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ORIGINAL PAPER

Collaborative study for the detection of toxic compounds in shellfish extracts using cell-based assays. Part I: screening strategy and pre-validation study with lipophilic marine toxins

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Abstract Human poisoning due to consumption of seafood contaminated with phycotoxins is a worldwide problem, and routine monitoring programs have been implemented in various countries to protect human consumers. Following successive episodes of unexplained shellfish toxicity since 2005 in the Arcachon Bay on the French Atlantic coast, a national research program was set up to investigate these atypical toxic events. Part of this program was devoted to fit-for-purpose cell-based assays (CBA) as complementary tools to collect toxicity data on atypical positive-mouse bioassay shellfish extracts. A collaborative study involving five laboratories was conducted. The responses of human hepatic (HepG2), human intestinal (Caco2), and mouse neuronal (Neuro2a) cell lines exposed to three known lipophilic phycotoxins—okadaic acid (OA), azaspiracid-1 (AZA1), and pectenotoxin-2 (PTX2)—were investigated. A screening strategy composed of standard operating procedures and a decision tree for dose–response modeling and assay validation were designed after a round of "trial-and-error" process. For each toxin, the shape of the concentration–response curves and the IC_{50} values were determined on the three cell lines. Whereas OA induced a similar response irrespective of the cell line (complete sigmoid), PTX2 was shown to be less toxic. AZA1 induced

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cytotoxicity only on HepG2 and Neuro2a, but not on Caco2. Intra- and inter-laboratory coefficients of variation of cell responses were large, with mean values ranging from 35 to 54 % and from 37 to 48 %, respectively. Investigating the responses of the selected cell lines to well-known toxins is the first step supporting the use of CBA among the panel of methods for characterizing atypical shellfish toxicity. Considering these successful results, the CBA strategy will be further applied to extracts of negative, spiked, and naturally contaminated shellfish tissues.

Keywords Cell-based assays · Collaborative study · Lipophilic phycotoxins · Cytotoxicity

Introduction

Marine biotoxins are metabolites produced by microalgae forming a large and diverse collection of compounds with different structures and mechanisms of action [1]. Through the food web, these toxins can accumulate in marine organisms including filter-feeding bivalves and fish, which may induce human seafood poisoning. Worldwide, the occurrence of harmful algal blooms in coastal waters increases, causing concern for human health, aquaculture and fishery activities, recreation, and tourism [2]. To protect the consumers' health, several phycotoxins are regulated within the EU [3], and routine monitoring programs have been implemented to control the sanitary status of the seafood destined to human consumption. A new European Commission regulation for shellfish entered into force on 1 July 2011. It prescribes the mouse bioassay (MBA) replacement by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) method for the monitoring of the four regulated lipophilic phycotoxin families (okadaic acid and dinophysistoxins group, pectenotoxins group, azaspiracids group, and yessotoxins group) [4]. Even though these LC-MS/MS methods are the most sensitive and specific for monitoring the targeted lipophilic toxins, they are inappropriate for detecting unexpected toxic compounds and for informing about any potential toxic effect of shellfish. To pick up atypical toxic events, other methods complementary to the MBA have to be considered [5].

In fact, various in vitro methodological approaches have been developed for the detection and the characterization of marine toxins in shellfish. They can be classified into biological, biochemical, and chemical assays [5]. Among the biological assays, the cell-based assays (CBA) were first used to study the cellular effects of numerous marine toxins [6]. They were further developed for detecting phycotoxins and evaluating their toxicity. To date, the cellular effects of phycotoxins have been studied on a range of cell types including neuroblastoma [7–14], fibroblasts [15, 16], myoblasts [17], and intestinal cells [18]. Some studies have been designed to characterize the toxic effect of one toxin on different cell types, like for AZA1 cytotoxicity [19–21]. The effects of pectenotoxins (PTXs) on the actin skeleton were first evidenced by Hori et al. [18]. The effects of PTXs on cell viability have also been compared on human intestinal Caco2 cells and immortalized and primary rat hepatocytes [22]. On the other hand, some comparative studies were conducted to investigate the toxicity of different toxin groups on one cell type, mainly on neuronal cells [23, 24]. But, to our knowledge, only one study on the suitability and sensitivity of two neuronal cell models (including Neuro-2a) to six phycotoxins has been published [12]. However, no information on the number of CBA, the number of rejected assays, or the intra-laboratory variability was provided in this study. Therefore, a larger study combining the toxic effects of different toxins on relevant target cell types and including both standard operating procedures and rigorous raw data validation is still missing [6, 25, 26].

To challenge this feature, the present study aimed at characterizing the responses of three lipophilic toxins [okadaic acid (OA), azaspiracid-1 (AZA1), and pectenotoxin-2 (PTX2)] on three different cell lines representative of the main target organs to phycotoxins. The cytotoxicity measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was chosen as the common endpoint. Following a validation scheme, the responses of intestinal (Caco2), hepatic (HepG2), and neuronal (Neuro2a) cells were first investigated in one laboratory. Then, a collaborative study involving five laboratories was carried out on the HepG2 cell line to study the inter-laboratory variability.

