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Ciguatoxin Detection Methods and High-Throughput Assays

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15.1 Introduction

15.1.1 Ciguatera

In the broad category of fish poisonings (ichthyosarcotoxaemias), ciguatera fish poisoning is the most prevalent and difficult to manage. The causative agents for this condition are known as ciguatoxins (CTXs), heat-stable lipophilic polyether neurotoxins produced by benthic dinoflagellate *Gambierdiscus* spp. and related species. The ciguatoxins are particularly prevalent in tropical and subtropical regions of the Pacific Ocean and Caribbean Sea, where two main variants of the toxin are found: P-CTX and C-CTX, respectively (Figure 15.1), while the less stable Indian Ocean variants (I-CTXs) remain to be structurally characterised (Murata *et al*. 1990; Lewis *et al*. 1991, 1998; Yasumoto *et al*. 1993; Hamilton *et al*. 2002a, 2002b). Ciguatera was first reported during the colonization of the Caribbean Islands in the 1700s, where settlers described neurological symptoms attributed (likely mistakenly, given contemporary reports do not suggest this species to be toxic) to the ingestion of the marine snail *Livona* sp. commonly known as cigua (Lee, 1980). In 1787, Don Antonio Parra in his exploration of the Antilles coined the term siguatera and later ciguatera, after his observations of clinical intoxication after consumption of fish (Parra, 1787). The first detailed description of the symptoms of ciguatera were recorded in the Pacific in 1786 in the writings of Captain James Cook of HMS Resolution, where he describes the bizarre neurological effects of distressing skin tingling, reversal of tactile heat sensation, and accompanying nausea attributed to the crew's fish dinner, which also poisoned pigs on board (Lee, 1980). However, it wasn't until 1977 that the link between a benthic dinoflagellate (*Gambierdiscus* spp.) and ciguatera was established, when a large dinoflagellate bloom associated with an ongoing ciguatera outbreak in Gambier Islands (French Polynesia) was identified in biodetritus samples (Yasumoto *et al*. 1977).

Ciguatera is endemic to tropical and circum-tropical regions circum-globally, with a prevalence of at least 10 000–50 000 annual cases (Abraham *et al*. 2012; Skinner *et al*. 2011) Due to the advancement of intercontinental commerce of reef products, improved cold transport technologies (Nicholson and Lewis, 2006), climate change and tourism, the global incidence is suspected to be on the increase, making ciguatera one of the most common forms of non-bacterial food poisoning. In addition to direct reef destruction that is thought to be associated with an increased incidence of *Gambierdiscus* blooms, global warming and associated rising sea

Analysis of Food Toxins and Toxicants, *Volume 2*. First Edition. Edited by Yiu-chung Wong and Richard J. Lewis.

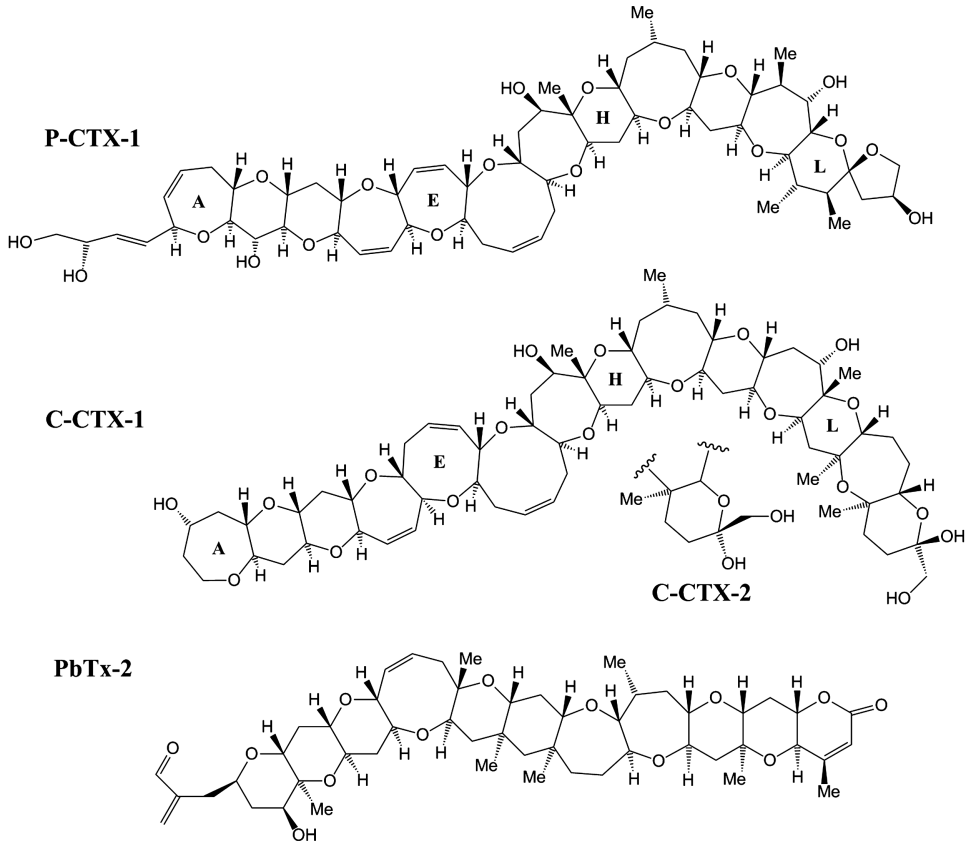

Figure 15.1 The structures of the most potent ciguatoxin isolated from the Pacific Ocean (P-CTX-1), the Caribbean isomers C-CTX-1 and C-CTX2 and brevetoxin 2 (PbTx-2). All ciguatoxins feature a long semi-rigid architecture comprising sequential trans/syn-fused ether rings and are structurally related to the brevetoxin which also activates sodium channel. *Source:* Murata (1990). Reproduced with permission of American Chemical Society.

water temperatures are predicted to extend the range where ciguatoxin blooms of *Gambierdiscus* spp. are likely to occur. Understandably, the greatest impact of ciguatera is on the inhabitants of island countries in the Pacific basin where fish is the primary source of dietary protein and the ciguatera prevalence approaches 10% of the population (Lewis, 1992). Overall, 0.01–10% of fish captured in these regions are estimated to be ciguatoxic (Nicholson and Lewis, 2006), often restricting dietary protein intake and posing a significant direct public health risk to inhabitants of the region.

