Production of Functionally Active Palytoxin-like Compounds by Mediterranean *Ostreopsis cf. siamensis*

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Key Words

Ostreopsis • Palytoxin analogues • Metabolic activity
• Depolarization • Cytosolic calcium • Ouabain

Abstract

Background and purpose: Dinoflagellates from the genus *Ostreopsis* have been related to the production of palytoxin and analogues. Based on that, this paper describes functional studies of crude extracts from *Ostreopsis cf. siamensis* collected in the Mediterranean Sea in order to biochemically characterize their toxic compounds. **Methods:** We compared the effects of 5 crude dinoflagellates extracts with a commercially available palytoxin and a purified *Ostreopsis ovata* extract on metabolic activity, membrane potential, and cytosolic calcium levels by using fluorescent dyes. **Results:** All the extracts resulted to be neurotoxic. In addition, all of them induced a membrane depolarization and a calcium increment that were abolished when preincubating with ouabain, an inhibitor of the Na⁺/K⁺ pump. **Conclusion:** The effects observed were quite close to those induced by palytoxin and the *Ostreopsis ovata* extract as well, suggesting that *Ostreopsis cf.*

siamensis is actually producing palytoxin-like compounds that are highly toxic and functionally active.

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Introduction

Palytoxin is one of the largest and highly potent marine toxins first isolated from zooanthids of the genus *Palythoa* [1-3]. It has been also found in sea anemones, polychaete worms, crabs and herbivorous fishes [4-6]. However, algae from the genus *Ostreopsis* have been proposed as the possible biogenetic origin of this toxin as well as some potent analogues, e.g. ostreocin-D [7-10].

Ostreopsis species are distributed worldwide, and even though are more common in tropical and subtropical waters, since 1994 these dinoflagellates started to appear in the Mediterranean Sea [11-14]. Several species such as *O. siamensis* [13, 15, 16] and *O. ovata* [17-19], which have been postulated as producers of palytoxin analogues, have been also involved in damaging effects in seafood or human toxic outbreaks.

Seafood poisoning in humans due to palytoxins, is characterized by abdominal cramps, nausea, diarrhea,

paresthesia, severe muscle spasms, and respiratory distress [20, 21]. Palytoxin-like compounds also cause human sufferings because of the exposure to the marine aerosols, with symptoms that include fever associated to serious respiratory disturbs, such as bronchoconstriction, mild dyspnea, wheezes, and in some cases conjunctivitis [17, 22].

At cellular level, it is widely accepted that palytoxin acts on the Na^+/K^+ -ATPase [23-25]. This pump hydrolyzes one molecule of ATP and transports 3 Na^+ ions out for 2 K^+ ions into the cell. Palytoxin seems to bind to the Na^+/K^+ -ATPase from the extracellular side and therefore inhibit the active transport of Na^+ and K^+ across the cell membrane by transforming the pump into a permanently open ion channel [24, 26-28]. The pump is also the specific target of heart glycosides, such as ouabain, and the reported data suggest that there is a competitive inhibition of the palytoxin actions by ouabain [3, 29-32].

One of the first effects induced by palytoxin is a membrane depolarization by increasing the sodium entry, potassium efflux, and altering the cationic permeability of the cells [33-37]. Another reported effect of this toxin is the induction of calcium influx [36, 38-40]. In excitable cells, at least three Ca^{2+} entry pathways are involved in this effect, though are not completely characterized: (i) voltage-dependent Ca^{2+} channels, which are activated by depolarization of the membrane; (ii) $\text{Na}^+/\text{Ca}^{2+}$ exchanger in reversed mode, which causes a Ca^{2+} accumulation in response to the increase in intracellular Na^+ and membrane depolarization, and (iii) an unknown pathway independent on its depolarizing action or on its action on intracellular pH [41, 42].

The rise in cytoplasmatic Na^+ , the Ca^{2+} increase, and, in general, the ionic disorder induced by palytoxin triggers a series of cellular events that lead to its cytotoxic effect [27, 43-49]. Palytoxin and its analogues exhibit extreme toxicity in mammals, with a LD_{50} value of 450 ng/kg and 750 ng/kg after intraperitoneal injection of palytoxin and ostreocin-D respectively [50].

Toxic compounds produced by *Ostreopsis spp.* are still not well known. Actually, a new palytoxin analogue, ovatoxin-A, has been described as produced by *O. ovata* collected in the Mediterranean coast [19], but almost nothing has been published with regard to their functional behavior. The aim of this work was to pharmacologically characterize the toxins from *O. cf. siamensis* collected in the Mediterranean Sea. Based on that, we compared their effects on viability, membrane potential and intracellular calcium, with the ones induced by a commercially available palytoxin and a purified extract

of *O. ovata*. Results allowed us to provide a deeper knowledge about the presence of palytoxin-like compounds in *O. cf. siamensis* produced by themselves.

Materials and Methods

Materials

Fluorescent dyes bis-(1,3-dibutylbarbituric acid) trimethine oxonol (bis-oxonol), and fura-2 acetoxymethyl ester (fura-2) were from Molecular Probes (Leiden, The Netherlands). Alamar Blue (AB) was from Biosource International (Nivelles, Belgium). Eagle's Minimum Essential Medium (EMEM) and Ham's F12 supplemented with glutamine, non-essential amino acids, gentamycin, and amphotericin B were purchased from Biochrom AG (Berlin, Germany). Fetal bovine serum (FBS) for neuroblastoma cell culture, trypsin-EDTA, Nutrient mixture F-12 Ham Kaighn's modification, streptomycin sulfate salt, penicillin G potassium salt, bovine serum albumine (BSA), and ouabain were from Sigma (Madrid, Spain). Fetal bovine serum for hepatocytes cell culture was from Gibco (Barcelona, Spain). Palytoxin from *Palythoa tuberculosa* was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). *Ostreopsis ovata* extract was purified by Dr. T. Yasumoto (Tama Laboratories, Japan). All other chemicals were reagent grade and purchased from Sigma (Madrid, Spain). 25 cm^2 tissue culture flasks and 60 mm tissue culture plates were from Nunc (Roskilde, Denmark) and 96 well plates were from Corning (Schiphol-Rijk, The Netherlands).

