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The total activity of a mixture of okadaic acid-group compounds can be calculated by those of individual analogues in a phosphoprotein phosphatase 2A assay

Clara Albano [a], Giuseppe Ronzitti [a], Anna Maria Rossini [b], Federica Callegari [a], Gian Paolo Rossini [a,*]

[a] Università di Modena e Reggio Emilia, Dipartimento di Scienze Biomediche, Via Campi 287, I-41100 Modena, Italy
[b] Liceo Scientifico Statale Leonardo da Vinci, Via Cavour 6, I-40033 Casalecchio di Reno (BO), Italy

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ABSTRACT

Monitoring of okadaic acid (OA)-group toxins in seafood is of paramount importance for the protection of consumer health from diarrheic shellfish poisoning. The property of OA-group compounds to inhibit type 2A serine/threonine phosphoprotein phosphatase (PP2A) has been exploited for the detection of OA in several experimental settings, but the performance of PP2A inhibition assays in the quantification of mixtures of OA-group compounds has not been reported yet. We have used a PP2A inhibition assay to analyze the total effect of mixtures including OA and one of its analogues, okadaol (OOH), by measuring the activity of individual compounds and of toxin mixtures through the inhibition they exert on the PP2A enzyme. We found that both OA and OOH inhibit PP2A under our experimental conditions, with IC_{50} values of 0.37 ± 0.04 nM and 4.3 ± 0.8 nM, respectively, confirming that OOH is about ten-fold less potent than OA. PP2A assays were also carried out with predefined mixtures of OA and OOH, covering the full dose–response of one compound in the presence of increasing concentrations of the other toxin. The experimental data we obtained were used to analyze their correlation with those that could be calculated by adding the relative effects exerted by individual analogues, and we found that a good correlation exists between the observed and the expected data, when the predicted effect was calculated on the basis of toxicity equivalence factors. Our findings show that an additive model based on the use of toxicity equivalence factors of individual toxins is appropriate for the calculation of the total activity of multi-component mixtures of OA-group compounds in unknown samples.

1. Introduction

Dinophysistoxins (DTXs) represent polyether acidic compounds produced by algae of the genera *Dinophysis* and *Prorocentrum* (Yasumoto and Murata, 1993), that are responsible for the contamination of filter feeding bivalve mollusks in several areas of the world (Hallegraeff, 2004). The presence of these toxins in edible shellfish poses risks to human health, because consumption of seafood contaminated by DTXs causes diarrheic shellfish poisoning (DSP), that is characterized by gastro-intestinal symptoms, including diarrhea, nausea and vomiting (Hallegraeff, 2004).

In order to protect consumer health, preventive measures are implemented in many countries, based on the

* Corresponding author. Tel.: +39 059 205 5388; fax: +39 059 205 5410.
E-mail address: gianpaolo.rossini@unimore.it (G.P. Rossini).

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measurement of DTXs in seafood, and prohibiting the harvesting and commercialization of edible materials when the levels of DTX contamination are above certain limits (European Commission, 2004, 2005).

Okadaic acid (OA) is the reference compound of DTXs, that comprise other OA analogues, differing with regard to the presence and positioning of several chemical groups, such as methyl and hydroxyl groups, as well as the presence of acyls esterified at the 7-hydroxy position of the compound (Yasumoto and Murata, 1993; Yasumoto et al., 1985).

The biological activity of OA and related compounds is related to their capacity to bind and inhibit several serine/threonine phosphoprotein phosphatases (Bialojan and Takai, 1988), and comparisons of enzyme isoforms have shown that type 2A phosphoprotein phosphatase (PP2A) is particularly sensitive to OA (Bialojan and Takai, 1988; Takai et al., 1992). Moreover, comparisons of potencies among some OA-group compounds have shown that the positioning of the methyl groups does not severely affect the activity of the analogue, whereas changes in the oxidation state of some of its carbon atoms substantially decrease the potency of the analogue, and 7-*O*-acylation of DTXs essentially obliterates their capacity to inhibit PP2A (Nishiwaki et al., 1990; Takai et al., 1992).