This is the first step of our approach proposing a panel of CBA for the study of the atypical toxicity events, like the one from Arcachon Bay, including a toxicity follow-up of shellfish chromatographic fractions as the ultimate step for the identification of toxic compound(s). However, it was first necessary to test and validate the proposed approach for some well-known lipophilic toxins. Thus, the aim of this study was to compare the responses of the three selected cell lines to three known lipophilic toxins as well as to determine the variability of such assays.

Experimental section

Standard operating procedures

The present study involved five laboratories. To limit the variability of the results, the assays were carried out according to standard operating procedures (SOP), including the use of the same batches of cell lines, culture media, sera, and certified reference calibration solutions of marine toxins.

SOP were written for both cell culture and cytotoxicity assays. A decision tree was elaborated to screen the assays through different validation criteria. A common Excel database was established to collect all metadata relative to experimental conditions, raw data, and data analysis for each assay performed within this study.

Preparation of marine toxin solutions

The certified reference calibration solutions of OA, AZA1, and PTX2 were purchased from the National Research Council Canada. The test solutions were prepared for each experiment and within each lab by serial twofold dilutions in serum-free medium; the final toxin concentrations ranged from 0.4 to 885 nM for OA, from 0.04 to 73.5 nM for AZA1, and from 0.24 to 500 nM for PTX2.

Cell maintenance

The human HepG2 hepatocarcinoma cell line (ATCC HB8065, passages 15–25) and the human Caco2 colorectal adenocarcinoma cell line (ATCC HTB-37, passages 18–40) were cultured in MEM-Glutamax containing 1 g/l glucose and supplemented with 10 % fetal calf serum (FCS), 50 U/mL penicillin and 50 µg/mL streptomycin, and 1 % nonessential amino acids. The mouse Neuro2a neuroblastoma cell line (ATCC CCL-131, passages 11–50) was cultured in RPMI 1640-Glutamax containing 2 g/l glucose and supplemented with 10 % FCS, 1 mM sodium pyruvate, 50 U/mL penicillin, and 50 µg/mL streptomycin. The three cell lines were routinely grown in 75-cm^2 flasks at 37 °C and 5 % CO_2. They were passaged every 5–6 days, when cells reached 75–80 % confluence.

MTT assays

Cells were seeded in 96-well plates at a density of 30,000 cells/100 µl per well (for Caco2) and 20,000 cells/100 µl per well (for HepG2 and Neuro2a) 24 h prior to treatment. After removing the medium, cells were exposed in triplicate to 12 concentrations of OA, AZA1, and PTX2 in serum-free medium (100 µL/well) for 48 h, except for OA and PTX2 on Neuro2a with only 24 h exposure. Based on the OECD guidance document no. 129 [27] on cytotoxicity tests, the template of each plate was designed as follows: marginal rows and columns were omitted, six wells were used for the control, and 12 wells (six wells on the right-hand side and six wells on the left) were used for the vehicle control (VeC). VeC corresponded to the medium containing 5 % methanol, a concentration tested in preliminary experiments that induced no more than 20 % of cytotoxicity on the cell lines (data not shown).

At the end of exposure, the cells were observed by light microscopy to detect possible morphological alterations. The cell cytotoxicity was measured using the MTT assay. After treatment, the medium was replaced by a FCS-free medium containing 0.5 mg/mL MTT (Sigma) for 2 h at 37 °C. The medium was discarded prior to the addition of 0.1 N HCl-acidified isopropanol to dissolve the formazan. The absorbance was read at 570 nm and was expressed as the percentage of mean absorbance (n=3) in VeC (100 % of viability).

Data analysis and assay validation

The different types of data collected in the database are listed in Table 1. The relative 50 % inhibition concentration (IC_{50}) corresponding to the concentration that caused a response midway between the minimum (bottom) and the maximum (top) observed viability [27] was calculated for each toxin on each cell line.

A decision tree, based on an iterative process, was elaborated according to two guidance documents, the Assay Guidance Manual [28] and the OECD guidance document no. 129 [27]. As summarized in Fig. 1, the decision tree defined the plate approval criteria for assay validation and the model of the dose–response curves for the calculation of IC_{50}. Five validation steps were successively applied:

1. The mean of each VeC (both right and left) should not differ by more than 15 % from the mean of all VeCs.
2. The cytotoxicity in VeC should be ≤20 % compared to the control.
3. The dose–response curves should be fitted using the four-parameter logistic model (4PL), also called the Hill slope model, to calculate the IC_{50} values according to the formula: $Y = \text{Bottom} + \frac{(\text{Top}-\text{Bottom})}{1+10^{(\log IC_{50}-\log X)\text{Hillslope}}}$. The GraphPad Prism software version 5.04 for Windows (GraphPad Software, San Diego, CA, USA) was used. When the compound induced no cytotoxicity (flat line), no IC_{50} could be calculated.
4. The fitted dose–response curves should have a R^2>0.85.
5. The percent fitting error (%FE) of IC_{50} should be <40 %. It is calculated according to the formula: %FE (IC_{50})=FE(logIC_{50})×ln 10×100, where FE(logIC_{50}) is the standard error of logIC_{50}.

All the assays performed in this study were assessed through the decision tree and were considered as validated when they met the five acceptance criteria.