The ciguatoxin precursors produced by *Gambierdiscus* spp. and related species are bioaccumulated up the marine food chain from smaller herbivorous species that graze on reef biodetritus to larger carnivorous species and finally to humans who develop ciguatera when ciguatoxins have accumulated to levels sufficient to cause human disease (>0.1 ppb of P-CTX-1) (Pearn, 2001; Lehane and Lewis; 2000, Gillespie *et al.* 1986b; Swift and Swift, 1993; Crump *et al.* 1999). Less polar forms including P-CTX-4A that are initially produced by *Gambierdiscus* spp. are biotransformed in fish into more polar and potent ciguatoxins through oxidative metabolism and acid-catalysed spiroisomerisation (Lewis and Holmes, 1993; Deslongchamps *et al.* 1981; Perron and Albizati, 1989). As the toxin moves through the different trophic levels, it concentrates, with large carnivores often having the highest concentrations of ciguatoxin in

their tissue (Lewis *et al*. 1999). Although more than 400 species of fish have been implicated in causing ciguatera, the primary species of fish that pose a significant risk for humans include moray eels, snappers, groupers, mackerels and trevally (Nations 2004; Schlaich *et al*. 2012). However, while most species pose a significant risk of toxicity only sporadically, some species in certain locations are toxic almost all the time, such as moray eel (*Gymnothorax javanicus*) from the southern side of Tarawa (Republic of Kiribati).

15.1.2 Clinical Presentation of Ciguatera

The characteristic symptoms of ciguatera involve gastrointestinal, neurological, and to a lesser extent cardiovascular effects (Table 15.1). The relative severity of each of these symptomatic clusters depends at least in part on the origin of the ciguatoxins consumed as well as the individual dose, with ciguatoxins from Caribbean origin causing predominantly gastrointestinal disturbances while the Indian and Pacific ciguatoxins elicit marked neurological disturbances.

The onset of symptoms usually occurs within 0.5–12 hours of consumption (Hokama, 1988), with victims experiencing both acute and chronic phases. In the acute phase, gastrointestinal symptoms such as nausea, vomiting and diarrhoea pervade (Lawrence *et al*. 1980), although these generally subside after several days. The additional symptoms of autonomic dysfunction such as bradycardia and hypotension comprise the cardiovascular effects; however, these are far less common and likely only associated with the consumption of high levels of ciguatoxins. In a large sample population, only 15% of ciguatera sufferers had low systolic blood pressures and even less presented with slowed heart rates (Dawson, 1977). Victims of ciguatera often become aware of neurological symptoms several hours after consumption of ciguatoxic fish. These neurological symptoms, which are often perplexing or distressing, include paraesthesia and dysaesthesia – specifically a paradoxical burning pain on cooling, or cold allodynia – numbness, heightened nociception and pruritus. These symptoms usually present themselves in the acute

Table 15.1 Common gastrointestinal and neurological symptoms associated with ciguatera.

Symptom	Onset	Duration
Gastrointestinal	0.5–24 h	Days
Diarrhoea		
Nausea/vomiting		
Abdominal pain		
Neurological	6–48 h	Weeks to months
Paresthesias, dysaesthesias		
Arthralgia, myalgia		
Pruritus		
Cold allodynia		
Cardiovascular	24–96 h	Weeks
Hypotension		
Bradycardia		

Adapted from: Bagnis *et al*. 1979; Gillespie *et al*. 1986a; Schnorf *et al*. 2002; Arena *et al*. 2004; Baumann *et al*. 2010.

phase of Pacific Ocean toxins (Abraham *et al*. 2012) and form the initial cause of concern for the victim in over 80% of cases (Pearn, 2001). Paraesthesiae and dysaesthesiae last for a minimum of several days, but in severe cases may last several months.

More than half of ciguatera victims display continued dysaesthesia 2 weeks after the initial exposure (Morris *et al*. 1982). In addition, chronic ciguatera is characterised by an intractable fatigue and weakness, evident in 3–20% of victims (Pearn, 1995). Less commonly, acute insomnia can translate into hypersomnolence (Friedman *et al*. 2007). This combination of fatigue, sleep disturbances, weakness and dysaesthesia often leads to transient depressive episodes in victims. It is important to note that sensitization to ciguatoxin is common (Narayan, 1980), and recurrence of ciguatera-like symptoms can also be triggered by exposure to alcohol and certain foods (Gillespie *et al*. 1986). While the underlying mechanisms are not entirely clear, it is likely that adipose and neuronal tissues may accumulate ingested ciguatoxins, sensitizing the victim to re-exposure to ciguatoxins (Lehane and Lewis, 2000). The clinical presentation of ciguatera and the delayed occurrence of neurological symptoms can be explained by the toxicokinetics of ciguatoxin. Local exposure leads to the rapid presentation of gastrointestinal symptoms, while the onset and duration of the acute phase is determined by slower distribution of CTX to systemic circulation and slow biphasic elimination with a terminal half-life of 4 days (Bottein *et al*. 2011). While CTX is predominantly cleared through the faeces, renal clearance may be a contributing factor in rare cases of painful urination. Overall, mortality from ciguatera is <0.1%, and the neurological symptoms mostly resolve within 1–2 months, although in some cases chronic symptoms develop that can last months and even years after initial exposure.

