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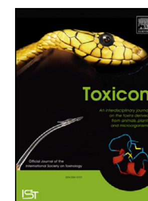

Detection of marine microalgal biotoxins using bioassays based on functional expression of tunicate xenobiotic receptors in yeast

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ABSTRACT

Marine microalgae can produce biotoxins that cause widespread poisoning in marine ecosystems and may also affect human health. While established microalgal biotoxins are detectable using chemical methods, a need remains for robust, inexpensive bioassays. Ligand-binding domains (LBDs) from a tunicate nuclear receptor, VDR/PXRα, which is orthologous to both the vertebrate pregnane X receptor (PXR) and the vitamin D receptor (VDR), can be activated by microalgal biotoxins when expressed in mammalian cell lines. Building on this observation, we developed a generic recombinant yeast bioassay platform that expresses chimeric proteins containing tunicate VDR/PXRα LBDs which mediate ligand-dependent transcription of a reporter gene (*lacZ*) encoding an easily assayed enzyme (β-galactosidase). Recombinant yeast strains expressing VDR/PXRα LBDs from two tunicate species, *Ciona intestinalis* and *Botryllus schlosseri*, were exposed to both synthetic and natural toxins. Structurally simple synthetic chemicals (*n*-butyl-*p*-aminobenzoate, carbamazepine, *p*-aminobenzoic acid, and bisphenol-A) generated EC_{50} values in the μM range, while more structurally complex marine biotoxins (okadaic acid, pectenotoxin-11, and portimine) activated the assays in the nM range. Given the large number of tunicate species, we propose that tunicate VDR/PXR LBDs may be used as 'sensor elements' in similar yeast-based high-throughput bioassays for detection of established microalgal biotoxins and uncharacterised marine bioactive compounds.

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1. Introduction

Some marine microalgal taxa can produce highly toxic chemicals that, on occasion, cause widespread poisoning within marine ecosystems and which may also affect human health, particularly when accumulated in filter-feeding shellfish consumed by humans (MacKenzie et al., 2011; Wang, 2008; Wear and Gardner, 2001; Zhang et al., 2013). Over the past two decades internationally accepted methodologies for the detection of such natural marine biotoxins have become increasingly based on chemical, rather than biological, assays (Gerssen et al., 2010; McNabb et al., 2012; Suzuki and Quilliam, 2011). While such chemistry-based detection methods are generally reliable and highly specific, they require specialised equipment and skills along with detailed structural knowledge regarding the targeted biotoxins, and so do not allow detection of unknown marine biotoxins (Humpage et al., 2010; Nicolas et al., 2014). Although a range of *in vitro* bioassays for microalgal biotoxins have been developed (Banerjee et al., 2013; Bovee et al., 2011; Nicolas et al., 2014), currently the most widely-used microalgal biotoxin bioassay remains the unreliable and ethically questionable mouse mortality bioassay (Botana et al., 2009; Buckland, 2010; Stewart and McLeod, 2014). Consequently,

Abbreviations: AD, activation domain; AF-2, transcription activation function domain; *Bs*VDR/PXRα, *Botryllus schlosseri* VDR/PXR orthologue α; BPA, bisphenol-A; CI, 95% confidence interval; *Ci*VDR/PXRα, *Ciona intestinalis* VDR/PXR orthologue α; CID, PubChem compound accession identifier; CPRG, chlorophenol red-β-D-galactopyranoside; DBD, DNA-binding domain; DSP, diarrhetic shellfish poisoning; GAL4, yeast DNA-binding transcription factor; GM, growth media; LBD, ligand-binding domain; MCS, multiple cloning site; MM, minimal media; NR, nuclear receptor; NR1I, nuclear receptor subfamily 1 class I; PTX-2, pectenotoxin-2; PTX-11, pectenotoxin-11; PXR, pregnane X receptor; SAM, seeded assay media; VDR, vitamin D receptor; VP16, viral protein 16; XR, xenobiotic receptor.