Statistical analysis

At least three MTT experiments were validated per laboratory for each cell line/toxin combination (except for Lab.5

Table 1 Type of data reported in the database for each assay

Metadata	Laboratory code and person who conducted the experiment
	Date of the experiment
	Cell line and passage number
	Marine toxin and range of concentrations tested
	Solvent final concentration in VeC
	Time of exposure
	Microplate and microplate reader references
Raw data	Mean absorbance of blanks
	Mean absorbance of control
	Mean absorbance of the right VeCs, the left VeCs, and all VeCs
	Cytotoxicity of the vehicle control (% of VeC)
	Quality code related to the global quality of the experiment
	Shape of dose–response curve
	Minimal cell viability observed and the corresponding toxin concentration
Data analysis	IC_{50} value
	Fitting error
	Number of experimental points used for the curve fitting
	Logistic model used for the curve fitting
Test acceptance criteria	Difference between the right VeCs and the left VeCs, expressed as percent of the mean of all VeCs
	Cytotoxicity of VeC (% of control)
	R^2
	% of fitting error
Assay validation	0: rejected; 1: accepted

See "Experimental section" for details

VeC vehicle control

with HepG2-PTX2 assays where only two IC_{50} values were validated). The results were expressed as the mean±SD of IC_{50}, and the variability of the data sets was expressed as the coefficient of variation (CV). Differences between the data sets were tested with the non-parametric Kruskal–Wallis test and Dunn's multiple comparison posttest and were considered statistically significant when $p<0.05$. Statistical analyses were carried out using GraphPad Prism.

Results and discussion

Experimental procedure and decision tree for dose–response modeling and assay validation

The sensitivity and reproducibility of CBA on the selected cell lines were determined for three lipophilic toxins (AZA1, OA, and PTX2). We first designed an experimental procedure as those mainly described in the biomedical literature [29]. Such a screening strategy had never been developed for marine toxins. Prior to the run of the assays presented in this paper, a round of "trial-and-error" process was conducted, leading to the construction of a decision tree (Fig. 1). Each assay included in this paper was accepted or rejected on the basis of quality and the modeling criteria detailed in this decision tree. A shared Excel database was established to collect all the data (experimental conditions, raw data, and analysis results) from each assay performed within this study.

Responses of the three cell lines to OA, AZA1, and PTX2 (Lab.1)

Percentage of validated assays

A total of 87 assays were performed in Lab.1, and 84 % of them ($n=73$ assays) were accepted according to the decision tree (Table 2). Comparing the cell lines, 76 % of the assays were validated for HepG2, 92 % for Caco2, and 83 % for Neuro2a. According to this parameter, no significant difference was noticed between the cell lines.

When comparing the toxins, it appeared that a high percentage of validated assays was observed for both OA and AZA1 (94 and 92 %, respectively), whereas a lower performance was reported for the PTX2 assays. Indeed, only 61 % of the PTX2 assays were validated, mainly because of the high rejection rate for HepG2 assays. The dose–response curves obtained with HepG2 did not fit well with the model, and as a consequence, 70 % of the PTX2 assays on HepG2 were rejected because they did not meet the last acceptance criterion of $R^2>0.85$ (step 4) or %FE<40 % (step 5) in the decision tree. For Caco2 and Neuro2a, the percentages of

