Identification of Caribbean ciguatoxins from benthic dinoflagellates advances knowledge on toxin chemistry and analysis

Ciguatera poisoning (CP) is a serious food-borne illness caused by the consumption of seafood from endemic tropical and sub-tropical regions, including the Pacific, Atlantic and Indian Oceans, and the Caribbean Sea, that contain highly toxic ciguatoxins (CTXs) that are present at very low levels [1]. Ciguatoxins are produced by *Gambierdiscus* spp. and transferred through the marine food web following consumption by reef herbivores. Microalgal species responsible for the production of CTXs in the Pacific Ocean (P-CTXs) were identified in the last decades of the 20th century, facilitating the production and purification of P-CTXs from culture, analytical method development, and leading a deeper understanding of the metabolism, trophic transfer, and toxicology of P-CTXs[2, 3].

Ciguatera poisoning in the Caribbean is associated with a class of CTXs that were initially isolated and identified in fish from the region. Lewis *et al.* first identified Caribbean CTX-1 (CCTX1) in the late 1990s[4]. Since that time, no additional C-CTXs were definitively identified, nor had an algal source of C-CTXs been confirmed with certainty, although *Gambierdiscus* spp. were suspected to be involved based on *in vitro* bioassays. It has since been shown that C-CTXs occur in fish not only in the Caribbean Sea, but throughout the tropical Atlantic Ocean and even in the Mediterranean Sea[5]. However, the lack of an identified algal source, and the difficulty of producing sufficient purified C-CTXs from fish, has impeded progress on the toxicology, analytical chemistry, and ecological risk assessments of this toxin group, and has hindered the development of effective mitigation strategies for CP management in these regions.

In an attempt to address these issues, an international collaborative project, CiguaPIRE, was initiated in 2017 funded by the US National Science Foundation with parallel support from the Norwegian Research Council, Center for the Environment, Aquaculture and Fisheries Sciences (UK), and the National Research Council of Canada[6]. The project includes global partners from the US, UK, Canada, Norway, and Australia, along with partnerships with a large group of both sovereign island nations and dependent island territories throughout the Caribbean and Western Atlantic. The overall objectives of this global initiative were to examine the dynamics and persistence of toxigenic benthic dinoflagellates and their metabolites in reefs around the globe to better understand the production and fate of C-CTXs. To enable this, it was necessary to develop improved analytical and purification methods for CTXs to allow identification and sensitive detection of an array of novel toxins in algae, invertebrates, and fish.

The initial focus of chemical research within CiguaPIRE included isolation of C-CTX1 from fish, and use of this semi-purified material for method development and *in vitro* metabolism studies that led to the identification of novel glucuronides of C-CTX1 [7]. The availability of C-CTX1 also led to the confirmation of the metabolites C-CTX3 and C-CTX4 as the C-56 reduction products of C-CTX1, through a series of chemical transformations combined with LC–HRMS comparisons with extracts from naturally contaminated fish [8]. Furthermore, the production of isotopically labelled C-CTX1[9], and development of methods for selective boronate extraction of C-CTXs (and a range of P-CTXs) from fish extracts, hold promise for improving analysis time while reducing the impacts of potential matrix effects and interferences[10].

The improved LC–HRMS method

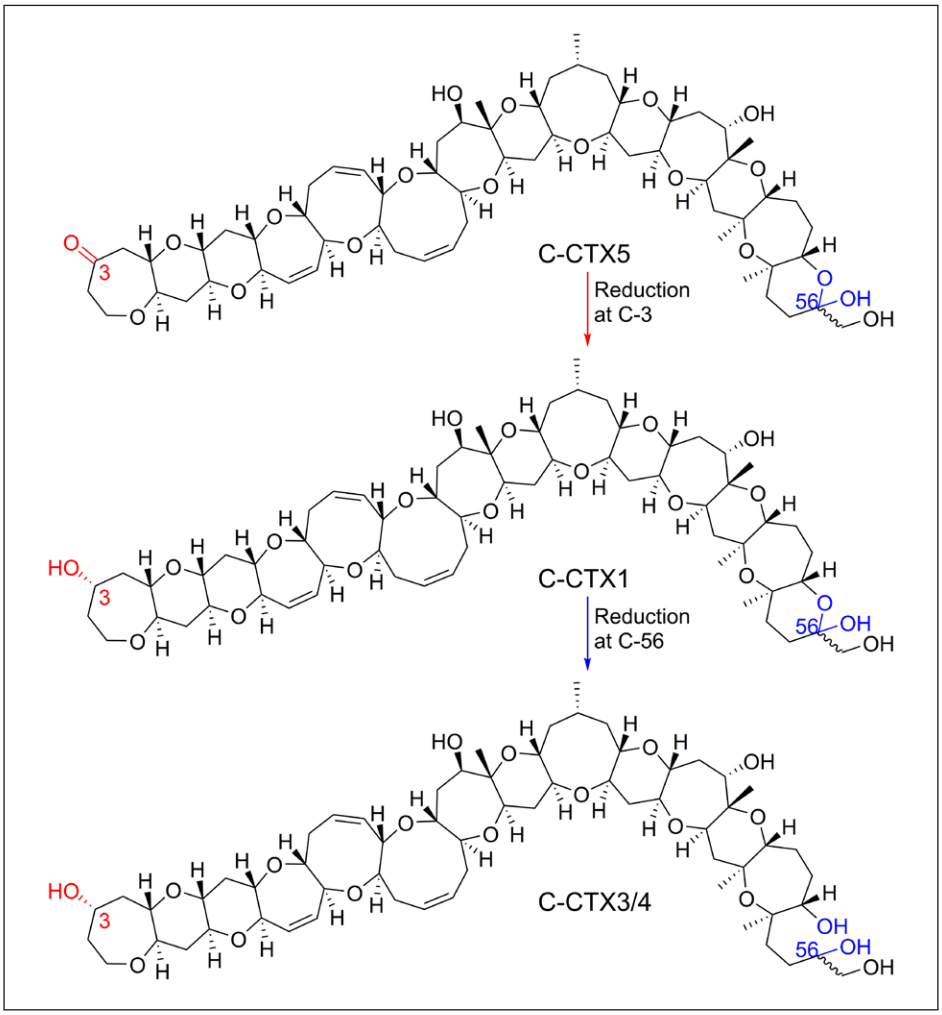

Fig. 1. Structures of the new algal precursor, C-CTX5, and its relationship to C-CTX1 and C-CTX3/4 previously identified in fish.