Experimental standard salt solution used for the experiments contained (in mmol/L): NaCl 137, KCl 5, CaCl_2 1, Glucose 10, MgCl_2 1.2, KH_2PO_4 0.44, NaHCO_3 4.2, pH 7.4.

Ostreopsis cf. siamensis Crude Extracts

Ostreopsis cf. siamensis were collected as epiphytic macroalgae at several locations along andalusian coast. Seawater containing the macroalgae sample was shaken vigorously to dislodge the epiphytic organisms. Clonal isolates were obtained by transferring individual cells through sterile seawater washes with a capillary pipette. After the washes, every single cell was similarly transferred into a microtiter containing 300 μL of sterile L1 medium.

Clonal cultures of *Ostreopsis cf. siamensis* were established in L1 media [51]. The cultures were grown at $18 \pm 1^\circ\text{C}$ and a 14:10 h light:dark photoperiod. Illumination was provided by cool white fluorescent lamps. The cultures were shaken twice a day.

Ostreopsis cells were collected from the culture using a 20 μm mesh plankton net. The extracts were newly concentrated by centrifugation. The supernatants were removed and pellets were resuspended with methanol [methanol:pellet 3:1 (v/v)]. These extracts were stored at -14°C .

Cell Line Culture

Human neuroblastoma cell line BE(2)-M17 used as excitable cellular model was cultured in EMEM:Ham's F12 (1:1) supplemented with 2 mM glutamine, 1% non-essential amino

acids, 50 mg/L gentamycin, 50 µg/L amphotericin B and 10% heat-inactivated FBS. Cells were grown in 25 cm² tissue culture flasks at 37°C in a humidified atmosphere containing 5% CO₂, and were subcultured by transferring cells released by the application of 0.1% trypsin-EDTA [52, 53].

Comparatively, rat hepatocytes from the cell line Clone 9 were used as non-excitable cellular model. Cells were cultured in a nutrient mixture F-12 Ham Kaighn's modification supplemented with 2.5 g/L NaHCO₃, 28 mg/L streptomycin sulfate salt, 17 mg/L penicillin G potassium salt and 10% FBS. Cells were grown on 60 mm tissue culture plates in a humidified atmosphere with 5% CO₂ at 37°C, and subcultured by transferring cells released by the application of 0.25% trypsin-EDTA [54].

For the fluorescence microscope assays, neuroblastoma cells were seeded on 22 mm glass coverslips at a density of 1.5-5 x 10⁴ cells/well and used after 4-7 days. For the metabolic activity assay, neuroblastoma and hepatocytes were seeded on 96 well plates at a density of 4 x 10⁴ cells/well, and were cultured for 24 h prior to treatment in order to allow them to attach to the bottom of the microplate.

Metabolic Activity Assay

Cell metabolic inhibition was tested as cytotoxic effect directly in the cell culture wells using the fluorescent probe Alamar Blue (AB). This redox indicator is a widely used nontoxic reagent that exhibits both fluorimetric and colorimetric properties in response to metabolic activity. The reduction-induced color change varies proportionately with cell number and time, changing from a nonfluorescent color blue to a reduced pink fluorescent form [55-60].

Toxins, extracts and controls were added in the wells by duplicate. Then, a 1:10 dilution of AB was aseptically added to the culture wells. AB fluorescence was measured using a FL600 fluorescence plate reader (Bio-Tek, Vermont, U.S.A.) at the excitation wavelength of 530 nm and the emission wavelength of 590 nm. Control cells were considered as the 100% viability, and results are expressed as the percentage of the fluorescence of treated cells versus the fluorescence in controls ones.

Measurement of Plasma Membrane Potential

Plasma membrane potential was measured with the slow potential-sensitive fluorescent dye bis-oxonol. Neuroblastoma cells were incubated in a thermostated chamber with the standard salt solution containing 5 nM bis-oxonol for 10 min at 37°C. Fluorescence recordings were performed with a Nikon Diaphot microscope with epifluorescence optics (Nikon 40x immersion fluor objective) at the wavelengths 490 nm excitation and 530 nm emission [61, 62]. Results are expressed as relative fluorescence as in Louzao et al. [63].

[Ca²⁺]_i Measurements

Neuroblastoma cells were incubated for 10 min at 37°C in 2 mL standard salt solution with 0.1% BSA and containing 0.5 µM fura-2. Then, the cells were washed three times and placed into the thermostated chamber at 37°C. Calcium measurements were made with the same microscope as above. Fluorescent images were collected by dual excitation wavelengths at 340

Fig. 1. Dose-dependent cell metabolism inhibition induced by palytoxin in neuroblastoma BE(2)-M17 cells (black) and Clone 9 hepatocytes (white). The cells were incubated for 24h in the presence of the toxin. Results are presented as % fluorescence vs control, which is equivalent to cell viability.