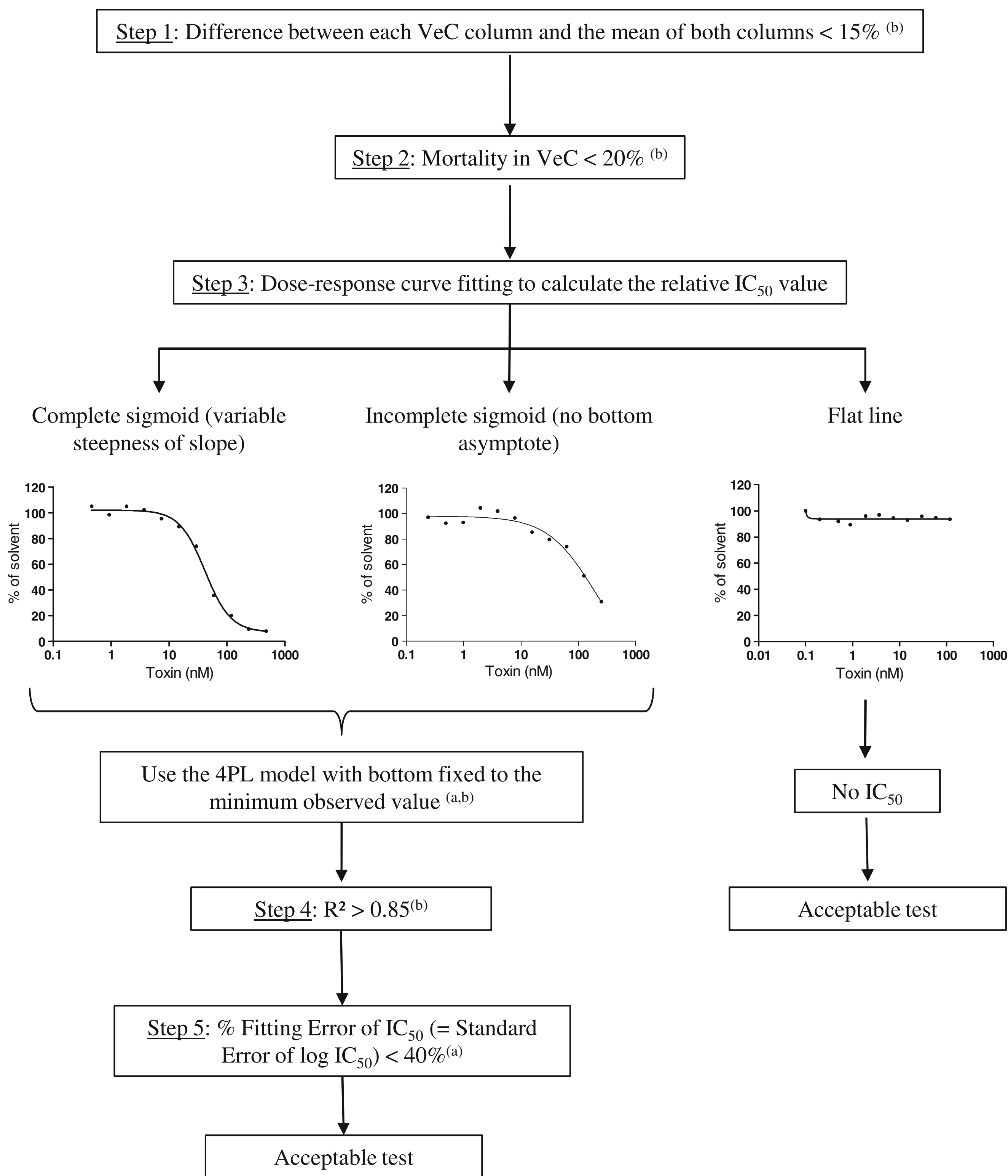


Fig. 1 Decision tree for dose–response modeling and assay validation. The evaluation of cytotoxicity induced by phycotoxins is based on the determination of the relative IC_{50} value. According to the shape of the dose–response curve, this parameter is calculated by data modeling using the Hill slope model (step 3). Based on the Assay Guidance Manual from the NIH Chemical Genomics Center (*a*) [28] and the OECD Guidance Document No. 129 (*b*) [27], several experimental criteria (steps 1 and 2) and modeling criteria (steps 4 and 5) are defined to validate the assay (see "Experimental section" for details). *VeC* vehicle control, *4PL* four-parameter logistic model

Table 2 Number of cytotoxicity assays performed on HepG2, Caco2, and Neuro2a cells exposed to OA, AZA1, and PTX2 in Lab.1

Toxin	HepG2	Caco2	Neuro2a	Total	Assay validation
OA	15/16	15/16	3/3	33/35	94 %
AZA1	10/11	11/11	3/4	24/26	92 %
PTX2	3/10	9/11	4/5	16/26	61 %
Total	28/37	35/38	10/12	73/87	
Assay validation	76 %	92 %	83 %		84 %

For each experimental condition, the ratio between the number of the validated assays and the total assays conducted is indicated. In addition, the percentage of assay validation was calculated for each cell line and each toxin

validation of the tests on PTX2 were 82 and 80 %, respectively.

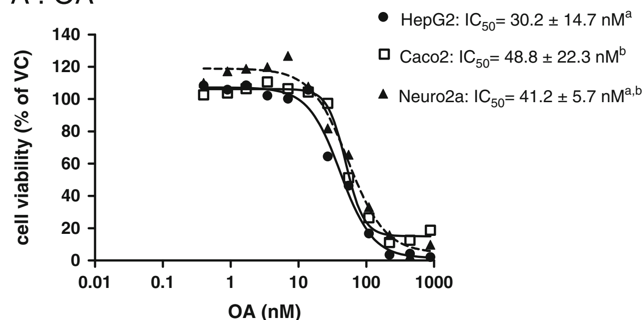
Two main causes for assay rejection within Lab.1 were identified from our database and were found to largely depend on the toxin considered. For AZA1 and OA, only four assays were rejected because they did not meet the quality acceptance criteria defined in steps 1 and 2 of the decision tree. Among the ten PTX2-rejected assays, seven were performed on the HepG2 cell line. Five out of these seven assays were rejected because of modeling criteria such as R^2 and %FE (steps 4 and 5 of the decision tree).

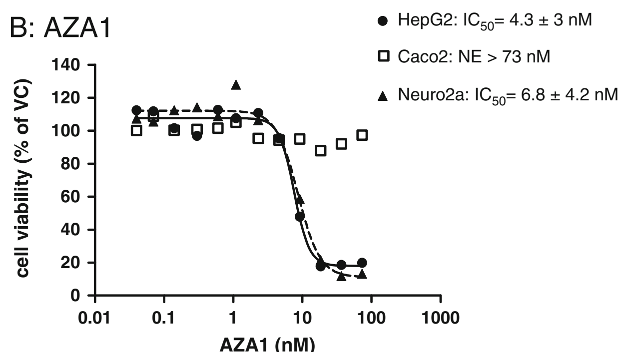
We could hypothesize that the variability in the toxin responses depends on their dissolution in the cell culture medium according to their physicochemical properties. However, it appears that the three toxins used in the study have a similar hydrophobicity, as expressed by their partition coefficient log*P* (AZA1: log*P*=3.7; OA: log*P*=3.4; PTX2: log*P*=3.8; http://pubchem.ncbi.nlm.nih.gov/). Therefore, we exclude that a difference in hydrophobicity would explain the great variability observed between PTX2 and the two other toxins.