15.1.3 Pharmacology of Ciguatoxins

The pathophysiology of ciguatera includes spontaneous or repetitive action potential firing in several neuronal types, increased neurotransmitter release, altered synaptic vesicle recycling, increased intracellular Na^+ and Ca^{2+} levels and dysregulation of processes dependent on these ions, as well as Schwann cell and axonal oedema (reviewed by Molgo *et al*. (1992)). These effects are attributable to the pharmacological effect of the ciguatoxins on neuronal voltage-gated sodium (Na_V) and potassium (K_V) channels, which are essential for the rising and falling phase of the action potential, respectively.

The family of Na_V channels consists of nine subtypes ($Na_V1.1$ to 1.9) with unique expression profiles and pharmacology that are critical for action potential generation and propagation in excitable cells. The ciguatoxins bind to site 5 of tetrodotoxin-sensitive and -resistant isoforms and activate Na_V channels through several mechanisms, making them some of the most potent sodium channel toxins known (Lehane and Lewis, 2000; Pearn, 2001). Specifically, the ciguatoxins cause hyperpolarising shifts in the voltage of activation and inactivation of Na_V channels, effectively decreasing the threshold of activation of these channels and increasing the likelihood of action potential generation at normal resting potentials. These electrophysiological effects increase Na_V channel activity and largely underlie the Na^+ dependent physiological effects of ciguatera.

The effects of ciguatoxins at Na_V channels have been assessed in detail in a number of cell types and expression systems, including primary neuronal cells and immortal cell lines. In cells heterologously expressing rat $Na_V1.8$, P-CTX-1 and CTX3C reduced the threshold of activation and hyperpolarised the voltage of inactivation. Similar effects were observed for cells expressing rat $Na_V1.2$, $Na_V1.4$ and $Na_V1.5$ (Yamaoka *et al*. 2004). In dorsal root ganglion cells, P-CTX-1 caused a hyperpolarising shift in the voltage of activation and inactivation and decreased peak current of both TTX-resistant and TTX-sensitive Na_V channels (Strachan *et al*. 1999), effectively decreasing the threshold of activation for these channels. Similar activity also

occurred in parasympathetic neurons, where P-CTX-1 increased action potential firing, membrane depolarisation and neuronal excitability (Birinyi-Strachan *et al.* 2005b; Hogg *et al.* 2002). These effects are consistent with ciguatoxin-induced spontaneous opening of Na_V channels that was observed during single channel recordings under steady-state conditions (Hogg *et al.* 1998).

Activity at voltage-gated potassium channels (K_V), which are responsible for the repolarisation of the membrane potential to resting state, also contributes to ciguatoxin-mediated increases in neuronal excitability (Birinyi-Strachan *et al.* 2005b; Hidalgo *et al.* 2002; Schlumberger *et al.* 2010). However, the precise effects on K_V channels, as well as the molecular identity of the channels affected by the ciguatoxins, are less clear. Inhibition of the $I_{K(DR)}$ and the I_{KA} currents by P-CTX-1 in dorsal root ganglion neurons leads to prolonged action potential and after-hyperpolarisation duration and contributes to increased neuronal excitability, altered membrane potential and spontaneous action potential firing (Birinyi-Strachan *et al.* 2005b). Similar effects were also elicited by P-CTX-4B in frog myelinated axons (Schlumberger *et al.* 2010), and CTX-3C was particularly potent at inhibiting I_K, although little effect on I_A was observed (Perez *et al.* 2011). The diverse pharmacological effects of the ciguatoxins on K_V channels was further illustrated by the observation that CTX-3C had no significant effect on K_V channels in mouse taste cells, albeit the ciguatoxin precursor gambierol potently blocked potassium currents (Ghiaroni *et al.* 2006). Overall, by inhibiting K_V channels and decreasing the threshold of activation of Na_V channels, the ability of ciguatoxins and related compounds to modulate neuronal activity is thereby mediated by two separate but closely related pharmacological entities.

15.1.4 Treatment

In the absence of clinical diagnostic tests able to detect ciguatoxins *in vivo*, diagnosis of ciguatera currently relies on detailed anamnesis and typically requires at least one type of neurological symptom in conjunction with a history of recent fish consumption. Nonetheless, ciguatera is frequently mistaken for a range of other diseases depending on clinical presentation: including misdiagnosis as influenza, bacterial seafood poisoning or gastroenteritis and neurological diseases such as multiple sclerosis or chronic fatigue syndrome (Mattei *et al.* 2014).

Treatment of ciguatera remains largely non-specific, symptomatic and supportive. Based on the pathophysiological mechanisms underlying ciguatera, in particular activation of neuronal sodium channels and a resulting increase in intracellular Ca^{2+}, compounds with activity at neuronal sodium and calcium channels (Ca_V) have been used in trials for treatment of ciguatera. Specifically, efficacy has been reported – albeit mainly as single or small series of case reports – for local anaesthetics such as tocainide (Lange *et al.* 1988), antidepressants including amitriptyline (Ruprecht *et al.* 2001; Calvert *et al.* 1987; Davis and Villar, 1986; Bowman, 1984) and Ca^{2+} channel modulators like nifedipine (Calvert *et al.*, 1987), gabapentin (Perez *et al.*, 2001) and pregabalin (Brett and Murnion, 2015). In addition, mannitol has been widely used based on the rationale that its osmotic effects reverse ciguatoxin-induced Schwann cell and axonal oedema (Allsop *et al.* 1986;, Pearn *et al.* 1989; Mattei *et al.* 1999; Birinyi-Strachan *et al.* 2005a; Blythe *et al.* 1992; Palafox, 1992). Indeed, correctly diagnosed and adequately hydrated ciguatera patients may benefit from mannitol, administered as an intravenous infusion of 1 g/kg over 30 minutes, with repeat doses given in case of symptom recurrence within 24 hours of treatment (Pearn *et al.* 1989). Unfortunately, animal studies failed to support the clinical benefit of mannitol (Lewis *et al.* 1993; Purcell *et al.* 1999), and a double-blind, placebo-controlled trial was unable to demonstrate significant improvement of mild cases of ciguatera (Schnorf *et al.* 2002). A similar lack of clinical evidence exists for traditional remedies which continue to be used

