Optimización de ensayos celulares para la detección de toxinas marinas responsables de intoxicaciones alimentarias. Aplicación en extractos lipofílicos de muestras naturales de *Mytilus galloprovincialis*

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ARTÍCULOS

3 PUBLICACIONES

3.1 Artículo 1

Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma x glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins

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RESUMEN

Se evalúa la idoneidad y sensibilidad de dos modelos celulares neuronales, NG108-15 y Neuro-2a, a diferentes toxinas marinas bajo distintos tiempos de incubación y exposición, y en presencia o ausencia de ouabaina y veratridina (O/V).

Las células NG108-15 fueron más sensibles a la pectenotoxina-2 que las células Neuro-2a. Para la saxitoxina, la brevetoxina-3, la palitoxina, el ácido okadaico y la dinofisistoxina-1 ambos tipos celulares demostraron ser sensibles y óptimos para la evaluación toxicológica. Se obtuvieron resultados preliminares para el ácido domoico. Las variaciones en el tiempo de incubación y de exposición demostraron ser críticas en el desarrollo de los ensayos. A fin de reducir la duración de los ensayos, es preferible reducir el tiempo de cultivo de las células previo a la exposición de la toxina que el propio tiempo de exposición. Para la palitoxina, después de 24 h de cultivo, ambos tipos celulares fueron sensibles en ausencia de O/V. Cuando el tiempo de cultivo previo a la exposición de la toxina se redujo, ambos tipos celulares fueron insensibles a la palitoxina en ausencia de O/V. Aunque

la dinofisistoxina-1 y el ácido okadaico son ambos inhibidores de fosfatasas, estas toxinas no respondieron de forma similar frente a las condiciones experimentales estudiadas. Ambos tipos celulares fueron capaces de identificar toxinas que actúan sobre canales de sodio dependientes de voltaje y capaces también de cuantificar el efecto de la saxitoxina, la brevetoxina-3, la palitoxina, el ácido okadaico, la dinofisistoxina-1 y la pectenotoxina-2 bajo diferentes condiciones experimentales.

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Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma × glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins

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ABSTRACT

The suitability and sensitivity of two neural cell models, NG108-15 and Neuro-2a, to different marine toxins were evaluated under different incubation and exposure times and in the presence or absence of ouabain and veratridine (O/V).
NG108-15 cells were more sensitive to pectenotoxin-2 than Neuro-2a cells. For saxitoxin, brevetoxin-3, palytoxin, okadaic acid and dinophysistoxin-1 both cell types proved to be sensitive and suitable for toxicity evaluation. For domoic acid preliminary results were presented. Setting incubation time and exposure time proved to be critical for the development of the assays. In order to reduce the duration of the assays, it was better to reduce cell time incubation previous to toxin exposure than exposure time. For palytoxin, after 24 h of growth, both cell types were sensitive in the absence of O/V. When growth time previous to toxin exposure was reduced, both cell types were unsensitive to palytoxin when O/V was absent. Although dinophysistoxin-1 and okadaic acid are both phosphatase inhibitors, these toxins did not respond similarly in front of the experimental conditions studied. Both cell types were able to identify Na-channel acting toxins and allowed to quantify the effect of saxitoxin, brevetoxin-3, palytoxin, okadaic acid, dinophysistoxin-1 and pectenotoxin-2 under different experimental conditions.

1. Introduction

Potent marine toxins can affect humans through food-borne intoxications mainly by consumption of contaminated shellfish, finfish and occasionally through direct exposure to seawater aerosols when some of these toxins are present in shore waters (Hallegraeff, 2003). In order to prevent intoxications and protect public health, the quantification of marine toxins in food and the environment is needed. The identification and quantification of marine toxins are valuable information that allows to conduct risk evaluation studies and that is necessary for the management of shellfish harvesting areas. Mammalian bioassays used for the quantification of toxins are supported by current legislation in Europe (European Union Commission Regulation, 2005) and several non-European countries. However, using mouse bioassays is also a matter of concern for ethical and practical reasons, and current legislation also favours its replacement by other methodologies (European Union Council Directive, 1986). In addition to analytical methods and functional assays, cell-based assays could contribute to reduce animal testing for toxin identification in shellfish.

Cell-based assays are routinely used to study the activity of bioactive compounds since these can affect cellular homeostasis leading to several types of observable effects such as physiological disruptions, the stimulation of

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cellular growth or cellular mortality (Stacey et al., 2001). The study of these responses can contribute to understand the mechanism of action of these compounds and also to quantify their potency. Cell-based assays have been used for the identification of bioactive compounds isolated from marine organisms (Mayer and Gustafson, 2003), and more precisely, marine toxins that affect food safety (Rossini, 2005).

Numerous marine toxins of very different nature (amino acid derivatives, complex polyethers, cyclic amines, ...) have been studied with the use of primary cell cultures (Larm et al., 1997; LePage et al., 2005; Shenfeng et al., 2005) and established cell lines (Aune et al., 1991; Diogène et al., 1995; Leira et al., 2001; Okumura et al., 2005; Tubaro et al., 1996).

Several mammalian cell types have been used to detect marine toxins, to evaluate their toxicity or to study their mechanisms of action (Rossini, 2005). One of the most stable methods used in cytotoxicity studies for voltage gated sodium channel (VGSC)-dependent neurotoxins such as saxitoxins (STXs), ciguatoxins, brevetoxins (PbTxs) and tetrodotoxins (Catterall, 1985) is the viability assay on Neuro-2a neuroblastoma cells (Gallacher and Birkbeck, 1992; Jellett et al., 1992; Kogure et al., 1988; Manger et al., 1995). Cell viability is commonly evaluated with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] cell proliferation assay which estimates mitochondrial activity. This assay is conducted after cell exposure to toxins (Manger et al., 1993), with or without the presence of ouabain and veratridine (O/V) in the case of Neuro-2a cells. The set-up of the Neuro-2a assay for the evaluation of the toxic effect of VGSC-dependent neurotoxins has supposed an important advancement in the applicability of cell cultures for toxic effect determination (Catterall, 1985). However, in 1998, a cell bioassay kit for PSP (Jellett et al., 1998) using Neuro-2a cells was presented as an alternative to the mouse bioassay for VGSC toxin determination. This kit was unsuccessful, probably due to cell unstability during shipment or unreliable laboratory conditions where it was used. In the present work, one of our goals is to contribute furtherly to demonstrate the suitability of the response of the Neuro-2a cell line to VGSC acting toxins, under different controlled laboratory conditions, in order to support future applicability of the assay.

