Specific and dynamic detection of palytoxins by *in vitro* microplate assay with human neuroblastoma cells

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Synopsis

Palytoxin is one of the most complex and biggest molecules known to show extreme acute toxicity. The dinoflagellate *Ostreopsis* spp., the producer organism of palytoxin, has been shown to be distributed worldwide, thus making palytoxin an emerging toxin. Rat-derived hepatocytes (Clone 9) and BE (2)-M17 human neuroblastoma cells were used to test palytoxin or palytoxin-like compounds by measuring the cell metabolic rate with Alamar Blue. The dose-dependent decrease in viability was specifically inhibited by ouabain in the case of BE (2)-M17 neuroblastoma cells. This is a functional, dynamic and simple test for palytoxins with high sensitivity (as low as 0.2 ng/ml). This method was useful for toxin detection in *Ostreopsis* extracts and naturally contaminated mussel samples. A comparative study testing toxic mussel extracts by LC (liquid chromatography)-MS/MS (tandem MS), MBA (mouse bioassay), haemolysis neutralization assay and a cytotoxicity test indicated that our method is suitable for the routine determination and monitoring of palytoxins and palytoxin-like compounds.

Key words: Alamar Blue, cell line, functional detection method, metabolic rate, viability assay, palytoxin (PLT)

INTRODUCTION

PLT (palytoxin) is one of the most potent toxins known. A wide range of fish and marine invertebrates used as food accumulate PLT and, through the food chain, this toxin has been responsible for numerous incidents of human morbidity and mortality [1]. PTL was initially isolated in 1971 from marine soft corals of the genus *Palythoa* [2]; however, dinoflagellates of the genus *Ostreopsis* have been proposed as possible biogenic origins of this toxin [3]. Originally, *Ostreopsis* species were localized in tropical and subtropical areas, similarly to *Gambierdiscus toxicus*, the producer organism of ciguatoxins. However, at present *Ostreopsis* has a worldwide distribution. A number of people have presented health problems related to exposure to marine aerosol in the Mediterranean Sea. The symptoms shown by the patients (fever, serious respiratory distress and conjunctivitis) were associated with *Ostreopsis ovata* blooms [4,5]. LC (liquid chromatography)-MS/MS (tandem MS) analyses of plankton samples from an *O. ovata* bloom from Ligurian coasts indicated the presence of putative PLT and a PLT-like molecule, named ovatoxin-a by Ciminiello et al. [6]. In this context, the expanding distribution of *Ostreopsis* spp. is potentially hazardous.

At present, the most common method for the detection of PLT is the MBA (mouse bioassay). On the basis of the reported LD$_{50}$ value of 450 ng/kg for PLT, 1 MU (mouse unit) is presumed to be 9 ng of PLT. However, the use of the MBA-detection method raises bioethical problems and this technique is not able to unequivocally distinguish the nature of the causative agent [7]. For these reasons, alternative assays, taking advantage of the functional properties of PLT, such as delayed haemolysis and the antibody-based HNA (haemolysis neutralization assay) have been developed [8,9]. LC-ESI (electrospray ionization)–MS/MS also has great potential for rapid, sensitive and unambiguous identification of PLT in contaminated material. One of the drawbacks for its use would be the necessity to relate toxin concentration to...
actual toxicity in order to evaluate the associated risk, whereas the fact that analytical methods require very expensive instruments and specialized personnel should also be taken into account.

In the present study, we generated a cell-viability assay to test PLT or PLT analogue activity in different naturally contaminated matrices by using two cellular models: a rat-derived hepatocyte cell line (Clone 9) and BE (2)-M17 human neuroblastoma cells.

EXPERIMENTAL

Materials
PLT standard from *Palythoa tuberculosa* was purchased from Wako. OA (okadaic acid) was from Alexis and Alamar Blue was purchased from Biochrom AG.

Culture medium for neuroblastoma cells, specifically EMEM (Eagle’s minimum essential medium) and Ham’s F12 medium supplemented with glutamine, non-essential amino acids, gentamicin and amphotericin B were obtained from Biochrom AG.

