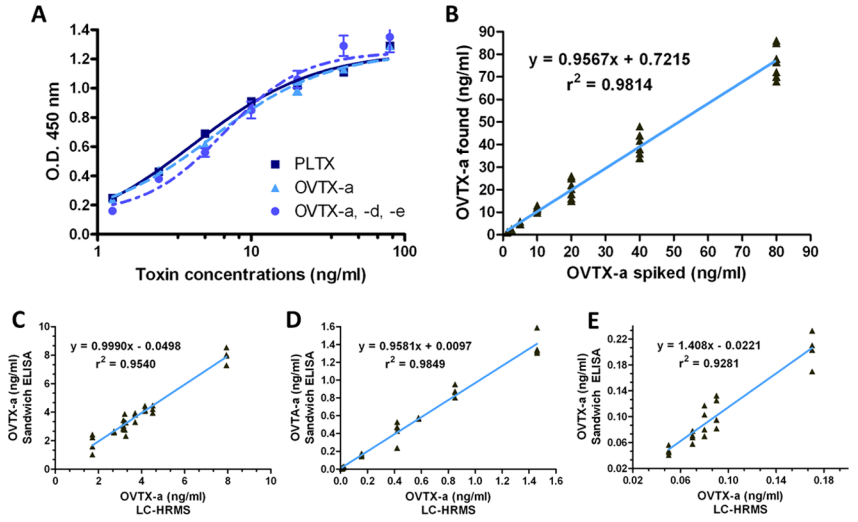


Figure 4. Detection of OVTX-a and a mixture of OVTX-a, -d, and -e by ELISA assay. (A) Calibration curve for OVTX-a and OVTXs mixture in comparison to that of PLTX. Each point represents the mean ± SE of five different experiments performed in duplicate. (B) The working range of OVTX-a was analyzed by linear regression, plotting the theoretic OVTX-a concentrations against OVTX-a concentrations measured by the assay (n = 6). (C) Detection of OVTX-a in *Ostreopsis* cf. *ovata* extracts (n = 11), (D) in *Ostreopsis* cf. *ovata* culture media (n = 9), and (E) in mussels (*Mytilus galloprovincialis*) extracts (n = 5) containing OVTX-a (as assessed by LC-HRMS analysis). Results obtained by the sandwich ELISA were plotted against the quantification carried out by LC-HRMS and analyzed by linear regression.

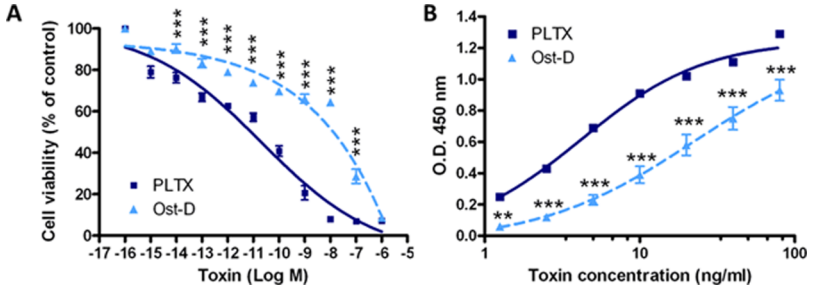


Figure 5. Cytotoxicity and detection of Ost-D. (A) Cells were exposed to Ost-D and PLTX (10^{-16}–10^{-7} M) for 4 h, and cell viability evaluated by MTT assay. Results are reported as % of control. (B) Detection of Ost-D by ELISA assay compared to PLTX. Each point represents the mean ± SE of five different experiments performed in duplicate. Statistical differences: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (Two-way ANOVA and Bonferroni's post test).

OVTXs that were detected directly in marine aerosols collected concomitantly with *O.* cf. *ovata* blooms along Tuscany (Italy) coasts.[34]

The detection of OVTX-a in *Ostreopsis* cells, seafood, and marine aerosols prompts the need to characterize its toxicological potential as well as to provide suitable detection methods for monitoring purposes. However, because limited amounts of purified toxin precluded animal studies, an in vitro approach was used.

