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Estudio in vivo e in vitro de los efectos de compuestos neuroactivos (ficotoxinas y pesticidas) que actúan sobre canales iónicos dependientes de voltaje

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ESTUDIO *IN VIVO* E *IN VITRO* DE LOS EFECTOS DE COMPUESTOS NEUROACTIVOS (FICOTOXINAS Y PESTICIDAS) QUE ACTÚAN SOBRE CANALES IÓNICOS DEPENDIENTES DE VOLTAJE

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Declaración de conflicto de intereses

El doctorando declara no tener ningún conflicto de interés en relación a la tesis doctoral.

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ABREVIATURAS Y ACRÓNIMOS

A: Amperio

AD: Ácido domoico

ANSES: Por sus siglas en francés Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail (Agencia nacional de seguridad sanitaria alimentaria de Francia)

AO: Ácido ocadaico

ASP: Del inglés, amnesic shellfish poisoning (Intoxicación amnésica por moluscos)

AZA: Azaspirácidos

BTX: Brevetoxina

BUN: Del inglés blood urea nitrogen (Nitrógeno ureico en sangre)

C-CTX: Ciguatoxina del Caribe

CI: Iminas cíclicas

CK: Creatina cinasa

CFP: Del inglés ciguatera fish poisoning (Intoxicación por ciguatoxina)

CTX: Ciguatoxina

DSP: Del inglés, Diarrheic Shellfish Poisoning (Intoxicación diarreica por consumo de moluscos)

EFSA: Del inglés, European Food Safety Authority (Autoridad Europea de Seguridad Alimentaria)

EUMOFA: Por sus siglas en inglés European Market Observatory for fisheries and aquaculture (Observatorio europeo del mercado de los productos de la pesca y de la acuicultura)

EURLMB: Por sus siglas en inglés EU Reference Laboratory for Monitoring of Marine Biotoxins (Laboratorio de referencia de la UE para biotoxinas marinas)

FANs: Floraciones algales nocivas

FAO: Organización de las Naciones Unidas para la Alimentación y la Agricultura

FDA: Por sus siglas en inglés, Food and Drug Administration (Administración de Alimentos y Medicamentos de los Estados Unidos)

FET/TEF: Factor de equivalencia tóxica/por sus siglas en inglés Toxicity Equivalency Factor

HAEDAT: Por sus siglas en inglés Harmful Algal Event Database (Base de datos de eventos de algas nocivas)

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I: Intensidad de la corriente

I-CTX: Ciguatoxina del Océano Índico

IDA: Ingesta diaria admisible

kDa: Kilodalton

LC-MS: Cromatografía líquida acoplada a espectrometría de masas

LD_{50}: Por sus siglas en inglés Lethal Dose 50 (Dosis letal 50)

LDH: lactato deshidrogenasa

LMR: Límite máximo de residuos

LOAEL: Por sus siglas en inglés Lower Observable Adverse Effects Level (Nivel más bajo al que se observan efectos adversos)

MAGRAMA: Ministerio de Agricultura, Alimentación y Medio Ambiente

MTT: Bromuro de 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazolio

MTX: Maitotoxina

mV: Milivoltios

MU: Por sus siglas en inglés mouse units (Unidades de ratón)

N2a: Neuro2a, neuroblastoma de ratón

Na_v: Canales de sodio dependientes de voltaje

NADH: Por sus siglas en inglés nicotinamida adenina dinucleótido (Dinucleótido reducido de nicotinamida y adenina)

NOAEL: Por sus siglas en ingles Non-Observable Adverse Effects Level (Nivel al que no se observan efectos adversos)

NSP: Por sus siglas en inglés, Neurotoxic shellfish Poisoning (Intoxicación neurotóxica por consumo de mariscos)

OCDE: Organización para la Cooperación y el Desarrollo Económico

OMS/WHO: Organización Mundial de la Salud / Por sus siglas en inglés World Health Organization

O/V: Ouabaína/Veratridina

PBS: Por sus siglas en inglés Phosphate Buffered Saline (Tampón fosfato salino)

P-CTX: Ciguatoxina del Pacífico

PLTX: Palitoxina

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PSP: Por sus siglas en inglés Paralytic Shellfish Poisoning (Intoxicación paralizante por moluscos)

PTX: Pectenotoxinas

SNC: Sistema nervioso central

SNP: Sistema nervioso periférico

SPX: Espirólido

STX: Saxitoxina

TTX: Tetrodotoxina

VGSC: Por sus siglas en inglés Voltaje Gated Sodium Channels (canales de sodio dependientes de voltaje)

UE: Unión Europea

UNESCO: Por sus siglas en inglés de United Nations Educational Scientific and Cultural Organization (Organización de las Naciones Unidas para la Educación, la Ciencia y la Cultura).

US EPA: Por sus siglas en inglés United States Environmental Protection Agency (Agencia de protección ambiental de Estados Unidos)

YTX: Yesotoxina

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RESUMEN, RESUMO, ABSTRACT

RESUMEN

Los canales iónicos dependientes de voltaje son proteínas transmembrana implicadas en múltiples funciones imprescindibles en el organismo como la conductividad eléctrica en el sistema nervioso y el corazón o el mantenimiento del pH. Dada su amplia distribución celular cualquier compuesto que actúe sobre ellos modificando su funcionamiento normal puede causar alteraciones importantes en la fisiología.

En el trabajo titulado "Estudio *in vivo* e *in vitro* de los efectos de compuestos neuroactivos (ficotoxinas y pesticidas) que actúan sobre canales iónicos dependientes de voltaje" se estudió la actividad de compuestos naturales y sintéticos cuya principal diana son estas proteínas. En concreto, las células con canales de sodio dependientes de voltaje se han empleado para estudiar el efecto de las ciguatoxinas reevaluando su potencia sobre los mismos, lo que permitió establecer sus factores de equivalencia tóxica *in vitro*. Estos datos se ampliaron con estudios toxicológicos *in vivo* para analizar su efecto agudo vía intraperitoneal y su efecto subcrónico por vía oral siguiendo las directrices internacionales establecidas por la Organización para la Cooperación y el Desarrollo Económico. En relación con este grupo de toxinas, en la presente tesis doctoral se estableció un método para mejorar y aumentar su detección empleando diferentes líneas celulares. Un segundo apartado de este trabajo se centró en el estudio de las brevetoxinas, cuya diana celular son también los canales de sodio dependientes de voltaje, analizando su efecto conjunto con las ciguatoxinas dado que la exposición a biotoxinas marinas no ocurre de forma aislada puesto que los alimentos de origen marino se encuentran contaminados con varios tipos de ficotoxinas. Del mismo modo, compuestos de origen sintético ampliamente empleados en la prevención y control de plagas que actúan sobre canales iónicos, pueden afectar la salud humana tras su ingesta a través de alimentos como frutas y hortalizas y agua. En la presente tesis doctoral se analizó la deltametrina, uno de los plaguicidas más abundantes en los alimentos que actúa sobre los canales de sodio y cloro dependientes de voltaje y se han evaluado sus efectos *in vitro* aisladamente y en presencia conjunta con las ciguatoxinas. Los resultados obtenidos en este trabajo ponen de manifiesto la presencia de sinergias entre diferentes contaminantes alimentarios y la necesidad de evaluar en detalle sus sinergias para establecer unos límites seguros para su presencia en alimentos, teniendo en cuenta que muchos de ellos comparten la misma diana molecular y garantizar de forma eficaz la salud de los consumidores.

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RESUMO

As canles iónicas dependentes de voltaxe son proteínas transmembrana implicadas en múltiples funcións esenciais do organismo como a condutividade eléctrica no sistema nervioso e no corazón ou o mantemento do pH. Dada a súa importancia no organismo, calquera composto que actúe sobre eles, modificando o seu normal funcionamento, provocará importantes alteracións na fisioloxía.

No traballo titulado "Estudo *in vivo* e in vitro dos efectos de compostos neuroactivos (ficotoxinas e pesciticidas) que actúan sobre as canles iónicas dependentes de voltaxe" estúdanse os efectos dos contaminantes dos alimentos cuxo obxectivo principal son estas proteínas. En concreto, empregáronse canles de sodio dependentes de voltaxe para avaliar o efecto directo dos compostos do grupo das ciguatoxinas sobre eles, reavaliando a potencia de diferentes análogos deste grupo de toxinas e establecendo os correspondentes factores de equivalencia tóxica para os análogos tanto pacíficos como caribeños. Os estudos *in vitro* ampliáronse mediante a realización de estudos toxicolóxicos *in vivo* que achegaron datos sobre os efectos agudos por vía intraperitoneal e o seu efecto subcrónico por vía oral seguindo as directrices internacionais establecidas pola Organización para a Cooperación e o Desenvolvemento Económico. En relación a este grupo de toxinas, nesta tese de doutoramento estableceuse un método para mellorar e aumentar a súa detección mediante o emprego de diferentes liñas celulares. Un segundo apartado deste traballo centrouse no estudo das brevetoxinas, cuxa diana celular tamén son as canles de sodio dependentes de voltaxe, analizando o seu efecto conxunto coas ciguatoxinas dado que a exposición a biotoxinas mariñas non se produce de forma illada xa que os alimentos de orixe mariña están contaminados con diversos tipos de ficotoxinas. Así mesmo, compostos de orixe sintético moi utilizados na prevención e control de pragas que actúan nas canles iónicas poden tamén afectar á saúde humana tras a súa inxestión a través de alimentos como froitas e verduras e auga. Nesta tese de doutoramento analizouse a deltametrina, un dos pesticidas máis abundantes nos alimentos que actúa sobre canles de sodio e cloruro dependentes da voltaxe, e avaliáronse os seus efectos *in vitro* só e en presenza conxunta de ciguatoxinas. Os resultados obtidos neste traballo revelan a presenza de sinerxias entre diferentes contaminantes alimentarios e a necesidade de avaliar detalladamente as súas sinerxias para establecer límites seguros para a súa presenza nos alimentos, tendo en conta que moitos deles comparten a mesma diana molecular e garanten de forma efectiva a saúde dos consumidores.

ABSTRACT

Voltage-gated ion channels are transmembrane proteins involved in multiple essential functions of the body such as electrical conductivity in the nervous system and heart or maintenance of pH. Given their importance in the body, any compound that acts on them, modifying their normal functioning, will cause important changes in their physiology.

In the work titled "In vivo and in vitro study of the effects of neuroactive compounds (phycotoxins and pesticides) that act on voltage-dependent ion channels" the effects of food contaminants whose main target are these proteins are studied. Specifically, cells with voltage-gated sodium channels were used to study the effects of ciguatoxins, reevaluating the potency of different analogues of this group of toxins and setting their in vitro toxicity equivalency factors. The *in vitro* studies were expanded with *in vivo* toxicological studies of its acute intraperitoneal effects and its subchronic oral effects following the international guidelines established by the Organization for Economic Cooperation and Development. Related to this group of toxins, in this doctoral thesis a method to improve and increase their detection using different cell lines was established. A second section of this work was focused on the study of brevetoxins, whose cellular target are also voltage-dependent sodium channels, studying their joint effect with ciguatoxins given that exposure to marine biotoxins does not occur in isolation since Foods of marine origin are contaminated with various types of phycotoxins. Similarly, compounds of synthetic origin widely used in the prevention and control of pests that act on ionic channels can affect human health after ingestion through foods such as fruits and vegetables and water. In this doctoral thesis, deltamethrin, one of the most abundant pesticides in foods that acts on voltage-dependent sodium and chloride channels was studied. Its *in vitro* effects have been evaluated alone and in combination with ciguatoxins. The results obtained in this work reveal the presence of synergies between different food contaminants and the need to evaluate their synergies in detail to establish safe limits for their presence in foods, taking into account that many of them share the same molecular target and effectively guarantee the health of consumers.

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1.INTRODUCCIÓN

1.1 CONTEXTUALIZACIÓN

Los canales iónicos dependientes de voltaje son proteínas transmembrana cuya función es permitir el paso de iones a través de la membrana celular. En este trabajo se han empleado células, que expresan los canales de sodio dependientes de voltaje, para el estudio de distintos compuestos y se han visto los efectos de los mismos sobre los canales de cloro dependientes de voltaje.

El transporte de los iones a través de los canales es pasivo, dependiendo del gradiente. Los canales de sodio dependientes de voltaje se consideran unos de los más importantes ya que se encuentran distribuidos en las células de todos los tejidos del cuerpo y regulan funciones vitales como la contracción del corazón o la generación y transmisión de impulsos nerviosos. Además, estos canales son la diana celular de varios fármacos (anestésicos locales, analgésicos, antiarrítmicos, antiepilépticos) y neurotoxinas. En la presente tesis doctoral, se estudiaron los efectos de algunas moléculas cuya principal diana son los canales de sodio dependientes de voltaje, incluyendo compuestos naturales como las biotoxinas marinas, sus efectos *in vivo* e *in vitro* y productos sintéticos con la misma diana celular, como es el caso de los pesticidas piretroides.