Clone 9 cell-culture medium, namely nutrient mixture Ham’s F12 Kaighn’s modification medium, streptomycin sulfate salt and penicillin G potassium salt were purchased from Sigma and fetal bovine serum was from Gibco. All other reagents were purchased either from Sigma or Panreac (Barcelona, Spain).

*Ostreopsis cf. siamensis* crude extracts
*O. siamensis* were collected epiphytic to macroalgae at several locations along the Andalusian coast in Spain. Seawater containing the macroalgae sample was shaken vigorously to detach the epiphytic organisms. Clonal isolates were obtained by transferring individual cells through sterile seawater washes with a 20-μm mesh plankton net. The extract was further concentrated by centrifugation at 3094 g for 8 min at 20°C, the supernatant was transferred into a volumetric flask. The second extraction was carried out using a high-speed homogenizer at 10000 rev./min for 1 min, then a further 9 ml of 100% methanol was added and the sample was centrifuged. This supernatant was transferred into the same volumetric flask containing the supernatant obtained in the first extraction and the volume was adjusted to a final volume of 20 ml using 100% methanol. These extracts were also used in the viability test.

Clone 9 rat hepatocytes
Rat hepatocytes from the Clone 9 cell line [ECACC (European Collection of Animal Cell Cultures) number 88072203] were grown on Nunc 60-mm-diameter tissue-culture plates with nutrient mixture Ham’s F12 Kaighn’s modification medium supplemented with 2.5 g/l NaHCO<sub>3</sub>, 28 mg/l streptomycin sulfate salt, 17 mg/l penicillin G potassium salt and 10% (v/v) fetal bovine serum (pH 7.2). Cells were maintained at 37°C in a humidified atmosphere at 5% CO<sub>2</sub>95% air and were subcultured weekly by transferring released cells with the addition of 0.1% trypsin/EDTA.

BE(2)-M17 neuroblastoma cells
BE(2)-M17 human neuroblastoma cells were routinely grown in EMEM/Ham’s F12 medium [1:1 (v/v)] with 2 mM L-glutamine, 1% non-essential amino acids, 10% (v/v) fetal bovine serum, 50 μg/ml gentamicin and 50 ng/ml amphotericin B. Cells were cultured in Nunc 25 cm<sup>2</sup> tissue-culture flasks, maintained at 37°C in a humidified atmosphere at 5% CO<sub>2</sub>95% air and were subcultured weekly by transferring released cells with the addition of 0.1% trypsin/EDTA.

Viability assay
The metabolic activity of Clone 9 or neuroblastoma cells was measured using Alamar Blue. This compound becomes fluorescent when reduced without affecting cell integrity [11]. Thus the cell metabolic rate can be evaluated proportionally by measurement of the fluorescence intensity.

Cells were seeded on to Corning Costar 96-well plates. After 24 h incubation, standard PLT (0.015, 0.075, 0.15, 1.5, 7.5, 15 or 75 nM), *O. siamensis* crude extracts (1:100 dilution of extract A–E) or mussel extracts (1:5 dilution) were added to the cells. In some experiments, cells were pre-incubated with 1 mM ouabain for 30 min before the addition of PLT or extracts. Finally, Alamar Blue (1:10 dilution) was added and fluorescence was measured by using a microplate fluorescence reader (FL600; Bio-Tek). Fluorescence was recorded using filters of 530 nm with a bandwidth of 25 nm for excitation and 590 nm with a bandwidth of 35 nm for emission.

Experiments testing OA were included in order to confirm the inhibition specificity of ouabain towards PLT-induced cytotoxicity. OA was used at a concentration of 500 nM based on the dose–response curve of the cytotoxic effect of OA (results not shown).
Results are presented as the percentage of fluorescence compared with the respective control (cells, culture medium and treatment vehicle); results are means ± S.E.M (n ≥ 3).

MBA
MBAs were conducted as described by Yasumoto et al. [12]. Samples 48, 54 and 56 which were also extracted in n-butanol as described by Aligizaki et al. [7] were tested using the HNA and the MBA.

All animal manipulations were performed in accordance with the EU Directive 86/609/EC (1986), under official license from the Prefectural Veterinary Service of Thessaloniki, Greece.

