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Short communication

High affinity for the rat brain sodium channel of newly discovered hydroxybenzoate saxitoxin analogues from the dinoflagellate *Gymnodinium catenatum*

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Abstract

The paralytic shellfish poison family has been recently extended by the discovery of several analogues possessing a hydoxybenzoate moiety instead of the carbamoyl group one finds in saxitoxin, the parent molecule of this toxin family. We have investigated the potency of these new analogues on a representative isoform of the pharmacological target of these toxins, the voltage gated sodium channel. These toxins were found to have K_I's in the low nanomolar range, only slightly less potent than saxitoxin. The hydroxybenzoate group may increase the lipophilicity of these toxins and improve their ability to pass through epithelia and therefore its uptake and elimination in both intoxication victims and animals that bioaccumulate paralytic shellfish toxins.

Keywords: Saxitoxin; Sodium channel; Paralytic shellfish poison; *Gymnodinium catenatum*; Hydroxybenzoate

Three new analogues of saxitoxin were recently reported from the dinoflagellate *Gymnodinium catenatum* (Dinophyceae) (Negri et al., 2003). These analogues are unique in possessing a hydroxybenzoate moiety instead of the carbamoyl side chain normally found in the parent saxitoxin molecule (STX) (Fig. 1). Two of these hydroxybenzoate analogues, GC1 and GC2, also possessed a sulfate at the C-11 position that one finds in gonyautoxins-2 and -3 and exist as an epimeric mixture Fig. 2). The analogue that differed from saxitoxin only in having the hydroxybenzoate was dubbed GC3 (Fig. 1; Negri et al., 2003). Saxitoxin and previously reported natural analogues mediate their toxicity to mammals by binding to the voltage activated sodium channel and prohibiting passage of sodium ions through the channel into the nerve, a process that underlies the generation of an action potential (Strichartz, 1986). The potential toxicity of these new hydroxybenzoate toxins is examined herein by measuring their ability to compete with radiolabelled saxitoxin for site 1 of the rat brain sodium channel.

Toxins GC1, GC2 and GC3 were prepared from *G. catenatum* strain GCDE09 as described in Negri et al. (2003). Stock toxin samples were quantified using chemiluminescence nitrogen detection (CLND), a technique that measures bound nitrogen with a high degree of specificity and allows the quantitation of nitrogen-containing compounds. The LC-CLND system comprised an Agilent model 1100 HPLC and a HP79994 data system interfaced with an Antek 8060R CLND (Houston, TX, USA). Oxygen and argon flow rates were set at 250 and 50 ml/min, respectively. Vacuum pressures between 21 and 23 Torr were used and an average capillary nebulizer pressure of 12 psi was obtained. The mobile phase was aqueous methanol with 2 mM heptanesulfonic acid on a Zorbax SB-C8 column (2.1 × 150 mm^2, 5 μm, AgilentTechnologies). The methanol was gradient programmed from 20 to 50% over 15 min. Concentrations were determined by calibrating the detector with an accurate solution of caffeine (manuscript in preparation).

Liquid chromatography with fluorescence detection (LC-FLD) was performed using a Waters (Milford MA, USA) model 600 HPLC unit in combination with a PCX 5100 Post-Column Reactor (Pickering, Mountain View CA, USA) and a Linear LC305 spectrofluorometric detector

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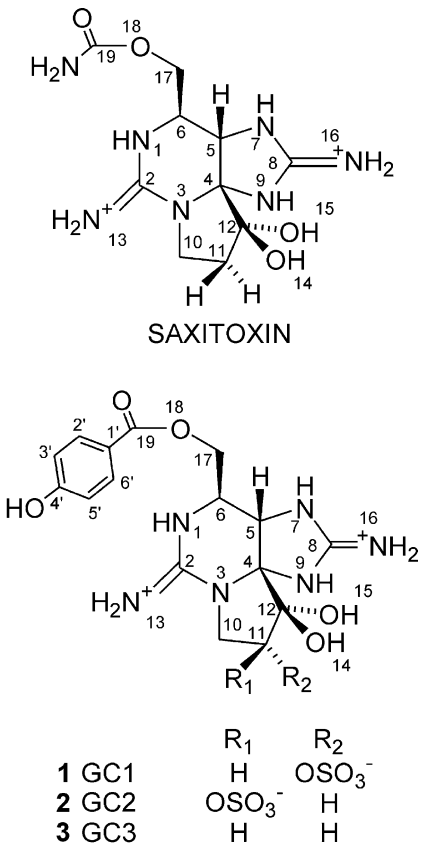