El mar ocupa más de un 70% de la superficie de la Tierra, constituye el hábitat más extenso del planeta y alberga una gran biodiversidad constituyendo por lo tanto una fuente de recursos para el hombre, especialmente una gran reserva alimentaria que ha sido explotada desde hace siglos a través de actividades como la pesca o el marisqueo (EUMOFA 2023). La Unión Europea (UE) se configura como la octava potencia pesquera a nivel mundial, ya que produce 4,7 millones de toneladas anuales de productos pesqueros según las últimas estadísticas reportadas del año 2023 (EUMOFA 2023). En concreto, España genera el 20 % de la producción pesquera de la UE (OECD 2022a) y en cuanto a la acuicultura, España es el estado miembro dentro de la UE con una mayor captura, produciendo un 25,3 % del total. Europa y España son regiones en primera línea de producción pesquera lo que genera un interés creciente por la seguridad alimentaria por parte de las autoridades competentes a nivel internacional que incluyen la Organización de las Naciones Unidas para la Alimentación y la Agricultura (FAO), la Autoridad Europea de Seguridad Alimentaria (EFSA) y las administraciones nacionales como la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) y el Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA), así como de los consumidores.

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Por este motivo se requiere el desarrollo de normas cada vez más enfocadas a la seguridad, legalidad y calidad de los productos alimenticios (EFSA 2010; WHO 2020).

En el ámbito de los productos del mar, el principal riesgo que puede comprometer la seguridad alimentaria es la contaminación por biotoxinas marinas que dan lugar a intoxicaciones en humanos, con importantes efectos perjudiciales para la biodiversidad, la salud pública y la economía causando pérdidas millonarias (Botana 2014; Karlson et al. 2021). En décadas anteriores, la intoxicación en humanos por consumo de alimentos procedentes del mar contaminados con biotoxinas marinas era un fenómeno típico de áreas tropicales y subtropicales, sin embargo, en la actualidad se está produciendo un aumento en la frecuencia, intensidad y expansión geográfica de todos los microorganismos fitoplanctónicos productores de biotoxinas marinas, principalmente como consecuencia del aumento de la temperatura y cambios en la disponibilidad de nutrientes, entre otros factores (Gobler 2020; Griffith and Gobler 2020; Trainer et al. 2020; Wurtsbaugh et al. 2019; Zhang et al. 2022). Este incremento de la proliferación de organismos productores de toxinas marinas a nivel global, y en concreto a nivel europeo, requiere de estudios tanto *in vivo* como *in vitro* para obtener información sobre la toxicidad de las biotoxinas marinas emergentes en aguas europeas ya que en la actualidad, no todas ellas están reguladas en la legislación y tampoco se consideran los posibles efectos combinados de toxinas que actúan sobre la misma diana celular, lo cual puede representar una amenaza añadida para la seguridad alimentaria. Además, aun a pesar de que estos compuestos se conocen desde hace años, su modo de acción molecular ha sido poco estudiado y se desconocen muchos aspectos de su toxicidad. Las opiniones de la EFSA sobre las toxinas marinas recaban en todos los casos la necesidad de profundizar en la comprensión del mecanismo de toxicidad de la mayoría de las ficotoxinas, y en particular las ciguatoxinas (EFSA 2010).

Los fitosanitarios son ampliamente utilizados en el tratamiento de cultivos por los beneficios que producen. Sin embargo, su uso puede suponer riesgos para la salud humana por ingestión de los residuos presentes en los productos agrícolas y en el agua de bebida (Sun et al. 2023; Zaganas et al. 2013; Zhao et al. 2014). Aunque el límite máximo de residuos (LMR) para estos compuestos en alimentos está legislado en Europa, su uso sigue siendo habitual en otros países a pesar de los riesgos que pueden ocasionar en la salud (Zaganas et al. 2013). Los análisis rutinarios realizados por la autoridad competente, en el caso de Europa la EFSA, indica en sus opiniones que, en los alimentos, se encuentran residuos de pesticidas en cantidades superiores

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a los LMR establecidos en la legislación (EFSA 2015). Teniendo en cuenta que la ingesta diaria recomendada de alimentos incluye productos que pueden contener biotoxinas como pescados y moluscos dos veces por semana y una ingesta diaria de frutas y verduras, con riesgo de contener restos de pesticidas (Serra-Majem et al. 2020), el estudio de los efectos que puede tener la exposición a estos compuestos que comparten mecanismo de acción es importante para garantizar la seguridad de los consumidores.

1.2 COMPUESTOS DE ORIGEN NATURAL PRESENTES EN ALIMENTOS QUE ACTUAN SOBRE CANALES IÓNICOS DEPENDIENTES DE VOLTAJE

1.2.1 Moléculas de origen natural: origen, expansión y transmisión de biotoxinas marinas

La mayor parte de los organismos y la biomasa del océano son microscópicos y constituyen la base de todas las redes tróficas marinas. Estos organismos microscópicos se pueden dividir en zooplancton (heterótrofos) y fitoplancton (autótrofo). El fitoplancton, formado por organismos fotosintéticos de origen vegetal, se encuentra distribuido en las aguas de todo el planeta, particularmente en la parte superficial del agua, la cual recibe más luz y es más abundante en zonas costeras que en zonas oceánicas abiertas (Muñiz et al. 2018). Dentro de los organismos fitoplanctónicos se encuentran los dinoflagelados y las diatomeas, algunos de los cuales pueden producir metabolitos secundarios con actividad tóxica. Estos microorganismos son los productores primarios más importantes del océano, constituyendo el alimento natural de moluscos bivalvos filtradores (ostras, mejillones, almejas y vieiras, entre otros), de peces herbívoros y de larvas de diferentes organismos marinos, tales como moluscos y crustáceos, que a su vez nutren a otras especies depredadoras mayores. En el momento en el que la concentración de estas microalgas unicelulares alcanza una alta densidad, se llega a producir un cambio en el color del agua del mar (rojo, amarillo, marrón) dependiendo del tipo de microalga presente y de su concentración. En general, las proliferaciones de fitoplancton mantienen el ecosistema marino saludable y determinan su riqueza, que se pone de manifiesto, por ejemplo, con la producción pesquera. Sin embargo, en ocasiones lo que proliferan son microalgas nocivas con capacidad de generar sustancias toxicas como producto de su metabolismo secundario, cuya función dista de ser comprendida, pero que se postula que podrían servirles a los organismos productores como una forma de defensa contra un crecimiento desmesurado de otros organismos, para combatir la depredación o para luchar por los espacios (Botana et al. 1996). En estos casos se habla de floraciones de algas nocivas (FANs)

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o "harmful algal blooms" (HABs, por sus siglas en inglés). El fenómeno también es comúnmente conocido como marea roja ya que, en los casos más llamativos, tiñen el agua de un tono rojizo muy característico. Son numerosos los casos de floraciones de algas que han sido asociadas a intoxicaciones severas e incluso muerte de humanos, mamíferos marinos, aves y peces (Ben-Gigirey et al. 2021; Fernandez-Figares et al. 2013; Fernandez-Ortega et al. 2010; Klontz et al. 2009; Lasley-Rasher et al. 2016; Lydon et al. 2022).

El aumento de la temperatura del planeta y el consecuente aumento de la temperatura del agua del mar por causa del cambio climático son los principales factores que han contribuido a la expansión e intensificación de las FANs (Gamarro and Englander 2023). El agua de los océanos se calienta de promedio unos 0,6 °C por década (Fox-Kemper 2021). En la Figura 1 se recogen dos imágenes que ilustran el aumento de la temperatura de la superficie del mar con datos recogidos en mayo de 1982 y en mayo de 2022. Tal y como se aprecia en las imágenes, la temperatura de las aguas superficiales es mucho más elevada actualmente, especialmente notable en el mar Mediterráneo (https://data.marine.copernicus.eu/viewer/expert?view=viewer&crs=epsg%3A4326&t=750092400000&z=0&center=-0.12499999999995257%2C1.8705309005962837e-14&zoom=10.095398625787936&layers=W3siaWQiOiJjMCIsIm9wYWNpdHkiOjEsImxheWVySWQiOiJNVUxUSU9CU19HTE9fUEhZX1RTVVZfM0RfTVlOUlRfMDE1XzAxMi9kYXRhc2V0LWFybW9yLTNkLXJlcC13ZWVrbHlfMjAyMDEyL3RvIiwiekluZGV4IjoyMCwibG9nU2NhbGUiOmZhbHNlfV0%3D&basemap=dark&tWin=6&zWin=25).

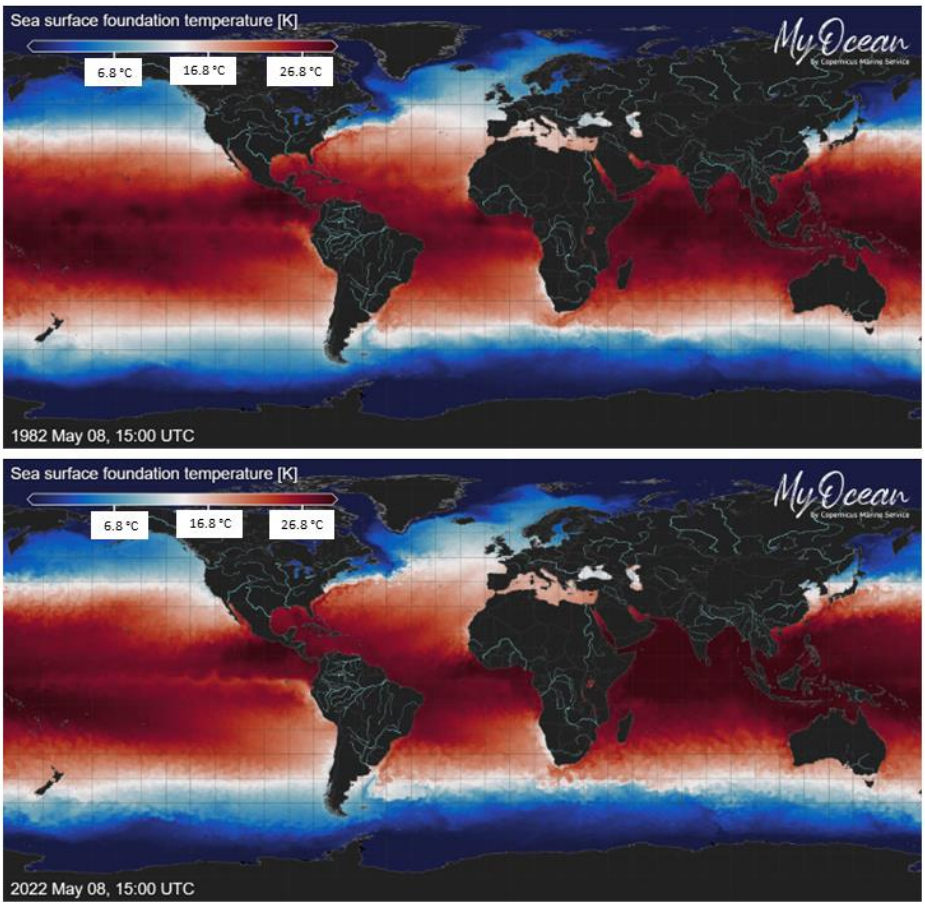


Figura 1. Temperatura de la superficie del agua de mar en mayo de 1982 y en mayo de 2022. Imagen realizada utilizando información del servicio marítimo de Copernicus E.U.

Como consecuencia directa del cambio climático en las últimas décadas, se ha constatado un aumento muy notable de la frecuencia y expansión espacial y temporal de las floraciones algales nocivas en las costas europeas (Fang et al. 2022; Karlson et al. 2021; Pörtner et al. 2019). De hecho, un estudio realizado por la Organización de las Naciones Unidas para la Educación, la Ciencia y la Cultura (UNESCO) donde se analizaron casi 10000 eventos de floraciones de algas nocivas en todo el mundo durante los últimos 33 años, muestra que los eventos de FANs registrados han aumentado. Durante los últimos años, el mayor número de registros de floraciones algales se realizó en Europa seguido del norte de Asia, el mar Mediterráneo, las costas de Norte América y por último del Caribe, ilustrando la alarmante situación de Europa y España ante este fenómeno (Dai et al. 2023; Hallegraeff 2021).

En concreto, en España el número de intoxicaciones por biotoxinas marinas en humanos registradas según la Base de Datos de Eventos de Algas Nocivas (HAEDAT) se ha incrementado de forma muy notable en las últimas décadas como se ilustra en la Figura 2. Entre

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los años 1990 y 2000 el número de intoxicaciones humanas por biotoxinas marinas reportadas en España fue de 61, en la siguiente década, entre los años 2000 y 2010 la cifra se triplicó, ascendiendo a 224. Esta tendencia ha continuado, aunque no tan pronunciada, en la siguiente década con un total de 249 intoxicaciones entre los años 2010 y 2020 pero una distribución más amplia por todas las costas del país. El incremento de los registros de FANs así como de las intoxicaciones producidas en humanos por consumo de productos del mar contaminados con biotoxinas marinas se debe también, en parte, al aumento de la monitorización y control y a los nuevos estudios que permiten diferenciar la sintomatología producida por estas toxinas de la ocasionada por otros compuestos.