The study of the toxic potential of neurotoxins has also been carried out with other neural cell lines such as PC12 cells (Perovic et al., 2000), neuroblastoma × glioma hybrid cells (NG108-15) (Molgó et al., 1993; Sheridan and Adler, 1989) or HEK-293 cells (Bottein Dechraoui and Ramsdell, 2003). NG108-15 cells have been employed for the study of neuronal processes and are frequently used for toxin detection and pharmaceutical screening (Mohan et al., 2006; Ohkuma et al., 1994). This cellular model, which contains VGSC (Molgó et al., 1993) and also glutamate receptors (Ohkuma et al., 1994), was selected in this study to improve the applicability of cellular models to a wider range of toxins. To our best knowledge, there have been no previous studies to determine the EC50s of STX, PbTx-3, palytoxin, pectenotoxin-2 (PTX-2), okadaic acid (OA), dinophysistoxin-1 (DTX-1) and domoic acid (DA) in NG108-15 cells.

On the other hand, since the toxic effect of some marine neurotoxins seems to be limited by the presence of receptors expressed on differentiated neurones, toxicity evaluation of marine neurotoxins has also been proposed in primary neural cell cultures as it is the case for DA (Novelli et al., 1992) where these receptors are more abundant than in stable neuroblastoma cell lines (LePage et al., 2005). For that reason, primary cell cultures may seem to be a more sensitive model to study the effect of some marine neurotoxins' toxic effect than established cell lines (LePage et al., 2005; Perovic et al., 2000). However, the use of primary cultures in routine toxin screening may have some difficulties associated with the isolation of cells and the establishment of cultures which will eventually have a negative incidence with the repeatability of the experimental conditions. Additionally, the applicability of these models for a continuous and rapid evaluation may not be possible for routine and intensive cytotoxicity screening. For these reasons, established cell lines may be a better choice for the routinely detection and quantification of the toxic effect of toxins.

To evaluate the cytotoxicity of other types of toxins that do not interact with VGSC, other cell types such as epithelial cells and fibroblasts have been proposed, for instance, for the evaluation of lipophilic toxins such as OA and yessotoxin (Diogène et al., 1995; Huynh-Delerme et al., 2003; Rossini, 2005). Although these cell systems have been proved to be efficient for these types of toxins, they may not respond to neurotoxins.

One of our research goals has been to propose a versatile toxicological cell-based assay with an established neural cell line for the evaluation of the toxic potential of standards of several marine toxins (neurotoxins and also other types of toxins) as an initial step for future development of cell-based assays to be used as a routine method for the screening of marine toxins. The present study focuses on the response of two established cell lines. The cytotoxic potency (i.e. the 50% effect concentration, EC50) of STX, PbTx-3, palytoxin, PTX-2, OA, DTX-1, and DA was evaluated on neuroblastoma Neuro-2a and NG108-15 cell viability. Particular attention has been placed on the experimental conditions required to run each cell-based assay, as these have to be cautiously set-up in order to have a stable cellular model. Precise conditions of the assays have been fixed to correctly understand the toxicological responses obtained and therefore favour the applicability of the assay. Several experimental conditions were studied in order to optimize the experimental protocol, including the presence and absence of O/V, incubation time and exposure time.

Results presented in this study contribute to a better understanding of the potency of these toxins and support the applicability of these two neural cell models for marine toxin detection and quantification. Differential toxicological behaviour of these cell types will help for the study of the toxin mechanism of action.

2. Material and methods

In the present study, different methods used to evaluate cytotoxic response of marine toxins on Neuro-2a cells (Dickey et al., 1999; Manger et al., 1993) were adapted to

be used in our laboratory with Neuro-2a and NG108-15 cells. Suitability of these two cellular models to evaluate toxic effect on toxins studied is determined by the possibility to generate dose–response curves and sensitivity was evaluated by the EC50s values.

2.1. Neuro-2a cell culture maintenance

Neuroblastoma cells, Neuro-2a (American Tissue Culture Collection (ATCC), CCL131), were cultured in 10% Foetal Bovine Serum (FBS)/Roswell Park Memorial Institute (RPMI)-1640 medium at 37 °C and 5.0% CO_2 in an incubator (Binder, Tuttlingen, Germany). Culture medium RPMI-1640 was supplemented with 1% sodium pyruvate solution (100 mM), 1% L-glutamine solution (200 mM) and 0.5% antibiotic solution (10 mg/mL streptomycin and 1000 U/mL penicillin). Cells' subcultures were made three times per week (dilution 1/4), in a 75 cm^2 flasks. An approximate confluence of 100% was obtained with 30 mL of 10% FBS/RMPI in 2 days. Trypsin solution (0.5 g/L) was used to dislodge cells from the flask.

2.2. NG108-15 cell culture maintenance

NG108-15 cells (ATCC, HB12317), were cultured in 10% FBS/Dulbecco's Modified Eagle's Medium (DMEM) at 37 °C and 5.0% CO_2 in an incubator (Binder, Tuttlingen, Germany). Culture medium DMEM was supplemented with 0.2% pyridoxine-HCl solution (2 g/L), 2% L-glutamine solution (200 mM), 0.5% antibiotic solution (10 mg/mL streptomycin and 1000 U/mL penicillin), 0.1 mM hypoxantine, 400 nM aminopterin, and 0.016 mM thymidine. Cells' subcultures were made three times per week (dilution 1/4), in 75 cm^2 flasks. An approximate confluence of 90% was obtained with 30 mL of 10% FBS/DMEM in 2 days. Trypsin solution (0.5 g/L) was used to dislodge cells from the flask.