Dose–response relationship and IC_{50} values

Overall, 84 % of the assays were validated and analyzed subsequently for determining the shape of the dose–response curves and estimating the IC_{50} for each toxin on HepG2, Caco2, and Neuro2a cells (Fig. 2). Interestingly, each of these toxins individually could be distinguished by combining the results (IC_{50} values and the shape of the dose–response curves) obtained on the three cell lines.

Whatever the cell line, the OA dose–response curve was a complete sigmoid, with a bottom reached at concentrations above 200 nM inducing a cell cytotoxicity up to 90 % (Fig. 2a). The IC_{50} of OA/HepG2 (IC_{50}=30.2±14.7 nM) was significantly different from the IC_{50} of OA/Caco2 (IC_{50}=48.8±22.3 nM, p<0.05). The IC_{50} of OA/Neuro2a was similar to the one obtained for the two other cell lines (IC_{50}=41.2±5.7 nM), but for a shorter exposure time (24 versus 48 h). Even if OA induced a complete sigmoid dose–response whatever the cell line, the calculated IC_{50} values were significantly different between cell lines. The main targets of OA are the protein phosphatases, considered as unspecific intracellular targets. The consequences are cytoskeleton changes and cell mortality whatever the cell types





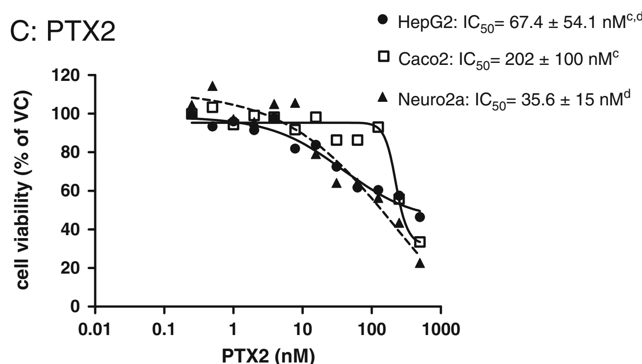


Fig. 2 Representative dose–response curves and relative IC_{50} values for OA (**A**), AZA1 (**B**), and PTX2 (**C**) on HepG2, Caco2, and Neuro2a cells obtained by MTT assay in Lab.1. HepG2 and Caco2 cells were exposed to the three phycotoxins for 48 h, whereas Neuro2a cells were exposed to OA and PTX2 for 24 h and to AZA1 for 48 h. IC_{50} are expressed as the mean±SD of validated assays (see Table 2 for the number of assays). The results *without common letter* were significantly different. *NE* no effect (the maximum concentration tested is reported)

[23], which could explain the similarity of the responses observed with this toxin.

AZA1 induced a strong cytotoxicity on HepG2 and Neuro2a cells above 5 nM after 48 h exposure (Fig. 2b). On both cell lines, the dose–response curves shaped a complete sigmoid with a level of cytotoxicity higher than 80 % for concentrations above 18 nM. The IC_{50} was equal to 4.3±3 nM on HepG2 and 6.8±4.2 nM on Neuro2a. This study refers for the first time to the toxicity of the pure toxin AZA1 on human hepatic cells. Even though no cytotoxicity could be observed on Caco2 cells with the MTT assay as previously shown [19, 20], obvious morphological changes with cell rounding have been observed for concentrations above 5 nM (data not shown). This effect is consistent with the in vivo effects inducing complete degradation of gastrointestinal lining and confirmed by TEER experiments on monolayer Caco2 model [30] or by disturbance of filament organization in Caco2 cells [31]. Even if some data indicated that AZA1 induced the fragmentation of E-cadherin [21] and inhibited endocytosis [32], its molecular target is still unknown.

PTX2 induced toxic effects on the different cell types without giving a complete sigmoid curve in the range of the tested concentrations (0.24–500 nM; Fig. 2c). The cytotoxicity on the three cell lines increased up to 70 % for the highest concentration (500 nM). The cytotoxic effects were two times higher on Neuro2a cells (IC_{50}=35.6±15 nM, 24 h exposure) compared to HepG2 (IC_{50}=67.4±54.1 nM), but not significantly different. The response of Neuro2a cells to PTX2 was significantly different ($p<0.05$) from that of Caco2 (IC_{50}=202±100.8 nM). The incomplete sigmoid of the dose–response curve observed on the three cell lines was previously reported by Cañete and Diogène on Neuro2a [12]. Our IC_{50} values were also similar to those previously obtained on neuronal cells [12, 33]. Moreover, similar dose–response curves were obtained on rat hepatocytes treated with PTX2, PTX1, and PTX9, with a maximum cytotoxicity ranging from 50 to 70 %, reached for toxin concentrations above 100 nM [22]. The effects of PTXs have been tested on primary and immortalized hepatocytes [22, 34]. Using X-ray crystallography and purified skeletal actin, it was established that the toxin forms a 1:1 complex with actin, affecting microfilament polymerization [35]. A recent study suggested that PTX2 can significantly suppresses cell proliferation due to cell cycle arrest in the G2/M phase on the human breast cancer MCF-7 cell line [36]. This finding could support our observations indicating that only 30–50 % cytotoxicity was obtained with PTX2.

