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# Development of a competitive fluorescence-based synaptosome binding assay for brevetoxins

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ABSTRACT

Brevetoxins are a family of ladder-frame polyether toxins produced during blooms of the marine dinoflagellate *Karenia brevis*. Inhalation of brevetoxins aerosolized by wind and wave action can lead to asthma-like symptoms in beach goers. Consumption of either shellfish or finfish exposed to *K. brevis* blooms can lead to the development of neurotoxic shellfish poisoning. The toxic effects of brevetoxins are due to activation of voltage-sensitive sodium channels (VSSCs) in cell membranes. Binding of brevetoxin analogs and competitors to site 5 on these channels has historically been measured using a radioligand competition assay that is fraught with difficulty, including slow analysis time, production of radioactive waste, and cumbersome and expensive methods associated with the generation of radioactive labeled ligands. In this study, we describe the development of a novel fluorescent synaptosome binding assay for the brevetoxin receptor. BODIPY®-conjugated to PbTx-2 was used as the labeled ligand. The BODIPY®–PbTx-2 conjugate was found to displace [$^3$H]–PbTx-3 from its binding site on VSSCs on rat brain synaptosomes with an equilibrium inhibition constant of 0.11 nM. We have shown that brevetoxin A and B analogs are all able to compete for binding with the fluorescent ligand. Most importantly, this assay was validated against the current site 5 receptor binding assay standard, the radioligand receptor assay for the brevetoxin receptor using [$^3$H]–PbTx-3 as the labeled ligand. The fluorescence based assay yielded equilibrium inhibition constants comparable to the radioligand assay for all brevetoxin analogs. The fluorescence based assay was quicker, far less expensive, and did not generate radioactive waste or need radioactive facilities. As such, this fluorescence-based assay can be used to replace the current radioligand assay for site 5 on voltage-sensitive sodium channels and will be a vital tool for future experiments examining the binding affinity of various ligands for site 5 on sodium channels.



## 1. Introduction

The dinoflagellate responsible for the majority of Florida red tides, *Karenia brevis* (formerly known as *Gymnodinium brevi* and *Ptychodiscus brevis*), produces numerous ladder framed polyether compounds, the most abundant of which are the brevetoxins (PbTxs) (Baden and Tomas, 1988). PbTxs bind to site 5 of voltage-sensitive sodium channels (VSSCs) resulting in persistent activation of the channel (Jeglitsch et al., 1998; Purkerson et al., 1999; Baden et al., 2005). The PbTxs are potent neurotoxins that are known to cause massive fish kills and have caused a large number of mortalities in seabirds, sea turtles, and marine mammals (O'Shea et al., 1991; Bossart et al., 1998; Flewelling et al., 2005; Fleming et al., 2011). In addition, humans who consume shellfish contaminated with PbTxs may develop neurotoxic shellfish poisoning (NSP). Symptoms of NSP include gastroenteritis, sensory abnormalities, cranial nerve dysfunction, and other neurotoxic effects. In cases of severe acute cases of NSP, victims require treatment in the emergency room and intensive care units to prevent respiratory failure (Watkins et al., 2008). Furthermore, aerosolized PbTxs in sea spray have been found to induce respiratory dysfunction and distress in beach visitors, especially those with preexisting conditions such as asthma. The most common symptoms of exposure to aerosolized brevetoxins are eye irritation and acute respiratory irritation of both the upper and lower respiratory tracts, including, nasal congestion, throat irritation, cough, chest tightness, wheezing, and shortness of breath (Backer et al., 2003). People that are particularly susceptible, such as asthmatics and those with COPD, may develop more severe symptoms and require hospitalization following exposure to inhaled brevetoxins (Kirkpatrick et al., 2006; Fleming et al., 2007). This is not surprising because brevetoxins have been found to induce bronchoconstriction and airway hypersensitivity in asthmatic sheep, with effects that are larger and lasting longer in susceptible animals as compared to control animals (Abraham et al., 2005a,b).

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The search for a treatment to alleviate the symptoms of brevetoxosis and NSP has led to the development of a competitive radioligand binding assay to examine the binding activity of various compounds in relation to brevetoxin (Poli et al., 1986). This type of assay was instrumental in characterizing the binding properties of the newly discovered antagonist to brevetoxin known as brevenal (Bourdelais et al., 2004, 2005). Since 1981, various assays have been developed to aid in detection and elucidation of the effects of brevetoxins during red tide events, including the development of a brevetoxin ELISA (Naar et al., 2002), LC–MS analysis and fluorescence monitoring of synapto-neruosomal membrane potentials (David et al., 2003) and calcium oscillations (Dravid et al., 2004). However, the standard for measuring binding affinity for compounds to the brevetoxin receptor remains the radioligand assay. With increasing restrictions placed on the use of radioactive materials and concomitant concerns regarding radioactive waste and contamination, in addition to cumbersome and expensive methodology associated with the generation of radioactive ligands, there is a great need for the development of a non-radioactive assay to examine the affinity of various compounds for the VSSC PbTx receptor.