2.3. Seeding of cells into 96-well plates for cytotoxicity assays

For cell viability assays, 96-well plates (flat bottom) were prepared with cells obtained from a 90–100% confluence flask with 8 mL of culture medium (5% FBS). For each plate, 2 mL of this cell suspension were diluted up to a final volume of 20 mL of 5% FBS culture medium per plate. Inocula of 200 µL cell suspension were added to each well. Cell densities were approximately 35 000 cells/well for Neuro-2a and in the range of 25 000–50 000 cells/well for NG108-15. For the whole study all conditions (controls included) were tested in triplicate.

2.4. Toxin preparation

Previous to the toxin exposure of cells, defined aliquots of toxin solutions were dispensed on glass vials and evaporated under gentle N_2 flux at 40 °C using a Turbovap (Zymark corp., Hopkinton, Massachusetts). Evaporated extracts were dissolved in 5% FBS culture medium (concentrated dose) and were added to the corresponding wells. Seven more concentrations were prepared (1:2 dilutions) from the concentrated dose, and added (10 µL/well) to three wells (triplicates). All plate's wells had the same final volume (230 µL); it was adjusted with PBS.

2.5. Toxin exposure of cells and response evaluation

Cells with or without O/V were exposed to increased concentrations of commercial toxins. For neurotoxins acting on VGSC, O/V concentrations (with a fixed proportional factor of 10:1, respectively) were selected to produce mortality in approximately 20 or 80% of the cell population depending on whether the toxins activate (PbTx-3) or inhibit (STX) the sodium channels (Table 1). All toxin assays were tested with or without O/V in the same 96-well plate.

For VGSC inhibiting toxins (e.g., STX) O/V concentrations that produce about 80% mortality were used to detect the VGSC inhibiting type effect that would counteract cell mortality. In the O/V 80% mortality treatment absolute and relative viability values were used to generate dose–response curves. The EC50 was calculated from the relative viability values' dose–response curve. A positive O/V control (O/V+) was used to determine the 0% response, since higher viability is expected in the presence of effective inhibiting toxin concentrations. The 100% response corresponds to the maximal viability obtained within the range of concentrations tested. For VGSC activating toxins (e.g., PbTxs) O/V concentration that produce around 20% mortality were used to detect the VGSC activating type effect that would increase cell mortality. In the O/V 20% mortality treatment, a positive O/V control (O/V+) was used to determine the 100% of viability, equivalent to the 0% toxic effect. A 0% viability corresponds to a 100% (toxic effect) response. For the evaluation of the response of other toxins the lowest O/V concentration was used (as for VGSC activating toxins) in the first step.

Table 1
Ouabain and veratridine (O/V) concentrations used in the different experimental conditions and cell models

	Cell type	% Viability	Ouabain (mM)	Veratridine (mM)
O/V addition in 24 h grown cells (experimental conditions 1 and 3)	Neuro-2a	80	0.1	0.01
		20	0.3	0.03
	NG108-15	80	0.3	0.03
		20	0.75	0.08
O/V addition in 1 h grown cells (experimental condition 2)	Neuro-2a	80	0.1	0.01
		20	0.45	0.05
	NG108-15	80	0.2	0.02
		20	0.87	0.09

The O/V concentrations were set to obtain an approximate viability of 20 or 80% on neuroblastoma cells (Neuro-2a) and neuroblastoma × glioma hybrid cells (NG108-15), in 96-well plate according to cell growth time before O/V addition.

For toxin exposure, the order in which the different reactives were used for plate preparation during the cytotoxicity assay was strict. First, the corresponding volume of PBS (from 0 to 30 μL, depending on the treatment) needed to obtain a final well volume of 230 μL was added to the 200 μL of medium in each well. After PBS addition, O/V treatment was dispensed in the corresponding wells and finally the standard of toxin.

Cell viability was estimated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] method as described elsewhere (Manger et al., 1993). Absorbances were read on an automated multiwell scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA) at 570 nm. Dose–response curves that allowed the determination of EC50 were analysed with the software Prism 4 (GraphPad, San Diego, California, USA). For the establishment of dose–response curves, cell viability for each toxin concentration was measured in triplicate ($n = 3$). Non-linear Regression for curve fit was applied using sigmoidal dose–response curve (variable slope) of the log X, X being toxin concentration. Curve fitness was described according to r^2 (r^2 ranged between 0.878 and 0.998). For each cellular model, each toxin and each experimental condition, one curve was used to obtain the EC50. Dose–response curves' ordinate axis consists of viability estimations using percentage of MTT reduction, using the corresponding controls for each experience and exceptionally negative O/V control (O/V−) for O/V treatment experience to estimate absolute values of viability.

2.6. Experimental conditions studied

Three experimental conditions regarding growth time previous to toxin exposure and exposure time were studied.

2.6.1. Twenty-four hours of cell growth in plates previous to 24 h of toxin exposure

In this experimental condition cells were grown without any treatment during 24 h, and after this time, cells were treated for additional 24 h with or without O/V and the toxins at different concentrations (1/2 dilution between concentrations). Absorbance plate reading was performed after 24 h of toxin exposure. Forty-eight hours were needed from the time plates were prepared to result reading.

2.6.2. One hour of cell growth in plates previous to 24 h of toxin exposure

In this experimental condition cells were grown without any treatment during 1 h, and after this time cells were treated for additional 24 h with or without O/V and the toxins at different concentrations (1/2 dilution between concentrations). Absorbance reading was performed after 24 h of toxin exposure. Twenty-five hours were needed from the time plates were prepared.