The recognition that DTX contamination of seafood can comprise multiple bioactive analogues (Yasumoto et al., 1985; Pavela-Vrančič et al., 2002; Vale and Sampayo, 2002), and the differing potencies of OA-group toxins (Nishiwaki et al., 1990; Aune et al., 2007) is taken into account in the legislation aimed at prevention of DSP. For instance, the existing regulation in the European Union postulates that live bivalve mollusks placed on the market must not contain okadaic acid and other dinophysistoxins at levels above 160 μg of OA equivalents/kg of shellfish meat (European Commission, 2004, 2005).

The measurement of total content of bioactive compounds can be carried out if functional assays that are selective for the class of compounds of interest are available, to yield an estimation of the total toxin content of samples in toxin equivalents (Rossini, 2005; Hess et al., 2006).

Based on the recognition that the mechanism of action of OA-group compounds involves the inhibition of serine/threonine phosphoprotein phosphatases, functional assays for the detection of compounds belonging to this toxin group have been developed (Holmes, 1991; Simon and Vernoux, 1994; Tubaro et al., 1996; Vieytes et al., 1997). Furthermore, the relative potencies of many compounds of the OA group have been determined (Nishiwaki et al., 1990; Aune et al., 2007).

We are not aware, however, of studies where the use of toxicity equivalence factors (TEFs) has been assessed with regard to its accuracy for the estimation of total toxin content in samples containing more than one biologically active compound of DTXs.

In this paper we have used the inhibition of PP2A by OA-group compounds as the model system to analyze the total activity of mixtures containing OA and one of its analogues, okadaol (OOH), and measured the activity of individual compounds and of predefined toxin mixtures through the inhibition they exert on the PP2A enzyme. We report that calculations based on simple addition of contributions of the two analogues using TEFs is mathematically equivalent to the use of an equation based on the general Langmuir isotherm (Langmuir, 1918) applied to the bimolecular interaction toxin-PP2A, upon competition between OA and OOH for the same binding site on the PP2A enzyme, providing a good correlation between observed and expected values, thereby showing that an additive model is appropriate for the calculation of the total activity of mixtures of OA-group compounds in unknown samples, according to their relative potencies.

2. Materials and methods

2.1. Materials

Okadaic acid (OA) was purchased from Alexis Biochemicals (Switzerland). Okadaol (OOH) was purchased from US Biological (Biochemicals & Biological Reagents, USA). Stock solutions of OA (50 μM) and OOH (25 μM) were prepared by dissolving the powders in absolute ethanol and stored in glass vials protected from light at −20 °C.

Purified PP2A from human red blood cells was obtained from Upstate Biotechnology Inc. (New York, USA). The concentrated enzyme preparation was diluted (1:10) using 20 mM MOPS, pH 7.4, 0.1 M NaCl, 60 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM $MnCl_2$, 1 mM DTT, 10% glycerol, 0.1 mg/ml BSA, and 25 μl aliquots were stored at −20 °C in a conical test tube.

Sodium 4-nitrophenylphosphate (*p*-NPP) was a product from Fluka Biochemika (USA). All other reagents were of analytical grade.

2.2. PP2A assay

The PP2A inhibition assays were carried out in either duplicate or triplicate, using 96-well plastic dishes. Dishes were prepared in order to contain the full standard curve of individual analogues (OA or OOH) and their respective mixtures, as described in Section 3. Each well contained 170 μl of the reaction buffer (40 mM Tris–HCl, pH 8.4 at 25 °C, 34 mM $MgCl_2$, 4 mM EDTA, 4 mM DTT), 50 μl of the *p*-NPP substrate (at a 25 mM final concentration) and 25 μl of the PP2A enzyme (0.025 units). The effect of OA and OOH on PP2A activity was assessed by including 2.5 μl of OA (in the 0.1–2 nM final concentration range) and/or 2.5 μl of OOH (in the 0.25–75 nM final concentration range) in the assay mix.

The reaction was monitored for 1 h at room temperature by recording the increase of A_{405}, with readings every 5 min, using a plate reader. The PP2A activity was then expressed as the relative increase of A_{405} over time (ΔA_{405}/min).

2.3. Calculations and data presentation

In most experiments the results of PP2A activity have been directly expressed as ΔA_{405}/min, and the graphical representation of this parameter *vs* the toxin concentration