HNA
HNAs were carried out as described previously [7] using sheep erythrocytes.

LC-MS/MS
PLT analysis was carried out using a HPLC system (Shimadzu; Kyoto, Japan) which consists of two pumps (LC-10ADvp), an auto-injector (SIL-10ADvp) and a column oven (CTO-10ACvp) coupled to a 3200QTrap LC-MS/MS system (Applied Biosystems), which consists of a hybrid quadrupole-linear-ion-trap mass spectrometer equipped with an atmospheric pressure ionization system fitted with an ESI source (Turbo V source). The LC conditions used were based on the method published previously by Ciminiello [5]. A C18 column (150 × 2 mm; 5 μm) was used at 20°C at a flow rate of 0.2 ml/min. Eluent A was water and eluent B was 95% (v/v) acetonitrile/water, with both eluents containing 30 mM acetic acid. Separations were performed by linear gradient elution, starting with 20–100% eluent B for 10 min, followed by a 4 min hold at 100% eluent B, decreasing to 20% eluent B over 5 min. MS detection was operated in positive mode, monitoring the ions in MRM (multiple-reaction monitoring). The transitions selected for toxin detection were: 912 → 327 [DP (de-clustering potential) 61 V/CE (collision energy) [13] 43 eV] and 1352 → 327 [DP 76 V/CE 75 eV]. The LOD (limit of detection) was calculated based on a signal/noise (S/N) ratio of 3. The LOD value corresponded to 50 ng PLT/ml.

Statistical analysis
All experiments were carried out in triplicate. Results were analysed using the Student’s t test for paired data where appropriate. P ≤ 0.05 was considered to be statistically significant.

RESULTS

Standard PLT detection
Initially, the effect of standard PLT on the metabolic activity of Clone 9 and neuroblastoma cells was evaluated. In both cell types, the experimental procedure was similar. Cells (40000) were seeded on to Costar 96-well plates. After incubation for 24 h to allow attachment, control samples and cells treated with 0.15, 1.5, 7.5, 15 or 75 nM PLT were incubated at 37°C with Alamar Blue for up to 24 h. Fluorescence measurements were taken at 3, 6, 8, 12 and 24 h of incubation.

Hepatocytes and neuroblastoma cells treated with PLT showed a dose-dependent reduction in fluorescence intensity (Figure 1). This fall in fluorescence intensity is related to a decrease in cell viability. An incubation time of 3 h with 15 nM or 75 nM PLT was enough to induce a rapid loss of viability (up to 80%) of hepatocytes (Figure 1a). However, neuroblastoma cells still maintained nearly 100% viability under these conditions (Figure 1b). This result clearly indicates different cytotoxicity effects depending on the cellular model. Nevertheless, in hepatocytes (Figure 1c) as well as in neuroblastoma cells (Figure 1d), 24 h incubation with 1.5 nM PLT was enough to trigger a significant and equal decrease in fluorescence compared with the control cells.

PLT binds to Na+/K+ pumps in the plasma membrane of animal cells and opens a cation pathway through the pumps. Ouabain is a potent inhibitor of the Na+/K+ pump and also inhibits the effects of PLT. In order to relate unequivocally the reduction in the metabolic rate of the cells with PLT activity, we performed ouabain inhibition assays (Figure 1). In neuroblastoma cells, ouabain pre-treatment blocked the decrease in viability induced by PLT (Figures 1b and 1d). However, in Clone 9 hepatocytes, ouabain only decreased the reduction in viability slightly (Figures 1a and 1c).

The dose–response curve for incubation of the cells with toxins for 6 h indicates that hepatocytes are more sensitive to the cytotoxic effects of PLTs (Figure 1e) than neuroblastoma cells (Figure 1f).

PLT or PLT-like compounds detection in O. cf. siamensis extracts
We evaluated the accuracy of the viability assay in identifying the presence of PLT-like compounds in naturally contaminated extracts. All extracts induced a reduction in viability in both Clone 9 and neuroblastoma cells. Moreover, ouabain inhibited this effect slightly in Clone 9 cells (Figures 2a–2e), but almost completely inhibited this effect in neuroblastoma cells, as shown for PLT (Figures 2f–2j).