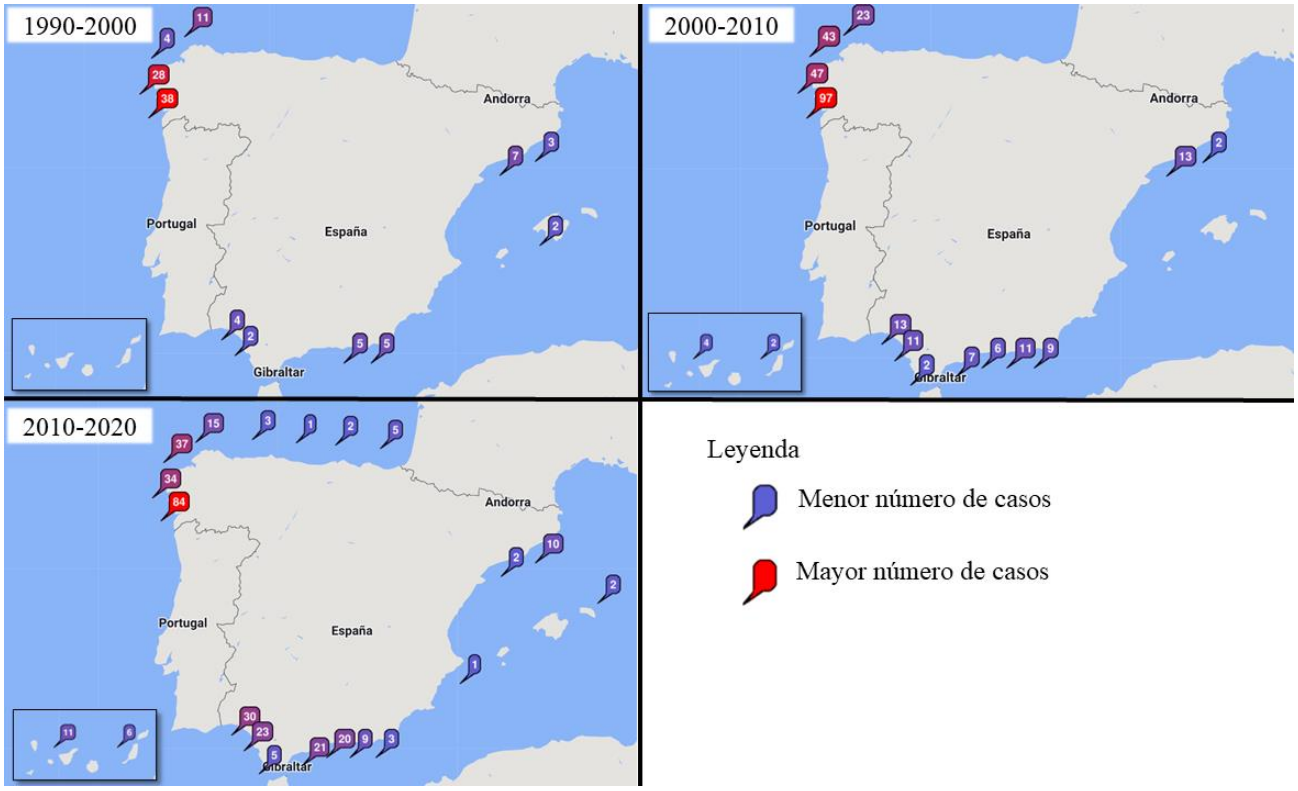


Figura 2. Intoxicaciones en humanos por biotoxinas marinas en España desde 1990 hasta 2020. Imagen generada utilizando datos de HAEDAT http://haedat.iode.org/.

Estas intoxicaciones se producen cuando las biotoxinas entran en la cadena trófica a través de organismos filtradores que las acumulan en sus tejidos (Figura 3) y no sufren sus efectos tóxicos. Los principales acumuladores de ficotoxinas son los moluscos bivalvos tales como mejillones, ostras, almejas y berberechos entre otros y en menor medida gasterópodos, crustáceos y peces herbívoros que se alimentan del fitoplancton productor y peces carnívoros que se alimentan de estos (Botana 2014). Las biotoxinas marinas se van acumulando a lo largo

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de la cadena trófica, por lo tanto, las mayores concentraciones pueden encontrarse en especies de mayor edad y tamaño.

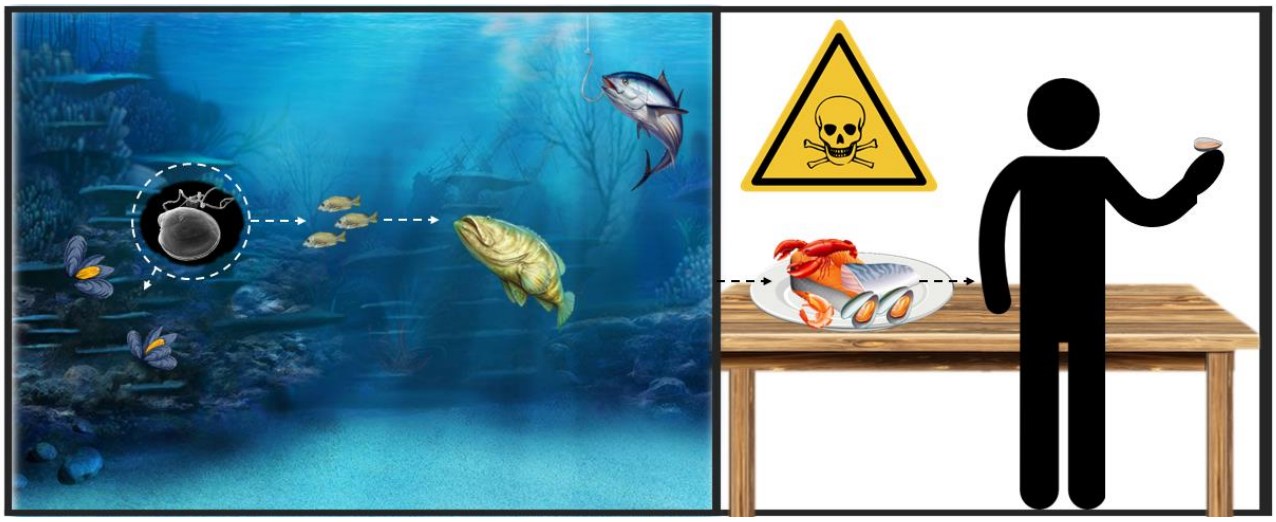


Figura 3. Biotoxinas marinas en la cadena trófica.

El mayor peligro de las biotoxinas marinas es que los organismos vectores las incorporan a los alimentos sin que se modifique ninguna de sus características organolépticas (olor, sabor, color, etc.), por ello sin un análisis de laboratorio es imposible detectar la contaminación de los alimentos por toxinas. Otro aspecto importante es que la mayoría de estas toxinas son termoestables, los procesos y las temperaturas habituales de cocinado de los alimentos no las inactivan (Warrell 2020). Por ese motivo, las intoxicaciones por consumo de mariscos y pescados que cursan con una sintomatología leve, en muchas ocasiones no se reportan como tal, y se realizan diagnósticos erróneos, llevando a una subestimación de la incidencia de estas intoxicaciones. En casos de intoxicaciones más graves, no existe un tratamiento específico ni antídoto para ninguna de ellas, lo único que se puede hacer es paliar los síntomas que producen hasta que sean eliminadas por el organismo (Louzao et al. 2022). Además, el control de toxinas en peces hace que su detección sea especialmente compleja debido a la naturaleza del producto, cuyo origen geográfico es difícil de trazar, y a que en la mayoría de los casos no hay materiales de referencia que permitan su análisis, por lo que se emplean métodos funcionales sin validar internacionalmente (Botana et al. 2012; Botana et al. 2009; Botana et al. 2017; Campbell et al. 2011; Greer et al. 2021; Louzao et al. 2022).

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1.2.2 Clasificación de biotoxinas marinas

Inicialmente, todas las biotoxinas marinas se clasificaron teniendo en cuenta los síntomas que producían en humanos como: intoxicación amnésica (ASP, amnesic shellfish poisoning), intoxicación neurotóxica (NSP, neurotoxic shellfish poisoning), intoxicación paralizante (PSP, paralytic shellfish poisoning), intoxicación diarreica (DSP, diarrheic shellfish poisoning) e intoxicación por ciguatoxina (CFP, ciguatera fish poisoning). Sin embargo, ante el avance de la investigación en este campo y el descubrimiento de nuevas toxinas, desde el año 2004 un informe de la FAO recomienda la clasificación de las toxinas marinas en función de su estructura química, que se resume en la Tabla 1 (FAO 2016).

Tabla 1. Resumen de los diferentes grupos de toxinas marinas, organismos productores y sus mecanismos de acción.

Grupo	Biotoxina	Microorganismo productor	Mecanismo de acción	Productos pesqueros implicados
Toxinas diarreicas (DSP)	Ácido ocadaico (AO), dinofisistoxinas (DTX) y pectenotoxinas (PTX)	*Dinophysis* *Prorocentrum*	OA y DTX: Inhiben fosfatasas PTX: Inhiben polimerización actina	Mariscos
Toxinas paralizantes (PSP)	Saxitoxina (STX)	*Alexandrium* *Gonyaulax* *Pyrodinium* *Cianobacterias*	Bloquean canales de sodio	Mariscos
Toxinas amnésicas (ASP)	Ácido domoico (AD)	*Pseudonitzschia*	Activan receptores de kainato	Mariscos y peces
Toxinas neurotóxicas (NTX)	Brevetoxinas 1, 2, 3 (BTX)	*Gymnodinium*	Activan canales de sodio	Mariscos
Toxinas ciguatera (CTX)	Ciguatoxina 1 (CTX)	*Gambierdiscus* *Fukuyoa*	Activan canales de sodio	Peces
Azaspirácidos (AZAs)	Azaspirácido 1-3 (AZA 1-3)	*Azadinium spinosum*	Desconocido	Mariscos

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Palitoxinas (PLTX)	Palitoxina (PLTX)	*Ostreopsis*	Altera Na^+/K^+ ATPasa	Mariscos
Yesotoxinas (YTXs)	Yesotoxina (YTX)	*Protoceratium Lingulodinium Gonyaulux*	Activan fosfodiesterasas	Mariscos
Tetrodotoxina (TTX)	Tetrodotoxina (TTX)	*Alexandrinium Vibrio Pseudomona*	Bloquea canales de sodio	Mariscos y peces
Iminas cíclicas (CI)	Gimnodimina, espirólidos (SPX)	*Alexandrium Karenia Gymnodinium Porocentrum*	Bloquean receptores nicotínicos	Mariscos

1.2.3 Legislación sobre biotoxinas marinas

Debido a la creciente expansión de las biotoxinas marinas y el consecuente incremento del riesgo de intoxicación alimentaria por las mismas, el control de los productos procedentes del mar constituye una preocupación cada vez mayor para las autoridades competentes, que cada vez demandan más información toxicológica sobre estos compuestos con el fin de proteger la salud pública y garantizar un nivel máximo de seguridad alimentaria. En términos generales, la gestión y regulación de las biotoxinas marinas en la cadena alimentaria se logra a través de la legislación y los controles rutinarios que se llevan a cabo. Los Laboratorios Nacionales de Referencia de la UE para Biotoxinas Marinas, coordinados por el EURLMB (por sus siglas en inglés EU Reference Laboratory for Monitoring of Marine Biotoxins), distribuidos por toda la Unión Europea, forman una red que lleva a cabo el control oficial de biotoxinas en moluscos bivalvos y otros alimentos de origen marino. El Laboratorio de Referencia de la UE para Biotoxinas Marinas tiene su sede en Vigo desde 1993 y el Laboratorio Nacional, recientemente designado, es el Intecmar (Vilaxoán, Pontevedra).

La actual normativa europea obedece al reglamento de higiene y seguridad alimentaria, Reglamento (CE) 853/04 por el que se establecen normas específicas de higiene en alimentos de origen animal (European Commission 2004). En éste se establecen los límites máximos de biotoxinas marinas en moluscos bivalvos vivos, exceptuando las yesotoxinas, cuyo límite máximo fue modificado por el Reglamento (UE) 786/2013 (European Commission 2013). Estos límites se recogen en la Tabla 2. No hay establecidos límites legales para el resto de biotoxinas marinas en Europa, pero sí se establece en el mismo reglamento que no se pueden poner en el mercado productos de la pesca que estén contaminados con ciguatoxinas, que son

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el principal objeto de estudio de esta tesis, ni comercializar partes procedentes de peces de las familias *Tetraodontidae*, *Molidae*, *Diodontidae* y *Canthigasteridae* (productores de tetrodotoxina). También se establecen los criterios para determinar tanto la seguridad de los alimentos puestos en el mercado por las empresas como la idoneidad de los procesos de producción llevados a cabo por los operadores en las industrias. Este reglamento constituye la base legislativa, a partir la cual se realiza la vigilancia y el control de la presencia de estos compuestos en los alimentos puestos en el mercado.

Tabla 2. Límites máximos de toxinas marinas establecidos para cada uno de los grupos de compuestos regulados en Europa por el Reglamento 853/2004 (y posteriores modificaciones) para moluscos bivalvos vivos (European Commission 2004).

Grupos de toxina	Límites máximos/kg de carne
Toxinas paralizantes	800 µg/kg
Toxinas amnésicas	20 mg/kg
Toxinas diarreicas	160 µg de equivalente de ácido okadaico/kg
Yesotoxinas	3,75 mg de equivalente de yesotoxina/kg
Azaspirácidos	160 µg de equivalentes de azaspirácido/kg
Toxinas neurotóxicas	Ausencia
Ciguatoxinas	Ausencia

El marco normativo europeo de las biotoxinas marinas se completa con el Reglamento (CE) 2019/627, que establece disposiciones para la realización de controles oficiales de los productos de origen animal destinados al consumo humano y que posteriormente fue modificado por el Reglamento (CE) 2021/1709 en lo que respecta a las disposiciones prácticas uniformes para la realización de controles oficiales de los productos de origen animal. Junto con estas disposiciones destaca también el Reglamento (CE) 852/2004 relativo a la higiene de los productos alimenticios, que establece la clasificación de las diversas zonas de recolección de los moluscos bivalvos. La Comisión del Codex Alimentarius tiene como objetivo principal proteger la salud de los consumidores y garantizar buenas prácticas en el comercio internacional de los productos alimenticios mediante el establecimiento de las normas recogidas en él. En este caso, se establece un nivel máximo para las brevetoxina (BTX-2) de 200 unidades de ratón

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(MU por sus siglas en inglés mouse units) equivalente a 800 µg/kg de pescado (Codex Alimentarius 2008).