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Table 1
EC_{50} values, with 95% confidence intervals (CI), of nine chemicals tested for induction of β-galactosidase enzymatic activity from yeast cells carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16, and pGAL4.*Bs*LBD.VP16. Structures of the chemicals can be viewed at http://pubchem.ncbi.nlm.nih.gov/using the PubChem compound accession identifier (CID) numbers listed.

CID	Comp. Name	Supplier (Catalogue No.)	GAL4.*Ci*LBDΔ31.VP16		GAL4.*Ci*LBD.VP16		GAL4.*Bs*LBD.VP16	
			EC_{50} (μM)	95% CI (μM)	EC_{50} (μM)	95% CI (μM)	EC_{50} (μM)	95% CI (μM)
2482	*N*-butyl-*p*-aminobenzoate	Sigma–Aldrich (B7753)	68	64–74	55	48–63	57	47–69
2554	Carbamazepine	Sigma–Aldrich (C4024)	7.1	4.9–9.9	6.2	5.3–7.3	7.0	4.9–9.8
978	*P*-aminobenzoic acid	Sigma–Aldrich (A9878)	1.8	1.2–2.9	3.0	1.1–8.8	~191	Very wide
6623	Bisphenol-A (BPA)	Sigma–Aldrich (B1760)	5.3	1.8–15.6	9.2	4.9–17.5	10	0–4108

CID	Comp. Name	Supplier (Catalogue No.)[c]	GAL4.*Ci*LBDΔ31.VP16		GAL4.*Ci*LBD.VP16		GAL4.*Bs*LBD.VP16	
			EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)	EC_{50} (nM)	95% CI (nM)
446,512	Okadaic acid	Sapphire Bioscience (AB120375)	19	1.1–327	27	15.4–46.4	16	8.3–30.4
64,37,385[a]	Pectenotoxin-11	MacKenzie, 2013.	633	117–3437	883	134–5834	553	3.4–90,840
N/A[b]	Portimine	Selwood et al., 2013.	143	114–180	130	105–162	124	77–202
6,438,357	Microcystin-RR	DHI Lab Products (PPS-MCRR)	NI	NI	NI	NI	NI	NI
431,734	Anatoxin-A	NRC Canada (IMB-CRM-ATX)	NI	NI	NI	NI	NI	NI

Abbreviations: NI, no induction; N/A, not available.
[a] The structure of pectenotoxin-11 (PTX-11) is not available on PubChem so the CID number listed is for the structurally related pectenotoxin-2 (PTX-2)
[b] The structure of portimine is not yet available on PubChem but can be viewed in Selwood et al., 2013 .
[c] Microcystin-RR was purchased from DHI Lab Products (Hørsholm, Denmark), anatoxin-A from the National Research Council Canada (Ottawa, Ontario, Canada), and okadaic acid from Sapphire Bioscience Pty. Ltd. (Waterloo, NSW, Australia). PTX-11 (MacKenzie, 2013) and portimine (Selwood et al., 2013) were sourced from within the Cawthron Institute (Nelson, New Zealand).

there is a need for novel simple, robust, and inexpensive bioassays for microalgal biotoxin detection (Hashimoto et al., 2011; Nicolas et al., 2014; Vilarino et al., 2010).

