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Development of a monoclonal antibody against the left wing of ciguatoxin CTX1B: Thiol strategy and detection using a sandwich ELISA

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ABSTRACT

Ciguatera fish poisoning (CFP) is a form of food poisoning caused by the ingestion of a variety of reef fish that have accumulated trace amounts of ciguatoxins produced by dinoflagellates of the genus *Gambierdiscus* through the food chain. CFP affects more than 50,000 people each year. The extremely low level of the causative neurotoxins, ciguatoxins, in fish has hampered the preparation of antibodies for detecting the toxins. In this paper, we describe a thiol strategy for synthesizing a keyhole limpet hemocyanin (KLH)-conjugate (**20**) of the ABCDE-ring fragment of the Pacific ciguatoxins, CTX1B (**1**) and 54-deoxyCTX1B (**4**). We succeeded in producing a monoclonal antibody (3G8) against the left wings of these ciguatoxins by immunizing mice with the hapten-KLH conjugate (**20**) as the synthetic antigen. The most promising mAb, 3G8, does not cross-react with other related marine toxins. Sandwich enzyme-linked immunosorbent assay (ELISA) utilizing 3G8 and the previously prepared monoclonal antibody (8H4) enabled us to detect **1** specifically at less than 0.28 ng/mL.

1. Introduction

Ciguatera fish poisoning (CFP) is caused by the ingestion of a variety of reef fish that have accumulated trace amounts of ciguatoxins produced by dinoflagellates of the genus *Gambierdiscus* through the food chain (Yasumoto and Murata, 1993; Yasumoto, 2001). In humans, the disease is characterized by severe gastrointestinal and neurological disorders, which may last for months or even years. Globally, more than 50,000 people are estimated to suffer annually from CFP, making it one of the most common non-bacterial types of food poisoning (Scheuer, 1994; Lewis, 2001). The spread of CFP causes tremendous damage to public health, fishery resources, and the economies of tropical and subtropical regions. Social and economic impacts of CFP in endemic regions are the avoidance of the consumption of seafood. For example, French Polynesia loses an estimated US $ 1 million annually due to banned sales of reef fish (Bagnis, 1992). As reef fish are increasingly exported to other areas, CFP has become a worldwide health problem. The difficulty in avoiding CFP arises from the normal appearance, smell and taste of fish contaminated with the causative toxins, along with the lack of a sensitive and reliable method for detecting ciguatoxic fish.

Pacific ciguatoxins, regarded as the principal causative toxins of CFP in Pacific regions, are produced by dinoflagellates of the genus *Gambierdiscus* and accumulate in various kinds of reef fish through the food chain (Yasumoto and Murata, 1993; Yasumoto, 2001). Pacific ciguatoxins, CTX1B and its congeners (**1**–**4**, Fig. 1), are highly toxic to mammals, and their lethal dose by intraperitoneal (i.p.)

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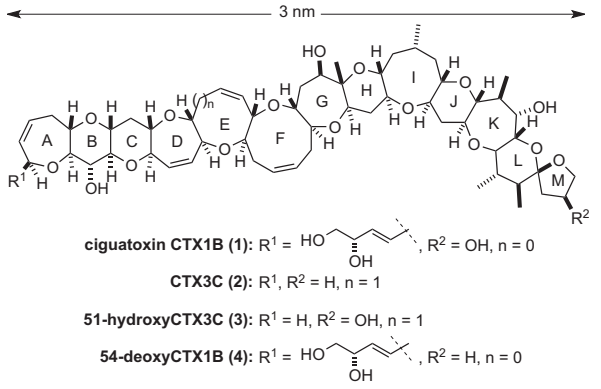

Fig. 1. Structures of the major Pacific ciguatoxin congeners, CTX1B (**1**), CTX3C (**2**), 51-hydroxyCTX3C (**3**), and 54-deoxyCTX1B (**4**); *n* shows the number of methylenes (CH_2) in the E-ring.

injection into mice [medium lethal dose (LD_{50}) 0.25–4 μg/kg] is much lower than that of the structurally related red-tide toxins, brevetoxins [$LD_{50} > 100$ μg/kg] (Murata et al., 1990; Lewis et al., 1991; Satake et al., 1993, 1998; Dechraoui et al., 1999).