En el caso de las ciguatoxinas, en Estados Unidos la Administración de Alimentos y Medicamentos (FDA, por sus siglas en inglés, Food and Drug Administration) estableció un límite recomendado de CTXs en pescado de 0,1 µg/kg del análogo caribeño C-CTX-1 y de 0,01 µg/kg del análogo pacífico P-CTX-1 (FDA 2020). A pesar de tratarse de un límite recomendado, la FDA podría emprender acciones legales si se sobrepasan, llevando a la retirada del producto del mercado. Esto implica que se permite una presencia 10 veces mayor de análogo caribeño con respecto al pacífico, asumiendo por lo tanto una potencia 10 veces mayor de este último. En este mismo documento se corroboran los límites máximos establecidos en el Codex Alimentarius para las BTXs en productos de la pesca (FDA 2020). En otros países como Japón y Méjico la regulación recoge que las ciguatoxinas pueden estar presentes en productos pesqueros en límites de hasta 0,025 MU/g (0,1 µg BTX-2/g de vianda) (Vilariño et al. 2021).

La evaluación y gestión de los riesgos asociados al consumo de biotoxinas marinas, conlleva la necesidad de conocer la cantidad de toxina presente en los productos del mar y la toxicidad relativa de cada uno de los análogos del grupo (Botana 2014). Esto se establece mediante el parámetro denominado factor de equivalencia tóxica (FET), definido como la toxicidad del compuesto objeto de estudio en relación a la de otro referente del mismo grupo con el que comparte el mismo mecanismo de acción. En la presente tesis doctoral se abordó el estudio de los FET del grupo de las ciguatoxinas y sus análogos, con el objetivo de poder definir la toxicidad relativa de cada uno de los compuestos y así estimar el riesgo potencial de la exposición a cada uno de ellos aisladamente o en combinación (FAO 2016). Esto es importante desde el punto de vista de la seguridad alimentaria ya que, en la mayoría de los casos, las personas no están expuestas únicamente a un compuesto tóxico, sino a una combinación de compuestos que pueden presentar un mecanismo de acción similar.

La transcendencia de los canales dependientes de voltaje justifica la investigación de compuestos que actúan sobre ellos alterando su funcionalidad. En la presente tesis doctoral se estudian compuestos tóxicos presentes en alimentos que pertenecen a distintos grupos de biotoxinas marinas y piretroides, que comparten la diana molecular. A continuación, se describen dos de los grupos de biotoxinas marinas estudiados en este trabajo. Por un lado, el grupo de las ciguatoxinas que compone la gran base de los estudios realizados y por otro, las

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brevetoxinas, cuyos efectos se han estudiado de forma complementaria al del grupo de las ciguatoxinas, teniendo en común ambos grupos los canales de sodio como diana molecular.

1.2.4 Ciguatoxinas

Las ciguatoxinas son potentes neurotoxinas liposolubles producidas por dinoflagelados de los géneros *Gambierdiscus* y *Fukuyoa.* Estos compuestos entrañan un peligro para la salud humana ya que se transfieren y transforman a través de las redes tróficas llegando a los consumidores.

Las microalgas productoras inicialmente generan compuestos menos potentes conocidos como gambierotoxinas, las cuales al entrar en la cadena trófica sufren procesos oxidativos y de isomerización dando lugar a numerosos análogos con diferentes propiedades y mayor toxicidad como son las ciguatoxinas (Ikehara et al. 2017; Lewis and Holmes 1993). Según su origen y estructura química, se han identificado tres grandes grupos de ciguatoxinas: ciguatoxinas del Mar Caribe (C-CTX), ciguatoxinas del Océano Índico (I-CTX) y ciguatoxinas del Océano Pacífico (P-CTX). Éste último grupo se subdivide en dos grupos, el de la CTX3C y sus análogos y el de la CTX4A y sus análogos, grupos caracterizados por las diferencias entre sus estructuras químicas. En la Figura 4 se resumen y agrupan de forma esquematizada los distintos grupos de ciguatoxinas según la clasificación del informe de la Organización de las Naciones Unidas para la Agricultura y la Alimentación y la Organización Mundial de la Salud (FAO/OMS) (WHO 2020) y se remarcan en negrita los principales análogos de la ciguatoxina estudiados en la presente tesis doctoral. Químicamente todas son grandes estructuras formadas por 13-14 poliéteres cíclicos que forman anillos unidos por enlaces éter que dan lugar a una estructura rígida en forma de escalera, aunque se desconocen las estructuras químicas del grupo índico.

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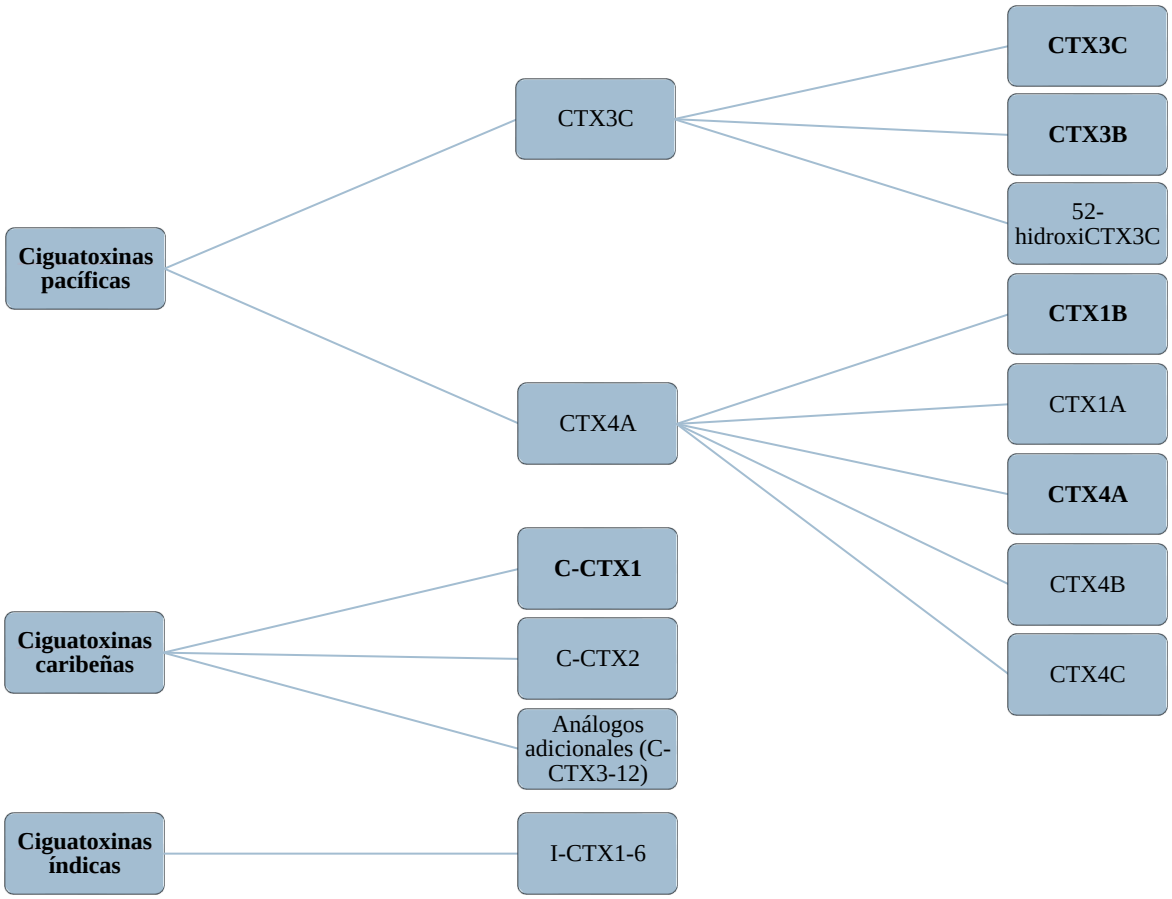


Figura 4. Clasificación esquemática de los grupos de las ciguatoxinas. Resaltados en negrita los análogos que se han estudiado en la presente tesis doctoral (WHO 2020).

El consumo de pescados y mariscos que contengan ciguatoxinas produce en las personas la intoxicación alimentaria conocida como ciguatera, una de las más ampliamente distribuidas a nivel mundial (Friedman et al. 2017; WHO 2020), endémica en muchas regiones, sobre todo tropicales y subtropicales. Más de 425 especies de peces se han relacionado con eventos de ciguatera, destacando los peces de arrecife como principales vectores de ciguatoxinas, entre ellos la barracuda (*Sphyraenidae*), el pez limón (*Seriola*), el mero (*Serranidae*), el pargo (*Lutjanidae*), los jureles (*Caranx spp*.) o la morena (*Muraenidae spp*.) entre muchos otros (WHO 2020). Además de peces, gran variedad de invertebrados marinos, entre los que se incluyen erizos, gasterópodos, bivalvos y equinodermos contienen CTXs, lo cual constituye una fuente adicional de intoxicación para los consumidores de productos del mar (WHO 2020).

La intoxicación por ciguatera se caracteriza porque incluye una gran variedad de síntomas, más de 175 descritos hasta el momento, entre los que se engloban síntomas neurológicos como parestesias, ataxia, dolor de cabeza, alteraciones de la percepción de la temperatura, síntomas

gastrointestinales como dolor abdominal, náuseas, vómitos y diarrea y, en casos más graves, pueden producirse alteraciones cardiovasculares, incluyendo hipotensión y bradicardia, aunque cabe destacar que la muerte por intoxicación por ciguatera es muy poco frecuente los síntomas pueden persistir en el tiempo (Friedman et al. 2017; L'Herondelle et al. 2020; WHO 2020). La sintomatología ocasionada por la ingestión de ciguatoxinas, para la cual no existe un tratamiento específico, suele comenzar entre 0,5 y 12 h tras la ingestión de pescado contaminado y su duración varía, remitiendo la sintomatología gastrointestinal en 1 o 2 días y las alteraciones cardiovasculares en 2 o 3 días. Sin embargo, la duración de las alteraciones neurológicas es variable y difícil de predecir pudiendo persistir desde 1 semana hasta años (Dickey and Plakas 2010; Friedman et al. 2007). Las ciguatoxinas actúan sobre el sitio 5 de los canales de sodio dependientes de voltaje, causando una hiperpolarización del voltaje de activación de los mismos (Lombet et al. 1987; Martin et al. 2014; Raposo-Garcia et al. 2022), lo cual produce la generación de potenciales de acción espontáneos en células excitables, desencadenando una sintomatología nerviosa (Nicholson and Lewis 2006).

Además de ciguatoxinas, las microalgas del género *Gambierdiscus* producen otro tipo de toxinas marinas, conocidas como maitotoxinas (MTXs), las cuales también están implicadas en la intoxicación por ciguatera (Traylor and Singhal 2023). Estos compuestos son poliéteres de elevado peso molecular. Actualmente, se han descrito 4 análogos de maitotoxinas: maitotoxina-1 (MTX1), maitotoxina-2 (MTX2), maitotoxina-3 (MTX3) y maitotoxina-4 (MTX4) (Holmes and Lewis 1994; Holmes et al. 1990; Pisapia et al. 2020). Su principal mecanismo de acción se asocia con un incremento de los niveles de calcio citosólico y una rápida muerte celular (Kutty et al. 1989; Reyes et al. 2014). Además, la toxina actúa sobre los canales de sodio causando una entrada de iones y una despolarización celular (Benoit et al. 1996). Históricamente la maitotoxina se ha considerado como una de las moléculas más tóxicas de la naturaleza con una letalidad tras la administración por vía intraperitoneal de 50 ng/kg en ratón (Murata et al. 1993). Sin embargo, debido a que estos primeros estudios fueron realizados sin identificar la pureza química de los compuestos administrados y sin realizar ninguna descripción de los procedimientos empleados en el bioensayo, junto con que en estudios posteriores se describió una amplia diversidad en el rango de dosis letales descritas para MTX1 (Legrand and Bagnis 1984; Munday et al. 2017; Murata et al. 1993; Murray et al. 2020; Reyes et al. 2014; Yokoyama et al. 1988), en la presente tesis doctoral se revaluaron estos datos.