Fig. 1. Structures of saxitoxin and *Gymnodinium catenatum* toxins **1**, **2** and **3**.

(Alltech Associates, Deerfield IL, USA). GC toxins were eluted isocratically at 0.25 ml/min on a 5 μm, 250 × 2.1 mm^2 Alltech Alltima C-18 (Alltech, IL, USA) column with 10 mM heptafluorobutyric acid:CH_3CN (82:18, v/v) and the fluorescent derivatives monitored using $\lambda_{excitation} = 330$ nm and $\lambda_{emission} = 390$ nm. LC-FLD analyses for C-toxins and gonyautoxins were performed as previously described (Negri and Llewellyn, 1998). The C toxin, GTX and STX standards were kindly donated by Prof. Y. Oshima of Tohoku University, Japan.

For sodium channel receptor binding assays, tritiated STX ([^{3}H]STX) was purchased from Amersham Pharmacia Biotech (UK). STX (Calbiochem, San Diego, CA, USA) was diluted in 1 mM citrate buffer (pH 5.0). Biological buffers and other general chemicals were obtained from Sigma. Water was deionised (≈18 MΩ) with a Barnstead system (IA, USA). Rat brain synaptosomes were prepared as the source of sodium channels, as described in Llewellyn (1997) and stored at −80 °C until use. [^{3}H]STX binding to rat brain sodium channels was measured as described in Llewellyn et al. (2001). Assay conditions were varied so that there was a total volume of 150 μl buffered with 20 mM of the biological buffer 3-[*N*-morpholino]propanesulfonic acid (MOPS) adjusted to pH 7.4 with NaOH, 200 mM choline chloride, 5 nM [^{3}H]STX and 26 μg protein of rat brain synaptosomes. Control samples containing 5 μM STX determined the level of background radioactivity. Values were then normalised to 100% using control samples containing no competitor. Competition curves were fit using the curve-fitting routine of Sigmaplot 7.0 (SPSS Scientific Software, Chicago, IL, USA).