Table 1. Cell metabolism inhibition of neuroblastoma BE(2)-M17 cells after 24h exposure to several *Ostreopsis cf. siamensis* extracts.

% Fluorescence vs Control <i>BE(2)-M17</i>	
Extract	
A	14.609 ± 5.942
B	21.030 ± 8.215
C	24.196 ± 6.121
D	24.253 ± 5.778
E	31.119 ± 6.387

and 380 nm, and a wavelength emission at 530 nm with a Lambda 10-2 Sutter Instrument Co equipment [61, 62]. Data are expressed as normalized ratio by dividing the fura-2 340/380 ratio at time t with respect to the 340/380 ratio at the baseline.

Statistical Analysis

All the experiments were carried out three times. Results were analyzed using the Student's t-test for paired data. A probability level lower than 0.05 ($p < 0.05$) was used for statistical significance. Results are expressed as the mean ± S.E.M.

Results

In this work we evaluate the functional behavior of five *Ostreopsis cf. siamensis* crude extracts compared with a commercially available palytoxin used as reference. Crude extracts are original from cultured cells collected from the South of Spain.

We first checked the cytotoxic effect at 24h of palytoxin and the *Ostreopsis* extracts in two cell lines, an excitable model (neuroblastoma BE(2)-M17) and a hepatic model (Clone-9) by using the metabolic activity dye Alamar Blue. 24h of incubation with palytoxin caused

Fig. 2. Time course of the influence of ouabain 1mM (first arrow) on the palytoxin induced depolarization (second arrow) in human neuroblastoma cells. Neuroblastoma cells were equilibrated with bis-oxonol. Once stabilization of the baseline was obtained, cells were treated only with palytoxin (filled symbols), or were preincubated with ouabain and then palytoxin was added to the cells (blank symbols).

a concentration-dependent decrease of fluorescence indicating a reduction of cell viability in both cellular models (Figure 1). Since an excitable model results a better model for our study, in Table 1 we present the effect of 1:100 dilutions of *O. cf. siamensis* crude extracts in neuroblastoma cells viability (Table 1). The samples induced a decrease in the cellular viability, showing that all the extracts contained toxic compounds, even though extract A was the most cytotoxic and E the less one.

It is widely accepted that palytoxin turns the sodium pump into a nonselective cation channel, resulting in a membrane depolarization. We used a previously developed assay to determine the effects of *O. cf. siamensis* crude extracts and palytoxin in neuroblastoma cells membrane potential, testing palytoxin as a positive control [63]. Results are presented by normalizing the fluorescence values obtained in the imaging microscope with respect to the baseline. Figure 2 shows that palytoxin induced a significant increase in bis-oxonol fluorescence, indicating a membrane depolarization of neuroblastoma cells. In addition, preincubation with 1mM ouabain for nearly 10 min, resulted in the complete abolishment of the depolarizing effect evoked by the toxin. This confirms the interaction between palytoxin and the specific inhibitor of the Na^+/K^+ -ATPase ouabain. We also evaluated the effect of a 1:1000 and 1:10000 dilution of an *Ostreopsis ovata* extract, finding a high membrane depolarization of neuroblastoma cells that was also inhibited by ouabain (Figure 3).

Fig. 3. Time course of the effect of a 1:1000 and 1:10000 dilution of an *Ostreopsis ovata* extract (second arrow) on the membrane potential of human neuroblastoma cells, and inhibition of the response by 1mM ouabain (first arrow).

Next, we tested a dilution 1:100 of the crude extracts of *O. cf. siamensis*. All of them evoked a significant increment of the fluorescence of bis-oxonol loaded cells, which pointed out a membrane depolarization (Figure 4). Furthermore, in order to determine if those extracts-evoked depolarizations were due to the presence of palytoxin-like compounds, cells were incubated with 1mM ouabain for nearly 10min before addition of each extract. As in the case of the palytoxin used as reference, the membrane depolarizing effect was inhibited. All the fractions were found to induce a similar behavior, specially extracts C (Figure 4C) and D (Figure 4D), while extract A (Figure 4A) and E (Figure 4E) were the less depolarizing ones, and extract B (Figure 4B) seemed to be the most depolarizing dilution. But, interestingly, this last one was the only extract where ouabain did not inhibit all the increment in bis-oxonol fluorescence induced by the toxic compounds.

A secondary action of palytoxin in excitable cells has been reported to be the activation of voltage-dependent Ca^{2+} channels and the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the reverse mode, inducing an increase in intracellular calcium ($[\text{Ca}^{2+}]_i$). Again as a positive control we first studied the effect of palytoxin on the basal state of intracellular calcium of neuroblastoma cells by using the ratiometric dye fura-2. Figure 5 shows that palytoxin induced a significant fluorescence increment in fura-loaded neuroblastoma cells, which was indicative of a cytosolic calcium influx. 75nM palytoxin stimulated a great and

Fig. 4. Time course of the effect of a 1:100 dilution of five *Ostreopsis cf. siamensis* extracts: A, B, C, D, E (second arrow) on membrane potential of human neuroblastoma cells, and influence of 1mM ouabain (first arrow) on the induced response.

Fig. 5. Time course of the influence of ouabain 1mM (first arrow) on the palytoxin induced calcium increase (second arrow) in human neuroblastoma cells. Intracellular calcium was monitored in fura-loaded neuroblastoma cells. Once stabilization of the baseline was obtained, cells were treated only with palytoxin (filled symbols), or were preincubated with ouabain and then palytoxin was added to the cells (blank symbols).

