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

Elisabet Cañete Ortiz

3.2 Artículo 2

Improvements in the use of neuroblastoma x glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins

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RESUMEN

Se presentan nuevos avances en el uso de células NG108-15 para la detección y cuantificación de toxinas marinas. Establecimos modificaciones en la proporción de ouabaina y veratridina para obtener buenas curvas dosis-respuesta, en esta línea celular, para neurotoxinas que actúan sobre canales de sodio dependientes de voltaje (VGSCs). Curiosamente, se observaron diferencias en la respuesta tóxica de dos toxinas activadoras de VGSCs, la brevetoxina-3 y la ciguatoxina-1 del pacífico. Para toxinas lipofílicas que no actúan sobre VGSCs, se analizaron varios factores que influyen en la detección y cuantificación de las toxinas. Cultivos celulares de una hora expuestos durante cuarenta y ocho horas, comparados con cultivos de veinticuatro horas y veinticuatro horas de exposición, incrementan la respuesta tóxica máxima de la yesotoxina (YTX) y el azaspirácido-1 (AZA-1) mientras que no se observan cambios en la respuesta del ácido okadaico (OA), la dinofisistoxina-1 y la pectenotoxina-2 en células NG108-15. Las curvas dosis-respuesta obtenidas para la YTX o para el AZA-1 muestran variabilidad en relación al día de experimentación mientras que hay una buena repetitividad de la respuesta para el OA. El tiempo de evaporación previo a la exposición celular, de las soluciones de toxinas podría ser

una importante fuente de variabilidad en la evaluación de la respuesta tóxica del AZA-1.

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Improvements in the use of neuroblastoma x glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins

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ABSTRACT

New advances in the use of NG108-15 cells for marine toxins detection and quantification are presented. We have established modifications on ouabain and veratridine proportion to obtain good toxins dose-response curves on this cell line for neurotoxins acting on voltage gated sodium channel (VGSC). Interesting, differences in the toxic response were observed between two VGSC activating toxins, brevetoxin-3 and pacific ciguatoxin-1. For non-VGSC acting lipophilic toxins, several factors that may influence toxin detection and quantification were analyzed. One hour cultures and forty-eight hours of exposure time, compared with 24 h of culture and 24 h of exposure, would increase NG108-15 cell maximal yessotoxin (YTX) and azaspiracid-1 (AZA-1) toxic response whereas no change was observed for okadaic acid (OA), dinophysistoxin-1 and pectenotoxin-2. Dose-response curves obtained for YTX or AZA-1 showed variability according to the day of the experiment while good reproducibility was obtained for OA. Evaporation time of toxin solutions before cell exposure could be an important source of variability in AZA-1 toxic response evaluation.

1. Introduction

Marine toxins from Harmful Algal Blooms (HAB) can have impact on coastal resources or implicate human health risk through food-borne intoxications (Hallegraeff, 2004; Sobel and Painter, 2005). Cell-based assays for toxicity evaluation contribute to the understanding of the potency of marine toxins, to the characterization of their mechanism of action (Botana, 2000) and, as other biological models, to identify and follow new toxic compounds through purification steps to contribute to develop analytical procedures for their identification. Additionally, cell-based assays could favour the reduction of animal testing in food safety monitoring programs which currently use for some marine toxins mouse bioassays.

Previous reviews regarding the specific use of cell-based assays for the evaluation of marine toxins illustrate their potency as toxicological models (Garthwaite, 2000; Rossini, 2005; Cañete and Diogène, 2008).

Regarding previous comparative studies on the use of two established neuronal cell lines; neuroblastoma × glioma hybrid cells (NG108-15) and Neuro-2a neuroblastoma cells (Cañete and Diogène, 2008) for the identification and quantification of marine toxins toxic effect, we have selected in the present study NG108-15 cells to evaluate the capacity of this cell line to be used in a toxicological cell-based assay for the evaluation of the toxic potential of neurotoxins and other types of toxins. In our previous studies, NG108-15 cells were more sensitive to pectenotoxin-2 (PTX-2) than Neuro-2a cells and a preliminary study with domoic acid (DA) seems to show

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a better capacity of NG108-15 than Neuro-2a cells to detect DA toxicity. NG108-15 used for cytoxicity 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 okadaic acid (OA), dinophysistoxin-1 (DTX-1) and palytoxin dose-response curves obtained for NG108-15 cells supports this model as a biological, suitable and sensitive tool to their toxicity detection and quantification.

First studies on NG108-15 cells to evaluate their capacity to detect toxicity of neurotoxins acting on VGSCs, saxitoxin (STX) and brevetoxin-3 (PbTx-3), were carried adapting the method used to evaluate cytotoxic response on Neuro-2a cells (Manger et al., 1993; Dickey et al., 1999) and ouabain and veratridine (O/V) concentrations were established based on Neuro-2a experience (with a fixed proportional factor of 10:1 respectively). Under these experimental conditions NG108-15 showed a narrow range of effect (quantitatively), with smaller differences in viability between the control and the concentration of toxin having the maximum effect. Preliminary results on O/V proportions in NG108-15 cells showed important improvement in offsetting O/V effect by VGSC inhibiting toxin or potentiate VGSC acting toxins effect by incrementing veratridine concentration in regard to ouabain.