widely in endemic areas, although some *in vitro* evidence for use of compounds such as rosmarinic acid from *Heliotropium foertherianum* (Boraginaceae) was recently reported (Braidy *et al.* 2014; Rossi *et al.* 2012). These effects warrant further exploration in relevant *in vivo* models. Several hundred patients have also reportedly been successfully treated with cholestyramine, a bile acid–binding resin that is speculated to reduce enterohepatic circulation of ciguatoxin soon after consumption (Shoemaker *et al.* 2010), although the benefits of this approach also remain to be validated in well-designed clinical trials. Given the lack of evidence-based treatment for ciguatera, identification of ciguatoxic fish and avoidance of consumption remain key to reducing the impact of ciguatera, highlighting the need for rapid, quantitative and highly sensitive assays that can detect ciguatoxins in fish.

15.2 Detection Methods

Given the sporadic and often unpredictable occurrence of ciguatera, possible approaches that may be useful to minimize the impact of this significant public health risk include limiting or restricting the sale and distribution of at-risk fish, minimizing permissible serving sizes, or introducing fishing bans in risk areas or of known fish species commonly associated with ciguatera. However, such restrictions have significant socioeconomic implications, making reliable detection of ciguatoxins – either in the form of large-scale, pre-sale screening or testing of at-risk fish prior to consumption – an attractive option. In addition, reliable highly sensitive and specific assays for the detection of ciguatoxins would also assist with the clinical diagnosis of ciguatera, which to date relies on thorough anamneses and clinical presentation, including at least one neurological symptom.

Several factors limit the simple detection of ciguatoxins in fish or biological samples. These include the low levels of ciguatoxins present in fish flesh as well as the very low levels required to cause disease, the generally limited quantities of CTX available as reference standards or for research, the presence of multiple structural CTX isoforms in ciguateric fish, the absence of useful chromophores, the difficult synthesis of CTX or even fragments of these molecules, as well as the unpredictable occurrence of CTX (Sasaki *et al.* 1994; Yamashita *et al.* 2015).

Several approaches have been described to detect and quantitate ciguatoxins, including *in vivo* assays, antibody-based assays, receptor binding assays, functional and cell-based assays, as well as analytical chemistry and mass spectrometry (MS) methods (Table 15.2). In general, the ideal ciguatoxin assay is cheap, simple, rapid, quantitative and highly sensitive, enabling detection of sub-picogram quantities of ciguatoxins in biological samples including fish flesh and patient samples. Ciguatoxin assays should be able to detect and identify multiple ciguatoxin congeners, be impervious to interference from organic solvents and sample matrix, be able to be performed by non-skilled operators without the need for specialised equipment, as well as be reproducible and robust. In addition, since ciguatoxic fish could potentially be contaminated with other marine biotoxins able to cause human disease (e.g., maitotoxin) (Yasumoto *et al.* 1971, 1976), distinguishing ciguatoxin contamination from other toxins is highly desirable. Unfortunately, no such assay exists to date, although significant advances have been made.

15.2.1 *In Vivo* Detection of Ciguatoxins

As ciguatoxins are undetectable in fish flesh by smell, taste or simple visual inspection, *in vivo* bioassays have been used historically to detect ciguatoxin contamination in fish and have been modified from simple observation of animal behaviour after ingestion of suspected ciguatoxic fish to more sophisticated quantification of symptoms following injection of serially diluted

Table 15.2 Summary of methods available for the detection of ciguatoxins.

Method	Assay duration	Throughput (sample no.)	Cost	Extract purity	Sensitivity (concentration)	Specificity
Mouse lethality	24 h	1–10	High	Low	nM	No
LC/S/MS	4 min	1	Low	Low	pM	Yes
Cytotoxicity	48 h	96–1536	Low	Low	nM	No
Fluorescent	2–4 h	96–1536	Low	Medium	nM	Yes
ELISA	2–4 h	96–1536	Intermediate	Unprocessed	pM	Yes
Radioligand binding	2–4 h	96–1536	High	Low	pM	No
HTS EPhys	2–4 h	8–153	High	High	nM	Yes

semi-purified or crude toxic extracts in species ranging from cats, mice, chickens and mongoose to brine shrimp, mosquito and diptera larvae (Vernoux *et al.* 1985; Banner *et al.* 1960; Granade *et al.* 1976; Hungerford, 1993; Bagnis *et al.* 1987; Lewis and Endean, 1984; Miller *et al.* 1986; Escalona de Motta *et al.* 1986). While these *in vivo* methods are not high throughput and only semi-quantitative, they provide a historical perspective on how ciguatera-causing toxins can be detected. In 1961, following difficulties in developing a reliable and robust assay for the detection of ciguatera-causing toxins using mongooses (Banner *et al.* 1960) or cats (Banner *et al.* 1960), Banner *et al.* (1961) described a bioassay that employed the use of mice. Toxic and non-toxic fish flesh could be differentiated after intraperitoneal injection of partially purified fish extract in mice. The end point in the assay was death, which was caused in a dose-dependent manner by extracts from toxic fish at a level of 0.2 mg/g (milligrams of extract per gram of mouse) (Figure 15.2).