Fig. 6. Time course of the effect of a 1:1000 and 1:10000 dilution of an *Ostreopsis ovata* extract (second arrow) on the intracellular calcium of human neuroblastoma cells, and inhibition of the response by 1mM ouabain (first arrow).

fast raise on $[Ca^{2+}]_i$, whereas the effect of 15nM palytoxin was lower and more gradual. Anyway, as it can be observed in the same figure, the calcium increase induced by both concentrations of palytoxin was completely abolished by preincubation with ouabain. Studies with the *O. ovata* extract showed a fast dose-dependent increase of intracellular calcium in neuroblastoma cells, and the same inhibitory effect by ouabain was observed (Figure 6). We analyzed the *O. cf. siamensis* extracts behavior

Fig. 7. Time course of the effect on intracellular calcium of a 1:100 dilution of five *Ostreopsis cf siamensis* extracts: A, B, C, D, E (second arrow) in human neuroblastoma cells, and influence of 1mM ouabain (first arrow) on the induced response.

on the fura-loaded neuroblastoma cells, and the calcium increase induced by them was significant in all the samples tested (Figure 7). Among them, extracts C (Figure 7C) and D (Figure 7D) evoked a similar calcium increase, while extract E (Figure 7E) induced the lower calcium increment, and extract B (Figure 7B) triggered a fast increase reaching a high calcium level. Again incubation with ouabain before addition of the samples significantly reverted the extracts-induced $[Ca^{2+}]_i$ raise to values close to those of control ones.

A sample of these crude extracts of *O. cf. siamensis* was analyzed by LC/MS with MRM technique, but nor palytoxin nor ovatoxin-a were detected (Fattorusso, E., personal communication).

Discussion

Although *Ostreopsis siamensis* is typical of tropical and subtropical waters, its distribution is not restricted to those areas. Actually, several articles have reported the expansion of these species during the last years and their occurrence in massive blooms in the Mediterranean Sea. Human symptoms after exposure to seaside areas where *Ostreopsis* blooms in the Mediterranean Sea occurred included: rhinorrhoea, cough, fever, bronchoconstriction and wheezing [17, 64]. Taniyama et al [65] observed that crude extracts of *Ostreopsis sp.* from Japan were toxic to mice, resulting in a potentially lethal syndrome with symptomatic episodes of convulsion and drowsiness. Those symptoms have been reported to be early manifestations in palytoxin induced symptoms [66]. Elsemore, Mediterranean strains of *O. siamensis* showed toxicity in mouse bioassays, delayed hemolytic activity, and chromatographic properties with similar characteristics to palytoxin. Despite the potential threat for human health of these dinoflagellates, very few is known about functional properties of the palytoxin analogues produced by *Ostreopsis* species.

This study provides a deeper insight into the pharmacological behavior of cultured crude *Ostreopsis*

cf. siamensis extracts from the South of Spain. The drop in cell viability observed in cells treated with the *O. cf. siamensis* samples indicates that all the extracts resulted to be toxic. The similar high mortality rate induced by all the extracts suggests the presence of potent or fast-acting toxins such as palytoxins. Although rat hepatocytes and human neuroblastoma were both sensitive to the cytotoxic action exerted by palytoxin, we decided to use the excitable human cellular model to make the pharmacological experiments. With the excitable model we could detect variations in membrane potential and ion fluxes, as well as use ouabain to confirm the palytoxin-like nature of the compounds produced by the dinoflagellates.

Palytoxin disturbs the function of the Na^+/K^+ -ATPase, and as a consequence, varies the cellular homeostasis and triggers activities of vital importance for the proper cellular function, as membrane depolarization, intracellular calcium increase and pH_i decrease.

We observed that the toxic *O. cf. siamensis* extracts induced a significant depolarization in human neuroblastoma cells, as well as the palytoxin used as reference and the purified extract from *O. ovata*. In fact, the crude extract B seems to evoke the higher depolarization, while extract C, D and E reached similar results, and the crude extract A was one that reached the lower level of fluorescence, indicative of a lower depolarization. Results suggest that depolarization may be due to an increase in cytosolic Na^+ as a primary effect evoked by the extracts, as it has been reported for palytoxin. This conclusion is supported by the fact that the depolarizing effect exerted by all the *Ostreopsis* extracts, as well as by palytoxin, was inhibited when preincubating with ouabain. Actually, no significant differences were observed between control cells and cells preincubated with ouabain, indicating that the depolarizing effect was completely abolished when ouabain was added before the toxin or the extracts. This fact was observed in all the extracts tested except in extract B. This one was the crude extract that showed a greater increment in bis-oxonol fluorescence, but ouabain was not able to completely abolish the initial depolarizing phase. This could be due to the presence of salts in the sample that could have enhanced the depolarizing effect evoked by the palytoxin-like compounds (since the most of the depolarizing effect resulted inhibited by ouabain). Even we can not discard the possible presence of other toxins that do not interact with ouabain.

Together with the depolarization observed, we also found an increase in cytosolic calcium levels induced by

the standard palytoxin, which was abolished when preincubated with ouabain. As we had previously published, this intracellular calcium rise is due to the influx of external Ca^{2+} [36]. This fact agrees with the cationic influx exerted by palytoxin due to the binding to the Na^+/K^+ -ATPase and with the competitive inhibition exerted by ouabain. In the same way, the *O. ovata* purified extract induced an even higher intracellular calcium increase that was also inhibited by ouabain.