2.6.3. Twenty-four hours of cell growth in plates previous to 3 h of toxin exposure

In this experimental condition cells were grown without any treatment during 1 h, and after this time cells were treated with or without O/V and 23 h later cells were exposed to the toxins tested at different concentrations (1/2 dilution between concentrations). Absorbance reading was performed after 3 h of toxin exposure. Therefore, 3 h of toxin exposure were initiated after 24 h of plate preparation and 23 h after O/V treatment in the corresponding wells. Twenty-seven hours were needed from the time plates were prepared to results reading.

This experimental condition was chosen since for a 3 h toxin exposure, it was not possible to obtain a stable response, adding the O/V at 24 h with the toxin treatment. Therefore, O/V was added 1 h after cell plate preparation (cell seeding) and consequently O/V had been added to the culture 23 h before toxin exposure.

2.7. Toxins

All the experiments of this study have been performed using commercially available, purified toxins: STX, PTX-2 and DA (National Research Council of Canada); PbTx-3 (Calbiochem); OA (Sigma); DTX-1 and palytoxin (Wako).

3. Results and discussion

3.1. Response of Neuro-2a and NG108-15 cells to different toxins

3.1.1. Neuro-2a and NG108-15 cell exposure to STX, PbTx-3, palytoxin, PTX-2, OA, and DTX-1 in experimental conditions 1–3

All these toxins were tested in both cell types and in the three experimental conditions with and without O/V treatment. Dose–response curves to all toxins were constructed and EC50s were determined (Table 2) for each of the toxins. In Figs. 1–4 dose–response curves obtained at the three experimental conditions described are presented for each toxin with O/V treatment (for STX, PbTx-3 and palytoxin) or without it (for PTX-2, OA and DTX-1).

3.1.1.1. Saxitoxin. Saxitoxin, a VGSC blocker toxin, counteracted the toxic O/V effect (in O/V 80% mortality treatment) (Fig. 1b, relative viability values) with an EC50 of 8.6 nM in Neuro-2a cells and 8.21 nM in NG108-15 cells in experimental condition 1. Detectable response was obtained within a range of concentrations of 0.91–58.41 nM in Neuro-2a cells and 4.2–67.17 nM in NG108-15 cells. There was no response to STX in cells without O/V treatment at the same STX concentrations (data not shown). Similar detection ranges (3–300 nM) (Jellett et al., 1995) were obtained in the same conditions than in experimental condition 1 in other laboratories. Dr R.W. Dickey's group of the Gulf Coast Seafood Laboratory (GCSL) – Food and Drug Administration (FDA), Alabama obtained an EC50 of about 3 nM (personal communication) with Neuro-2a cells.

The EC50s for STX in experimental condition 2 were about 20 nM for Neuro-2a and 29 nM for NG108-15 (Table 2). In Fig. 1a (absolute viability values), cell viability dose–response curves for STX in relation to control without O/V treatment (O/V−) are presented. In experimental conditions 1 and 2, Neuro-2a cells showed a maximum of 40–50% viability increase (with regard to control O/V−), whereas NG108-15 cells had a maximum increase of 14%. In experimental

Table 2
Toxin potency on Neuro-2a and NG108-15 according to different experimental conditions

Experimental condition	Toxin	EC50 (nM)			
		Neuro-2a		NG108-15	
		Without OV	With OV	Without OV	With OV
1, 24 h growth, 24 h exposure	Saxitoxin	NE (>58.4)	8.6	NE (>268.7)	8.2
	Brevetoxin-3	NE (>335.2)	8.2	NE (>335.16)	90.0
	Palytoxin	0.1	0.0	0.2	0.1
	Pectenotoxin-2	28.3	17.4	1.0	5.5
	Okadaic acid	21.9	8.8	8.9	7.3
	Dinophysistoxin-1	20.6	23.1	11.25	9.0
2, 1 h growth, 24 h exposure	Saxitoxin	NE (>58.4)	20.6	NE (>268.7)	28.6
	Brevetoxin-3	NE (>90.9)	9.8	NE (>90.9)	No quant[a]
	Palytoxin	NE (>0.6)	0.1	NE (>0.6)	0.1
	Pectenotoxin-2	58.6	140.0	2.0	5.0
	Okadaic acid	16.7	14.4	17.6	12.3
	Dinophysistoxin-1	8.6	6.7	10.2	7.3
3, 24 h growth, 3 h exposure	Saxitoxin	NE (>116.8)	NE (>116.8)	NE (>116.8)	NE (>116.8)
	Brevetoxin-3	NE (>181.8)	NE (>181.8)	NE (>181.8)	NE (>181.8)
	Palytoxin	NE (>1.2)	NE (>1.2)	NE (>1.2)	NE (>1.2)
	Pectenotoxin-2	79.4	90.4	11.8	13.0
	Okadaic acid	113.7	168.1	197.8	218.1
	Dinophysistoxin-1	18.0	17.8	24.1	23.9

Corresponding 50% effect concentrations (EC50s) obtained from dose–response curves on neuroblastoma cells (Neuro-2a) and neuroblastoma × glioma hybrid cells (NG108-15) in experimental condition 1 (24 h of cell growth in plates previous to 24 h of toxin exposure), experimental condition 2 (1 h of cell growth in plates previous to 24 h of toxin exposure) and experimental condition 3 (24 h of cell growth in plates previous to 3 h of toxin exposure) with or without ouabain and veratridine (O/V) treatment. For those toxins where no effect (NE) was observed, the maximum concentration used is reported in parenthesis.

[a] No quantifiable EC50.

condition 3, there was no effect of STX on Neuro-2a or NG108-15 cell viability (with regard to control O/V+).

NG108-15 cells may have more VGSC than Neuro-2a cells (actually, higher O/V concentrations are needed in NG108-15 cells). Therefore, fewer VGSC would be activated in NG108-15 and for that reason STX may not offset O/V effect as highly in NG108-15 cells as in Neuro-2a cells, even if a O/V concentration that produced an elevated cell mortality was used. In order to better quantify the VGSC inhibitor toxin STX effect in NG108-15 cells, the O/V concentrations should be modified, increasing the veratridine proportion in the O/V addition that produces 80% of mortality.