Intra-laboratory variability of cell-based assays

All MTT assays carried out in Lab.1 were performed using SOP and showed a steady shape of the dose–response curves from one experiment to another. Nonetheless, the IC_{50} values collected from each toxin/cell line conditions were scattered (Table 3 and Fig. 3). For the three cell lines, the coefficients of variation of the IC_{50} values ranged from 14 % (OA/Neuro-2a) to 80 % (PTX2/HepG2), with a mean of 52 % (Table 3).

The main causes of CBA variability within one laboratory could be explained by (1) the different physiological states of cells from one experiment to another; (2) the dissolution behavior of the toxins in the cell culture medium; (3) the possible adsorption of the toxins to plastic microplates; and (4) the variability inherent to several experimenters. Further work is required to explore these sources of variability and improve their control.

Despite this intra-laboratory variability, the responses of the three cell lines to the three toxins showed some significant differences (Fig. 3). The three lipophilic toxins affected the viability of HepG2 cells, showing that AZA1 was significantly more cytotoxic than OA and PTX2. The Caco2 cell line was sensitive to OA and PTX2, but not to AZA1. Finally, the Neuro2a cell line showed significant differences between OA and AZA1.

Collaborative study on HepG2 (Lab.1 to Lab.5)

The reproducibility of the CBA for the three lipophilic marine toxins was studied between five laboratories. The

Table 3 IC_{50} values (expressed in nanomolars) obtained in Lab.1 for OA, AZA1, and PTX2 assessed on HepG2, Caco2, and Neuro2a cells

Cell lines	OA		AZA1		PTX2	
	Mean±SD	CV%	Mean±SD	CV%	Mean±SD	CV%
HepG2	30.2±14.7 (*n*=15)	49	4.3±3 (*n*=10)	71	67.4±54.1 (*n*=3)	80
Caco2	48.8±22.3 (*n*=15)	47	NE (>73) (*n*=11)	–	202±100.8 (*n*=9)	50
Neuro2a[a]	41.2±5.7 (*n*=3)	14	6.8±4.2 (*n*=3)	62	35.6±15 (*n*=4)	42

Results are expressed as the mean±SD of validated assays (*n*) and the variability of data expressed as CV (%)

NE no effect (the maximum concentration tested is reported in parenthesis)

[a] The shape of the dose–responses curves and the IC_{50} values obtained on the Neuro2a cell line were confirmed within Lab.4, with a higher number of replicates (*n*=6) than in Lab.1 (data not shown)

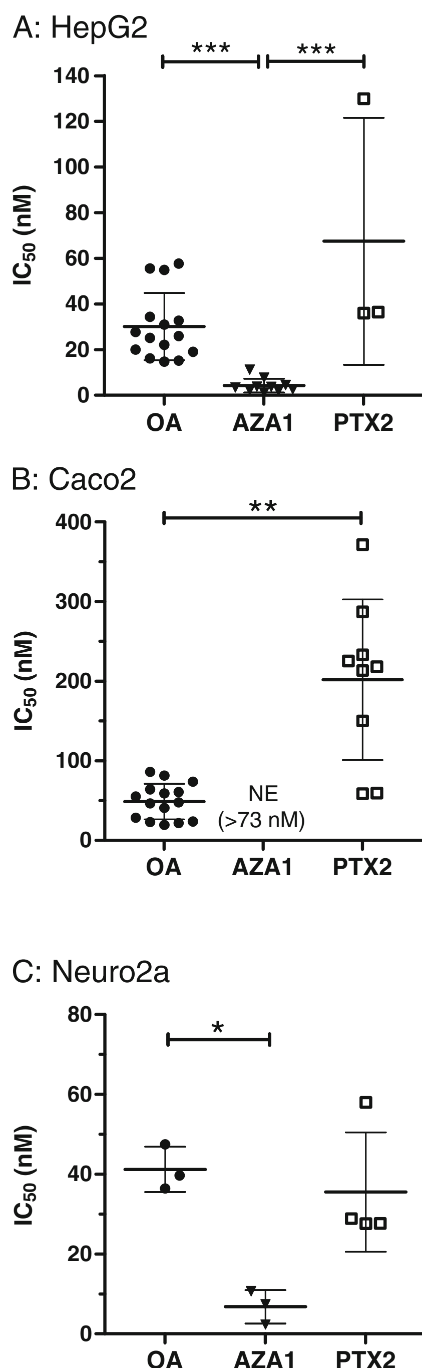


Fig. 3 Distribution of IC_{50} values for OA, AZA1, and PTX2 determined from all validated assays in Lab.1 per cell line: HepG2 (**A**), Caco2 (**B**), and Neuro2a (**C**). The mean value is represented by *horizontal lines* and the standard deviation by *barred vertical lines*. Non-parametric Kruskal–Wallis test with Dunn's multiple comparison posttest were performed. Differences were considered statistically significant when $*p<0.05$, $**p<0.005$, and $***p<0.001$. *NE* no effect (the maximum concentration tested is reported in *parenthesis*)

HepG2 cell line was chosen because the comparison of the cytotoxic responses between the three toxins reached the highest level of significance on this cell line (Fig. 3).