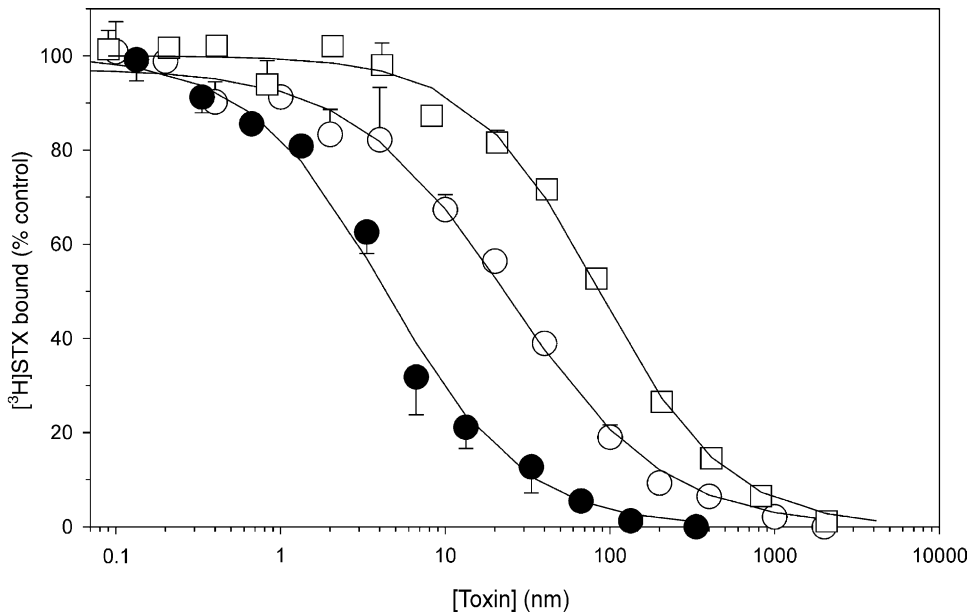

Fig. 2. Competition curve of unlabelled saxitoxin (●), GC3 (○) and GC1 and 2 epimeric mixture (□) versus ^{3}H-STX binding for rat brain sodium channel. Curves are drawn to the curve described in the text using the stated values for the IC_{50}'s and slope.

Analysis of a late log phase culture of *G. catenatum* strain GCDE09 by LC-FLD revealed that the GC toxins made up a total of 37% of the PSTs on a molar basis (Fig. 3). GC3 accounted for 18.4% of the total molar toxin composition while GC1 and GC2 accounted for 18 and 0.5%, respectively. The remaining toxins included the C1 (6%), C2 (10%), C3 (4%), C4 (43%), dcGTX3 (0.3%) and dcGTX2 (0.2%), along with trace levels of dcSTX. The total toxin concentration of this culture was 12 fmol/cell.

The IC_{50} of unlabelled STX versus ^{3}H-STX should be equivalent to the concentration of ^{3}H-STX used (Cheng and Prusoff, 1973). Using the assay conditions described above, unlabelled saxitoxin produced an IC_{50} of 4.3 ± 0.4 nM, demonstrating the viability of the assay system used for measurement of the relative affinity of the GC analogues of STX. GC3 possessed an IC_{50} of 22 ± 2 nM and IC_{50} of 86 ± 6 nM was obtained for the epimeric mixture of GC1 and GC2. The latter IC_{50} is calculated assuming that both GC1 and GC2 are equally potent and their concentrations were added together. In all curves, a Hill slope close to unity was obtained which would be expected with a single toxin population and a single receptor population (Table 1). For GC3, an estimate of the K_D (K_I) for the sodium channel can be calculated using the Cheng Prusoff equation $K_I = IC_{50}/(1 + \{[^{3}\text{H-STX}]/K_D^{3\text{H-STX}}\})$ (Cheng and Prusoff, 1973) which relates the IC_{50} of a competitor to the known K_D of the reference ligand, namely [^{3}H]STX. Using rat brain sodium channels in brain membrane vesicles, Weigele and Barchi, 1978 measured the K_D of [^{3}H]STX to be 0.5 nM under the same conditions used here. Using this value, the K_I of GC3 would be 2.2 nM. It is difficult to do the same determinations for GC1 and GC2 that exist as an epimeric mixture. At best, calculations of their maximal potency can be derived if one assumes that only one of the epimers is biologically active. If one does assume that GC2 is inactive and all of the observed bioactivity is elicited by GC1 alone, then it has a K_I of 4.4 nM. If the opposite is assumed, that is all of the observed bioactivity was due to GC2, then this toxin may have a K_I of 3.4 nM. In all of the above, if one uses the value of 0.19 nM reported for [^{3}H]STX by Moczydlowski et al. (1986) (Table 2), then the calculated values of K_I's for GC1-3 will be even more potent by a factor of approximately 2.5.