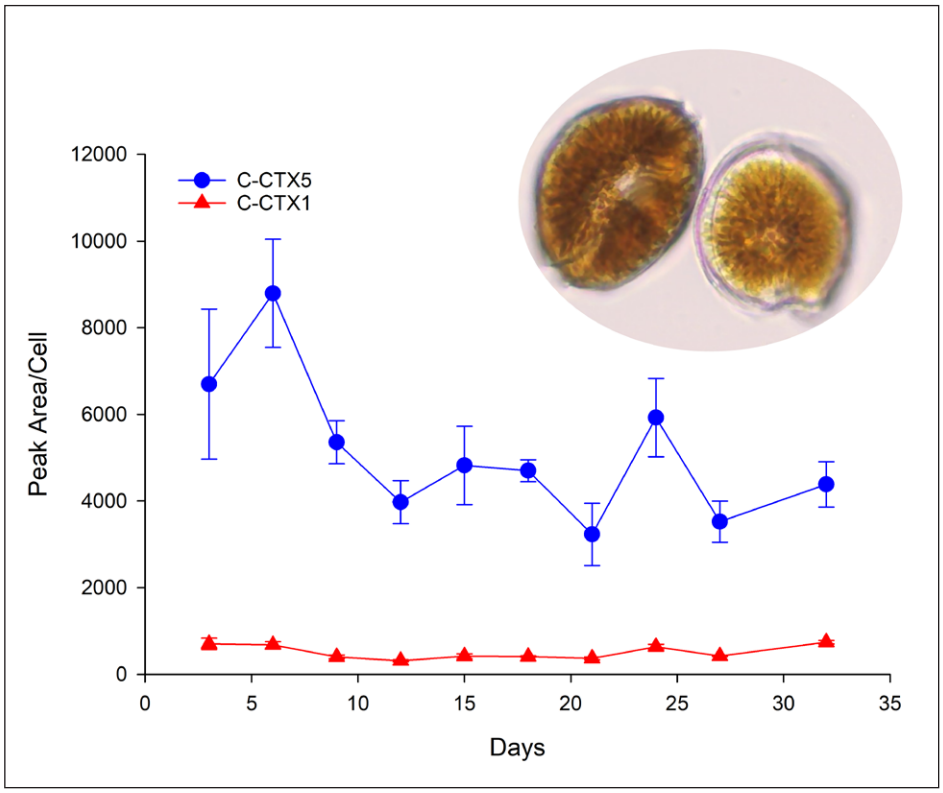

Fig. 2. Toxin content per cell (peak area per cell) grown in L1 medium at a salinity of 35‰ over 32-days (error bars: standard deviation of three culture replicates). Inset: Light microscopy image of Gambierdiscus silvae cells in culture (×400 magnification)

was used to identify a putative C-CTX1 analogue, named C-CTX5 (Fig. 1), in established cultures of *Gambierdiscus silvae* and *G. caribaeus* from the Caribbean Sea[11]. The chemical transformation and isotopic labelling procedures developed in the project for C-CTX1 and C-CTX3/4 were applied to C-CTX5 to unambiguously show that it was 3-oxoC-CTX1. It was then demonstrated that C-CTX5 was biotransformed to C-CTX1 by fish liver microsomes, showing that it is a precursor to the C-CTX1 found in fish. In a semiquantitative study, C-CTX5 was shown to have slightly lower ciguatoxin-like activity than C-CTX1 in an *in vitro* mouse neuroblastoma assay (N2a-MTT), suggesting that it has qualitatively similar sodium channel activity to other C-CTXs. These findings are significant and pave the way for a wide range of studies that were previously not possible.

Initial efforts focused on large-scale culturing of the strain of *G. silvae* (1602 SH-6) that was confirmed to produce C-CTX5 along with lower levels of C-CTX1, with the goal of purifying sufficient material for structural elucidation and studying the stability of the compounds. Over the time course of large-scale culture C-CTX production per cell was consistent (Fig. 2). Large-scale culturing and subsequent isolation of C-CTXs will allow structural confirmation by nuclear magnetic resonance spectroscopy and enable production of small amounts of much-needed quantitative reference materials. Progress in these aspects is vital for subsequent development and validation of *in vitro* bioassays, immunoassays and LC–MS-based methods for C-CTX analysis. Application of such methods in conjunction with previously developed sample clean-up techniques will greatly improve the tools available to the scientific community for understanding and managing the risks presented by C-CTXs in the marine foodweb.

Acknowledgements

This research was part of the CiguaPIRE project funded by the National Science Foundation (NSF), grant number 1743802 to A.R. and the Research Council of Norway, grant number 279247 (to S.U.). Additional support was provided from the Greater Caribbean Center for Ciguatera Research co-funded by NSF (1841811) and the National Institutes of Environmental Health Sciences (P01ES028949). We appreciate the support of all CiguaPIRE partners and students who have contributed to this and broader project goals, and for prior funding from the National Oceanic and Atmospheric Administration from which this work was founded.

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