This method was further refined and used by Lewis and Endean (1983) to detect, for the first time, the presence of ciguatoxin in Spanish mackerel caught in Queensland, Australia. The presence of ciguatoxin was initially confirmed in a traditional bioassay assessing effects of fish in

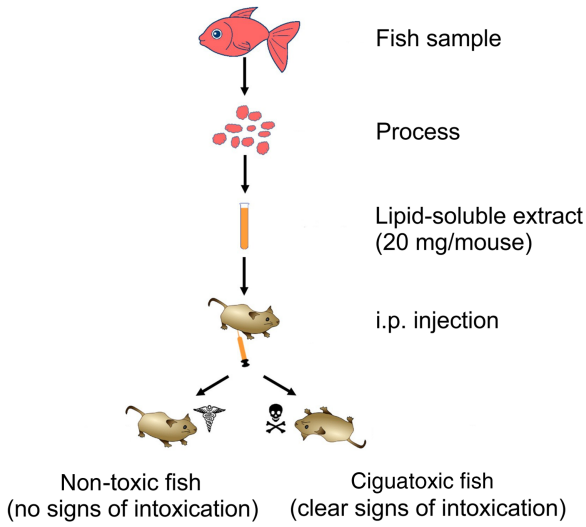

Figure 15.2 Outline of the methodology underlying *in vivo* assays for the detection of ciguatoxins. Fish flesh is extracted using acetone, and the lipid-soluble ciguatoxins are enriched using liquid–liquid partition (Adapted from: Lewis *et al.* 2009; Lewis and Endean, 1983). The enriched extract is dried and solubilized in Tween 20/saline and injected into intraperitoneally into ~20 mice at a dose of 20 mg per mouse. Ciguatoxin-containing extracts from highly toxic fish are lethal, while less toxic fish extracts produce signs of intoxication (diarrhea, loss of activity, hypersalication, lachrymation, reduced body temperature) but recover after 1–24 hours.

cats. After ingestion of contaminated fish, the animals displayed signs of ciguatera, including hypersalivation, lachrymation, dyspnoea, ataxia, irregular heartbeat, paralysis and death in severe cases of poisoning. While cats are relatively sensitive to toxicity from ciguatoxins, regurgitation of contaminated fish makes accurate determination of dose, and thus ciguatoxin levels in fish flesh, difficult. Thus, crude fractions and purified extracts of toxin were injected in mice via the intraperitoneal route to determine LD_{50} values. Similar to the effects observed in cats, the extracted toxin caused piloerection, diarrhoea, lachrymation, hypersalivation, dyspnoea, cyanosis and convulsive spasms prior to death, with the LD_{50} of the crude toxin extract and the purified toxin determined as 510 mg/kg and 0.72 mg/kg respectively, confirming that *in vivo* biodetection of ciguatera-causing toxins is a viable though semi-quantitative method of identifying ciguatoxin-like compounds in contaminated fish.

While these *in vivo* assays have also been adapted for detection of ciguatoxins in a variety of animal species, the low throughput, poor sensitivity, high cost and large amount of toxin required limit the utility of this approach. In addition, ethical concern about the use of animals restrict the large-scale implementation of *in vivo*, as well as tissue-based or *ex vivo* assays. Thus, while bioassays represent a reliable method to detect large amounts of toxin in contaminated samples, other methods of detection, such as radioligand binding and immunoassays, were developed to address these issues.

15.2.2 Immunochemical Assays

In 1977 at the University of Hawaii, a radioimmunoassay for the detection of ciguatoxin was developed by the Hokama group (Hokama *et al*. 1977), representing a major breakthrough in the development of a high-throughput, sensitive and selective assay for detecting ciguatoxins. The assay was based on detection of ciguatoxins by ^{125}I-labelled antibodies raised against ciguatoxin conjugated to human serum albumin and permitted quantitative detection of ciguatoxins from fish flesh. A benefit of this approach, which performed well compared with the traditional *in vivo* mongoose assay, lies in its high specificity, although false negatives may arise from insensitivity of these antibodies to other ciguatoxin isoforms. In addition to safety concerns and the need for specialised equipment, the prohibitive costs related to generating radiolabelled antibodies limit its more widespread use.

Hokama *et al.* (1983) further modified this approach and developed an enzyme-based immunoassay in an effort to provide a simpler, more cost-effective ciguatoxin detection. Horseradish peroxidase-conjugated ciguatoxin antibodies permitted detection of ciguatoxin contamination in Hawaiian reef fish using a competitive enzyme-linked immunosorbent assay (ELISA) (Hokama, 1985), leading to subsequent development of commercially available solid-phase immunobead assay test kits known as Ciguatect™ and Cigua Check® (Hokama, 1990; Hokama *et al*. 1998a, 1998b). Ciguatect was subject to a patent purchased by Hawaii Chemtect International for use in the detection of toxins causing ciguatera (Park, 1994), but the patent has since been abandoned, possibly due to controversial issues relating to test performance and poor agreement with previously established detection methods (Bienfang *et al*. 2011; Ebesu and Campora, 2012; Dickey *et al*. 1994). Cigua Check was developed and marketed by Oceanit for the detection of ciguatoxins and related polyethers. The test kit was available commercially before being withdrawn from the market, presumably due to inconsistent results or a lack of uptake. During development, no false negatives were reported, but further test validation is required to confirm that the testing procedure is robust and accurate before this type of method can be re-released commercially.