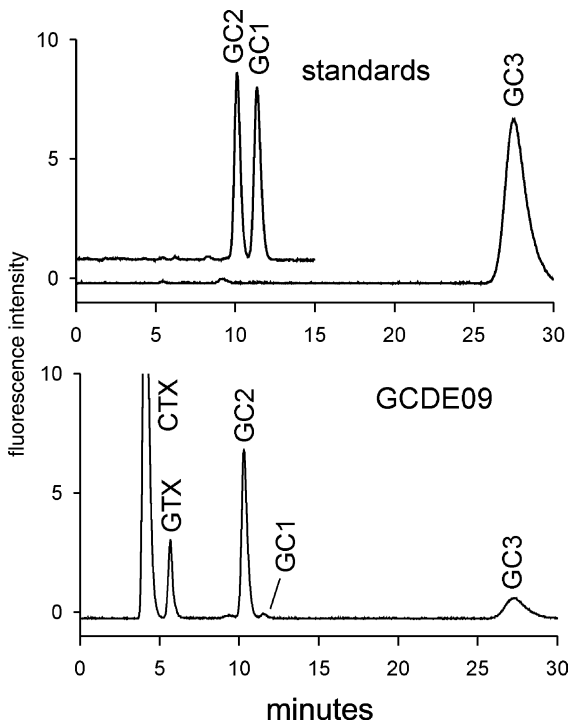

Fig. 3. LC-FLD chromatograms of GC1-3 standards and *G. catenatum* strain GCDE09.

Table 1
Values derived from fitting inhibition curves depicted in Fig. 2 with the equation: fraction ^{3}H-STX bound = $1/(1 + [\text{competitor}]/IC_{50}^{n})$, where IC_{50} is concentration at which competitor causes 50% inhibition, and n is slope of curve and can be considered as a pseudo Hill coefficient (Doyle et al., 1993)

Curve parameter	STX	GC3	GC1 and 2
IC_{50} (nM)	4.3 ± 0.4	22.0 ± 2.2	86.1 ± 6.2
Hill slope	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.1

It transpires that these benzoate analogues of saxitoxin bind strongly to the sodium channel but are somewhat less potent than saxitoxin (Table 1). Whole animal toxicity is the end result of a toxin being able to not only elicit an effect on a molecular pharmacological target but to be able to be absorbed and distributed to the site of action within the intoxication victim's body. The possession of a less polar hydroxybenzoate moiety raises the possibility that GC1-3 may possess a greater ability than saxitoxin and other paralytic shellfish poisons to cross epithelia and so may possess greater absorption across the mammalian gut and vascular epithelia to pose a significant public health risk. Further still, lipophilicity may reduce the rate of elimination of the toxins similar to their retention within the fat tissue as previously observed for brevetoxins in rats (Poli et al., 1990).

This increased lipophilicity can also increase the potential for bioaccumulation and retention in lipid containing tissues in dinoflagellate consumers, such as bivalves. As with intoxication, bioaccumulation from a dietary item requires uptake across the epithelium of the digestive and circulatory systems. Once located within a tissue, toxins may be retained in lipid compartments, a process again reported for the lipophilic dinoflagellate toxin, brevetoxin (Poli et al., 1990; Ishida et al., 1996). Onodera et al. (1997) discovered a suite of saxitoxin analogues with an acetyl substitution at the C19 position. The potency of those analogues was between 6 and 14% of STX in mouse bioassay. Although the acetyl substitution provides

Table 2
Reported values for mammalian toxicity of saxitoxin determined by intra-peritoneal injection into mice and potency in binding to rat brain sodium channels using conditions similar to those used in this study

Parameter	Toxin							Reference
	STX	neoSTX	GTX3	C2	acetyl STX	GC1,2	GC3	
Specific toxicity (mouse units/μmol)	2483	2295	1584	239				Oshima, 1995
	2046	1038						Genenah and Shimizu, 1981
	2100	2300						Sullivan et al., 1985
					52–346			Onodera et al., 1997
Rat brain Na channel K_D (nM)	0.19	0.067						Moczydlowski et al., 1986
	0.5							Weigele and Barchi, 1978
						3.4–4.4	2.2	This study

the closest relationship with the hydroxybenzoate toxins, the likely difference in uptake makes direct comparisons of potency unreliable. Until enough material can be obtained for whole animal studies, the potential impact of the benzoate moiety on mammalian toxicity and bioaccumulation remains to be determined.

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