Non-VGSC acting lipophilic toxins such as yessotoxin (YTX) and azaspiracid-1 (AZA-1) have been demonstrated to increase strongly their maximum toxic effect on mammalian cell cultures in 48 h exposure time compared with 24 h (Twiner et al., 2005; Pérez-Gómez et al., 2006). In these studies, dose-response curves obtained after 48 h exposure allow to estimate, in all cases, the 50% effect concentration (EC50) which is around 20 nM for YTX (Pérez-Gómez et al., 2006) and around 1.1–7.9 for AZA-1 (Twiner et al., 2005).

In the present study a better O/V proportion obtained for NG108-15 cells to evaluate neurotoxins acting on VGSCs is presented. Dose-response curves of STX, PbTx-3 under O/V optimal conditions on NG108-15 are presented and compared to dose-response curves obtained under first O/V concentrations selected. Pacific ciguatoxin-1 (P-CTX-1), another VGSC activating toxin, was exposed under both O/V conditions (proportion 10/1 and 1/1). Results are presented and compared with those obtained for STX and PbTx-3.

For non-VGSC acting lipophilic toxins OA, DTX-1, PTX-2, YTX and AZA-1, several assays are proposed in order to optimize the experimental protocol including incubation time and exposure time. Variability in toxic response estimation in different days as well as the importance of evaporation time of toxin solutions was evaluated for OA, YTX and AZA-1.

2. Material & methods

In the present study, different methods used to evaluate cytotoxic response of marine toxins on NG108-15 cells have been developed according to previous work on Neuro-2a cells (Manger et al., 1993; Dickey et al., 1999) and NG108-15 cells (Cañete and Diogène, 2008). Suitability of this cellular model to evaluate the toxic effect of toxins studied is determined by the possibility to generate dose-response curves. Sensitivity of the model to different marine toxins was evaluated by the EC50s values obtained and maximal toxic effects observed.

2.1. 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 μ/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 two days.

2.2. 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 to 100% confluence flask. Inocula of 200 μL cell suspension were added to each well. Cell densities were approximately in the range of 25 000–50 000 cells/well for NG108-15. For the whole study all conditions (controls included) were tested at least in duplicate.

2.3. Toxin preparation

Previous to toxin exposure of cells, defined aliquots of toxin solutions were dispensed on 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. Dilutions from the concentrated dose were prepared, and added to a minimum of two wells (duplicates). Volume in each well was adjusted with 5% FBS culture medium (as complementary medium) to a final volume of 230 μL.

2.4. Toxin exposure of cells and response evaluation

Toxins studied in this work included neurotoxins acting on VGSCs (STX, PbTx-3 and P-CTX-1) and other toxins which mechanism of action does not involve VGSCs (OA, DTX-1, PTX-2, YTX and AZA-1). For neurotoxins acting on VGSCs cells with or without O/V were exposed to increased concentrations of commercial toxins. Ouabain and veratridine concentrations were selected to produce mortality in approximately 20% or 80% of the cell population depending on whether the toxins activate (e.g., PbTx-3 and P-CTX) or inhibit (e.g., STX) sodium channels. Two different proportional factors of O/V concentration were studied. A 10:1 (O/V) proportion was chosen based on experience on Neuro-2a cells. A 1:1 O/V proportion was also used in this study after a series of assays with NG108-15 to determine the optimal O/V proportion causing the highest increment of response for toxins acting on VGSCs. These different proportional factors of O/V (10:1 and 1:1) were obtained using concentrations of O/V at 0.75/0.075 mM or 0.35/0.35 mM in VGSC inhibiting

toxin exposures and 0.3/0.03 mM or 0.13/0.13 mM in VGSC activating toxins. These concentrations were slightly readjusted periodically in order to obtain the 20% or 80% mortality. 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. A negative O/V control (O/V−) (without O/V) was used to determine the 100% viability. For VGSC activating toxins (e.g., PbTxs and P-CTX) O/V concentration that produce around 20% mortality (positive O/V control, O/V+) were used to detect the VGSC activating type effect, that would increase cell mortality. For toxin exposure, the order in which the different solutions were used for plate preparation during the cytotoxicity assay was strict: 5% FBS as complementary medium, O/V and finally toxins. Details as described elsewhere (Cañete and Diogène, 2008).

Cell viability was estimated using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium] method as described elsewhere for Neuro-2a cells (Manger et al., 1993). Absorbances were read on an automated multiwell scanning spectrophotometer (Biotek, Synergy HT, Winooski, Vermont, USA) at 570 nm. Statistical analyses were developed with the software Prism 4 (GraphPad, San Diego, California, USA). For the establishment of dose-response curves, that allowed the determination of EC50, cell viability for each toxin concentration was measured at least in duplicate ($n = 2$). 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. For each toxin and each experimental condition, EC50 was calculated from the absolute viability values dose-response curves and for STX according to relative viability values. Graphic ordinate axis consists of viability estimations using percentage of MTT reduction, using the corresponding controls for each experience and the negative O/V control (O/V-) for O/V treatment experience to estimate absolute values of viability. Significant differences between means were evaluated using unpaired t-test ($p < 0.05$ was considered statistically significant).