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Las microalgas del género *Gambierdiscus* producen también otros compuestos como la gambierona y el gambierol ambos objetos de estudio de esta tesis, el gambieróxido y el ácido gambiérico. La contribución de todos estos compuestos a la intoxicación por ciguatera está aún por determinar ya que no han sido estudiados en profundidad hasta la fecha (Canals et al. 2021; Murray et al. 2020). Al igual que las ciguatoxinas, la gambierona actúa principalmente sobre el canal de sodio dependiente de voltaje, activándolo a potenciales de membrana negativos (Raposo-Garcia et al. 2022; Rodriguez et al. 2015) mientras que el gambierol actúa principalmente bloqueando los canales de potasio dependientes de voltaje (Kopljar et al. 2013). Sin embargo, este último también actúa como agonista parcial de canales de sodio dependientes de voltaje a concentraciones más elevadas (Louzao et al. 2006). Sintomatológicamente, el gambierol causa efectos similares a los de la ciguatoxina, lo que ha llevado a sugerir que este compuesto está también implicado en la intoxicación alimentaria por ciguatera (Ito et al. 2003).

Para la monitorización de los niveles de CTXs en productos de la pesca se han desarrollado una gran cantidad de métodos a lo largo del tiempo, desde pruebas *in vivo* (en desuso) hasta ensayos basados en el uso de células, ensayos de unión a receptores, cromatografía líquida de alta resolución acoplada a la espectrometría de masas (LC-MS, por sus siglas en inglés liquid chromatography–mass spectrometry) o inmunoensayos (Pasinszki et al. 2020). Entre todos estos, los métodos más ampliamente utilizados son los ensayos basados en el uso de células porque son rápidos, prácticos, baratos y evitan el problema de la falta de calibrantes analíticos certificados. En la parte *in vivo*, el bioensayo en ratón fue uno de los métodos más utilizados para evaluar la potencia relativa de las CTXs y para detectar su presencia en los alimentos (Bottein Dechraoui et al. 2005; Vernoux and Lewis 1997) a pesar de sus múltiples desventajas y consideraciones éticas (EFSA 2010). Uno de los principales problemas en el uso del bioensayo en ratón como método para detectar CTXs es que los datos obtenidos se derivan del establecimiento de la relación entre la dosis y el tiempo de supervivencia, generalmente después de la administración intraperitoneal del compuesto. Sin embargo, el efecto principal de la intoxicación por ciguatera no es la muerte, sino los síntomas que ocasiona a corto y largo plazo (WHO 2020).

La detección de CTX en alimentos se basa en la capacidad de las toxinas para unirse al sitio 5 de los VGSC, desencadenando la hiperpolarización celular (EFSA 2010). La viabilidad celular se analizó comúnmente en la línea celular de neuroblastoma de ratón Neuro2a (N2a) mediante el uso de 1-(4,5-dimetiltiazol-2-il)-3,5-difenilformazán (ensayo MTT). Sin embargo, estas

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toxinas marinas no tienen efecto citotóxico *per se* sobre las células y su detección requiere la adición del bloqueador de la bomba de sodio/potasio (Na^+/K^+ATPasa) ouabaína (O), junto con el activador de los canales de sodio veratridina (V). Bajo este tratamiento de ouabaína/veratridina (O/V) se potencia el efecto citotóxico de las CTXs permitiendo detectar su presencia en las muestras. Sin embargo, a pesar de usarse en bajas concentraciones, la ouabaína y la veratridina tienen un efecto tóxico propio sobre las células (Coccini et al. 2017; Viallon et al. 2020). No obstante, el ensayo MTT en células N2a es el método utilizado de forma rutinaria por la FDA para analizar pescados sospechosos de estar contaminados con CTXs (Friedman et al. 2008). Se han desarrollado otros métodos de detección basados en la medida de diferentes parámetros, como la despolarización de la membrana mediante indicadores fluorescentes (Louzao et al. 2004) o el uso de otras líneas celulares como el neuroblastoma humano SH-SY5Y (Coccini et al. 2017) consideradas más adecuadas debido a su origen humano en contraste con el origen murino de la línea celular N2a. El estudio y evaluación de las distintas metodologías empleadas para la detección de ciguatoxinas se abordarán en la presente tesis doctoral.

1.2.5 Brevetoxinas

Las brevetoxinas son neurotoxinas producidas por dinoflagelados marinos del género *Karenia brevis.* Químicamente son éteres policíclicos formados por diez u once anillos distribuidos en forma de escalera con una lactona en uno de sus extremos. Las brevetoxinas se dividen en dos grandes grupos, brevetoxinas tipo A (esqueleto flexible de 10 anillos poliéter) y tipo B (esqueleto rígido de 11 anillos poliéter) dentro de las cuales se engloban el resto de los análogos como se esquematiza en la Figura 5 (Hort et al. 2021).

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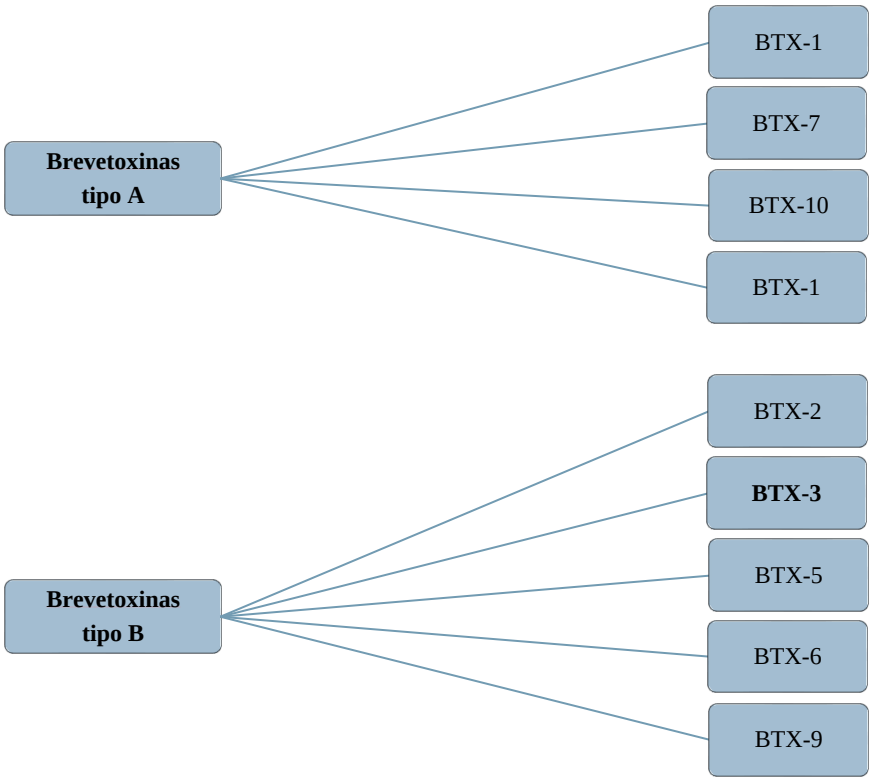


Figura 5. Clasificación esquemática de las brevetoxinas según su estructura química (Hort et al. 2021). Resaltado en negrita el análogo que se ha estudiado en la presente tesis doctoral.

El consumo de productos del mar que contengan brevetoxinas causa la intoxicación neurotóxica por consumo de molusco conocida como NSP. Las brevetoxinas comparten diana celular con las ciguatoxinas, el sitio 5 de canales de sodio dependientes de voltaje, donde inhiben su inactivación favoreciendo el flujo de iones Na^+ hacia el interior celular (Lombet et al. 1987). La consecuencia fisiológica de esto es la apertura permanente de los canales de sodio dependientes de voltaje a potenciales de reposo. La sintomatología es similar a la originada por la intoxicación por ciguatera, iniciándose ésta entre 1 y 3 horas después de la ingesta de la toxina e incluyendo síntomas neurológicos, gastrointestinales y/o cardiovasculares (Watkins et al. 2008). Gastrointestinalmente produce dolor abdominal, vómitos y diarrea. Los síntomas neurológicos incluyen parestesias, alteraciones en la percepción de la temperatura, vértigo y ataxia (Arnich et al. 2021). La muerte por intoxicación NSP no es frecuente, habitualmente la recuperación tiene lugar en 2-3 días tras la intoxicación con tratamiento sintomático (Watkins et al. 2008).

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Hasta el momento los datos sobre toxicidad oral aguda en animales eran muy limitados y no permitían determinar un nivel sin efectos adversos observados (NOAEL, por sus siglas en inglés no observed adverse effect level) o el nivel de efecto adverso observado más bajo (LOAEL, por sus siglas en ingles lowest observed adverse effect level). Del mismo modo, en humanos, los datos eran demasiado limitados para permitir el establecimiento de una dosis aguda de referencia. Sin embargo, recientemente varios estudios completos de toxicidad crónica han aportado datos de toxicidad a partir de los cuales se pudieron obtener estos parámetros y establecer niveles adecuados (Costas et al. 2023). Debido a la escasez de datos toxicológicos relacionados con la brevetoxina, la Agencia nacional de seguridad alimentaria de Francia (ANSES por sus siglas en francés Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail), reclamó estudios con el fin de obtener datos fiables que permitiesen establecer en el futuro niveles seguros para la presencia de estas toxinas en productos pesqueros ya que ni en Francia ni en el resto de Europa se encuentran reguladas y su presencia es cada vez mayor (Arnich et al. 2021). La ANSES propuso como método de detección de BTX en productos del mar una técnica de inmunoensayo ELISA (por sus siglas en ingés enzyme-linked immunosorbent assay) (ANSES 2021; Arnich et al. 2021; Flewelling et al. 2020). La BTX-3 se considera como biomarcador para monitorizar la presencia de toxinas neurotóxicas en mariscos, ya que se ha reportado su presencia en niveles elevados (Abraham et al. 2008). La misma agencia recomendó un nivel de 180 µg de BTX-3 eq/kg de carne de mariscos (Arnich et al. 2021), sin embargo, sigue remarcando la necesidad de estudios toxicológicos para asegurar el establecimiento de un nivel seguro y una regulación a nivel europeo. Además de casuar intoxicaciones en humanos, las brevetoxinas se han asociado a episodios de mortalidad masiva de peces, aves y mamíferos marinos suponiendo un peligro para los ecosistemas además de para la seguridad alimentaria (Fernández et al. 2022; Kirkpatrick et al. 2004).

1.3 COMPUESTOS SINTÉTICOS QUE ACTÚAN SOBRE CANALES DE SODIO DEPENDIENTES DE VOLTAJE: PESTICIDAS PIRETROIDES

La creciente preocupación del hombre por conseguir un aumento en la cantidad y calidad de las producciones agrícolas debido al aumento de la demanda mundial de alimentos propició el aumento del empleo masivo de pesticidas en las cosechas de forma rutinaria para el control de las plagas. Alrededor de un 47% del producto aplicado en suelos ingresa en el medio ambiente

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(Ramírez and Lacasaña 2001) y constituye una fuente de contaminación tanto para los sistemas bióticos (animales y plantas) como abióticos (suelo, agua y atmósfera) aumentando la exposición a estos compuestos y comprometiendo la salud humana. Los pesticidas ingresan en la cadena alimentaria acuática acumulándose en primer lugar en el plancton, posteriormente en pequeños peces acumulándose los residuos en sus tejidos y llegando a organismos mayores de la cadena trófica cuando éstos se alimentan de los pequeños (Banach et al. 2020; Lushchak et al. 2018). Los pesticidas también se pueden acumular en alimentos vegetales pasando directamente a los humanos o bien acumularse en los animales que los ingieren ingresando del mismo modo en la cadena alimentaria (Gupta and Gupta 2020; Katagi 2010; Sun et al. 2023; Zhao et al. 2014). Como se representa en la Figura 6, los pesticidas afectan a la salud humana por la contaminación de alimentos y agua debido a su uso generalizado y a su persistencia en el medio ambiente.

Durante las últimas décadas la exposición humana a pesticidas a través de los alimentos se ha incrementado, debido a factores que incluyen un aumento de su uso en la agricultura, el aumento en la temperatura de las aguas, cambios demográficos, la contaminación y las modificaciones en los hábitos alimenticios de las personas (Lu et al. 2019; Schreiner et al. 2016). Los pesticidas llegan tanto al agua dulce como a los ecosistemas marinos a través de la deposición atmosférica, la escorrentía de los ríos o las descargas de tratamiento municipal (Aznar-Alemany et al. 2017). Los piretroides se han encontrado de forma ubicua en el ambiente acuático marino (Moschet et al. 2017; Van Metre et al. 2017) y en aguas de ríos (Lu et al. 2019; Maguire 1992).

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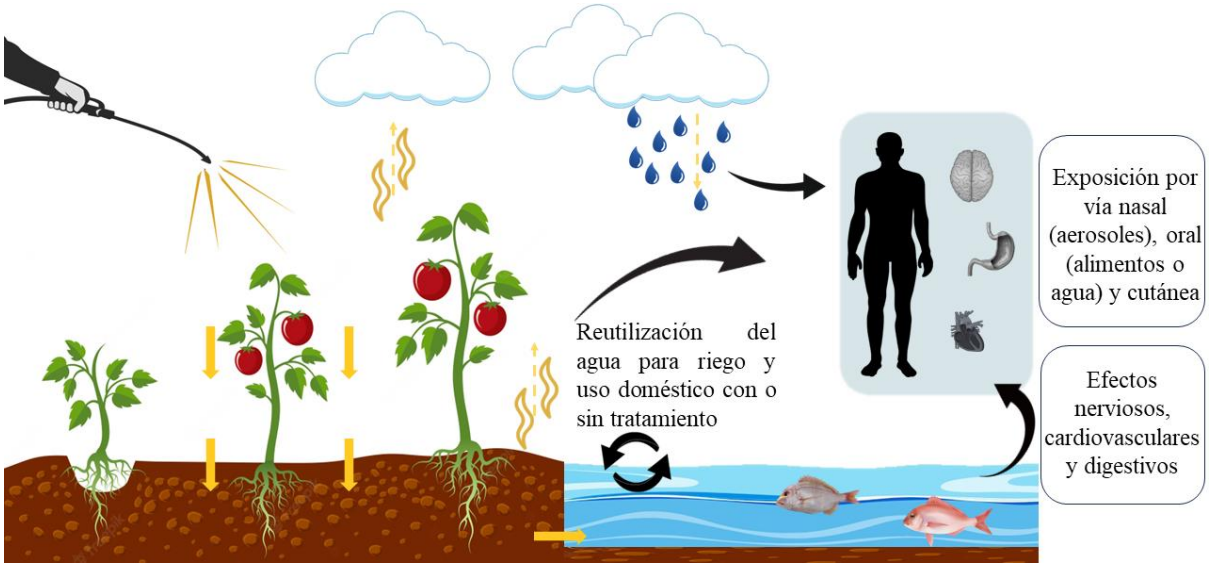


Figura 6. Piretroides en la cadena trófica, vías de exposición de piretroides en seres humanos (Bilal et al. 2019; Nowell 2019).