More recent advances in the development of antibodies to synthetic fragments of ciguatoxins promise opportunities for further improvement of immunoassays, although affinity of these

antibodies require further optimisation, and the high specificity of antibodies developed to date is prohibitive for the broad detection of ciguatoxin congeners in fish flesh (Pauillac *et al*. 2000; Oguri *et al*. 2003). These problems could be overcome by production of antibodies based on additional synthetic ciguatoxin fragments and congeners (Yamashita *et al*, 2015; Kobayashi *et al*. 2004). Indeed, the use of ciguatoxin ELISAs was further improved with creation of the first monoclonal antibodies that could be used to detect and differentiate between various isoforms of Pacific and Caribbean ciguatoxin (Campora *et al*. 2006).

15.2.3 Receptor Binding Assays

As detailed above, the ciguatoxins bind to site 5 of the α-subunit of Na_V channels expressed throughout the body (Lombet *et al*. 1987; Vetter *et al*. 2014; Vetter and Lewis, 2014). The high potency, near irreversible binding and pharmacological selectivity of the ciguatoxins have enabled the development of several receptor binding assays based predominantly on displacement of the cyclic polyether neurotoxin brevetoxin (PbTx) which binds to the same site on Na_V channels. The most common assay variant involved competitive displacement of [^{3}H]-PbTx-3 from native tissue or cells expressing high levels of Na_V channels. For example, ciguatoxin isomers from the Pacific (Lewis *et al*. 1991), Caribbean (Poli *et al*. 1997) and Indian (Hamilton *et al*. 2002a, 2002b) oceans all competed with [^{3}H]-PbTx-3 for binding to Na_V channels in a rat brain membrane preparation. The ability to detect ciguatoxin contamination in a semi-purified lipid extract of a portion of cooked fish enabled further use of this assay to confirm that a severe case of food poisoning among U.S. soldiers in Haiti was a case of ciguatera (Poli *et al*. 1997) due to the presence of C-CTX-1.

Cost and safety concerns associated with radioligand binding assays lead to the development of a fluorescence-based binding assay utilising a fluorophore-conjugated brevetoxin-2 ligand (McCall *et al*. 2014) with similar assay performance and sensitivity. However, while a major advantage of receptor binding assays lies in the high specificity and the ability to quantitate the composite potency of a sample, identification or differentiation of related ciguatoxin isoforms in a sample is not possible, nor is detection of other toxic components like maitotoxin that do not affect Na_V channels (Fleming, 1997). These factors, coupled with difficulties in obtaining or synthesizing sufficiently pure labelled brevetoxin, limit these assays to being useful tools as opposed to appropriate high-throughput methods for the detection of ciguatoxins in contaminated fish.

15.2.4 Cell-Based Assays

15.2.4.1 Tetrazolium Cell Viability Assay

This cell-based assay is probably the most widely utilised method of ciguatoxin detection to date and is routinely use by the Federal Drug Administration for the testing of fish suspected of being contaminated with ciguatoxins (Friedman *et al*. 2008). The assay was first outlined in 1993 by Manger *et al.* (1993, 1995) who investigated the cytotoxic effects of neurotoxic molecules, including saxitoxins, brevetoxins and ciguatoxins, by taking advantage of the conversion of tetrazolium to a coloured formazan product in metabolically active cells which can be simply quantified using a standard spectrophotometer. The tetrazolium cell viability routinely employs a mouse neuroblastoma cell line (Neuro2a) that natively expresses various Na_V channel subtypes and assesses synergistic cell death following treatment with veratridine (a site 2 Na_V channel activator), ouabain (a Na^+/K^+ ATPase inhibitor) and the toxin of interest. The combined effect of these compounds is an elevation of intracellular Na^+ ions to toxic levels

and a resultant decrease in cell viability that can be measured as a function of toxin concentration.

The assay is reliable, relatively simple to execute and is able to detect ciguatoxins at levels present in disease-causing fish. In addition, assay miniaturization and conversion to high-throughput format is easily achieved, providing a cost-effective ciguatoxin detection method that requires only very small sample amounts. However, like most cell-based assays, interference from other toxins that affect cell viability reduces assay specificity, and identification of the causative toxins is not possible, albeit showing that the effects that are reversed with TTX can provide evidence of sodium-dependent toxicity. In addition, cell viability assays cannot discriminate between ciguatoxin and brevetoxin activity. This issue was addressed, to an extent, by Bottein-Dechraoui *et al.* (2005) by combining a receptor binding assay with the cell viability assay which showed that brevetoxins are more readily detected by the receptor binding assay than ciguatoxins. However, although a slight improvement in assay selectivity was achieved by combining these two techniques, the resultant additional level of complexity limits wider application of this technique.