The crude extracts also evoked a fast and significant calcium entry, with a profile quite similar to that one evoked by palytoxin and by the *O. ovata* purified extract. Extracts B, C and D induced the higher increase in calcium, while extract E was the less effective in raising the cytosolic calcium concentration. Interestingly, during the linear period obtained in the first 100 s after addition of the extract A, the mean slope seems to be lower than in the other cases. Anyway, in all the samples tested the intracellular calcium increment was completely abolished when ouabain was added before, though the extracts produced a higher or lower effect. This specific inhibitor of the Na^+/K^+ pump was able to block the calcium increase induced by the extracts since no differences were found with respect to the controls, indicating that this effect was probably due to the action of the toxic extracts on that receptor.

This hypothesis is based on the fact that the plasma membrane sodium-potassium pump is the only clearly recognized receptor for ouabain, despite several signaling pathways has been described for it [67-70]. In addition, ouabain and palytoxin are known to competitively interact. Taking advantage of such interaction, we conclude a similar functional behaviour of the extracts when compared to those of palytoxin.

Ouabain is a compound used in our set of experiments just as a tool. It resulted surprising that ouabain did not induce a significant depolarization, but as can be observed on the graphs, its effect is quite variable among the experiments. These variations are reflected by the S.E.M. and even among the different assays. But what can be deduced from the graphs is that ouabain effectively abolishes the effect of the extracts in the same way as those of palytoxin. In fact, the concentration and time of exposure used were effective against palytoxin, the toxin used as positive control. So, although ouabain failed to induce neither a clear depolarization nor a calcium influx, it was certainly able to interfere with palytoxin action, and similarly, with the action of the extracts, not only in one of the effects observed (depolarization), but also in the calcium increase evoked.

Despite not being exerting a depolarizing effect, it doesn't mean that ouabain can not be targeting the pump. In fact, there are more examples, like Sorrentino et al., who did not observe a membrane depolarization induced by 5mM ouabain in LA-N-1 human neuroblastoma cells either [71]. And Kulikov et al. just observed a Na^{+2} increase of only 33% with 10 μM ouabain after 6h [70]. The reason for concluding a possible functional interaction of the extracts with the $\text{Na}^{+}/\text{K}^{+}$ -ATPase is that although several signaling pathways have been described for ouabain, the plasma membrane sodium-potassium pump is its only clearly recognized receptor. Since ouabain and palytoxin are known to competitively interact [3, 29-32], and the inhibition of the palytoxin effects by ouabain has been considered a specific method for detecting this toxin [14, 32, 72], we took advantage of that interaction by applying it to other effects induced by palytoxin, and then concluding a similar functional behaviour of the extracts when compared to those of palytoxin with respect to the $\text{Na}^{+}/\text{K}^{+}$ -ATPase. In addition, as commented before, since palytoxin as well as ouabain have effects on several signaling pathways [36, 70, 73-77], it will be also interesting as a future project to test if the extract share with them other common functional effects.

Detection of palytoxin-like compounds on crude extracts could also be accomplished by analytical methods such as LC-MS, but no presence of palytoxin or ovatoxin-a was detected. However it is well-known that functional assays are more sensitive. Also due to the complex stereostructure of palytoxin, there are many possible isomers that can be being produced by *O. cf. siamensis*, among them, ostreocin-D, but due to the scarcity of this toxin, it could not be tested as a positive control. Anyway, this work compares the functional activity of palytoxin with that one of a purified extract from *O. ovata* and 5 crude extracts from *O. cf. siamensis*, leaving no doubt that all of them share a common functional activity.

Results obtained in this work provide the first evidence that *Ostreopsis cf. siamensis* from the Mediterranean Sea produce active compounds that were detected not to be palytoxin nor ovatoxin-a, but with similar functional characteristics than palytoxin and toxins from *O. ovata*: A) these compounds are highly cytotoxic to neuronal cells, and B) since a pharmacological point of view these compounds trigger a membrane depolarization and a Ca^{2+} overload that can be suppressed by the preincubation with ouabain, suggesting an interaction with the $\text{Na}^{+}/\text{K}^{+}$ -ATPase.

Hence, the present findings confirm that *Ostreopsis cf. siamensis* contains highly active palytoxin-like compounds, and thus point out the possible human risk caused by *Ostreopsis sp.* blooms, demonstrating the requirement of control measures and biomonitoring of these species, that are becoming not just a tropical threat, but a worldwide problem.

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References

- 1 Moore RE, Scheuer PJ: Palytoxin: A new marine toxin from a coelenterate. *Science* 1971;172:495-498.
- 2 Beress L, Zwick J, Kolkenbrock HJ, Kaul PN, Wassermann O: A method for the isolation of the caribbean palytoxin (C-PTX) from the coelenterate (zooanthid) *Palythoa caribaeorum*. *Toxicon* 1983;21:285-290.
- 3 Ishida Y, Takagi K, Takahashi M, Satake N, Shibata S: Palytoxin isolated from marine coelenterates. The inhibitory action on (Na,K)-ATPase. *J Biol Chem* 1983;258:7900-7902.