In recent years, a variety of fluorescence techniques have revolutionized the study of receptor–ligand interactions. The development of fluorescent probes has allowed researchers to develop vital techniques for life science research, including fluorescence correlation spectroscopy, fluorescence polarization spectroscopy, and in vivo fluorescence imaging. In addition, many studies have investigated the use of fluorescent probes as a substitute for radioligands in competition assays (as reviewed in Leopoldo et al., 2009; Middleton and Kellam, 2005). These techniques are of particular importance for therapeutically important receptors involved in drug discovery research where the ability to measure and quantify receptor–ligand binding remains important. Fluorescent competition assays historically have the advantage of lower non-specific binding and background, which plagues radioligand assays. In particular, fluorescent based assays may be able to detect interactions between receptors and natural product ligands which may be overlooked in the conventional radioligand assays (Pramanik, 2004). The use of fluorescent probes and fluorescence-based competition assays, for example, has revolutionized the study of G-protein coupled receptors (GPCRs) in drug discovery and has allowed researchers to elucidate complex receptor interactions (Verkaar et al., 2008; Daly and McGrath, 2003; Arterburn et al., 2009).

As fluorescent ligands and probes offer safer, less expensive, more powerful, and more versatile alternatives to radioligands, it was our intent to develop an effective fluorescence-based assay for the study of inhibition of binding at the brevetoxin receptor. This assay allows specific detection of compounds that bind to site 5 of VSSCs and allows for development of novel compounds that may be used as a treatment for the effects of these neurotoxins. In this report, we describe the development and validation of a non-radioactive fluorescence-based receptor assay for site 5 of the VSSC.

## 2. Materials and methods

### 2.1. Reagents and materials

Reagent grade sucrose, sodium phosphate, Trizma base, HEPES, choline chloride, glucose, EGTA, bovine serum albumin (BSA), protease inhibitor cocktail, and polyethyleneimine solution (PEI) were purchased from Sigma–Aldrich (St. Louis, MO). Reagent grade potassium chloride, magnesium sulfate, and ethyl alcohol were purchased from ThermoFisher Scientific. Alkamuls detergent was purchased from Rhone-Poulenc (Cranbury, NJ).

### 2.2. Isolation of brevetoxins

PbTx-1, PbTx-2, PbTx-3, and PbTx-9 were purified from unialgal cultures of *K. brevis* (Wilson strain) as previously described (Bourdelais et al., 2004; Truxal et al., 2010). Brevetoxins were extracted from *K. brevis* cultures using 1 L of choroform/10 L culture through liquid:liquid extraction. The chloroform was added to the carboy containing *K. brevis* culture and homogenized using an IKA ultra turrex. The homogenate was allowed to stand until the two layers separated. The chloroform layer (lower) was removed, filtered and dried under vacuum. The polyethers were then separated from lipophilic pigments and cell debris using petroleum ether partitioning with a 90:10:1 (petroleum ether:-methanol:water) mixture. The methanol layer containing the target compounds was collected and washed with petroleum ether (2×). The petroleum ether layer was backwashed with 90% MeOH (2×). A Kromaton Fast Centrifugal Partition Chromatograph (Kromaton, France) was then used on the methanol:water extract to effect a rapid liquid:liquid partitioning into 72 fractions based on compound polarity. The fractions containing the brevetoxins were identified (by thin layer chromatography) and further purified using a series of two different HPLC columns.

The fractions from the Kromaton containing PbTx-1, PbTx-2, PbTx-3 and PbTx-9 were purified using two different reversed phase HPLC columns. The first step in HPLC purification used a Phenomenex™ phenyl-hexyl column, 10 mm × 250 mm, 100A, 5 μm column with isocratic elution using MeOH/$H_2O$; 98:2; with UV detection at 215 nm. The fractions eluting from the column containing PbTx-1, PbTx-2, PbTx-3, and PbTx-9 were collected separately and run individually on a second column: Varian, MVP $C_{18}$, 10 mm × 250 mm, 100A, 5 μm column using isocratic elution with MeOH/$H_2O$; 90:10; with UV detection at 215 nm. After the second HPLC separation the compounds had a purity of >99% by LC/MS and NMR analysis.

### 2.3. Synthesis of the BODIPY®–PbTx-2 conjugate

BODIPY® FL hydrazide (D-2371, Invitrogen, Eugene, OR) was conjugated to PbTx-2 using a modified Fischer reaction (Heydari et al., 2007). A one pot reaction was performed using a 1:1 molar ratio of PbTx-2:BODIPY® FL hydrazide in DMF. The reaction conditions were carried out using a catalytic amount of tungstophosphoric acid at 60 °C for 4 h with stirring. The DMF was evaporated to dryness under vacuum at 60 °C then resuspended in a small amount of methanol and then filtered (0.2 mM nylon filter). After filtration the reaction mixture was dried again under vacuum to a volume of about 1 mL and then the BODIPY®–PbTx-2 conjugate was purified from the starting materials using a two step reversed phase HPLC procedure. The first step utilized a Phenomenex™ phenyl-hexyl column, 10 mm × 250 mm, 100A, 5 μm column with isocratic elution using ACN:$H_2O$; 55:45, 3.4 mL/min; UV detection at 215 nm and 360 nm was used. The fraction eluting from the column containing the BODIPY®–PbTx-2 conjugate was collected and further purified using a Varian, MVP $C_{18}$, 10 mm × 250 mm, 100A, 5 μm column using isocratic elution with MeOH:$H_2O$; 74:26; and detected using an UV detector set to 215 nm and 360 nM. The purified PbTx–BODIPY® hydrazone derivative was confirmed using LC/MS and 1D and 2D-NMR, as described below.

### 2.4. MS and NMR spectra measurements

A liquid chromatography–tandem mass spectrometry method was used to confirm the mass (M + 1) of PbTx-2 and BODIPY®–PbTx-2. The sample was run under acidic conditions (MP 80:20:0.1% acetonitrile:$H_2O$:formic acid) 100 μL/min over 3 min using an Agilent 1100 LC (Agilent Technologies, Santa Clara, USA)