15.2.4.2 Cell-Based Fluorescent Imaging Assays

While cell-based cytotoxic assays measure a single end point that is typically only achieved by very high toxin concentrations or in synergy with veratridine and ouabain after prolonged incubation, cell-based fluorescent imaging assays aim to detect ciguatoxin-induced cellular responses in real time. These assays are typically performed in neuronal cell lines such as mouse Neuro2a cells and take advantage of the pharmacological effects of ciguatoxins on Na_V and K_V channels, which result in membrane depolarisation and an increase in intracellular Ca^{2+} levels through downstream activation of voltage-gated calcium channels (Ca_V) and accumulation via the Na^+/Ca^{2+} exchanger. These effects allow, potentially, for the use of a number of different approaches to measure ciguatoxin activity, including fluorescence detection of changes in membrane potential or intracellular Na^+ and Ca^{2+} ions using suitable fluorescence dyes. Specifically, a range of fluorophores exist for Na^+ and Ca^{2+} ions, permitting the direct measurement of intracellular accumulation of these ions, while membrane potential or voltage-sensitive dyes respond to global changes in charge distribution across biological membranes with a change in fluorescence and thus enable quantitation of changes in membrane voltage that occur as a result of modulation of Na_V or K_V channels by ciguatoxins. The kinetic changes in fluorescence induced by sequential addition of sample and the Na_V activator veratridine can additionally provide insights into potential toxin contaminants. Maitotoxin, a toxin contaminant frequently found in ciguateric fish, is a potent Ca^{2+} channel toxin that converts the plasmalemmal Ca^{2+} ATPase (PMCA) pump into a Ca^{2+}-permeable channel and elicits dramatic, rapid increases in cytosolic free Ca^{2+} concentration (Sinkins *et al.* 2009). These responses can easily be distinguished from ciguatoxin-induced increases in intracellular Ca^{2+}, which are typically small in comparison, transient in nature and potentiated considerably by subsequent stimulation with Na_V activators like veratridine (Figure 15.3). An additional advantage of this approach lies in the ability to readily adapt cell-based fluorescence assays into high-throughput format, permitting simultaneous assessment of samples in 384- or 1536-well format and thus reducing the amount of sample needed, as well as accelerating ciguatoxin detection for commercial screening.

A platform that is particularly well suited to this approach is the industry standard Fluorescent Imaging Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA). However, while FLIPR assays represent a robust and reproducible ciguatoxin detection method (Vetter *et al.* 2012; Zimmermann *et al.* 2013), this technology is not widely available, and the presence of maitotoxin could potentially obscure ciguatoxin responses due to saturation of fluorescence

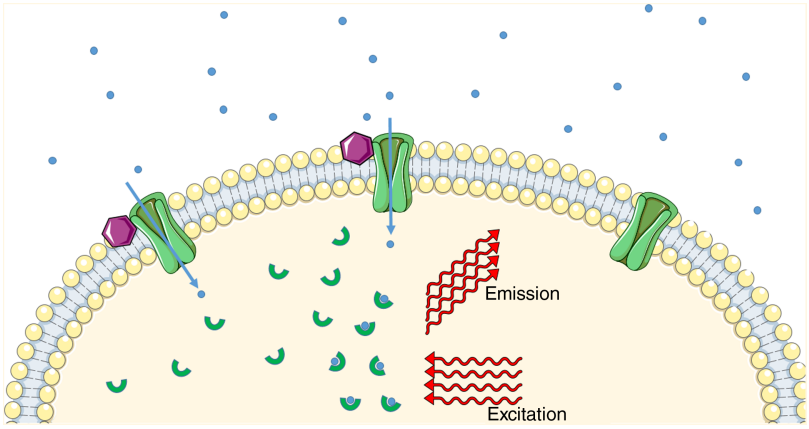

Figure 15.3 Basic principle of a functional cell-based fluorescence assay. Ciguatoxin (purple hexagon) binds to an ion channel allowing sodium ions (blue sphere) to move down their concentration gradient from outside to inside the cell. The pre-loaded fluorophore (green semi-circle) binds to the specific ion entering the cell, which alters the fluorescent properties of the fluorophore. This change in fluorescence can be measured, and toxin activity can be confirmed as a function of channel modulation (figure produced using Servier Medical Art, www.servier.com).

by maitotoxin-induced increases in intracellular Ca^{2+}. This may lead to a false negative assessment of ciguatoxin levels. Like all of the cell-based bioassays discussed in this chapter, FLIPR assays require specialised equipment (the FLIPR^{TETRA} or equivalent systems), and the required fluorescent dyes (Figure 15.4) can be quite costly.

An adaption of fluorescence-based bioassays utilising voltage-sensitive dyes assessed the effects of ciguatoxin, saxitoxin and brevetoxin in Neuro2a cells using flow cytometry (Manger *et al.* 2014, 2007). While relative toxicity, based on changes in fluorescence, can be determined using this approach, the assay lacks specificity in terms of identifying ciguatoxins or maitotoxins since both of these compounds can alter the membrane potential of neuronal cells.

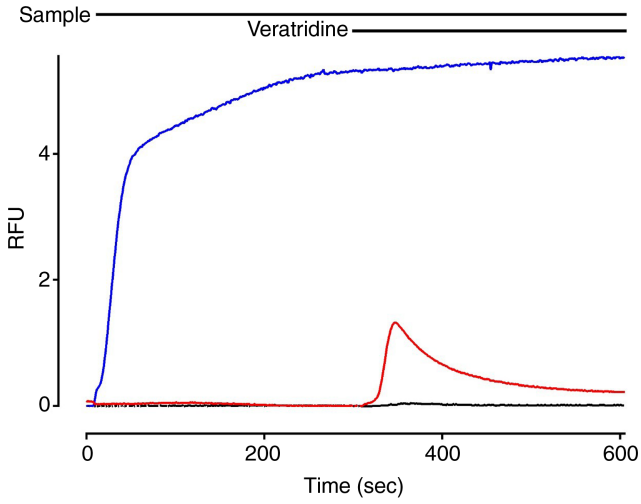

Figure 15.4 Typical fluorescent responses to ciguatoxin and maitotoxin in SH-SY5Y cells. Neuronal cells were loaded with fluorescent Ca^{2+} dye and changes in fluorescence in response to addition of sample and veratridine were monitored using the FLIPR^{TETRA}. Maitotoxin-like activity resulted in large, rapid changes in fluorescence (blue trace), whereas veratridine (5 μM) addition 300 seconds later identified CTX-like activity (red trace). The control (black trace) response is veratridine (5 μM) after saline addition (unpublished results).