- 4 Gleibs S, Mebs D: Distribution and sequestration of palytoxin in coral reef animals. *Toxicon* 1999;37:1521-1527.
- 5 Mebs D: Occurrence and sequestration of toxins in food chains. *Toxicon* 1998;36:1519-1522.
- 6 Yasumoto T: Fish poisoning due to toxins of microalgal origins in the pacific. *Toxicon* 1998;36:1515-1518.
- 7 Ukena T, Satake M, Usami M, Oshima Y, Fujita T, Naoki H, Yasumoto T: Structural confirmation of ostreocin-D by application of negative-ion fast-atom bombardment collision-induced dissociation tandem mass spectrometric methods. *Rapid Commun Mass Spectrom* 2002;16:2387-2393.
- 8 Ukena T, Satake M, Usami M, Oshima Y, Naoki H, Fujita T, Kan Y, Yasumoto T: Structure elucidation of ostreocin d, a palytoxin analog isolated from the dinoflagellate *Ostreopsis siamensis*. *Biosci Biotechnol Biochem* 2001;65:2585-2588.
- 9 Usami M, Satake M, Ishida S, Inoue A, Kan Y, Yasumoto T: Palytoxin analogs from the dinoflagellate *Ostreopsis siamensis*. *J Am Chem Soc* 1995;117:5389-5390.
- 10 Rhodes L, Towers N, Briggs L, Munday R, Adamson J: Uptake of palytoxin-like compounds by shellfish fed *Ostreopsis siamensis* (Dinophyceae). *NZ J Mar Freshwat Res* 2002;36:631-636.
- 11 Tognetto L, Bellato S, Moroi I, Andreoli C: Occurrence of *Ostreopsis ovata* (Dinophyceae) in the thyrrhenian sea during summer 1994. *Bot Mar* 1995;38:291-295.
- 12 Riobó P, Paz B, Franco JM: Analysis of palytoxin-like in *Ostreopsis* cultures by liquid chromatography with precolumn derivatization and fluorescence detection. *Anal Chim Acta* 2006;566:217-223.
- 13 Vila M, Camp J, Garcés E, Masó M, Delgado M: High resolution spatio-temporal detection of potentially harmful dinoflagellate in confined waters of the NW Mediterranean. *J Plankton Res* 2001;23:497-514.
- 14 Aligizaki K, Katikou P, Nikolaidis G, Panou A: First episode of shellfish contamination by palytoxin-like compounds from *Ostreopsis* species (Aegean Sea, Greece). *Toxicon* 2007
- 15 Penna A, Vila M, Fraga S, Giacobbe MG, Andreoni F, Riobo P, Vernesi C: Characterization of *Ostreopsis* and *Coolia* (Dinophyceae) isolates in the western Mediterranean Sea based on morphology, toxicity and internal transcribed spacer 5.8S rDNA sequences. *J Phycol* 2005;41:212-225.
- 16 Vila M, Garcés E, Masó M: Potentially toxic epiphytic dinoflagellate assemblages on macroalgae in the nw Mediterranean. *Aquat Microbiol Ecol* 2001;26:51-60.
- 17 Ciminiello P, Dell'Aversano C, Fattorusso E, Forino M, Magno GS, Tartaglione L, Grillo C, Melchiorre N: The Genoa 2005 outbreak. Determination of putative palytoxin in mediterranean *Ostreopsis ovata* by a new liquid chromatography tandem mass spectrometry method. *Anal Chem* 2006;78:6153-6159.
- 18 Monti M, Minocci M, Beran A, Ivesa L: First record of *Ostreopsis* cfr. *ovata* on macroalgae in the Northern Adriatic Sea. *Mar Pollut Bull* 2007;54:598-601.
- 19 Ciminiello P, Dell'aversano C, Fattorusso E, Forino M, Tartaglione L, Grillo C, Melchiorre N: Putative palytoxin and its new analogue, ovatoxin-a, in *Ostreopsis ovata* collected along the Ligurian Coasts during the 2006 toxic outbreak. *J Am Soc Mass Spectrom* 2007
- 20 Alcalá AC, Alcalá LC, Garth JS, Yasumura D, Yasumoto T: Human fatality due to ingestion of the crab *Demania reynaudii* that contained a palytoxin-like toxin. *Toxicon* 1988;26:105-107.
- 21 Yasumoto T, Yasumura D, Ohizumi Y, Takahashi M, Alcalá AC, Alcalá L: Palytoxin in two species of xanthid crab from the Philippines. *Agric Biol Chem* 1986;50:163-167.