Because no human cases of seafood poisoning ascribed to PLTXs have been recorded in these areas to date, but dermotoxic effects are recurrently and commonly reported, OVTX-a was evaluated for its cytotoxicity on human HaCaT skin keratinocytes, a suitable cell model for the in vitro characterization of dermotoxic agents[35] and one of the most sensitive cell line to PLTX.[36−38] The in vitro assay demonstrated, for the first time, that OVTX-a cytotoxicity is lower than that of PLTX, reducing cell viability with an EC_{50} (1.1×10^{-9} M) about 2 orders of magnitude higher than that of PLTX ($EC_{50} = 1.8 \times 10^{-11}$ M). This result is in perfect agreement with the reduced binding affinity of OVTX-a to HaCaT cells. Indeed, the K_d value of OVTX-a is about 2 orders of magnitude higher than that of PLTX. Moreover, the reduced cytotoxicity of OVTX-a is confirmed by the reduced hemolytic activity. The latter result seems to be in disagreement with previous investigations reporting the same hemolytic activity for PLTX and OVTXs. However, these conclusions were achieved using *Ostreopsis* crude extracts containing a mixture of OVTXs and, in some cases, also traces of isobaric PLTX, among other compounds. These crude extracts may potentially contain other unidentified compounds that could tentatively be hemolytic, thus contributing to the total hemolytic effect.[11,16] However, since OVTX-a seems to be the major toxin produced by *Ostreopsis* cf. *ovata* in the Mediterranean Sea, these results, if confirmed by in vivo studies, could have a significant impact in the evaluation of the actual risk for human health associated with *Ostreopsis* cf. *ovata* in these areas.

Even though lower than that of PLTX, OVTX-a cytotoxicity falls in the nanomolar concentration range after an exposure time as short as 4 h, suggesting that this PLTX analog represents a real risk for human health after cutaneous exposure. In fact, several people have developed moderate to severe dermatitis after exposure to marine aerosols and/or accidental contact with *Ostreopsis* cf. *ovata* blooms. The severity of these exposures was sufficient to warrant treatment with steroidal and nonsteroidal anti-inflammatory drugs.

For this reason methods to detect OVTX-a are required. A sandwich ELISA has been recently developed to quantify PLTX in different matrices.[29] This simple ELISA assay was characterized for its ability to quantify OVTX-a as well as an OVTX mixture (OVTX-a, -d, and -e). These analogues are detected by the sandwich ELISA in an overlapping manner in comparison to PLTX. Moreover, the assay is sensitive (LOD and LOQ of 4.2 and 5.6 ng/mL, respectively) and accurate (Bias of 0.3%). This could suggest a good efficiency in the quantitation of OVTX-a also in contaminated natural samples. Indeed, the ELISA quantitation of OVTX-a in contaminated samples is comparable to that of LC-HRMS. In a total of 25 contaminated natural samples (11 *Ostreopsis* cf. *ovata*, 9 *Ostreopsis* cf. *ovata* cell culture media, and 5 *Mytilus galloprovincialis* extracts) submitted for analyses, OVTX-a was quantified in a similar manner with respect to that quantified by LC-HRMS, as shown by the high r^2 values. These data suggest an excellent applicability of the sandwich ELISA for OVTX-a quantitation in complex biological samples of marine origin.

Interestingly, two *Ostreopsis* cf. *ovata* cell extracts, not containing PLTXs by LC-HRMS analysis, were negative also in the ELISA analysis, suggesting the specificity of the assay in the quantitation of PLTXs.