In addition to the traditional mouse bioassay of lipid extracts of potentially contaminated fish, several methods have been developed to detect ciguatoxins, including assays based on cytotoxicity (Manger et al., 1995), radioligand binding (Dechraoui et al., 1999; Poli et al., 1997), high performance liquid chromatography (Yasumoto et al., 1995), mass spectrometry (Lewis and Jones, 1997; Lewis et al., 1999; Yasumoto et al., 2000), and LC-MS/MS analysis (Lewis et al., 2009; Otero et al., 2010; Yogi et al., 2011). However, there are no rapid and reliable methods for detecting ciguatoxins at fisheries. An antibody-based immunoassay would likely provide the best method for accurate, sensitive, routine, and portable detection. However, although Hokama et al. (1977) claimed that anti-CTX1B monoclonal antibody (mAb) was produced by immunization with natural CTX1B, the antibody showed cross-reactivity with another marine toxin, okadaic acid (Hokama et al., 1992, 1998). Moreover, immunochemical tests using their antibody have been in a controversy (Hokama, 1993; Lehane and Lewis, 2000; Wong et al., 2005). Our own research has focused on using synthetic haptens for producing anti-Pacific ciguatoxin mAbs. After achieving a fundamental understanding of anti-Pacific ciguatoxin mAbs (Oguri et al., 1999; Pauillac et al., 2000; Nagumo et al., 2001, 2004), we found that haptenic groups with a surface area larger than 400 $Å^2$ are required to produce mAbs that can bind strongly to CTX3C (**2**). Immunization of mice with KLH-conjugates (**5** and **6**) of the pentacyclic ABCDE-ring and IJKLM-ring fragments (Hirama et al., 2001; Inoue et al., 2004, 2006; Yamashita et al., 2011) of **2** elicited the production of mAbs 10C9 and 3D11 which exhibited high and specific affinities with K_d of 2.8 nM and 122 nM, respectively, for **2** (Oguri et al., 2003) (Fig. 2). These mAbs did not show cross-reactivity with other related marine toxins, including brevetoxin A (BTX-A), brevetoxin B (BTX-B), okadaic acid (OA), and maitotoxin (MTX). Furthermore, we also produced a specific mAb, 8H4, against the right wing of CTX1B (**1**) and 51-hydroxyCTX3C (**3**, $K_d = 75$ nM) by immunizing mice with KLH-conjugate (**7**) of a synthetic hexacyclic HIJKLM-ring fragment (Tsumuraya et al., 2006, 2010). We used mAbs 10C9 and 3D11 to develop a sandwich enzyme-linked immunosorbent assay (ELISA) to detect **2** [detection limit: 5 ng/mL (nM)], and then by using mAbs 10C9 and 8H4, we were able to detect **3** at less than 1 ng/mL (1 nM) (Tsumuraya et al., 2006, 2010).

However, preparation of a highly specific mAb against the left wing of the most common Pacific ciguatoxin, CTX1B (**1**) (Otero et al., 2010; Yogi et al., 2011), was unsuccessful in spite of our extensive studies with a similar strategy using synthetic fragments with a cyclic acetal linker such as **8** (Fig. 3). In this paper, we employed thiol strategies to synthesize protein-conjugates of the synthetic ABCDE-ring of **1**, and produced a mAb against the left wing of **1** by immunizing mice. These achievements culminated in the development of a specific sandwich ELISA for detecting CTX1B (**1**).

2. Materials and methods

2.1. General methods

Unless otherwise noted, all reactions sensitive to air were carried out in an argon or nitrogen atmosphere under anhydrous conditions. Analytical thin-layer chromatography was performed using plates precoated with E. Merck Silica gel 60 F254 (Merck & Co., Inc., Whitehouse Station, NJ, USA). Flash column chromatography was performed using 40–50 μm Silica Gel 60N (Kanto Chemical Co., Inc., Chuo-ku, Tokyo, Japan). Optical rotations were recorded on a JASCO (Hachioji, Tokyo, Japan) P-2200 polarimeter. IR spectra were recorded on a PerkinElmer (Waltham, MA, USA) Spectrum BX FT-IR spectrometer. ^1H NMR and ^{13}C NMR spectra were recorded using a Varian (Palo Alto, CA, USA) 400 MR (400 MHz) spectrometer. Chemical shifts are reported in δ (ppm) down field from tetramethylsilane and referenced to residual solvent signals [^1H NMR: $CHCl_3$ (7.26), C_5HD_4N (7.56); ^{13}C NMR: $CDCl_3$ (77.16), C_5D_5N (123.5)]. Signal patterns are indicated as s, singlet; d, doublet; m, multiplet; br, broad peak. MALDI-TOF MS spectra were measured on an Applied Biosystems (Foster City, CA, USA) Voyager DE STR SI-3 instrument. High resolution mass spectra were measured on a Themo Fisher Scientific (Waltham, MA, USA) Orbitrap Discovery (ESI LTQ Orbitrap).

2.2. Synthesis of *11*

Compound **10** was synthesized as reported previously (Kobayashi et al., 2004). To a solution of Fmoc-Gly (32.1 mg, 108.1 μmol) in CH_2Cl_2 (1.1 mL) at room temperature were added DMAP (9.89 mg, 81.0 μmol) and DCC (22.3 mg, 108.1 μmol). After being stirred for 5 min, the mixture was added to a solution of **10** (5.0 mg, 5.4 μmol) in CH_2Cl_2 (1.0 mL). After being stirred for an additional 14 h, the reaction mixture was quenched with phosphate buffer (pH = 7) and extracted three times with EtOAc. The organic layer was washed with saturated aqueous NaCl and dried