- 22 Gallitelli M, Ungaro N, Addante LM, Procacci V, Silver NG, Sabbà C: Respiratory illness as a reaction to tropical algal blooms occurring in a temperate climate. *JAMA* 2005;293:2599-2600.
- 23 Terao KK, Ito E, Yasumoto T: Light and electron microscopic observation of experimental palytoxin poisoning in mice. *Bull Soc Pathol Exot* 1992;85:494-496.
- 24 Habermann E: Palytoxin acts through Na^+, K^+ -ATPase. *Toxicon* 1989;27:1171-1187.
- 25 Hirsh JK, Wu CH: Palytoxin-induced single-channel currents from the sodium pump synthesized by in vitro expression. *Toxicon* 1997;35:169-176.
- 26 Artigas P, Gadsby DC: Na^+/K^+ -pump ligands modulate gating of palytoxin-induced ion channels. *Proc Natl Acad Sci U S A* 2003;100:501-505.
- 27 Hilgemann DW: From a pump to a pore: How palytoxin opens the gates. *Proc Natl Acad Sci U S A* 2003;100:386-388.
- 28 Scheiner-Bobis G: Ion-transporting ATPases as ion channels. *Naunyn Schmiedebergs Arch Pharmacol* 1998;357:477-482.
- 29 Artigas P, Gadsby DC: Large diameter of palytoxin-induced Na/K pump channels and modulation of palytoxin interaction by Na/K pump ligands. *J Gen Physiol* 2004;123:357-376.
- 30 Ishida Y, Satake N, Habon J, Kitano H, Shibata S: Inhibitory effect of ouabain on the palytoxin-induced contraction of human umbilical artery. *J Pharmacol Exp Ther* 1985;232:557-560.
- 31 Bottinger H, Habermann E: Palytoxin binds to and inhibits kidney and erythrocyte Na^+, K^+ -ATPase. *Naunyn Schmiedebergs Arch Pharmacol* 1984;325:85-87.
- 32 Artigas P, Gadsby DC: Ouabain affinity determining residues lie close to the Na/K pump ion pathway. *Proc Natl Acad Sci U S A* 2006;103:12613-12618.
- 33 Ito K, Karaki H, Ishida Y, Urakawa N, Deguchi T: Effects of palytoxin on isolated intestinal and vascular smooth muscles. *Jpn J Pharmacol* 1976;26:683-692.
- 34 Habermann E: Action and binding of palytoxin, as studied with brain membranes. *Naunyn Schmiedebergs Arch Pharmacol* 1983;323:269-275.
- 35 Castle NA, Strichartz GR: Palytoxin induces a relatively non-selective cation permeability in frog sciatic nerve which can be inhibited by cardiac glycosides. *Toxicon* 1988;26:941-951.
- 36 Louzao MC, Ares IR, Vieytes MR, Valverde I, Vieites JM, Yasumoto T, Botana LM: The cytoskeleton, a structure that is susceptible to the toxic mechanism activated by palytoxins in human excitable cells. *Febs J* 2007;274:1991-2004.
- 37 Rakowski RF, Artigas P, Palma F, Holmgren M, De Weer P, Gadsby DC: Sodium flux ratio in Na/K pump-channels opened by palytoxin. *J Gen Physiol* 2007;130:41-54.
- 38 Satoh E, Ishii T, Nishimura M: Palytoxin-induced increase in cytosolic-free Ca^{2+} in mouse spleen cells. *Eur J Pharmacol* 2003;465:9-13.
- 39 Ito K, Karaki H, Urakawa N: [increased ^{45}Ca uptake by the vascular smooth muscles caused by palytoxin]. *Nippon Heikatsukin Gakkai Zasshi* 1976;12:225-226.
- 40 Amano K, Sato K, Hori M, Ozaki H, Karaki H: Palytoxin-induced increase in endothelial Ca^{2+} concentration in the rabbit aortic valve. *Naunyn Schmiedebergs Arch Pharmacol* 1997;355:751-758.
- 41 Frelin C, Van Renterghem C: Palytoxin. Recent electrophysiological and pharmacological evidence for several mechanisms of action. *Gen Pharmacol* 1995;26:33-37.
- 42 Ishii K, Ikeda M, Ito K: Characteristics of palytoxin-induced cation currents and Ca^{2+} mobilization in smooth muscle cells of rabbit portal vein. *Naunyn Schmiedebergs Arch Pharmacol* 1997;355:103-110.