Percentage of validated assays

A total of 126 assays were performed in the five laboratories and were assessed through the decision tree for validation or rejection. Overall, 66 % of the HepG2 assays were validated, with a validation proportion ranging from 52 % (Lab.2) to 83 % (Lab.5; Table 4).

The causes of assay rejection were analyzed per lab. Overall, 82 and 76 % of the OA and AZA1 assays were validated, respectively, while only 36 % of the PTX2 assays were validated. This could be explained by the low performance observed for PTX2 assays within Lab.2 (and to a lesser extent in Lab.3, resulting from a %FE>40 %). This outcome led to the rejection of all PTX2 assays for Lab.2 (n=10) and four out of seven PTX2 assays for Lab.3 (Table 4). This low percentage of acceptance was clearly linked to the non-respect of the SOP for some experiments performed in these two labs. Indeed, some PTX2 assays were conducted with a narrower range of concentrations (from 3.9 to 500 nM instead of 0.24–500 nM), resulting in truncated dose–response curves. Therefore, these assays did not allow the determination of relative IC_{50} and failed to pass the decision tree.

For Lab.4, the assay validation performance was found to largely depend on the experimenter, resulting in the rejection of assays mainly at step 1. For Lab.5, steps 4 and 5 of the decision tree were equally involved in the rejection of the assays.

The consequence was that numerous assays were rejected depending on the toxin or the laboratory. The number of experimental points is a critical point for model fitting. To increase the data validation percentage, the decision tree should be modified by adding other criteria, such as a minimal OD absorbance value.

Dose–response effects of OA, AZA1, and PTX2 on HepG2 and reproducibility of the IC_{50} values

The OA IC_{50} measured per lab ranged from 27.4 nM (Lab.2) to 61 nM (Lab.5), and the inter-laboratory mean IC_{50} was 40.7±14.9 nM (Fig. 4 and Table 5). The results from Lab.4 and Lab.5 were higher than the others and significantly different from Lab.1 ($p<0.05$). The coefficients of variation ranged from 35 to 49 %, except for Lab.2 which showed a very low CV (9 %; Fig. 4).

Interestingly, for Lab.2, all the five validated assays were performed only on two different runs of experiments, thus minimizing the inter-day variability. This could explain why this laboratory reported the lowest variability for OA. Finally, the average value of the five intra-laboratory variability was 35 %; this value is very close to the inter-laboratory variability (CV=37 %; Table 5).

Table 4 Proportion of number of validated versus number of performed assays on the HepG2 cell line for the three toxins within each lab

Toxin	Lab.1	Lab.2	Lab.3	Lab.4	Lab.5	Total	Assay validation
OA	15/16	5/6	6/6	10/17	5/5	41/50	82 %
AZA1	10/11	8/9	4/5	3/8	3/4	28/37	76 %
PTX2	3/10	0/10	3/7	6/9	2/3	14/39	36 %
Total	28/37	13/25	13/18	19/34	10/12	83/126	
Assay validation	76 %	52 %	72 %	56 %	83 %		66 %

The percentage of validated assays for each lab and toxin are reported in marginal rows and columns

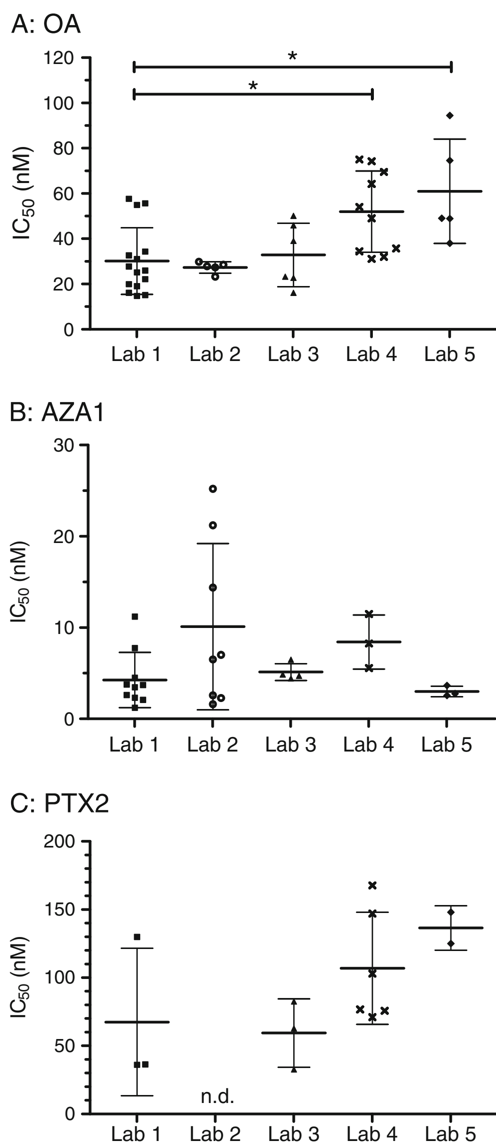


Fig. 4 Distribution of IC_{50} values for OA (**A**), AZA1 (**B**), and PTX2 (**C**) on HepG2 cells measured in Lab.1 to Lab.5. All IC_{50} values (in nanomolars) validated through the decision tree are represented. The mean value is represented as *horizontal lines* and the standard deviation by *barred vertical lines*. Non-parametric Kruskal–Wallis test with Dunn's multiple comparison posttest were performed. Differences were considered statistically significant when $*p<0.05$. *n.d.* not determined