We also found cytotoxicity in hepatocytes and neuroblastoma cells with other toxins that could be associated to PLT, such as OA (Figure 3). The specificity of our test for PLTs was demonstrated by the lack of effect of ouabain on the decrease in viability induced by 500 nM OA in both cell lines (Figure 3). Although ouabain plus OA compared with OA alone seems to induce a slight reduction in cell viability in Clone 9 cells and increases cell viability in neuroblastoma cells, there are no significant differences between the results of both treatments (P > 0.05).

Even though hepatocytes seem to be more sensitive to the effects of PLT, we chose neuroblastoma cells to improve our test based on the high inhibition induced by ouabain of the effect of PLT in this cellular model. In Figure 4, we decreased the number of cells seeded to 5000 cells/well. After incubating the cells for 24 h to allow attachment, the experimental procedure was performed as stated above. Control samples and cells treated with
Figure 1  Dose-dependent effect of PLT on Clone 9 rat hepatocytes and BE(2)-M17 human neuroblastoma cells

Rat hepatocytes (a, c) or neuroblastoma cells (b, d) were incubated with PLT and Alamar Blue for 24 h. Pre-treatment with 1 mM ouabain for 30 min was included for all concentrations tested where indicated. Dose–response curves of PLT on Clone 9 rat hepatocytes (e) and BE(2)-M17 neuroblastoma cells (f) after 6 h incubation. All results are expressed as the percentage of fluorescence compared with controls (set as 100%) and are means ± S.E.M. (n = 3).

various concentrations of PLT were incubated at 37°C with Alamar Blue for up to 24 h. However, in this case, we found a loss of viability after a 24 h incubation with very low concentrations of PLT (0.015 nM) (Figure 4b). A loss in viability of 20% was induced with 0.15 nM PLT after an incubation time of 4 h (Figure 4b). The dose–response curve corroborates the increase in sensitivity of the test after reducing the number of cells (Figure 4c). We also confirmed the specificity of the method in these experimental conditions by the lack of effect of ouabain on the decrease of viability induced by 500 nM OA (Figure 4d).

O. cf. siamensis crude extracts were tested again using the new conditions, and a clear increase in sensitivity was obtained (Figure 5). All of the extracts triggered a sharp decrease in the metabolic rate of the cells from the first fluorescent measurement (4 h incubation). At this incubation period, they showed a largely increased cytotoxicity which could be inhibited completely when
Figure 2  Effect of different _O. cf. siamensis_ crude extracts on the viability of Clone 9 rat hepatocytes (a–e) and BE(2C)-M17 neuroblastoma cells (f–j) and its inhibition by ouabain

Both cell types were incubated for 24 h with 1:100 dilutions of 5 _O. cf. siamensis_ crude extracts. Pre-treatment with 1 mM ouabain for 30 min was included where indicated. All results are expressed as the percentage of fluorescence compared with controls (set as 100%) and are means ± S.E.M. (n = 3).
neuroblastoma cells were pre-treated with 1 mM ouabain (Figure 5). It is interesting to point out that in the case of extract A, inhibition by ouabain after 6 h incubation was incomplete (Figure 5a). A similar lack of inhibition was observed with 15 nM and 75 nM PLT (Figure 4a).

In Figure 5(f), we present the quantity of PLT equivalents per cell for each extract, calculated using the linear relationship shown in Figure 4(c). Taking into account these results, an average of 4.103 ± 1.2 pg of PLT equivalents/cell was calculated using the average number of cells in all crude extracts.

**PLT or PLT-like compound detection in mussel extracts**

We also analysed some methanol extracts from mussels collected along the North Aegean coast in Greece (48–56), where *Ostreopsis* spp. have been found previously. All extracts displayed a clear reduction in the metabolic rate of neuroblastoma cells after 48 h incubation, but only the effects of extracts 48, 49, 50, 51, 52 and 53 were significantly inhibited by ouabain (Figure 6a). The cytotoxic effect of extracts 54 and 55 was only partially inhibited by ouabain, whereas no inhibition was observed after pre-treatment with ouabain before the addition of extract 56. Although some protective effects of pre-treatment with ouabain can be observed for extracts 54, 55 and 56 (mainly extract 55), no significant differences were observed with respect to treatment of the extracts (P > 0.05, Student’s t-test). However, the effect of extracts 48, 49, 50, 51, 52 and 53 was significantly different from the effect of these extracts after pre-treatment (P < 0.05).