2.5. Toxin exposure time

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

For OA, DTX-1, YTX, AZA-1 and PTX-2 two experimental conditions regarding growth time previous to toxin exposure and exposure time were studied:

2.5.1. Experimental condition 1

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 the toxins at different 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.

2.5.2. Experimental condition 2

In this experimental condition, cells were grown without any treatment during 1 h, and after this time, cells were treated for additional 48 h with the toxins at different concentrations. Absorbance reading was performed after 48 h of toxin exposure. Forty-nine hours were needed from the time plates were prepared to result reading.

These two conditions were selected considering that both conditions resulted in approximately a 50 h (including MTT assay) toxin-evaluation assay.

2.6. Toxins

All the experiments of this study have been performed using commercially available, purified toxins: STX, YTX, AZA-1, PTX-2, (National Research Council of Canada); PbTx-3 (Calbiochem); OA (Sigma); DTX-1 (Wako); P-CTX-1 (Dr. R. Lewis, Univ. Queensland).

2.7. Additional parameter evaluation for OA, YTX and AZA-1

For assay optimization, further analysis regarding evaporation time of samples and reproducibility of toxin standard curves was assessed with OA, YTX and AZA-1. In order to estimate the reproducibility of the toxin effect evaluation in NG108-15 cells, between 7 and 9 dose-response curves for each of the toxins OA, YTX and AZA-1 obtained in different days were compared in experimental condition 1 (24 h cell growth previous to 24 h toxin exposure) and experimental condition 2 (1 h cell growth previous to 48 h toxin exposure) according to the estimation of r^2. In order to study evaporation time, we evaluated the possible variability induced by toxin handling previous to cell exposure. Two different evaporation times for standard toxin solutions were evaluated for the preparation of OA, YTX and AZA-1 treatments: Toxin evaporation time between 2 and 15 min versus 1 h (The range 2–15 min was considered the minimum evaporation time depending on the volume of standard solution to be evaporated).

3. Results

3.1. NG108-15 cell exposure to STX, PbTx-3 and P-CTX at different O/V proportions

Saxitoxin, PbTx-3 and P-CTX were tested with and without O/V treatment. No response to STX, PbTx-3 or P-CTX in cells without O/V treatment was observed at the concentrations tested (data not shown). In Fig. 1, dose-responses curves obtained with O/V treatment at different O/V proportions are presented for each toxin.

For STX, the use of O/V in a 10:1 proportion resulted in no more than 20% viability increase (with regard to control O/V-) at the highest STX concentration (Fig. 1a), whereas using a 1:1 O/V proportion resulted in a maximum viability increase of about 40%.

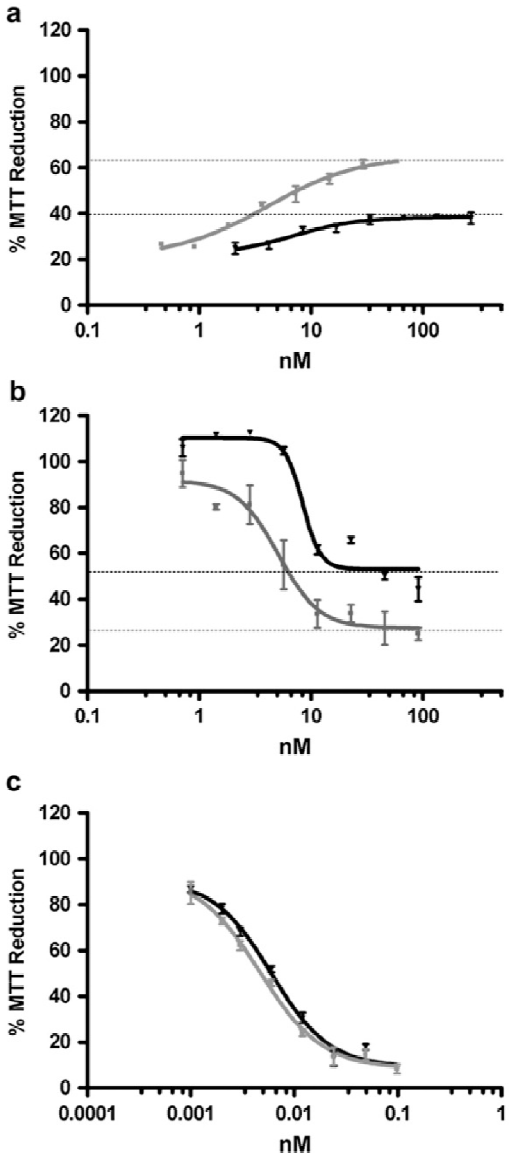

Fig. 1. Dose-response curves (r^2 ranged between 0.899 and 0.989) of a) saxitoxin (STX), b) brevetoxin-3 (PbTx-3) and c) pacific ciguatoxin-1 (P-CTX-1) on neuroblastoma × glioma hybrid cells (NG108-15). In every graph two curves corresponding to two treatments with different ouabain and veratridine (O/V) proportions: 10:1 O/V proportion (black) and 1:1 O/V proportion (grey). The ordinate axis represents viability estimations using percentage of MTT reduction. Three repetitions were performed per dose in 96 wells plates, at a cell density between 25 000 and 50 000 cells/well on NG108-15 cells. Cells were placed in plates 24 h previous to 24 h of toxin exposure.