Los piretroides son pesticidas sintéticos cuyo mecanismo de acción es la interacción con los canales de sodio. El sitio de unión de los piretroides se encuentra en el segmento transmembrana seis de los dominios dos y tres, en la parte interna del poro (O'Reilly et al. 2014). Los piretroides se dividen en dos tipos, tipo I y tipo II, según la estructura del compuesto. La consecuencia funcional de la unión de estos compuestos a los canales de sodio es la prolongación del tiempo de apertura y la hiperpolarización del voltaje de activación del canal dando lugar a una despolarización prolongada de las células, lo cual en células excitables provoca hiper excitación y posterior bloqueo del impulso eléctrico (Nicholson and Lewis 2006). En el caso de los piretroides de tipo II la prolongación del tiempo de apertura de los canales de sodio dependientes de voltaje es mayor que tras la exposición a los piretroides tipo I (Narahashi et al. 1998). La deltametrina, objeto de estudio en esta tesis doctoral, es un insecticida piretroide tipo II muy utilizado tanto en agricultura como en el control de vectores en salud pública y animal (Chrustek et al. 2018; EFSA 2015; EFSA 2020). Aunque inicialmente se consideró un insecticida de baja toxicidad para los mamíferos, múltiples estudios han demostrado que, tras su ingesta oral, la deltametrina se absorbe de forma rápida causando efectos neurotóxicos ya que debido a su liposolubilidad atraviesa rápidamente la barrera hematoencefálica y entra en el sistema nervioso central (SNC) (Amaraneni et al. 2017), afecta al sistema inmune, reproductor, muscular y tiene efectos hepato y nefrotóxicos (Kaneko 2011; Khalatbary et al. 2016; Sharma et al. 2014; Tewari et al. 2018).

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En el año 2000, en la Reunión Conjunta del Panel de Expertos en Residuos de Plaguicidas en los Alimentos y el Medio Ambiente de la FAO y WHO se calculó un valor de ingesta diaria admisible (IDA) para la deltametrina de 0.01 mg/kg (McGregor 2000), valor determinado en base al NOAEL de 1 mg/kg peso, obtenido por la EFSA y la U.S. EPA (United States Environmental Protection Agency) en base a estudios de toxicidad crónica que identificaron como punto crítico para evaluar el riesgo de toxicidad de este compuesto el descenso de la actividad motora obtenido en dos estudios en perros tratados con deltametrina por vía oral, de 90 días y un año de duración, respectivamente (EFSA 2009).

Por los riesgos que entraña la exposición humana a piretroides se establecieron en la legislación europea (Reglamento CE 396/2005) unos límites máximos de residuos permitidos en alimentos. Sin embargo, los informes de la EFSA indican que se encuentran residuos de deltametrina en los alimentos en cantidades superiores al LMR establecido (EFSA 2015). Esta problemática es muy relevante ya que el uso de algunos de estos pesticidas está prohibido en la UE por el peligro que entrañan para la salud humana (EFSA 2015; EFSA 2020). De hecho, se ha propuesto el uso de principios farmacológicos tales como su potencia sobre la diana celular para establecer un LMR que garantice la salud del consumidor y el control de plagas (Connolly et al. 2022).

Una dieta equilibrada incluye el consumo recomendado de pescado y productos del mar al menos 2 veces por semana así como el consumo diario de fruta, verdura y carne, existiendo el riesgo de exposición simultánea a múltiples contaminantes de estos grupos de alimentos y hasta el momento, existe una carencia absoluta de datos sobre el posible efecto aditivo que pueden tener las combinaciones de contaminantes biológicos (toxinas marinas) y contaminantes químicos (pesticidas) que comparten el mismo mecanismo de acción ingeridos habitualmente en la dieta. Esto es un hecho importante para garantizar la salud de los consumidores y evitar la aparición de alteraciones crónicas como pueden ser las enfermedades endocrinas (Kumar et al. 2020; Sirohi et al. 2021) o neurodegenerativas (Li et al. 2021; Zhang et al. 2021), por ello en la presente tesis doctoral se abordó por primera vez el efecto de la presencia de biotoxinas marinas y pesticidas sobre canales iónicos, imprescindibles para el funcionamiento celular y que se describen a continuación.

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1.4 CANALES IÓNICOS DEPENDIENTES DE VOLTAJE

Los canales iónicos dependientes del voltaje son proteínas transmembrana que responden a cambios de potencial de membrana permitiendo el paso selectivo de iones. Los canales iónicos son imprescindibles y están presentes en todas las células del organismo ya que regulan funciones como la contracción muscular, participan en múltiples vías de señalización y en la generación y propagación de impulsos nerviosos entre otras muchas. En concreto, en esta tesis doctoral se evaluarán compuestos cuya principal diana molecular son los canales de sodio y los canales de cloro dependientes de voltaje en los que se centra esta sección.

1.4.1 Canales de sodio dependientes de voltaje

Los canales de sodio dependientes de voltaje se consideran una de las familias de canales iónicos más importantes debido a su notable papel fisiológico en el potencial de reposo y la generación y propagación de potenciales de acción. Estas proteínas están formadas por una subunidad α grande (260 kDa) y una o dos subunidades β más pequeñas (30 – 40 kDa) (Catterall et al. 2020).

La subunidad α del canal de sodio está compuesta por aproximadamente 2000 residuos de aminoácidos organizados en cuatro dominios homólogos (DI – DIV) que constituyen la estructura del poro central, cada dominio contiene seis segmentos transmembrana (Catterall 2014; Catterall et al. 2020). El segmento S4 de cada dominio contiene el sensor de voltaje del canal, cambiando su conformación al detectar los cambios en el voltaje. Entre los segmentos S5 y S6 se encuentra un segmento aminoacídico extracelular que puede ser alterado por distintos contaminantes alimentarios. Las subunidades β del canal de sodio están compuestas por un pliegue extracelular con un extremo N-terminal, un único segmento transmembrana y un segmento intracelular corto como se representa en la Figura 7.

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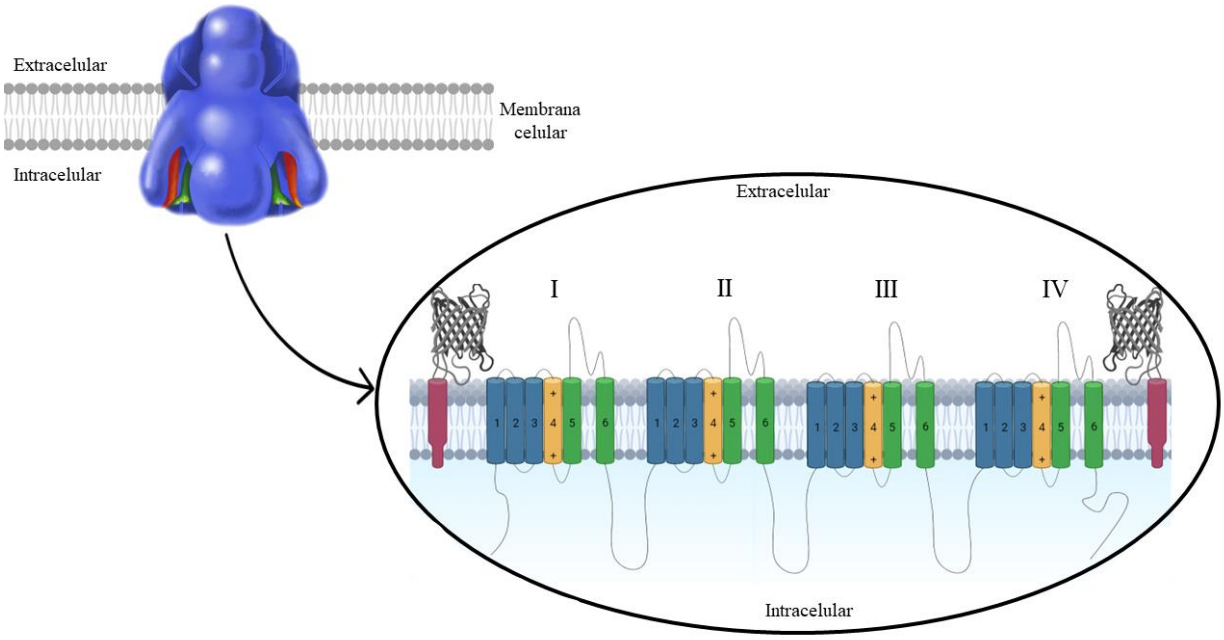


Figura 7. Representación adaptada de la arquitectura tridimensional del canal de sodio dependiente de voltaje humano(Sato et al. 2001) y desglose de las subunidades a y ß del canal de sodio humano formado por cuatro dominios transmembrana indicados con números romanos cada uno con 6 segmentos transmembrana. El segmento S4 contiene el sensor de voltaje representado en amarillo. Las dos subunidades beta se representan como pliegues extracelulares.

Hasta la fecha, se han identificado nueve isoformas distintas de la subunidad α de los canales de sodio ($Na_v1.1$-$Na_v1.9$) que varían en su localización en el organismo (Catterall et al. 2005). Esta distribución diferencial de los subtipos de canales de sodio sugiere que tienen funciones distintas, relevantes en el mantenimiento de la funcionalidad del organismo (Whitaker et al. 2000). Así, $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ y $Na_v1.6$ predominan en el sistema nervioso central, mientras que en el sistema nervioso periférico se expresan principalmente las isoformas $Na_v1.7$, $Na_v1.8$ y $Na_v1.9$. Los subtipos $Na_v1.4$ y $Na_v1.5$ se encuentran en mayor cantidad en el músculo esquelético y en el corazón, respectivamente (Vacher et al. 2008; Wang et al. 2017). En la presente tesis doctoral todos los estudios se han realizado empleando canales de sodio con la subunidad alfa $Na_v1.6$ debido a su amplia distribución en el sistema nervioso central y el papel crucial que estos desempeñan en el inicio y transmisión del impulso nervioso (Wang et al. 2017). En la Tabla 3 se recoge la distribución tisular y las principales funciones fisiológicas de los principales subtipos de la subunidad α de los canales de sodio dependientes de voltaje.

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Tabla 3. Distribución tisular y funciones fisiológicas de las distintas subunidades a de los canales de sodio dependientes de voltaje en el sistema nervioso central (SNC) y el sistema nervioso periférico (SNP) (Alexander et al. 2021).

Subunidad α	Localización	Función
$Na_v1.1$	SNC (soma neurona) SNP	Inicio de potenciales de acción e integración de señales eléctricas
$Na_v1.2$	SNC (axones no mielinizados)	Conducción del potencial de acción
$Na_v1.3$	SNC (soma neurona)	Control de la excitabilidad neuronal
$Na_v1.4$	Músculo esquelético	Control de la excitabilidad de los miocitos esqueléticos
$Na_v1.5$	Corazón	Control de la excitabilidad de los miocitos cardiacos
$Na_v1.6$	SNC (Soma y dendritas celulares, tronco y médula espinal)	Inicio y transmisión de potenciales de acción
$Na_v1.7$	SNP y células neuroendocrinas	Inicio y conducción del potencial de acción (implicado en dolor neuropático)
$Na_v1.8$	SNP	Fase despolarizante de potenciales de acción en neuronas de la raíz espinal dorsal
$Na_v1.9$	SNP	Despolarización, incremento de excitabilidad

La subunidad α de los canales de sodio es la diana molecular para distintos compuestos que son objeto de estudio en esta tesis doctoral, donde los efectos producidos por contaminantes alimentarios que les afectan se han analizado. Hasta el momento se han identificado cinco principales sitios de unión de toxinas, actuando cada compuesto sobre un sitio específico del canal como se ilustra en la Figura 8. Cabe destacar que algunos compuestos como los tóxicos sintéticos (insecticidas piretroides, DDT) o anestésicos locales no actúan sobre los mismos sitios de unión de las toxinas, sino sobre otras partes del canal donde no actúan los compuestos naturales. En concreto en esta tesis nos centramos en el estudio de toxinas liposolubles que se unen al sitio 5 del canal (S5 del dominio IV), como son las ciguatoxinas y las brevetoxinas. La unión de estas toxinas naturales al canal causa la activación a potenciales de membrana más negativos, así como una disminución en la intensidad máxima de la corriente de entrada de sodio para las ciguatoxinas (Martin et al. 2014; Raposo-Garcia et al. 2022), mientras que no se han observado efectos significativos causados por la BTX en este parámetro (Sheridan and Adler 1989; Yates et al. 2021). Por otra parte, el lugar de unión de los piretroides contaminantes alimentarios, como la deltametrina, se encuentra en el segmento 6 de los dominios II y III (O'Reilly et al. 2006).