Applying a dose–response curve for PLT after 48 h incubation (Figure 6b), we could estimate the toxin concentration in the mussel extracts that were positive in our test (samples 48–53). This concentration was expressed in ng/ml of PLT equivalents (Table 1).

As a comparative study, the extracts from mussels were analysed by LC-MS/MS, MBA and HNA (Table 1). No PLT was detected in any sample by LC-MS/MS. However, OA was detected in extract 56 (13 ng/ml). All samples were tested and found to be positive using MBA protocol (A) [12] when acetone extracts were used. n-Butanol extracts from samples 48, 54 and 56 were found to be clearly positive for PLT-like compounds, as determined by both MBA protocol (B) and delayed HNA.

**DISCUSSION**

The Na⁺/K⁺ pump is a common and specific target of two kinds of natural toxins, heart glycosides and PLT [14]. Heart glycosides, such as ouabain, bind to the pump when its sodium-binding sites are open to the extracellular side, thereby locking two sodium ions into the pump. Ouabain was also demonstrated to inhibit the action of PLT [15]. PLT binds to the Na⁺/K⁺ ATPase pump and converts it into a cation-selective ion channel [16–19]. This action triggers numerous cytotoxic effects, such as disturbance of the cytoskeleton [20] or intracellular Ca²⁺ increase [21]. We found that pre-incubation with ouabain prevents the cytotoxic effects induced by PLT. On this basis, we present a viability method to test PLTs using the metabolic-rate indicator Alamar Blue [11]. This dye allows continuous measurement of cell viability because it is not toxic for the cells, unlike other cytotoxicity indicators [22,23].

PLT effects were comparatively tested in two cellular models: a rat hepatic cell line (Clone 9) widely used in metabolic studies [24] and human neuroblastoma BE-(2)-M17 cells, excitable cells with a great reference potential for the toxicity evaluation of many compounds [25,26]. In this case, we took advantage of the ease of obtaining cells from a cell line compared with the laborious isolation procedure of erythrocytes for the delayed HNA [7].

Changes in the cell metabolic rate indicated a dose-dependent decrease in the viability of both cell types. However, rat hepatic cells displayed a higher sensitivity to the action of the toxin. Habermann et al. [16] demonstrated a species-specific PLT potency relationship, showing that rat erythrocytes were more sensitive to PLT than those of humans, cattle or dogs.

The usefulness of the viability assay with Alamar Blue for the detection of PLT or PLT-like compounds was tested with crude
extracts of O. cf. siamensis obtained after culture of dinoflagellates harvested on the southern coast of Spain. Unequivocally, all crude extracts tested had a high cytotoxic effect that was inhibited by ouabain in neuroblastoma cells. However, as observed previously with PLT, this glycoside did not manage to completely inhibit the toxic effect of extracts in rat hepatocytes. This lack of effectiveness of ouabain in the rat hepatic cell line can be attributed to the reported species- and isoform-dependent potency of this glycoside [27]. There are four α-subunit isoforms of the Na\(^+\)/K\(^+\)-ATPase, the binding site of ouabain, which are expressed in organisms in a tissue- and species-specific manner. The four α peptide isoforms have similar high ouabain affinities, with a \(K_d\) of approx. 1 nM in almost all mammalian species. However, rodents express an α1 isoform [28] which is 100-fold less sensitive to ouabain [13]. It could be considered that the α1 isoform in rats and a few other rodents is nearly ouabain insensitive [29]. This may explain the poor inhibition of PLT-induced cytotoxicity by ouabain in rat hepatocytes, but not the higher toxicity that we found in comparison with the neuroblastoma cells. This is an interesting effect that must be studied further. On the other hand, nervous tissue expresses α2 and α3 isoforms of the Na\(^+\)/K\(^+\)-ATPase [30], which are highly sensitive to cardiac glycosides. Neuroblastoma cells are therefore the ideal cell model for a specific PLT-detection assay.