3.1.1.2. Brevetoxin-3. Brevetoxin-3, a voltage gate sodium channel activator toxin, was able to kill O/V pre-treated cells with a dose-dependent response (Fig. 1c). Neuro-2a cells presented a more sensitive response in experimental conditions 1 and 2 (EC50s around 9 nM) than NG108-15 with an EC50 of about 90 nM in experimental condition 1 and no quantifiable effect on experimental condition 2 (Table 2). Since NG108-15 may have more VGSC than Neuro-2a cells, sensitivity in NG108-15 would increase if veratridine proportion was increased in the O/V concentration that produces 20% mortality. In order to improve the toxic effect quantification of VGSC activator and blocker toxins on NG108-15 cells, an increase of veratridine proportion in the O/V concentration used may be necessary.

There was no response at 3 h cell exposure to PbTx-3 (experimental condition 3) in both cell types. Dr R.W. Dickey's group using similar experimental conditions to experimental condition 1 with Neuro-2a cells obtained an approximate EC50 of 10 nM (personal communication).

Experiments carried by others groups in Neuro-2a cells obtained EC50s of about 20 nM for PbTx-3 after 16 h exposure (Manger et al., 1995), and in a range of 1–60 nM using specific techniques with different primary cell cultures, like calcium monitorization (EC50: 27.1 nM) (LePage et al., 2003) or membrane potential monitorization (EC50: 2.5 nM) (David et al., 2003).

3.1.1.3. Palytoxin. Dose–response curves obtained for palytoxin in experimental condition 1 prove a high sensitivity in both cell types, with an EC50 of 0.04 nM for Neuro-2a cells and 0.05 nM for NG108-15 cells with the O/V treatment. Sensitivity was very slightly reduced without O/V treatment (Table 2) with an EC50 of 0.1 nM for Neuro-2a cells and 0.16 nM for NG108-15 cells.

In experimental condition 2 (1 h grown cells, and 24 h exposure to palytoxin) a very sensitive response (EC50s around 0.1 nM) was also obtained as in condition 1 in both cell types, only when cells were treated with O/V. However, in this experimental condition, there was no response in both cell types in the absence of O/V.

Palytoxin affect ionic cell equilibrium, increasing cell permeability for Na^+, K^+ but not for Ca^{2+}. Higher toxin concentrations (100 nM) inhibit Na^+/K^+-ATPase (Habermann, 1989). Ouabain and veratridine treatment affect the ionic cell equilibrium; therefore, cells treated with O/V were more sensitive to palytoxin than non-treated cells. Palytoxin has haemolytic activity in an approximate concentration of 3 pM (Riobó et al., 2004). Toxic effects could be detected between 5 pM and 10 nM in cardiac muscle and above 10 nM in skeletal muscle cells (Habermann, 1989). After a 3 h toxin exposure in 24 h grown cells (experimental condition 3), no effects were recorded on

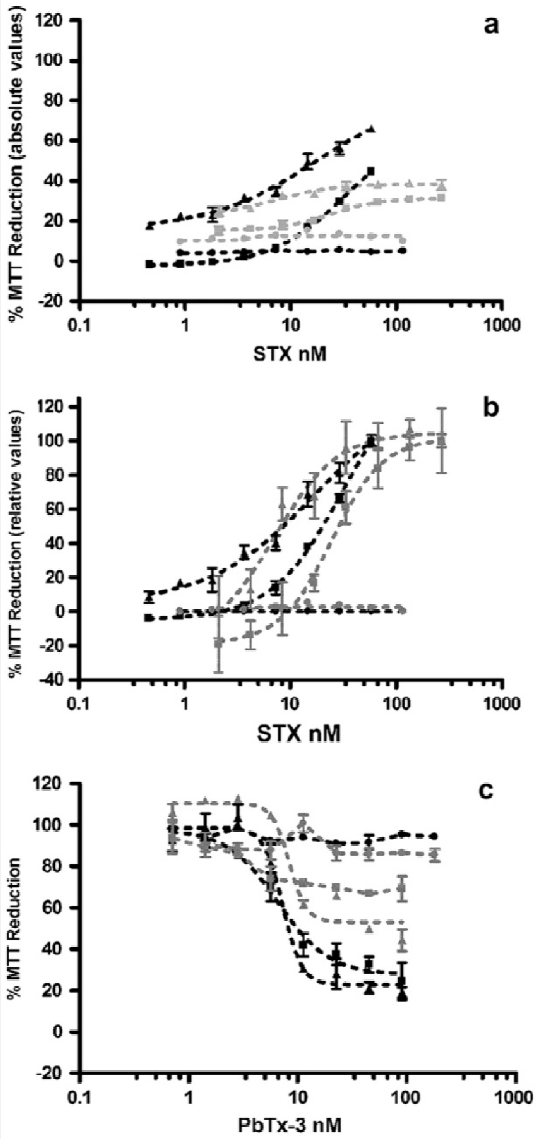

Fig. 1. Dose–response curves of saxitoxin (STX) and brevetoxin-3 (PbTx-3) on neuroblastoma cells (Neuro-2a; black) and neuroblastoma × glioma hybrid cells (NG108-15; grey). In every graph three curves corresponding to three experimental conditions are represented: 24 h of cell growth in plates previous to 24 h of toxin exposure (▲), 1 h of cell growth in plates previous to 24 h of toxin exposure (■), 24 h of cell growth in plates previous to 3 h of toxin exposure (●). Graphs correspond to ouabain and veratridine (O/V) treated cell exposures (discontinuous lines). The ordinate axis represents viability estimations using (a) percentage of MTT reduction (absolute values), (b) percentage of MTT reduction (relative values) and (c) percentage of MTT reduction. Three repetitions were performed per dose in 96-well plates, at a cell density close to 35 000 cells/well on Neuro-2a cells and between 25 000 and 50 000 cells/well on NG108-15 cells.

cell viability. Dr R.W. Dickey's group in similar conditions to experimental condition 1 obtained EC50s in Neuro-2a cells with or without O/V treatment close to 0.4 and 0.04 nM, respectively (personal communication).