For PbTx-3, the use of O/V in a 10:1 proportion resulted in a maximum mortality increase of about 50% (with regard to control O/V+) (Fig. 1b), whereas for a 1:1 O/V proportion, PbTx-3 caused a maximum mortality increase of 75% at the same concentration.

For P-CTX-1, the use of O/V in a 1:1 proportion did not increase maximum toxic effect in respect to the 10:1 O/V proportion (Fig. 1c).

Selecting the 1:1 O/V proportion dose-responses curves obtained, resulted in EC50s about 4.5, 6.6 and 0.006 nM for STX, PbTx-3 and P-CTX-1, respectively.

3.2. NG108-15 cell exposure to OA, DTX-1, PTX-2, YTX and AZA-1

In order to detect and quantify non-VGSC acting lipophilic toxins, five standard toxins were selected to evaluate NG108-15 response.

3.2.1. Importance of exposure time

Two experimental conditions (1 and 2) were designed in order to compare NG108-15 toxins response to different exposure times. Dose-response curves for OA, DTX-1, PTX-2, YTX and AZA-1 were obtained in both experimental conditions (Fig. 2).

For OA, dose-response curves showed a maximum toxic effect about 100% mortality in both experimental conditions and EC50s about 15.6 and 19.3 nM were obtained in experimental condition 1 and 2 respectively (Fig. 2a).

Dinophysistoxin-1 showed in both experimental conditions a maximum response about 100% mortality, and EC50s were about 11.3 and 12.5 nM at experimental condition 1 and 2, respectively (Fig. 2b).

Pectenotoxin-2 in experimental condition 1 and 2 showed a maximum response of about 90% mortality, with EC50s about 1.3 and 1 nM, respectively (Fig. 2c).

For YTX, dose-response curves in experimental condition 1 showed a maximum response of about 50% mortality whereas mortality close to 100% was obtained in experimental condition 2 at the same concentration (Fig. 2d). The EC50s were about 15, 2 and 5.4 nM in experimental condition 1 and 2 respectively.

For AZA-1, dose-response curves in experimental condition 1 showed a maximum response of about 40% mortality whereas mortality close to 100% was obtained in experimental condition 2 at the same concentration (Fig. 2e). The EC50 at 48 h of toxin exposure was 2.1 nM. A good dose-response curve was also obtained at 24 h with an intermediate effect (in this case, the EC80) obtained at a concentration of 4.1 nM.

3.2.2. OA, YTX and AZA-1 toxic effect quantification

Variability in the toxic response estimation of OA, YTX and AZA-1 were evaluated in different days and according to different evaporation times of standard toxin solution.

3.2.2.1. Dose-response curves fitness according to experimental day. For OA, YTX and AZA-1 dose-response curves in experimental condition 1 and 2 obtained in different days were analyzed (Fig. 3).

For OA good dose-response curves were obtained in all experiments in both experimental conditions, r^2 of this curves ranging between 0.969 and 0.998 (Fig. 3a and b).

For YTX, in experimental condition 1 it was not possible to obtain good dose-response curves in all the experiences, r^2 obtained ranging between 0.433 and 0.985 (Fig. 3c). In