The inhibition by ouabain of the cytotoxic effect induced by crude extracts of O. cf. siamensis is complete after incubation for 4 h. However, a time-dependent loss of the inhibitory effect of ouabain indicates the presence of high concentrations of PLT or PLT-like compounds in the sample, as found in extract A. In this context, a viability assay at a low density of cells performed over a long time period would be appropriate for routine assays, taking into account the high sensitivity of this kind of test. In routine assays, we expect the concentrations of PLT or PLT-like compounds to be similar to toxins in natural samples (lower than 40 or 200 ng/ml respectively). However, in the presence of unexpected high concentrations of PLT or PLT-like toxins in the samples, a shorter incubation period may be more reliable for specific detection. However, more precise determination of the

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**Figure 4** Dose-dependent effect of PLT on BE(2)-M17neuroblastoma viability at a low cell density

(a, b) Alamar Blue viability assays were carried out as described in the Experimental section. Pre-treatment with 1 mM ouabain for 30 min was included where indicated. (c) Dose–response curve for 4 h incubation with PLT in neuroblastoma cells at a low cell density. (d) Lack of inhibition of ouabain on the effect of OA. Neuroblastoma cells at a low density were incubated for 4 h with 500 nM OA. Pre-treatment with 1 mM ouabain for 30 min was included where indicated. Results are expressed as the percentage of fluorescence compared with controls (set as 100%) and are means ± S.E.M. (\(n = 3\)).
Figure 5  Effect of different O. cf. siamensis crude extracts on the viability of neuroblastoma cells seeded at low cell density

(a–e) Neuroblastoma cells were incubated for 24 h with 1:100 dilutions of five O. cf. siamensis crude extracts. Pre-treatment with 1 mM ouabain for 30 min was included where indicated. (f) The concentration of the PLT-like compounds was calculated for each O. cf. siamensis crude extract, taking into account its effect on the viability assays and the dose–response curve. Results are expressed as the percentage of fluorescence compared with controls (set as 100%) and are means ± S.E.M. (n = 3).

Toxin concentration can be made by testing the samples with a high density of cells.

Two principal characteristics are required for an effective detection method: (i) specificity and (ii) sensitivity, which have been addressed as follows. (i) A decrease in viability induced by PLT and PLT-like toxins is specifically inhibited by ouabain.

The specificity of our assay was confirmed by the lack of effect of ouabain on the reduction in viability induced by OA. In this case, we chose OA because it has a different structure and mechanism of action from PLTs, even though it is a cytotoxic compound. Also, both kinds of toxins could appear in the same geographic zone and be accumulated in the same fish and mollusc.
species, since *Prorocentrum* spp., a known benthic producer organism of OA, often co-exists with *Ostreopsis* spp. [31,32].

(ii) We set up the number of cells and toxin incubation time to improve the method and increase sensitivity. This way, we can detect 0.15 nM PLT after a 4 h incubation (0.4 ng/ml). Also, since Alamar Blue is a non-toxic dye, this is a dynamic method of detection, as cells may be incubated with toxins for up to 72 h, allowing detection of lower concentrations of toxins [up to 0.075 nM PLT (0.2 ng/ml)]. As a result, we obtained a very wide detection range of 0.2–40 ng/ml PLT equivalents, which includes the MU (9 ng). Under these conditions, *O. cf. siamensis* crude extracts and even extracts from mussels can be tested for the presence of PLT and PLT-like compounds.

Table 1: Comparison of PLT detected by viability assays, LC-MS/MS analysis, MBA and delayed HNA in mussel extracts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cytoxicity (μg PLT eq./ml)</th>
<th>LC-MS/MS</th>
<th>MBA</th>
<th>HNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>11.43 ± 2.21</td>
<td>None</td>
<td>++++§</td>
<td>Yes</td>
</tr>
<tr>
<td>49</td>
<td>7.88 ± 2.59</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>50</td>
<td>5.00 ± 1.45</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>51</td>
<td>13.41 ± 0.60</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>52</td>
<td>13.79 ± 2.66</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>53</td>
<td>13.03 ± 4.11</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>54</td>
<td>Yes*</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>55</td>
<td>Yes*</td>
<td>None</td>
<td>+++</td>
<td>n.d.</td>
</tr>
<tr>
<td>56</td>
<td>Yes†</td>
<td>OAT†</td>
<td>+++</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Not completely inhibited by ouabain.
†Not inhibited by ouabain.
§13 ng/ml OA.
§Tested positive when extracted with n-butanol as well.