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En la Figura 8 se representan compuestos tóxicos y su sitio de unión a los canales de sodio, en algunos de los cuales no se entra en detalle ya que no atañen al objetivo principal de esta tesis doctoral, pero comparten diana celular con los estudiados (Cestele and Catterall 2000; Stevens et al. 2011).

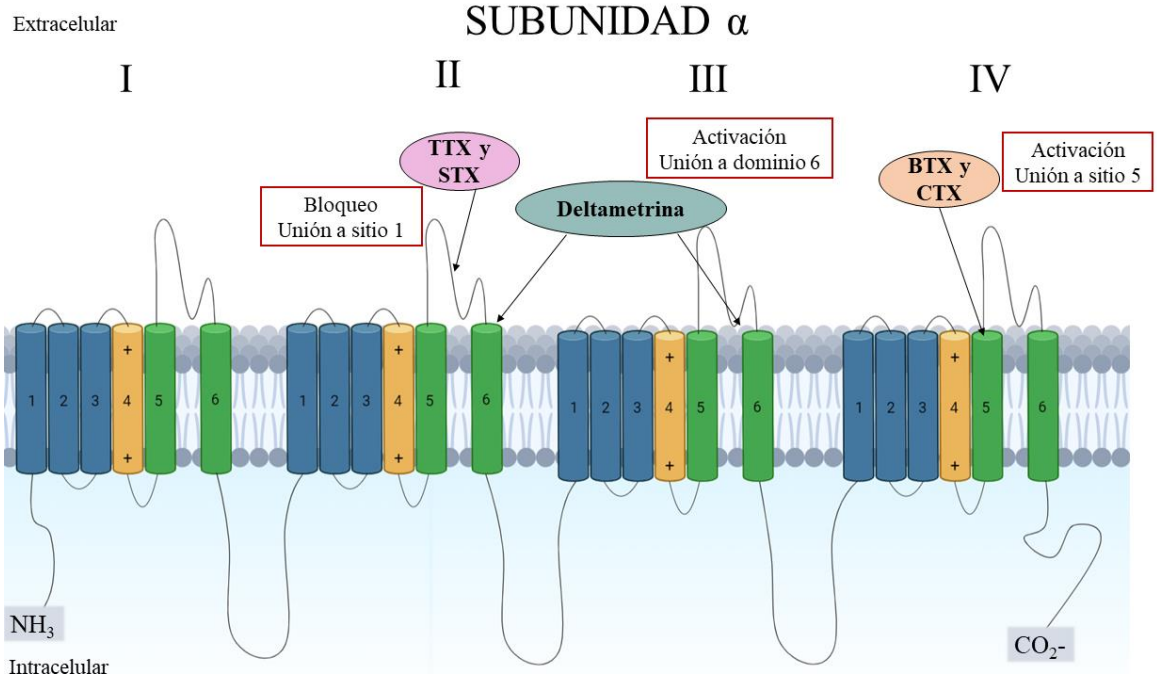


Figura 8. Representación esquemática de la estructura de la subunidad a de los canales de sodio dependientes de voltaje y de los lugares de unión de diferentes compuestos a los mismos.

Los canales de sodio dependientes de voltaje son los encargados de la generación y propagación del potencial de acción. El canal de sodio varía entre tres estados funcionales: reposo (cerrado), activado (abierto) e inactivo (no responde). La activación del canal ocurre cuando hay una despolarización en la membrana celular que provoca un cambio de los residuos cargados positivamente en los segmentos S4 hacia el lado extracelular de la membrana. Este cambio conformacional en el sensor de voltaje abre el poro que mantenía el canal en estado de reposo permitiendo el paso de iones de sodio a su través. Tras unos milisegundos el canal de sodio se inactiva y sufre otro cambio conformacional asociado en este caso al bucle intracelular que conecta los dominios III y IV, bloqueando el poro del canal, impidiendo la entrada de iones y llevando así al canal a su forma inactiva (Catterall 2014). En el estado inactivo, los canales no responderán a estímulos, no podrán volver a abrirse hasta que la célula se repolarice a un potencial de reposo que varía según el tipo de célula.

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1.4.2 Activación dependiente de voltaje de los canales

La dependencia de voltaje de la activación de los canales de sodio fue demostrada por primera vez por Hodgkin y Huxley al realizar estudios empleando el axón gigante de calamar, donde observaron que la activación de los canales de sodio requería el movimiento de partículas cargadas eléctricamente a través de la membrana celular (Hodgkin and Huxley 1952). La activación dependiente de voltaje depende del segmento S4, que como ya hemos mencionado constituye el sensor de voltaje, que detecta los cambios en el potencial de membrana. Independientemente de los movimientos conformacionales que sufre el canal, el S4 se encuentra siempre en una posición transmembrana tanto en el estado de reposo como en el estado activado (Catterall 1979; Catterall 1986; Cestele et al. 1998).

1.4.3 Inactivación rápida y lenta del canal de sodio

Los canales de sodio se abren en respuesta a una despolarización y 1-2 ms después se inactivan. La inactivación del canal de sodio es un proceso crítico que ocurre tras unos milisegundos de la apertura del canal, y es necesaria para el control de la excitabilidad en las células nerviosas y musculares con el fin de evitar la hiper excitabilidad (Cestele and Catterall 2000). Los canales de sodio presentan dos tipos de inactivación las cuales difieren en duración y mecanismo. El bucle intracelular que conecta los dominios III y IV de la subunidad α del canal de sodio es responsable de la inactivación rápida que dura unos milisegundos, sirviendo como partícula de bloqueo intracelular, ya que se pliega en la estructura del canal y bloquea el poro (Stuhmer et al. 1989; Vassilev et al. 1988). La estimulación repetitiva o prolongada de las células, puede resultar en una inactivación lenta de los canales de sodio que puede llegar a durar segundos. Durante la inactivación lenta, los sensores de voltaje experimentan un movimiento adicional, y dos de los segmentos S6 opuestos se mueven hacia el eje central del poro. Esta asimetría provoca un colapso estructural del canal que no responderá a estímulos hasta que la célula se repolarice (Ghovanloo et al. 2016). En la Figura 9 se resume el funcionamiento de los canales de sodio dependientes de voltaje.

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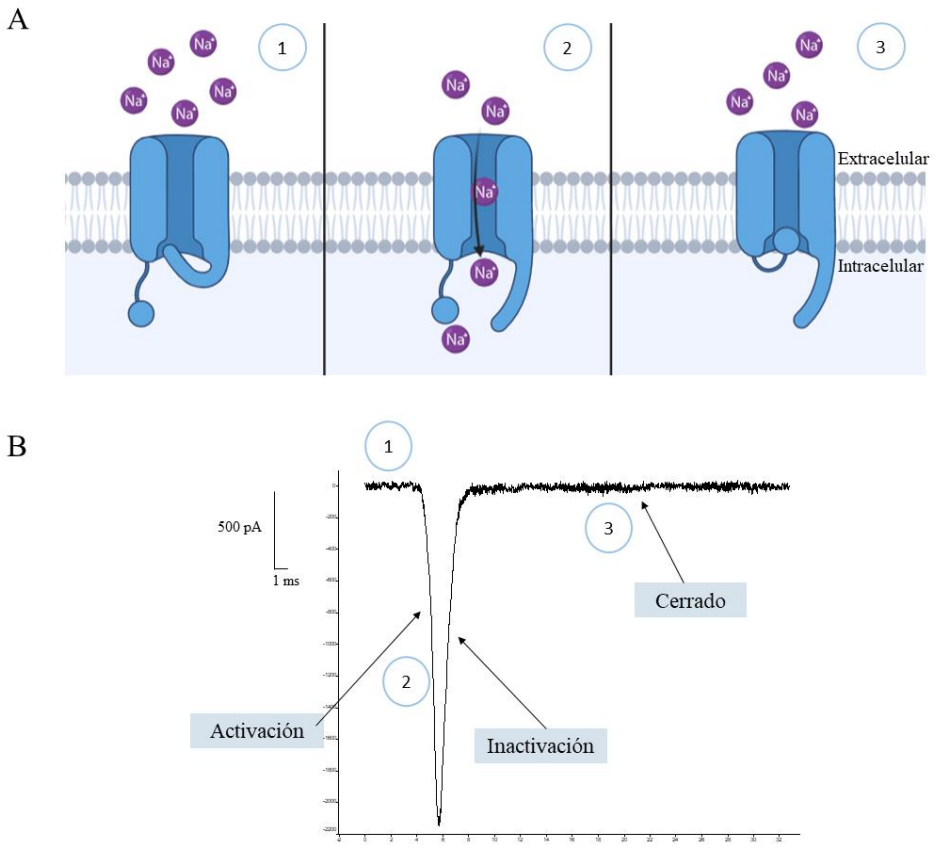


Figura 9. Canales de sodio dependientes de voltaje y su funcionamiento. A. Representación esquemática del funcionamiento del canal de sodio dependiente de voltaje. B. Representación de la intensidad de la corriente de entrada de sodio. Los canales de sodio están cerrados en estado de reposo a potenciales de membrana de -70 mV (1). Los canales se activan en respuesta a una despolarización de la membrana celular que supere el umbral, generando corrientes de entrada de sodio (2). Después de unos milisegundos, el canal se inactiva y posteriormente se recupera de la inactivación volviendo su conformación cerrada (3).

1.4.4 Canales de cloro dependientes de voltaje

Los canales de cloro están presentes en todos los tipos de células, con gran variedad de funciones fisiológicas entre las que destacan la regulación del volumen celular, el mantenimiento de la homeostasis iónica y la regulación de la excitabilidad celular (Martinez and Mohiuddin 2023). Existen canales de cloro localizados en la membrana plasmática, mientras que otros miembros de esta familia se distribuyen en orgánulos y vesículas intracelulares. Previamente ha sido demostrado que las alteraciones en la homeostasis de la concentración intracelular de Cl^- (además de la de Na^+) causa alteraciones importantes de la excitabilidad alterando el mantenimiento del potencial de reposo y la hiperpolarización (Berndt

et al. 2011; Currin et al. 2020; Sorensen et al. 2017). En esta tesis doctoral se aborda el grupo de canales unidos a la membrana plasmática.

Existen 5 familias de canales de cloro; canales de cloruro regulados por AMPc, canales de cloruro activados por calcio (CaCC), canales de cloruro dependientes de voltaje (ClC), canales cloruro regulados por ligando (activados por GABA (ácido γ aminobutírico), glicina, β-alanina y taurina) y canales de cloruro regulados por volumen (Martinez and Mohiuddin 2023). Durante el desarrollo de la investigación de los efectos de los distintos contaminantes alimentarios sobre canales iónicos dependientes de voltaje, se han observado efectos sobre los ClC. En mamíferos, hasta la fecha se han descrito 9 ClC diferentes, cuatro de ellos se expresan y actúan como canales en la membrana plasmática, cuya distribución y principal función se resume en la Tabla 4 y los otros cinco que se localizan principalmente en membranas intracelulares y actúan como intercambiadores de Cl^-/H^+ (Poroca et al. 2017).

Tabla 4. Distribución y función de los canales de cloro dependientes de voltaje localizados en la membrana plasmática de las células. Adaptado de (Poroca et al. 2017).

Canal de cloro dependiente de voltaje de la membrana celular	Localización	Función
ClC 1	Músculo esquelético	Recuperación del potencial de membrana en reposo
ClC 2	Cerebro; riñón; hígado; corazón; páncreas; músculos esqueléticos; pulmones y tracto gastrointestinal	Homeostasis de iones intra y extracelular
ClC Ka	Oído interno; Riñón	Transporte transepitelial
ClC Kb	Oído interno; Riñón	Transporte transepitelial

Los canales ClC son dímeros formados por dos subunidades idénticas de 18 hélices transmembrana, cada una de las subunidades está formada por dos mitades que atraviesan la membrana con orientaciones opuestas y que se agrupan formando en cada subunidad un poro central (Jentsch and Pusch 2018; Ludewig et al. 1996; Middleton et al. 1996; Weinreich and Jentsch 2001). Esta arquitectura antiparalela permite la selectividad al ion Cl^- (Dutzler 2004b; Stolting et al. 2014).

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Los ClC se abren en respuesta a una despolarización, sin embargo, tienen un comportamiento de activación complejo en la que intervienen también otros mecanismos como la concentración de iones o el pH (Dutzler 2004a). Los ClC participan en importantes funciones fisiológicas como la regulación de la excitabilidad eléctrica de la membrana, el manteniendo del potencial de reposo o la regulación del pH entre muchas otras, por ello, cualquier compuesto que cause una alteración en estas corrientes llevará a una alteración de la excitabilidad celular, que, junto con los efectos producidos por los contaminantes alimentarios que afectan a la funcionalidad de los canales de sodio dependientes de voltaje, ponen en riesgo la salud de los consumidores.