- 43 Sheridan RE, Deshpande SS, Adler M: Cytotoxic actions of palytoxin on aortic smooth muscle cells in culture. *J Appl Toxicol* 2005;25:365-373.
- 44 Rouzaire-Dubois B, Dubois JM: Characterization of palytoxin-induced channels in mouse neuroblastoma cells. *Toxicon* 1990;28:1147-1158.
- 45 Yoshizumi M, Nakanishi A, Houchi H, Morita K, Katoh I, Oka M: Characterization of palytoxin-induced catecholamine secretion from cultured bovine adrenal chromaffin cells. Effects of Na⁺- and Ca²⁺-channel blockers. *Biochem Pharmacol* 1991;42:17-23.
- 46 Yoshizumi M, Ishimura Y, Masuda Y, Houchi H, Kato I, Oka M: Inhibition by ouabain of palytoxin-induced catecholamine secretion and calcium influx into cultured bovine adrenal chromaffin cells. *Biochem Pharmacol* 1994;48:1047-1049.
- 47 Nakanishi A, Yoshizumi M, Morita K, Murakumo Y, Houchi H, Oka M: Palytoxin: A potent stimulator of catecholamine release from cultured bovine adrenal chromaffin cells. *Neurosci Lett* 1991;121:163-165.
- 48 Tosteson MT, Scriven DR, Bharadwaj AK, Kishi Y, Tosteson DC: Interaction of palytoxin with red cells: Structure-function studies. *Toxicon* 1995;33:799-807.
- 49 Schilling WP, Snyder D, Sinkins WG, Estacion M: Palytoxin-induced cell death cascade in bovine aortic endothelial cells. *Am J Physiol Cell Physiol* 2006;291:C657-667.
- 50 Onuma Y, Satake M, Ukena T, Roux J, Chanteau S, Rasolofonirina N, Ratsimaloto M, Naoki H, Yasumoto T: Identification of putative palytoxin as the cause of clupeotoxism. *Toxicon* 1999;37:55-65.
- 51 Guillard RRL, Hargraves PE: *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia* 1993;32:234-236.
- 52 Vilarino N, Nicolaou KC, Frederick MO, Cagide E, Ares IR, Louzao MC, Vieytes MR, Botana LM: Cell growth inhibition and actin cytoskeleton disorganization induced by azaspiracid-1 structure-activity studies. *Chem Res Toxicol* 2006;19:1459-1466.
- 53 Ares IR, Louzao MC, Espina B, Vieytes MR, Miles CO, Yasumoto T, Botana LM: Lactone ring of pectenotoxins: A key factor for their activity on cytoskeletal dynamics. *Cell Physiol Biochem* 2007;19:283-292.
- 54 Louzao MC, Espina B, Vieytes MR, Vega FV, Rubiolo JA, Baba O, Terashima T, Botana LM: "Fluorescent glycogen" Formation with sensibility for *in vivo* and *in vitro* detection. *Glycoconj J* 2008;25(6):503-10.
- 55 O'Brien J, Wilson I, Orton T, Pognan F: Investigation of the alamar blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 2000;267:5421-5426.
- 56 Larson EM, Doughman DJ, Gregerson DS, Obritsch WF: A new, simple, nonradioactive, nontoxic *in vitro* assay to monitor corneal endothelial cell viability. *Invest Ophthalmol Vis Sci* 1997;38:1929-1933.
- 57 Abe T, Takahashi S, Fukuuchi Y: Reduction of alamar blue, a novel redox indicator, is dependent on both the glycolytic and oxidative metabolism of glucose in rat cultured neurons. *Neurosci Lett* 2002;326:179-182.
- 58 Vanitha JD, Paramasivan CN: Evaluation of microplate alamar blue assay for drug susceptibility testing of *Mycobacterium avium* complex isolates. *Diagn Microbiol Infect Dis* 2004;49:179-182.
- 59 Hamid R, Rotshteyn Y, Rabadi L, Parikh R, Bullock P: Comparison of alamar blue and MTT assays for high through-put screening. *Toxicol In Vitro* 2004;18:703-710.
- 60 Schreer A, Tinson C, Sherry JP, Schirmer K: Application of alamar blue/5-carboxyfluorescein diacetate acetoxymethyl ester as a noninvasive cell viability assay in primary hepatocytes from rainbow trout. *Anal Biochem* 2005;344:76-85.
- 61 Cagide E, Louzao MC, Ares IR, Vieytes MR, Yotsu-Yamashita M, Paquette LA, Yasumoto T, Botana LM: Effects of a synthetic analog of polycavernoside A on human neuroblastoma cells. *Cell Physiol Biochem* 2007;19:185-194.
- 62 Louzao MC, Cagide E, Vieytes MR, Sasaki M, Fuwa H, Yasumoto T, Botana LM: The sodium channel of human excitable cells is a target for gambierol. *Cell Physiol Biochem* 2006;17:257-268.
- 63 Louzao MC, Vieytes MR, Yasumoto T, Yotsu-Yamashita M, Botana LM: Changes in membrane potential: An early signal triggered by neurologically active phycotoxins. *Chem Res Toxicol* 2006;19:788-793.
- 64 Brescianini C, Grillo C, Melchiorre N, Bertolotto R, Ferrari A, Vivaldi B, Icardi G, Gramaccioni L, Funari E, Scardala S: *Ostreopsis ovata* algal blooms affecting human health in Genova, Italy, 2005 and 2006. *Euro Surveill* 2006;11:E060907060903.
- 65 Taniyama S, Arakawa O, Terada M, Nishio S, Takatani T, Mahmud Y, Noguchi T: *Ostreopsis* sp., a possible origin of palytoxin (PTX) in parrotfish *Scarus oviifrons*. *Toxicon* 2003;42:29-33.
- 66 Vick JA, Wiles JS: The mechanism of action and treatment of palytoxin poisoning. *Toxicol Appl Pharmacol* 1975;34:214-223.
- 67 Ferrandi M, Manunta P, Ferrari P, Bianchi G: The endogenous ouabain: Molecular basis of its role in hypertension and cardiovascular complications. *Front Biosci* 2005;10:2472-2477.
- 68 Ferrandi M, Molinari I, Bianchi G, Ferrari P: Ouabain-dependent signaling in caveolae as a novel therapeutic target for hypertension. *Cell Mol Biol (Noisy-le-grand)* 2006;52:15-18.
- 69 Aperia A: New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *J Intern Med* 2007;261:44-52.
- 70 Kulikov A, Eva A, Kirch U, Boldyrev A, Scheiner-Bobis G: Ouabain activates signaling pathways associated with cell death in human neuroblastoma. *Biochim Biophys Acta* 2007;1768:1691-1702.
- 71 Sorrentino G, Monsurro MR, Singh IN, Kanfer JN: Membrane depolarization in La-N-1 cells. The effect of maitotoxin is Ca²⁺- and Na⁺-dependent. *Mol Chem Neuropathol* 1997;30:199-211.
- 72 Espina B, Cagide E, Louzao MC, Fernandez MM, Vieytes MR, Katikou P, Villar A, Jaen D, Maman L, Botana LM: Specific and dynamic detection of palytoxins by *in vitro* microplate assay with human neuroblastoma cells. *Biosci Rep* 2009;29:13-23.
- 73 Li S, Wattenberg EV: Differential activation of mitogen-activated protein kinases by palytoxin and ouabain, two ligands for the Na⁺,K⁺-ATPase. *Toxicol Appl Pharmacol* 1998;151:377-384.
- 74 Ares IR, Louzao MC, Vieytes MR, Yasumoto T, Botana LM: Actin cytoskeleton of rabbit intestinal cells is a target for potent marine phycotoxins. *J Exp Biol* 2005;208:4345-4354.
- 75 Louzao MC, Ares IR, Cagide E: Marine toxins and the cytoskeleton: A new view of palytoxin toxicity. *Febs J* 2008;275:6067-6074.
- 76 Edwards A, Pallone TL: Ouabain modulation of cellular calcium stores and signaling. *Am J Physiol Renal Physiol* 2007;293:F1518-1532.
- 77 Rajasekaran SA, Palmer LG, Moon SY, Peralta Soler A, Apodaca GL, Harper JF, Zheng Y, Rajasekaran AK: Na,K-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. *Mol Biol Cell* 2001;12:3717-3732.