All tested extracts from the dinoflagellates were clearly positive for the presence of PLT or PLT-like compounds. However, although all of the extracts from mussels were cytotoxic, two of them displayed a partially PLT-independent effect. Moreover, sample 56 exerted complete PLT-independent cytotoxicity. This fact could be explained by the presence in the methanol extract of a different sort of toxin, such as OA, as indicated by LC-MS/MS analysis. Sample 56 (as well as samples 48 and 54) displayed clear positive results when analysed using the MBA and delayed HNAs, pointing to the presence of PLT or PLT-like compounds. In this case, MBA and delayed HNAs were carried out in the same group of mussels, but using n-butanol extraction. PLT-like compounds could be more concentrated in this fraction, and this could be responsible for the differences in the results obtained with this sample by LC-MS/MS analysis and our assays. However, a routine assay for testing natural samples of shellfish may require initial simple extraction, such as with 100% methanol, so not to exclude other toxins, such as DSP (diarrheic shellfish poisoning).

Calculation of PLT equivalents for each extract was also possible using our method. The average of all of the *O. cf. siamensis* crude extracts was 4.103 pg PLT-equivalents/cell. This quantity is very similar to the content of PLT and PLT analogues obtained in another recent study using crude extracts from *Ostreopsis* spp. [6]. Currently, we use this method in our laboratory to evaluate the production of PLT and PLT-like compounds by *O. cf. siamensis* cultures, with concentrations detected in the range 2.76 ± 1.18 to 17.23 ± 5.50 μg PLT equivalents/ml.

The new method presented here allows the sensitive detection of PLT or PLT-like compounds in a natural sample of...
dinoflagellates or mussels, but also indicates the presence of other toxins and differentiates them from PLT. The action mechanism of PLT is specific and well defined. PLT binds to the membrane Na\(^+\)/K\(^+\)-ATPase pump [16,17,19]. Many studies have reported that the action of PLT is blocked totally by ouabain, a cardiac glycoside that binds to the Na\(^+\)/K\(^+\)-ATPase [15]. This property is unique in the marine toxin world and is the starting point for our detection method. Many toxic compounds result in an acute fall in the metabolic rate, followed by a decrease in cell viability. However, only a group of toxins trigger high cytotoxicity which can be completely inhibited by ouabain: PLT and PLT-like compounds, such as ostreocin D or ovatoxin A. The complexity of the PLT molecule makes the existence of many PLT analogues possible. Using our detection method, three possibilities can occur in cytotoxic samples: (i) The cytotoxicity is not inhibited by ouabain, meaning that the sample contains non-PLT-like toxins. (ii) The cytotoxicity is partially inhibited by ouabain, therefore the sample contains at least two types of compounds: PLT-like and non-PLT-like compounds. In this case, we can detect the presence of PLT based on the partial inhibition of the cytotoxic effect by ouabain; however we cannot infer a PLT-equivalent concentration because of the impossibility of assigning the proportion of cytotoxicity for which PLT-like compounds are responsible. (iii) The cytotoxicity is completely inhibited by ouabain, so the sample contains only PLT or PLT-like compounds and so their concentration in the sample can be calculated. In short, in a sample we can differentiate the cytotoxic effect resulting from PLT or PLT-like compounds from that of any other toxin based on its specific inhibition by ouabain.

In the present study, we have produced an easy, fast, sensitive, specific and dynamic viability assay in neuroblastoma cells to test PLTs and to detect PLT-like compounds in crude extracts. The assay has many advantages, such as the possibility of simultaneous testing of many samples in microtitre plates and the wide detection range that it offers.

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