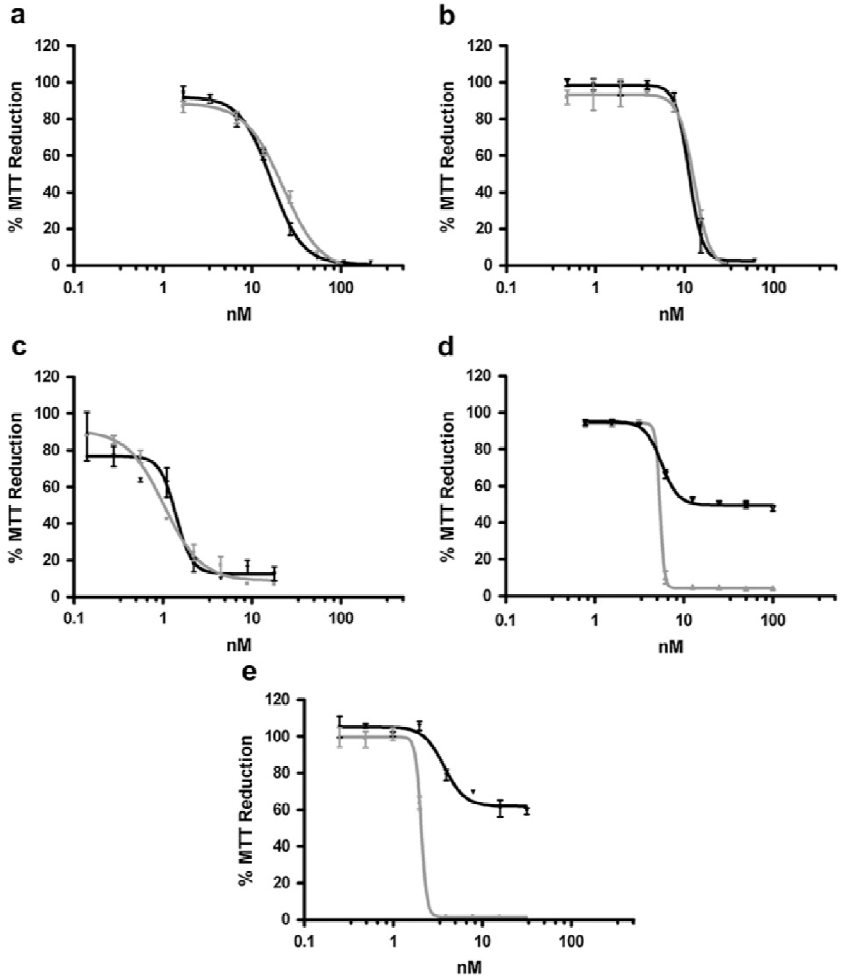

Fig. 2. Dose-response curves (r^2 ranged between 0.934 and 0.999) of a) okadaic acid (OA), b) dinophysistoxin-1 (DTX-1), c) pectenotoxin-2 (PTX-2), d) yessotoxin (YTX) and e) azaspiracid-1 (AZA-1) on neuroblastoma × glioma hybrid cells (NG108-15). In every graph two curves corresponding to two experimental conditions performed in the same experimental day are represented: 24 h of cell growth in plates previous to 24 h of toxin exposure (black) and 1 h of cell growth in plates previous to 48 h of toxin exposure (grey). The ordinate axis represents viability estimations using percentage of MTT reduction. Two repetitions at least were performed per dose in 96 wells plates, at a cell density between 25 000 and 50 000 cells/well on NG108-15 cells.

experimental condition 2, in all experiments a good dose-response curve was obtained, r^2 ranging between 0.957 and 0.998 (Fig. 3d).

For AZA-1, in experimental condition 1, a dose-response was obtained for every experiment but r^2 of theses curves ranged between 0.250 and 0.985 (Fig. 3e). In experimental condition 2r^2 in all curves has ranged between 0.927 and 0.997 (Fig. 3f).

3.2.2.2. Simple and pooled dose-response curves. For OA, a good overlap of curves in both experimental conditions was obtained, with the exception of two curves on experimental condition 1 (Fig. 3a and b). All values were analysed in a unique dose-response curve, the "pooled D-R" (pooled data from different experiments performed in different days) curve, in order to obtain a theoretical curve of all experiences. In both experimental conditions acceptable r^2 were obtained (0.876 and 0.906 in experimental condition 1 and 2 respectively). Regarding comparison of responses in the two experimental conditions, the combined pooled D-R curve for OA, with all data from both experimental conditions, presented an r^2 of 0.879 with an EC50 around 17.5 nM.

For YTX, no good overlap of dose-response curves was obtained (Fig. 3c and d). The pooled D-R curve for YTX obtained with all the experiences presented an r^2 about

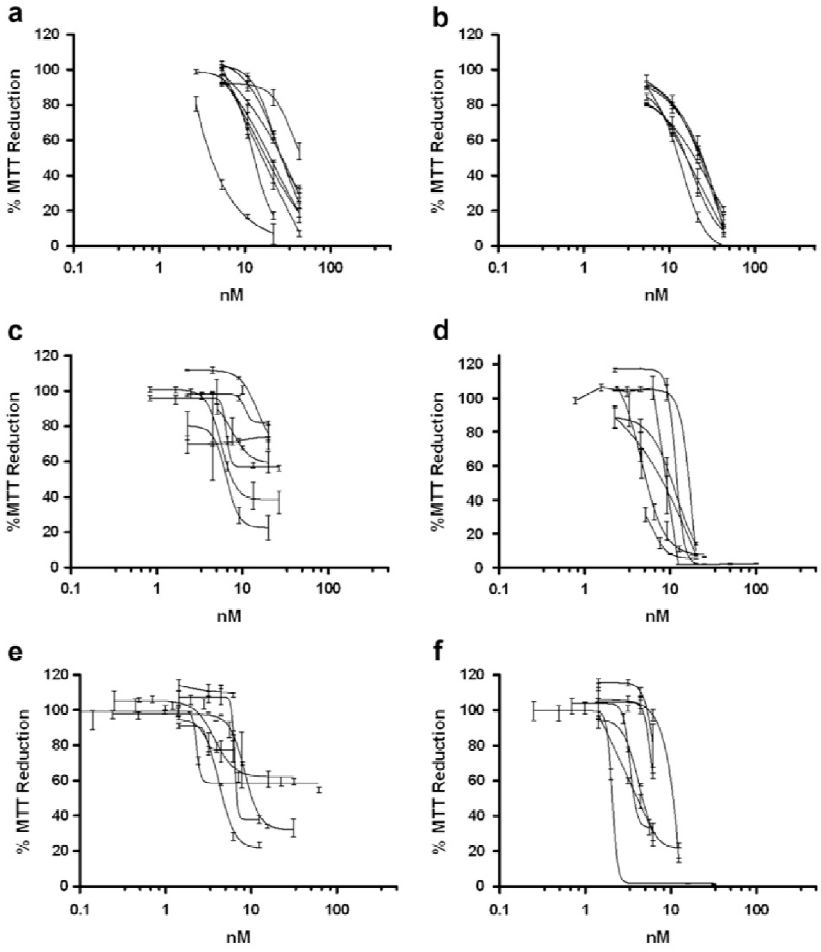

Fig. 3. Dose-response curves of a) & b) okadaic acid (OA), c) & d) yessotoxin and e) & f) azaspiracid-1 (AZA-1) on neuroblastoma × glioma hybrid cells (NG108-15) at two different experimental conditions: a), c) & e) 24 h of cell growth in plates previous to 24 h of toxin exposure and b), d) & f) 1 h of cell growth in plates previous to 48 h of toxin exposure. In every graph several curves corresponding to different experimental days in the same conditions. The ordinate axis represents viability estimations using percentage of MTT reduction. Two repetitions at least were performed per dose in 96 wells plates, at a cell density between 25 000 and 50 000 cells/well on NG108-15 cells.