3.1.1.4. Pectenotoxin-2. Cell exposure to PTX-2 always resulted in a quantifiable response in the three experimental conditions in both cell types, although a higher sensitivity was recorded in NG108-15 than in Neuro-2a (Fig. 3). The EC50s obtained in NG108-15 experiments ranged between 0.99 and 13 nM depending on the experimental condition and the presence or absence of O/V, while EC50s in Neuro-2a cells ranged from 17.4 to 140 nM (Table 2). NG108-15 cells without O/V treatment were more sensitive to PTX-2 than cells treated with O/V. If the three experimental conditions are compared according to cell sensitivity to PTX-2, the experimental condition 1 gives the lowest EC50 (highest sensitivity), subsequently experimental condition 2 and finally experimental condition 3 (the less sensitivity). Although exposure and cell growth time have definitive implications in toxin sensitivity to PTX-2, all the experimental conditions studied permitted to establish dose–response curves and allowed EC50 quantification in NG108-15 cells. In Neuro-2a cells, the experimental condition has an influence on differential response to O/V treatment (Table 2). In experimental condition 1, O/V treated Neuro-2a cells were slightly more sensitive to toxin exposure than non-treated cells, while in experimental conditions 2 and 3, O/V untreated cells seem to be more sensitive than treated cells, especially in experimental condition 2 (Table 2). Very few cytotoxicity studies exist on PTXs, and reported toxic effects range between 571.7 nM (Hamano et al., 1985) and 5.7 μM (Aune et al., 1991).

3.1.1.5. Okadaic acid. Okadaic acid had an effect in both cell types in all the experimental conditions studied with or without O/V treatment (Fig. 4; Table 2). Cell sensitivity to OA was strongly reduced in experimental condition 3 where the EC50 obtained was around 150 nM while in the other two situations EC50s were approximately around 15 nM. In Neuro-2a cells, O/V treated cells were slightly more sensitive to OA exposure than cells not treated with O/V in the experimental condition 1. This was not so evident in condition 2, while in experimental condition 3, the contrary was observed (Table 2). Ouabain and veratridine treatment seems not to be so important in the response of NG108-15 cells to OA in experimental condition 1. Dr R.W. Dickey's group in similar conditions to experimental condition 1 in Neuro-2a cells with or without O/V treatment obtained dose–response curves with EC50s around 40 nM (personal communication). Okadaic acid cell sensitivity in this study is similar to other cell type sensitivities reported in other experiments that define EC50s between 5 and 100 nM (Diogène et al., 1995; Huynh-Delerme et al., 2003; Leira et al., 2001; Tubaro et al., 1996). In primary cultures, the EC50 for okadaic acid has been established in 4.5 nM after 24 h exposure in the first study using okadaic acid in neurons (Fernández et al., 1991). In the same study, an EC50 of approximately 7.5 nM was observed for okadaic acid in glial cells.

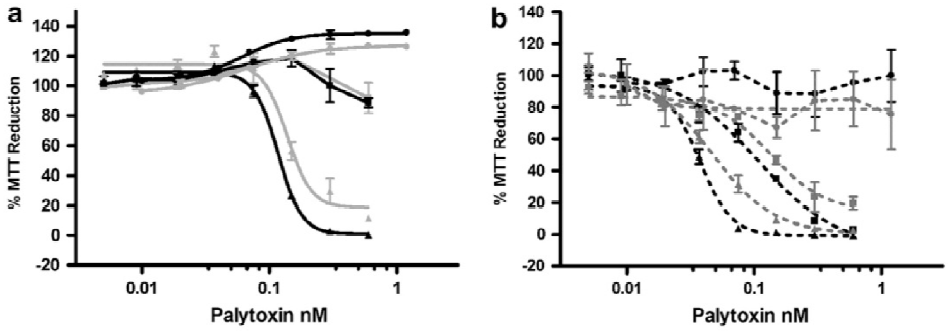

Fig. 2. Dose–response toxic effect curves of neuroblastoma cells (Neuro-2a; black) and neuroblastoma × glioma hybrid cells (NG108-15; grey) exposed to palytoxin (a) with ouabain and veratridine (O/V) treatment and (b) without O/V treatment. In every graph three curves corresponding to three experimental conditions are represented: 24 h of cell growth in plates previous to 24 h of toxin exposure (▲), 1 h of cell growth in plates previous to 24 h of toxin exposure (■), 24 h of cell growth in plates previous to 3 h of toxin exposure (●). Three repetitions were performed per dose in 96-well plates, at a cell density close to 35 000 cells/well on Neuro-2a cells between 25 000 and 50 000 cells/well on NG108-15 cells.

3.1.1.6. Dinophysistoxin-1. Small EC50s variability was obtained for DTX-1 regarding the estimated EC50s (ranging from 6.74 to 24.1 nM) in both cell types, with or without O/V treatment, in the three experimental conditions (Fig. 4; Table 2). Dr R.W. Dickey's group in similar conditions to experimental condition 1 in Neuro-2a cells, with or without O/V treatment, obtained dose–response curves with EC50s of about 20 nM (personal communication). In other cell types, DTX-1 effects have been observed at concentrations ranging between 20 (Fladmark et al., 1998) and 60 nM (Hamano et al., 1985).