All laboratories reported a strong cytotoxic effect of AZA1 on HepG2 cells. The inter-laboratory mean IC_{50} was equal to 6.2±3 nM. The mean IC_{50} values ranged from 3 nM (Lab.5) to 10.1 nM (Lab.2), without a significant difference among laboratories (Fig. 4b). Data from Lab.1 and Lab.2 were dispersed, resulting in CVs>70 %. Conversely, the variability was low in Lab.3 and Lab.5 (CV< 20 %). For Lab.3, the assays were performed within the same day, which could explain the small variability of the results. The inter-laboratory CV calculated for the five participating labs was 48 %; this is similar to the average of the IC_{50} intra-laboratory variability (47 %; Table 5).

Regarding the toxic effects of PTX2 on HepG2 cells, four laboratories obtained an analogous incomplete sigmoid curve, but with an erratic steepness of the slope. The mean IC_{50} ranged from 59.4 nM (Lab.3) to 106.9 nM (Lab.4). The inter-laboratory mean IC_{50} for PTX2 was equal to 92.5± 36 nM, with a CV of 39 %. The average of the intra-laboratory CVs was 54 %. Within each lab, the IC_{50} data varied largely, with intra-laboratory CV ranging from 42 % to 80 %.

Overall, the five laboratories obtained a similar classification based on the IC_{50} values of the three lipophilic toxins. AZA1 appeared to be the most toxic compound and PTX2 the less toxic. Interestingly, the variability of the assays was identical from one toxin to another as well as within and between the laboratories, except for PTX2.

The means of the coefficient of variation of the assays were around 40 %. Such CVs have been previously reported in the literature on in vitro bioassays. For example, cytotoxicity tests performed with cosmetic ingredients on HeLa and CHL cells revealed inter-laboratory CVs of 25 and 35 %, respectively, including CVs>50 % for some laboratories [37, 38]. Recently, a variability of 35 % for the IC_{50} value of palytoxin on Neuro2a cells using the MTT assay was reported [39].

Zimmermann et al. [40] investigated the variance components for bioassays carried out in microtiter plates. Clearly, assay reproducibility was impacted firstly by experimental design parameters (e.g., design of the 96-well plate, i.e., position of controls, standards and samples, numbers of replicates, days of measurements) and, secondly, by the choice of the parametric logistic model.

Table 5 Comparison of IC_{50} obtained in the five laboratories for OA, AZA1, and PTX2 on HepG2 cells

Laboratory code	OA		AZA1		PTX2	
	Mean±SD	CV (%)	Mean±SD	CV (%)	Mean±SD	CV (%)
Lab.1	30.2±14.7	49	4.3±3.0	71	67.4±54.1	80
Lab.2	27.4±2.5	9	10.1±9.1	90	n.d.	
Lab.3	32.9 ±14.0	43	5.1±0.9	18	59.4±25.1	42
Lab.4	52.0±17.9	35	8.4±3.0	35	106.9±41.2	39
Lab.5	61.0±23.0	38	3±0.6	20	Only two validated IC_{50}: 125 and 148	
Mean of intra-laboratory CV (%)		35		47		54
Inter-laboratory mean±SD and CV (%)	40.7±14.9	37	6.2±3	48	92.5±36	39

The results indicate the relative IC_{50} of toxin (in nanomolars), expressed as the mean±SD (the number of validated assays is indicated in Table 3) and the variability of data, expressed as CV (in percent), within each laboratory. Additionally, the inter-laboratory mean of the IC_{50} values for each toxin and the inter-laboratory variability are presented. The mean of the five intra-laboratory CV is also indicated and represents the average variability of each data set in one laboratory

n.d. not determined

In our study, we noticed that the variability of CBA results was higher when the plates were carried out under reproducibility conditions (>35 %) compared to repeatability conditions (<20 %). For this reason, the SOP used in this study should mention that the assay replicates have to be performed under reproducibility conditions (on different days).

Conclusion

Integrated cytotoxicity assays on three cell lines enabled detecting and characterizing the toxicity of the three lipophilic toxins: OA, AZA1, and PTX2. The variability of the method was similar from one toxin to another and was similar within and between laboratories.

This pre-validation work highlighted that SOP are essential and that not following the key points (range of toxin concentrations for example) can be considered as a rejection criterion. The decision tree can be improved by including additional acceptance criteria. For example, as proposed by the OECD guidance document no. 129 [27], a minimal absorbance value should be fixed for the control and VeC wells in order to reduce the variability of cell seeding and cell density at the beginning of toxin exposure.

Overall, this pre-validation study enabled characterizing the sensitivity and the reproducibility of some CBA exposed to three known lipophilic toxins. The next steps will be to apply this approach (1) to uncontaminated and spiked shellfish extracts for investigating the responses of these assays to complex biological matrices and (2) to shellfish chromatographic fractions for their toxicity follow-up in the toxic compound(s) identification process. These steps are necessary to support the use of cell-based assays as a tool to study atypical shellfish toxicity events, such as those observed in the Arcachon Bay.

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