0.567 and 0.568 in experimental condition 1 and 2 respectively. In most dose–response curves obtained in experimental condition 1 an EC50 could not be calculated. However in experimental condition 1 when an EC50 could be calculated this was in the similar range as the EC50s obtained in experimental condition 2, from 5 to 17 nM.

For AZA-1, no good overlap of dose-response was obtained (Fig. 3e and f). The pooled D-R curve for AZA-1 obtained with all the experiences presented an r^2 of about 0.710 and 0.640 in experimental condition 1 and 2 respectively. In most dose-response curves obtained in experimental condition 1 an EC50 could not be calculated. However in experimental condition 1 when an EC50 could be calculated this was in the similar range as the EC50s obtained in experimental condition 2, from 2 to 10.6 nM.

3.2.2.3. Importance of toxin evaporation time. Responses of five toxin concentrations in experimental condition 2 for OA, YTX and AZA-1 were analysed in the two different evaporation times selected (2–15 min and 1 h) (Fig. 4).

For OA and YTX, no significant differences were observed at different dilutions prepared with the two different evaporation times of toxin standard solution (Fig. 4a and b).

For AZA-1, significant loss of toxicity of the extract was observed in the serial dilutions of the toxin evaporated at

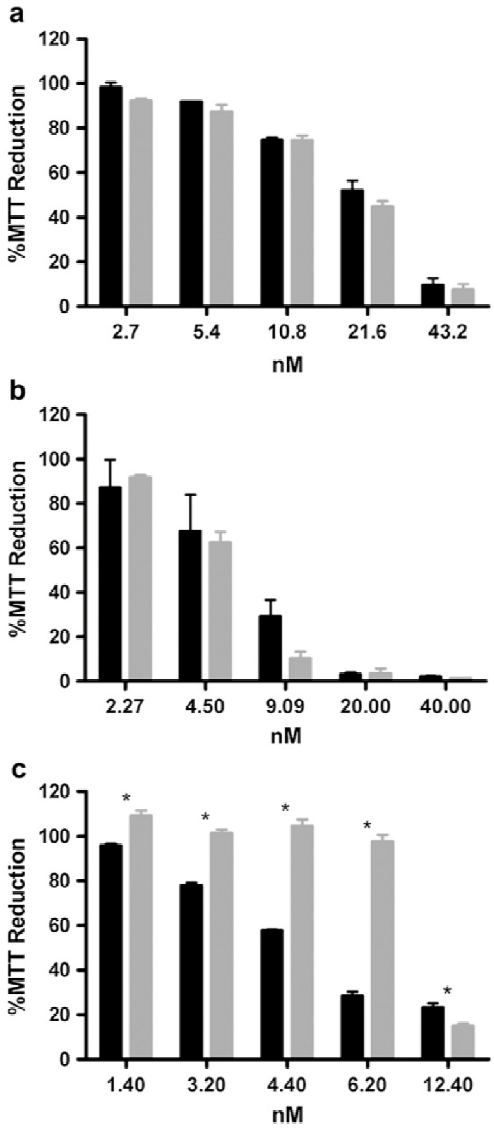

Fig. 4. Dose–response bar graphs of a) okadaic acid (OA), b) yessotoxin (YTX) and c) azaspiracid-1 (AZA-1) on neuroblastoma × glioma hybrid cells (NG108-15). In every toxin concentration two bars corresponding to two treatments with different toxin evaporation time under gentle N_2 flux at 40 °C: 2–15 min (black) and 1 h (grey) evaporation time. The ordinate axis represents viability estimations using percentage of MTT reduction. Two repetitions at least were performed per dose in 96 wells plates, at a cell density between 25 000 and 50 000 cells/well on NG108-15 cells. Cells were placed in plates 1 h previous to 48 h of toxin exposure. Significant differences between means were evaluated using unpaired *t*-test ($p < 0.05$ was considered statistically significant *).

1 h in relation to the toxicity values detected with 2–15 min evaporation times. At the highest concentration studied (12.4 nM), a higher toxic effect was observed with the longest evaporation time (Fig. 4c).

4. Discussion

NG108-15 cells (ATCC, HB12317) proved to be a suitable and stable model for routine culture with no handling difficulties. According to our results, this model is reliable and as it will be discussed further, stable responses for toxin evaluation are obtained for different toxins. The sensitivity of NG108-15 to the different toxins reported in our study demonstrates that this model is sensitive enough as compared to other cell models (Fernández et al., 1991; Bottein Dechraoui et al., 2005; Twiner et al., 2005; Pérez-Gómez et al., 2006; Bottein Dechraoui et al., 2007; Cañete and Diogène, 2008). The suitability of the assay for each specific toxin, as reported here, will have to be modulated according to important variables such as exposure time, O/V proportion or processing of samples.