3.1.2. Neuro-2a and NG108-15 cell exposure to DA in experimental condition 1

Domoic acid toxicity was only analysed in experimental condition 1 for both cell types. In O/V treated Neuro-2a and NG108-15 cells, DA effect on cell viability could not be observed below 2 μM in both cell types. Nevertheless, O/V untreated cells exposed to 6 μM DA concentration (log[DA, nM] ± 3.8) presented mortality around 95% in NG108-15 cell population, while Neuro-2a O/V untreated cells only presented a 10% of mortality at 6 μM. An EC50 of 3 μM was obtained for NG108-15 O/V untreated cells. This experiment was repeated in the same conditions and no toxic response was obtained for both cell types. Dr R.W. Dickey's group in similar conditions to experimental condition 1 did not obtain effect of DA on viability of Neuro-2a cells with or without O/V treatment at a maximal dose of approximately 90 μM (personal communication). In primary cultures, an EC50 of 7–8 μM was determined for domoic acid in neurons without O/V and no toxicity was observed in glial cells (Novelli et al., 1992). In DA toxic action it is known the possible implication of NMDA (*N*-methyl D-aspartate) and non-NMDA (AMPA–kainate) glutamate receptors (Berman and Murray, 1997; Sharp et al., 2003; Van Dolah et al., 1997). NMDA receptors presence has been reported on NG108-15 cells (Ohkuma et al., 1994), while they seem not to be present in high amounts on Neuro-2a cells (LePage et al., 2005). More work has to be done to control DA sensitized factors on NG108-15 cells toxic response.

3.2. Importance of exposure time and growth time

3.2.1. Importance of exposure time in toxin effect quantification. Dose–response curves comparison in the experimental conditions 1 and 3

Decreasing exposure time from 24 to 3 h (comparison between experimental conditions 1 and 3) did not allow to observe the toxic potential of STX, PbTx-3 and palytoxin, since no effect was recorded after 3 h exposure. For PTX-2 and OA, EC50s increased when exposure time was reduced. For DTX-1 little change was observed.

For palytoxin, in condition 1 (cells exposed to the toxin for 24 h), toxic effect was recorded regardless of the presence or absence of O/V, although a lower EC50 was

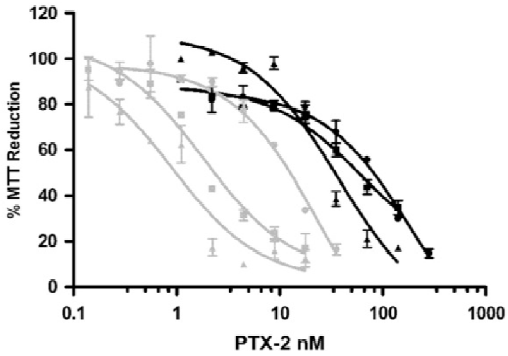

Fig. 3. Dose–response toxic effect curves of pectenotoxin-2 on neuroblastoma cells (Neuro-2a; black) and neuroblastoma × glioma hybrid cells (NG108-15; grey). In every graph three curves corresponding to three experimental conditions are represented: 24 h of cell growth in plates previous to 24 h of toxin exposure (▲), 1 h of cell growth in plates previous to 24 h of toxin exposure (■), 24 h of cell growth in plates previous to 3 h of toxin exposure (●). Graphs correspond to ouabain and veratridine (O/V) untreated cell exposures (continuous lines). Three repetitions were performed per dose in 96-well plates, at a cell density close to 35 000 cells/well on Neuro-2a cells and between 25 000 and 50 000 cells/well on NG108-15 cells.

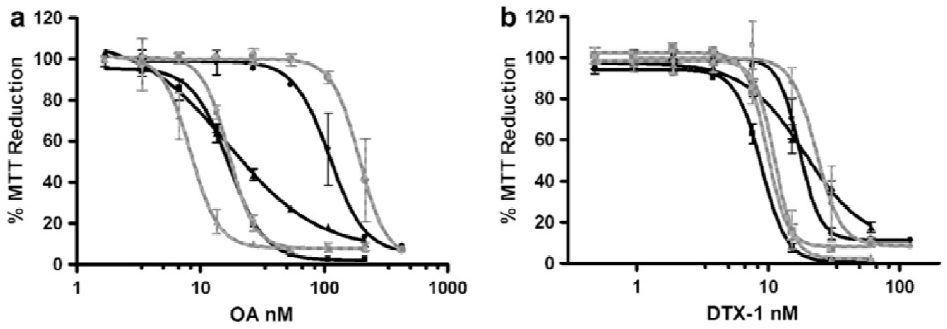

Fig. 4. Dose–response toxic effect curves of (a) okadaic acid (OA) and (b) dinophysistoxin-1 (DTX-1) on neuroblastoma cells (Neuro-2a; black) and neuroblastoma × glioma hybrid cells (NG108-15; grey). In every graph three curves corresponding to three experimental conditions are represented: 24 h of cell growth in plates previous to 24 h of toxin exposure (▲), 1 h of cell growth in plates previous to 24 h of toxin exposure (■), 24 h of cell growth in plates previous to 3 h of toxin exposure (●). Graphs correspond to ouabain and veratridine (O/V) untreated cell exposures (continuous lines). Three repetitions were performed per dose in 96-well plates, at a cell density close to 35 000 cells/well on Neuro-2a cells and between 25 000 and 50 000 cells/well on NG108-15 cells.

obtained in the presence of O/V for both cell types. In condition 3 (cells were exposed to palytoxin only for 3 h) no response was obtained in the absence of O/V, indicating that certainly exposure time was insufficient to obtain a toxic response in the studied concentrations. The addition of O/V in this condition (3 h exposure) did not influence toxicity, and palytoxin still did not show any response. It is clear that exposure time influences the response of palytoxin, and that our results suggest that O/V did not increase the sensitivity of the cells after a 3 h exposure to the toxin. The delay between O/V addition and toxin treatment may eventually explain why O/V may not have sensitized the cells to palytoxin. As we will see below, O/V definitively sensitized the cells to palytoxin in condition 2 (1 h growth and 24 h exposure).

For STX and PbTx-3, a 3 h exposure with O/V was not adequate to obtain a toxic response, indicating the importance of exposure time. However, again, the delay between O/V addition and toxin exposure may also explain why no response was obtained after a 3 h exposure of cells to these toxins.