The vertebrate pregnane X receptor (abbreviated PXR, NR1I2 (nuclear receptor subfamily 1, group I, member 2)), is a ligand-dependent nuclear receptor (NR) which regulates transcription of multiple genes involved in detoxification pathways after being activated by potentially harmful compounds, including exogenous chemicals (i.e., xenobiotics), in an animal's diet (Bertilsson et al., 1998; di Masi et al., 2009; Kliewer et al., 2002; Nakata et al., 2006; Sachar and Ma, 2013; Wallace and Redinbo, 2013; Xie and Chiang, 2013; Zhou et al., 2009). In contrast to most NRs, which tend to have structurally constrained cognate ligands, vertebrate PXR ligands are structurally diverse and include both endogenously synthesized chemicals (e.g., bile acids, steroid hormones, vitamins) and exogenous xenobiotics (e.g., both synthetic and herbal drugs, environmental chemicals) (Biswas et al., 2009; Chang and Waxman, 2006; Hernandez et al., 2009; Manez, 2008; Staudinger et al., 2006; Zhou et al., 2009). Comparisons of the ligand-binding domain (LBD) sequences of vertebrate PXR orthologues suggest that they may have adaptively evolved to bind those xenobiotics typically encountered by a given taxa (Krasowski et al., 2011a, 2011b, 2005a, b; Moore et al., 2002, 2000; Zhang et al., 2004). This observation suggested the possibility that the LBDs of PXR orthologues could be utilised as biosensor elements whose structures, and ligand specificities had been moulded by adaptive evolution to detect those bioactive compounds typically present in an animal's diet (Fidler et al., 2012).

Filter-feeding marine tunicates (Deuterostomia; Phylum: Chordata; sub-phylum: Urochordata) are exposed to a wide range of microalgal biotoxins associated with the microalgae accumulating in their diet (Roje-Busatto and Ujević, 2014; Sekiguchi et al., 2001). Tunicates form the sister clade to the Vertebrata (Delsuc et al., 2006, 2008). Annotated genomes of two ascidian tunicates, *Ciona intestinalis* (*Ci*) and *Botryllus schlosseri* (*Bs*), encode at least two genes orthologous to the vertebrate PXR and vitamin D receptor (VDR), abbreviated as VDR/PXR (Dehal et al., 2002; Voskoboynik et al., 2013; Yagi et al., 2003). One such VDR/PXR gene from *C. intestinalis*, *Ci*VDR/PXRα (GenBank accession number: NM_001078379), has been characterized in terms of its LBD mediating ligand-dependent luciferase reporter gene expression when fused to the generic GAL4 DNA-binding domain (GAL4-DBD) and expressed in a mammalian cell line (Ekins et al., 2008). Using this bioassay, three synthetic chemicals and two microalgal biotoxins (okadaic acid and pectenotoxin-2) were identified as *Ci*VDR/PXRα agonists and a common pharmacophore defined (Ekins et al., 2008; Fidler et al., 2012; Reschly et al., 2007). Together these studies established that the *Ci*VDR/PXRα receptor has ligand-binding characteristics consistent with a natural role in detecting marine xenobiotics, including microalgal biotoxins, making it a potential sensor element for microalgal biotoxin bioassays (Fidler et al., 2012).

Here we report the development of recombinant yeast (*Saccharomyces cerevisiae*) strains expressing the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD as fusion proteins combined with the GAL4-DBD and a generic transcription activation domain (VP16-AD). Such expressed fusion proteins were coupled with the easily assayed *lacZ* reporter gene, which generated yeast strains that facilitate detection of probable ligands for tunicate VDR/PXRα receptors. The bioassay strains were tested with synthetic toxicants (n = 4) and natural toxins (n = 5). Results indicate that such tunicate VDR/PXR LBD-based recombinant yeast bioassays are technically feasible and that these bioassays may find application in high-throughput, robust, and inexpensive screens for microalgal biotoxins and for novel marine bioactive chemicals in general.

2. Materials and methods

2.1. Chemicals tested in the bioassay

Chemicals tested in the bioassay are listed in Table 1. All chemicals were dissolved in analytical grade ethanol (Merck, Whitehouse Station, NJ, U.S.A.) to form stock solutions. Serial dilutions were added to the bioassay media at a final ethanol concentration of 1% (v/v).

2.2. Media

All media ingredients were purchased from Sigma–Aldrich (St. Louis, U.S.A.) unless stated otherwise. Minimal media (MM) was

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