On the contrary, the ELISA assay detected Ost-D to a lower extent than PLTX. However, Ost-D has been detected in Japanese strains of *O. siamensis*, while Mediterranean/Atlantic strains of *O.* cf. *siamensis* have been found to be devoid of any appreciable toxin content.[39,40] In addition, the ability of the assay to quantify not only OVTX-a but also a mixture of OVTXs, in an overlapping manner, highlights its applicability in a real-life situation, in which mussels are likely to be contaminated by a mixture of OVTXs rather than by OVTX-a alone. Thus, the ELISA assay could be used as a screening method to detect OVTXs, before confirmatory analysis by LC-HRMS. This could be particularly important and helpful at the beginning of an *Ostreopsis* cf. *ovata* bloom, since the presence of OVTXs could be confirmed within some hours, allowing time to issue an alert to avoid sanitary problems but also economic losses to the tourism industry due to possible false alarmism. Previously, using the same monoclonal antibodies employed in the sandwich ELISA, we proposed an immunocytochemical assay directly on *Ostreopsis* cf. *ovata* cells, which is much more complicated, costly and time-consuming.[41]

Intriguingly, the presented data suggests that even small changes in the complex PLTX molecular structure, such as three missing hydroxy groups on C17, C44, and C64, one additional hydroxy group on C42, and a configurational inversion at C26 in OVTX-a (Figure 1), significantly reduce its toxicity with respect to PLTX. So far, it is only known that the N-terminal portion is required for PLTX toxicity, possibly allowing the dimerization of the toxin.[42] To investigate the structural features necessary for the PLTX cytotoxic effect, data regarding PLTX and OVTX-a have been compared to those obtained exposing HaCaT cells to Ost-D (a 42-OH-PLTX lacking two hydroxy groups on C19 and C44 and two methyl groups on C3 and C26). Compared to PLTX, the common features between OVTX-a and Ost-D are an additional hydroxyl group on C42 and a missing hydroxyl group on C44. On the other hand, compared to PLTX, OVTX-a lacks the hydroxyl group at C17, while Ost-D lacks the hydroxyl group at C19. Cell viability reduction by Ost-D was 3 orders of magnitude lower than that induced by PLTX and 1 order of magnitude lower than that induced by OVTX-a, with an order of potencies of PLTX > OVTX-a > Ost-D. This allows us to make some observations regarding the relationship between the structure and cytotoxic activity of these toxins: (i) the additional hydroxyl group at C42 seems not to be important for the cytotoxic effect since 42-OH-PLTX and PLTX show similar cytotoxicities[6,8] (ii) the missing hydroxyl group on C44, a common feature between OVTX-a and Ost-D, but present in PLTX and 42-OH-PLTX, seems to be determinant for PLTX cytotoxic activity since both of them are largely less toxic than the parent compound; (iii) the missing methyl and hydroxy groups at C3, C19 and C26 of Ost-D, in comparison to OVTX-a, could further reduce the toxin cytotoxicity. However, we cannot exclude that other factors such as hydroxyl group lacking at C17 or at C19, stereochemical changes, and consequent conformational changes, could affect the total cytotoxic activity.

In conclusion, this study provides the first characterization of the cytotoxic effects of OVTX-a. In particular, OVTX-a appears to be less toxic in vitro compared to the reference compound PLTX, displaying lower binding affinity and cytotoxicity on HaCaT cells as well as a lower hemolytic activity on human erythrocytes. Structure−activity considerations suggest that the lack of a hydroxy group at C44 is determinant for PLTX cytotoxicity. Despite OVTX-a cytotoxicity is lower than that of PLTX, it is exerted at nanomolar concentrations after a short exposure time, suggesting that OVTX-a is a legitimate concern for human health. The sandwich ELISA for PLTX detection can also quantify OVTX-a in contaminated marine samples suggesting that it can be a simple method for self-monitoring by shellfish producers as well as monitoring programs for the detection of *Ostreopsis* cf. *ovata* toxins.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by a grant of the University of Trieste (Università degli Studi di Trieste - Finanziamento di Ateneo per progetti di ricerca scientifica - FRA 2012). Chemical analyses were carried out in the framework of Programme STAR Linea 1 2013 (VALTOX, Napoli_call2013_08) financially supported by University of Naples and Compagnia di San Paolo. The authors are grateful to Prof. Takeshi Yasumoto (Japan Food Research Laboratories) for kindly supply ostreocin-D.

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