15.2.5 High-Throughput Electrophysiology

Electrophysiological techniques are arguably the gold standard for measuring effects of ligands on voltage-gated ion channels. However, until recently, this laborious technique required a high degree of expertise as well as delicate and sophisticated equipment that did not lend itself to high-throughput screening. In recent times, high-throughput electrophysiological screening platforms have been developed which can automatically perform whole-cell patch clamp experiments in a 96- or 384-cell format. However, while this approach may represent a particularly sensitive, selective and specific technique for detection of ciguatoxins, it has not been applied to high-throughput screening of ciguatoxin contamination as yet, and both quantification as well as identification of toxins are likely difficult using this technology.

15.2.6 Mass Spectrometry

MS has been used since early 1991 to detect, characterise and confirm the presence of ciguatoxins. Historical analyses of ciguatoxins employed the technique of fast atom bombardment ionisation with glycerol/thioglycerol matrices to detect and characterise these difficult-to-ionise molecules (Lewis *et al.* 1991). The combination of high performance liquid chromatography (HPLC), MS and MS/MS has the potential of providing a rapid, specific and sensitive assay enabling detection and quantification of ciguatoxin plus conformers (Lewis *et al.* 1994; Yogi *et al.* 2011). A number of issues have prevented the development of a robust, high-throughput, low-cost, accurate, sensitive and reliable assay, as mentioned previously. Specifically for MS assay development, progress has been slow due to issues including the lack of availability of standards and the lipophilic nature of the toxins, making the development and optimisation of sample extraction and high-sensitivity MS techniques difficult. In addition to this, the lack of a suitable functional group within the toxins makes it difficult to easily and reproducibly protonate, deprotonate or derivatise for enhancing MS sensitivity. Limited molecular backbone collision-induced dissociation MS/MS fragmentation information, with typically only water losses observed, are all factors affecting the development of a robust, sensitive, cost-effective high-throughput screening method for ciguatoxins and ciguatoxin conformers. The early establishment of a MS-based analysis was developed for the confirmation of the presence of ciguatoxin conformers from contaminated ciguateric fish at below 4 parts per billion (ppb) concentration and used the application of HPLC positive ion electrospray ionisation (HPLC ESI/MS) and selected ion monitoring, covering the expected mass range of the ciguatoxin conformers (Lewis and Jones, 1997; Pottier *et al.* 2002). This method proved more sensitive than the mouse bioassay and had the added advantage of providing information about different conformers.

Ciguatoxin extraction and MS analysis improved in 2009 with the development of a ciguatoxin rapid extraction method (CREM/LC/MS) that provided greater sensitivity to more clinically relevant concentrations. Sample requirement was reduced from typically 50–100 g of fish flesh to 2 g, and the application of HPLC/MS MS/MS analysis provided increased specificity to reduce detection and quantification limits from around 1–5 ppb to 0.1 ppb (Lewis *et al.* 2009). Over the next 6 years, a number of improvements to the CREM methods were made by adding an additional chloroform extraction step and inclusion of HILIC or aminopropyl column chromatography to reduce MS ion suppression (Wu *et al.* 2011; Solino *et al.* 2015; Caillaud *et al.* 2010). The CREM LC/MS method can process approximately 12 samples in a batch over 2–3 days, including toxin extraction and quantification at clinically relevant concentrations above 0.1 ppb with only 2 g of fish tissue (Meyer *et al.* 2015).

With recent advances in HPLC/MS MS/MS assays, the two-tiered methods of detection using traditional bioassays followed by MS confirmation are no longer the only option for

ciguatoxin detection and identification. This is mainly a result of the ability of HPLC/MS MS/MS to detect and quantify clinically relevant concentrations of ciguatoxin directly from fish extracts. In light of these new, rapid, specific and sensitive MS experimental protocols, the use of analytical chemistry to detect ciguatoxin contamination is becoming more and more relevant.

15.3 Conclusion

In the absence of viable treatment strategies for ciguatera, prevention remains key to minimizing the health and economic impacts of this foodborne illness. This is particularly pertinent given the increased global incidence of ciguatera, resulting from spread of toxin-producing dinoflagellates due to ocean warming as well as increased global trade and travel. Accordingly, there has never been a more critical time to ensure that people consuming fish are protected from the dangers of ciguatera and detection of ciguatoxins in fish flesh by reliable and robust methods that are cost-effective and easy to implement. While significant advances have been made in the technology and techniques available for the detection of ciguatoxins, no assay currently meets all requirements of high specificity and sensitivity, excellent reproducibility, low cost and ease of use. In addition, it is likely that different detection strategies will be required for private consumers who may wish to confirm food safety of a limited number of samples before consuming their catch and commercial or government agencies who will likely be involved in larger scale safety monitoring.

In vivo detection methods which were widely used towards the later part of the 20th century to detect the effects of ciguatera-causing compounds in whole animals have been superseded by *in vitro* assays with greater sensitivity, including receptor binding assays based on displacement of radiolabelled or fluorescently labelled brevetoxin. However, ELISAs using monoclonal antibodies specific for various ciguatoxin congeners are particularly powerful tools in the detection of ciguatoxin contamination and show real promise as a high-throughput platform if issues of reproducibility can be overcome. Irrespective of the advantages and disadvantages of specific assays or detection methods, sensitivity, reliability and accessibility to authorities responsible for the screening of fish suspected of causing ciguatera are most important to reduce the global burden of ciguatera.

Acknowledgements

This work was supported by a National Health and Medical Research Council (NHMRC) project grant (APP1045592) (I Vetter), an NHMRC Senior Principle Research Fellowship (RJ Lewis) and an Australian Research Council Future Fellowship (I Vetter).

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