3.2.2. Importance of growth time previous to toxin exposure in toxin effect quantification. Dose–response curves comparison in the experimental conditions 1 and 2

Decreasing growth time previous to toxin exposure from 24 to 1 h (comparison between experimental conditions 1 and 2) still allows the quantification of the toxic potential of all the toxins tested.

In Fig. 2, palytoxin dose–response curves for Neuro-2a and NG108-15 cells corresponding to the experimental conditions 1 and 2 with and without O/V treatment are presented. Fig. 2 shows the importance of cell growth time previous to toxin exposure. In experimental condition 1, cells were exposed during 24 h to palytoxin after 24 h of cell growth in 96-well plates. Therefore, at the time cells were exposed to the toxins, cells were firmly fixed to the plate surface creating a high confluence monolayer. Palytoxin in this condition caused toxic effects with and without O/V treatment in both cell types. In experimental condition 2, cell exposure to the toxin was performed 1 h after cell plate preparation. After only 1 h growth, cells may not present the same properties, cell confluence may not be so high and probably cells do not present the same membrane receptors disposition than in experimental condition 1. In this condition (2), evident palytoxin toxic effects could be seen on Neuro-2a and NG108-15 O/V treated cells (more toxin was needed than in experimental condition 1) but there were no effect on O/V untreated cells (at the studied concentrations). This is the type of response obtained for VGSC activator toxins.

For PTX-2, in Neuro-2a cells, in condition 2 higher PTX-2 concentrations were needed to produce the same effect than in condition 1. Little change was observed between both experimental conditions on NG108-15 cells.

Neuro-2a cells seem to be more sensitive to DTX-1 when growth time previous to toxin exposure is decreased from 24 to 1 h. This response could not be seen on NG108-15 cells. For OA little change was observed.

4. Conclusions

4.1. Reducing time for cytotoxic evaluation

It is important to note that 3 h toxin exposure in cells previously grown for 24 h with O/V is not a suitable method for STX, PbTx-3 and palytoxin cytotoxicity detection, while this approach is good for DTX-1 in Neuro-2a cells as well as in NG108-15 cells. Reducing exposure time from 24 to 3 h is also applicable to PTX-2 and OA quantification, but it reduces the sensitivity of the assay.

Setting exposure initiation at different moments of cell culture has important implications on toxic effect responses. Palytoxin response is an evident example. This toxin seems to act in a similar mode to VGSC activator toxins at 24 h exposure in 1 h grown cells, since no appreciable effects were observed on O/V untreated cells while a well-defined dose–response curve was obtained on O/V treated cells. After a 24 h exposure in 24 h grown cells, palytoxin had an effect with or without O/V. This clearly demonstrates that O/V was not a requirement to observe the palytoxin effect on cell viability at the tested concentrations.

Our results prove that in order to reduce the duration of a classical cytotoxicity assay lasting 24 h of exposure after 24 h of cell growth (a total of 48 h), it is better to use 1 h grown cells and maintain a 24 h exposure time (a total of 25 h) than growing cells for 24 h and reducing exposure time to 3 h (a total of 27 h). Reducing growth time to 1 h previous to toxin exposure still requires to take into consideration possible changes in cell sensitivity, as it is the case for palytoxin.

Similarities in the DTX-1 EC50s obtained for the two cell lines tested in this study show the stability of the toxic effect evaluation in different experimental conditions. Nevertheless, for OA, which is also a phosphatase inhibitor, cells exposed for 3 h needed a much higher concentration of OA (in the order of 7–22 nM at 24 h exposure, instead of approximately 114–218 nM at 3 h exposure) to obtain the same toxic effect than cells exposed at 24 h. This result suggests that OA and DTX-1 do not share the same mechanism of action.

4.2. Comparing Neuro-2a and NG108-15 dose–response curves

For STX and PbTx-3, under O/V exposure, Neuro2-a showed a wider range of effect (quantitatively) than NG108-15 cells, with greater differences in viability between the control and the concentration of toxin having the maximum effect.

For palytoxin and DTX-1 no obvious differences existed between the two cell lines tested.

NG108-15 cells were more sensitive to PTX-2 and DA toxins than Neuro-2a cells. Differences in toxin response in both cell types seem to show that NG108-15 cells have a better structural disposition which permits a better toxic effect evaluation for these toxins. NG108-15 used for the cytotoxicity evaluation of PTX-2 would represent an important reduction of the use of expensive standard toxin and could be used in a routinely marine toxin detection laboratory. For DA, more work needs to be done to modify experimental conditions and eventually increase suitability and sensitivity of NG108-15 to DA.

For OA, differences in Neuro-2a toxic response in the presence or absence of O/V treatment are slightly higher than differences in NG108-15 cells.

At this stage, the reliable dose–response curves obtained for NG108-15 cells support this model as a biological, suitable and sensitive tool that complements the use of neuroblastoma Neuro-2a cells.

4.3. Toxin quantification with cell-based assays

Neuro-2a and NG108-15 cells responded to all the tested toxins, with the exception of Neuro2-a which was not sensitive to DA, as expected. Both cell models allowed to obtain reliable dose–response curves for STX, PbTx-3, palytoxin, OA, DTX-1 and PTX-2 under different experimental conditions. These assays allowed to quantify the toxic effects of marine toxins and therefore our results support the applicability of these two neural cell models for marine toxin detection and quantification under thoroughly controlled conditions. Differential toxicological behaviour of these cell types and the use of O/V can contribute to study the mechanism of action of these toxins. Results in this study may eventually improve the acceptance of the use of mammalian cytotoxicity assays for marine toxin detection and favour the development of further studies on marine toxin quantification in shellfish samples using cell-based assays.

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Conflict of interest

We would like to state that all the research involved in the study and all information written in our manuscript "Comparative study of the use of neuroblastoma cells (Neuro-2a) and neuroblastoma × glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins" have no conflict of interest with any person or institution.

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