4.1. NG108-15 cell-based assay for VGSC acting toxins

In this study STX and PbTx-3 caused an increment of maximal response about 20% approximately by incrementing by 10 fold the veratridine proportion with the O/V treatment (the use of a 1:1 O/V proportion compared to a 10:1 O/V proportion). For both toxins this improvement supposes an increase on the response range obtained and consequently a refinement of the toxin effect quantification. No increment of P-CTX-1 response was observed by incrementing the veratridine proportion.

4.1.1. Why the O/V proportions tested did not affect the P-CTX-1 response as it was observed on STX or PbTx-3?

Saxitoxin, PbTx-3 and P-CTX-1 target on VGSCs at receptor site 1 (STX) and receptor site 5 (PbTxs and CTXs) (Catterall, 1980; Wang and Wang, 2003). Saxitoxin acts from the extracellular side of the membrane and block sodium conductance (Cestèle and Catterall, 2000). Lipid-soluble brevetoxins and ciguatoxins enhance sodium channel activity by binding to receptor site 5 and cause "a shift in activation to more negative membrane potentials and a block of inactivation" (Cestèle and Catterall, 2000) with differences on their binding affinity (Lombet et al., 1987; Bottein Dechraoui et al., 1999; Bottein Dechraoui and Ramsdell, 2003). Moreover, ciguatoxins have been suggested to have more differences with brevetoxin according to their mode of action than according to their affinity for the sodium channel (Hogg et al., 2002; Bottein Dechraoui et al., 2005; Sauviat et al., 2005). The different results obtained between PbTx-3 and P-CTX-1, regarding O/V proportion, could be explained by a difference in the mode of action between PbTx-3 and P-CTX-1. The toxic effect of PbTx-3 in NG108-15 cells would be more dependent of veratridine VGSC activation than that of P-CTX-1.

4.2. NG108-15 cell-based assay for non-VGSC acting lipophilic toxins

4.2.1. Importance of exposure time

Implementing NG108-15 cells for toxic effect evaluation of the non VGSC acting lipophilic toxins OA, DTX-1, PTX-2, YTX and AZA-1 proved to be suitable according to the results obtained, taking into account that assay conditions

had to be carefully selected for some of these toxins. For YTX and AZA-1, in our study, 1 h incubation time followed by a 48 h exposure resulted in a higher toxicity in relation to 24 h incubation followed by a 24 h exposure as was expected in regard to differences on YTX and AZA-1 toxic response related to 24 or 48 h time exposure (Twiner et al., 2005; Pérez-Gómez et al., 2006). Differences between 24 and 48 h exposure for OA, DTX-1 or PTX-2 toxic response at established cell lines, to our best knowledge have not been reported previously and were not observed in our study. Nevertheless, using primary cultures of rat cerebellar neurons and sensitive techniques for toxicity evaluation, differences between 24 and 48 h exposure for OA were observed (increasing effect after 48 h exposure with respect to 24 h exposure) at low concentrations (1–5 nM) (Fernández et al., 1991; Fernández et al., 1993). However, higher concentrations (5–50 nM) of OA, at the same sensitive CBA, have been demonstrated to act more quickly (Fernández-Sánchez et al., 1996; Ferrero-Gutiérrez et al., 2008). All these results suggest that OA time depending response, as for other toxins, probably, could be related to CBA sensitivity and in that context differences between time exposure have to be related to the experimental system used.

4.2.2. OA, YTX and AZA-1 toxic effect quantification

4.2.2.1. Simple and pooled dose-response curves. The reproducibility of the assays was studied by analysis of multiple dose-response curves for OA, YTX and AZA-1 obtained at different days. Several studies of NG108-15 cells exposure at the two experimental conditions of incubation and exposure time revealed that for OA exposures, toxic responses was reproducible in different assays.

These results favour the use of a theoretical dose-response curve (pooled dose-response curve) obtained from several experiments to conduct OA equivalent estimations and therefore reduce the number of OA control concentrations used in every experiment.

4.2.2.2. Importance of toxin evaporation time. For YTX and AZA-1, the toxic response estimation varied in different days. The possible physiological status or density of cells may be relevant in the quantification of their toxic effect. As a consequence our recommendation is to evaluate YTX or AZA-1 toxicity with control curves with standards obtained the same day of the experiment.

Toxin evaporation time has been proved by our results to be important on toxicity determination for AZA-1 but no so important for OA and YTX evaluation. This factor could be a source of variability of the dose-response curves between different experiments for AZA-1 toxicity evaluation. For AZA-1 toxic effect evaluation it will be advisable to control evaporation time.

4.3. Future prospects of NG108-15 cell-based assay for the toxic effect evaluation of marine toxins

The different experimental approaches presented herein regarding the use of NG108-15 cells for the evaluation of different toxin standards will presumably strengthen the use of this model in toxicity evaluation strategies of marine toxins. Within the possible application of the assay, future work will contemplate its' applicability to natural samples including shellfish.

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Conflicts of interest

The authors would like to state that all the research involvement in the study and all information written in the manuscript "Improvements in the use of neuroblastoma × glioma hybrid cells (NG108-15) for the toxic effect quantification of marine toxins" have no conflict of interest with any person or institution.

References

Botana, L.M., 2000. Seafood and freshwater toxins: pharmacology, physiology, and detection. In: Botana, L.M. (Ed.). CRC Press.
Bottein Dechraoui, M.Y., Naar, J., Pauillac, S., Legrand, A.M., 1999. Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels. Toxicon 37, 125–143.
Bottein Dechraoui, M.Y., Ramsdell, J.S., 2003. Type B brevetoxins show tissue selectivity for voltage-gated sodium channels: comparison of brain, skeletal muscle and cardiac sodium channels. Toxicon 41, 919–927.
Bottein Dechraoui, M.Y., Tiedeken, J.A., Persad, R., Wang, Z., Granade, H.R., Dickey, R.W., Ramsdell, J.S., 2005. Use of two detection methods to discriminate ciguatoxins from brevetoxins: application to great barracuda from Florida Keys. Toxicon 46, 261–270.
Bottein Dechraoui, M.Y., Wang, Z., Ramsdell, J.S., 2007. Optimization of ciguatoxin extraction method from blood for Pacific ciguatoxin (P-CTX-1). Toxicon 49 (1), 100–105.
Cañete, E., Diogène, J., 2008. 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. Toxicon 52, 541–550.
Catterall, W.A., 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. Annual Review of Pharmacology and Toxicology 20, 15–43.
Cestèle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.
Dickey, R.W., Jester, E., Granade, R., Mowdy, D., Moncreiff, C., Rebarchik, D., Robl, M., Musser, S., Poli, M., 1999. Monitoring brevetoxins during a gymnodinium breve red tide: comparison of sodium channel specific cytotoxicity assay and mouse bioassay for determination of neurotoxic shellfish toxins in shellfish extracts. Natural Toxins 7, 157–165.
Fernández-Sánchez, M.T., García-Rodriguez, A., Díaz-Trelles, R., Novelli, A., 1996. Inhibition of protein phosphatases induces IGF-1-blocked neurotrophin-insensitive neuronal apoptosis. FEBS Letters 398, 106–112.
Fernández, M.T., Zitko, V., Gascón, S., Novelli, A., 1991. The marine toxin okadaic acid is a potent neurotoxin for cultured cerebellar neurons. Life Sciences 49, 157–162.
Fernández, M.T., Zitko, V., Gascón, S., Torreblanca, A., Novelli, A., 1993. Neurotoxic effect of okadaic acid, a seafood-related toxin, on cultured cerebellar neurons (Markers of Neuronal Injury and Degeneration). Annals of the New York Academy of Sciences 679, 260–269.
Ferrero-Gutiérrez, A., Pérez-Gómez, A., Novelli, A., Férnandez-Sánchez, M. T., 2008. Inhibition of protein phosphatases impairs the ability of astrocytes to detoxify hydrogen peroxide. Free Radicals in Biology and Medicine 44, 1806–1816.
Garthwaite, I., 2000. Keeping shellfish safe to eat: a brief review of shellfish toxins, and methods for their detection. Trends in Food Science & Technology 11, 235–244.

Hallegraeff, G.M., 2004. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae. UNESCO, pp. 25–49.

Hogg, R.C., Lewis, R.J., Adams, D.J., 2002. Ciguatoxin-induced oscillations in membrane potential and action potential firing in rat parasympathetic neurons. European Journal of Neuroscience 16, 242–248.

Lombet, A., Bidard, J.N., Lazdunski, M., 1987. Ciguatoxin and brevetoxins share a common receptor site on neuronal voltage-dependent Na channel. FEBS Letters 219 (2) 355–359.

Manger, R.L., Leja, S., Lee, S.Y., Hungerford, J.M., Wekell, M.M., 1993. Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins. Analytical Biochemistry 214, 190–194.

Pérez-Gómez, A., Ferrero-Gutierrez, A., Novelli, A., Franco, J.M., Paz, B., Fernández-Sánchez, M.T., 2006. Potent neurotoxic action of the shellfish biotoxin yessotoxin on cultured cerebellar neurons. Toxicological Sciences 90 (1), 168–177.

Rossini, G.P., 2005. Functional assays in marine biotoxin detection. Toxicology 207, 451–462.

Sauviat, M.P., Boydron-Le Garrec, R., Masson, J.B., Lewis, R.L., Vernoux, J.P., Molgó, J., Laurent, D., Benoit, E., 2005. Mechanisms involved in the swelling of erythrocytes caused by Pacific and Caribbean ciguatoxins. Blood Cells, Molecules & Diseases 36, 1–9.

Sobel, J., Painter, J., 2005. Illnesses caused by marine toxins. Clinical Infectious Diseases 41, 1290–1296.

Twiner, J.M., Hess, P., Bottein Dechraoui, M.Y., McMahon, T., Samons, M.S., Satake, M., Yasumoto, T., Ramsdell, J.S., Doucette, G.J., 2005. Cytotoxic and cytoskeletal effects of azaspiracid-1 on mammalian cell lines. Toxicon 45, 891–900.

Wang, S., Wang, G.K., 2003. Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. Cellular Signalling 15, 151–159.