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2 . OBJETIVOS

Dada la amplia distribución en el organismo y la importancia de los canales iónicos dependientes de voltaje, cualquier sustancia que actúe sobre ellos modificando su funcionalidad puede conllevar importantes repercusiones en la salud. Muchas de las sustancias que actúan sobre estos canales entran al organismo a través del agua y los alimentos, por ello la compresión de sus mecanismos de toxicidad es un factor crucial. Actualmente, factores como el aumento de la demanda de alimentos y la globalización, la contaminación ambiental, el cambio climático o los cambios en los hábitos alimenticios aumentan el riesgo de sufrir una intoxicación alimentaria. Dentro de éstas, el riesgo de intoxicación por consumo de biotoxinas marinas emergentes es cada vez mayor en Europa, siendo la intoxicación por ciguatera la principal causa de intoxicación alimentaria, no bacteriana, por consumo de pescado a nivel mundial. La escasez de información toxicológica junto con su elevada frecuencia pone de manifiesto la necesidad de nuevos estudios. Sin embargo, la falta de material de referencia y calibrantes certificados dificulta estas tareas. Otro de los retos a los que se enfrenta en la actualidad la seguridad alimentaria es el notable incremento del uso de pesticidas y productos químicos en las cosechas buscando obtener una mayor rentabilidad, sin tener en cuenta que los residuos de estos compuestos se depositan y acumulan en los alimentos y el medio ambiente y suponen una fuente de contaminación. Algunas biotoxinas marinas junto con otros contaminantes alimentarios comparten como diana celular los canales de sodio dependientes de voltaje. Los riesgos que implica el consumo de sustancias contaminantes de alimentos para la salud humana han sido siempre evaluados por separado, no teniendo en cuenta posibles efectos conjuntos de diferentes compuestos que comparten la misma diana celular tras la ingesta de una dieta equilibrada. En este contexto, en la presente tesis doctoral se pretendió establecer la posible relación de sinergias o antagonismos entre estos compuestos con el fin último de proporcionar datos *in vivo* e *in vitro* que permitan establecer regulaciones y límites seguros para su presencia en alimentos.

El objetivo general de esta tesis se centró en evaluar los riesgos para la seguridad alimentaria que supone el consumo en la dieta por separado y en conjunto de contaminantes alimentarios que actúan sobre canales iónicos dependientes de voltaje. En el primer bloque de resultados se presentan datos toxicológicos sobre las biotoxinas marinas implicadas en la intoxicación por ciguatera como son los distintos análogos de las ciguatoxinas, gambierol, gambierona y maitotoxinas. Todos estos compuestos tienen en común que actúan sobre los canales de sodio dependientes de voltaje. En estos estudios se determinaron las diferencias entre los análogos

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pacíficos y caribeño de las ciguatoxinas que hasta el momento habían sido muy poco estudiados, además de establecer factores de equivalencia tóxica para distintos análogos pacíficos de la ciguatoxina y para otras toxinas que como mencionamos anteriormente también se encuentran implicadas en la intoxicación por ciguatera (gambierol, gambierona y MTX3). Estos datos de toxicidad *in vitro* han sido completados con estudios *in vivo* de toxicidad aguda y subcrónica de ciguatoxinas y los compuestos relacionados con la intoxicación por ciguatera. Dado que no existe una regulación específica sobre cómo se han de realizar los estudios de toxicidad *in vivo* con toxinas marinas, en esta tesis se emplearon los protocolos internacionalmente recomendados por la OECD para la determinación de la toxicidad de los compuestos químicos anteriormente mencionados en el apartado de métodos (OECD 2008; OECD 2022b). Además, en la presente tesis doctoral se reevalúa el método MTT (bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolio) de detección de ciguatoxinas en la línea celular de neuroblastoma de ratón N2a basado en el uso de ouabaína y veratridina para potenciar la toxicidad de las ciguatoxinas, ya que a pesar de ser el método más comúnmente empleado y recomendado para la detección de estas toxinas, todavía existe cierto desacuerdo en las condiciones en las que se debe realizar el ensayo y una gran variabilidad en los resultados obtenidos por los distintos laboratorios por lo que este método no ha llegado a ser validado ni estandarizado.

A continuación, en un segundo bloque de resultados se describen los efectos combinados de ciguatoxinas junto con otras moléculas presentes en alimentos que actúan sobre la misma diana celular, los canales de sodio dependientes de voltaje. Se estudian las sinergias entre compuestos que actúan con el mismo mecanismo de acción sobre las mismas dianas celulares como son brevetoxinas y ciguatoxinas, dos grupos de compuestos naturales que aparecen como contaminantes de productos del mar y entre las ciguatoxinas y la deltametrina, una molécula sintética que pertenece al grupo de los piretroides, aparece como contaminante en los alimentos y actúa sobre los canales de sodio dependientes de voltaje.

Objetivos específicos

Para conseguir el objetivo general se plantearon los siguientes objetivos específicos junto con la publicación en la que se encuadran:

- Determinar las diferencias que existen en el modo de acción entre los análogos pacíficos y caribeños de las ciguatoxinas.

- In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins

- Establecer factores de equivalencia tóxica para los compuestos implicados en la intoxicación por ciguatera.
  - Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels.

- Determinar los efectos agudos y subcrónicos de la ingestión de ciguatoxinas y compuestos implicados en la intoxicación por ciguatera: maitotoxina, gambierol y gambierona.
  - *In vivo* subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity.

- Reevaluar el método empleado para la detección de las ciguatoxinas y proponer posibles alternativas.
  - Mouse N2a neuroblastoma assay: Uncertainties and comparison with alternative cell-based assays for ciguatoxin detection

- Estudiar la existencia de posibles sinergias entre distintos compuestos que actúan sobre canales de sodio.
  - Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins.
  - Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C in human voltage-gated $Na_v1.6$ sodium channels.

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3 . MÉTODOS

Para el desarrollo de la presente tesis doctoral se han combinado múltiples metodologías que se resumen en la Figura 10.

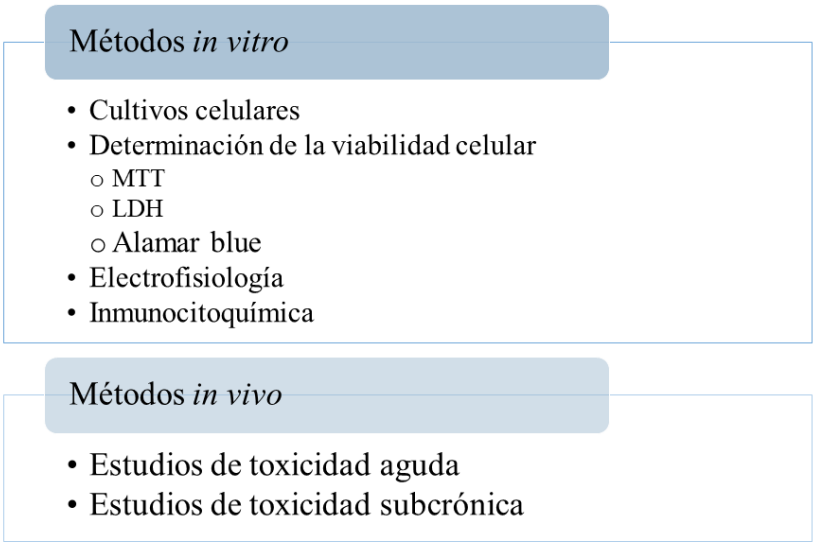


Figura 10. Resumen de la metodología empleada.

3.1 LÍNEAS CELULARES PARA ESTUDIOS *IN VITRO*

Los ensayos *in vitro* se han llevado a cabo a lo largo de toda la tesis doctoral constituyendo los principales métodos para el estudio de los compuestos. Dado que esta tesis se centra en el estudio de los efectos de diversos compuestos sobre los canales de sodio dependientes de voltaje, se han empleado células embrionarias de riñón humano denominadas HEK293 transfectadas con la subunidad α 1.6 del canal de sodio dependiente de voltaje ($Na_v1.6$). Debido a que en la actualidad la detección de las ciguatoxinas se basa en el empleo de células de neuroblastoma, se han realizado estudios adicionales en las líneas de neuroblastoma humano SH-SY5Y y la comúnmente empleada neuroblastoma de ratón N2a.

3.1.1 Células HEK293 $Na_v1.6$

La línea de células embrionarias de riñón humano que expresa de forma estable la subunidad α $Na_v1.6$ se obtuvo bajo un acuerdo de transferencia de material establecido GlaxoSmithKline I+D, Stevenage, Reino Unido. Las células HEK293 $Na_v1.6$ se mantuvieron empleando medio DMEM/F12 suplementado con glutamina, aminoácidos no esenciales (1 %), suero fetal bovino (10%) y 400 µg/ml del antibiótico geneticina. La línea celular se mantuvo en una atmósfera

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húmeda con 5% de CO_2 y 95% de O_2 a 37 °C renovando el medio cada 2 días. Antes de realizar los experimentos las células se incubaron a 30 ºC al menos durante 24 h con la finalidad de maximizar la expresión de los canales de sodio dependientes de voltaje (Burbidge et al. 2002).

3.1.2 Neuroblastoma humano SH-SY5Y

La línea de neuroblastoma SH-SY5Y se obtuvo comercialmente de American Type Culture Collection (ATC, CRL-2266, pase 182). El mantenimiento del cultivo celular se realizó en medio DMEM/F12 suplementado con suero fetal bovino (10%), glutamax (1%), y antibiótico penicilina y estreptomicina (1%). El cultivo se mantuvo en incubación a 37 °C en una atmósfera húmeda con 5% de CO_2 y 95% O_2.

3.1.3 Neuroblastoma humano de ratón N2a

La línea de neuroblastoma de ratón N2a se obtuvo comercialmente de American Type Culture Collection (CCL-131). Las células N2a, se mantuvieron en medio RPMI-1640 suplementado con glutamina (1%), suero fetal bovino (10%) y antibiótico penicilina y estreptomicina (1%). El cultivo se mantuvo a 37 °C en una atmósfera húmeda con 5% de CO_2 y 95% O_2.

3.2 ENSAYOS PARA DETERMINACIÓN DE LA VIABILIDAD CELULAR

Existen una amplia variedad de métodos que se emplean para determinar la viabilidad celular tras la exposición a compuestos tóxicos. Estos ensayos se basan en la exposición de las células a un compuesto de prueba y tras un período de incubación, se mide un marcador para reflejar el número de células viables presentes en comparación con las no expuestas al tratamiento. En esta tesis doctoral, uno de los ensayos empleados ha sido el ensayo del MTT que permite evaluar la capacidad de las deshidrogenasas mitocondriales celulares para reducir el colorante vital MTT (bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difenil-tetrazolio) o azul de tiazolilo, ya que hay una correlación entre la capacidad de un compuesto para disminuir la función mitocondrial y su citotoxicidad (Mosmann 1983). Como se muestra en la Figura 11, la actividad de la deshidrogenasa mitocondrial depende de la concentración intracelular de NADH (nicotinamida adenina dinucleótido), durante este proceso, el NADH es reducido a NAD^+ y así el MTT se convierte en formazán (Khor Goot et al. 2017).

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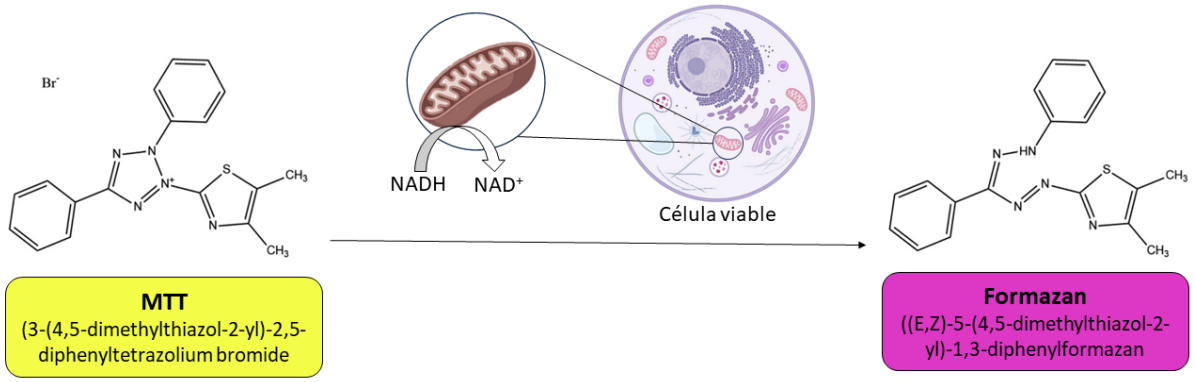


Figura 11.Esquema que muestra la estructura química del MTT (amarillo), del formazán (violeta) y el proceso de reducción del MTT por las deshidrogenasas mitocondriales (van Meerloo et al. 2011).

Después de exponer las células a los compuestos objeto de estudio durante un periodo de 24 h, se aspiró el medio y se realizó un lavado con tampón salino. Tras esto, se añadió el colorante MTT a una concentración final de 500 μg/ml disuelto en tampón salino Locke's que contenía, en mM: 154 de NaCl, 5,6 de KCl, 1,3 de $CaCl_2$, 1 de MgCl, 5,6 de glucosa y 10 de HEPES. El pH del tampón se ajustó a 7,4. Las células se incubaron durante 50 minutos a 37 ºC. Tras la incubación, se aspiró el MTT y se añadió DMSO para romper las membranas celulares y liberar el colorante. Las placas se mantuvieron en la oscuridad a temperatura ambiente durante 24 horas permitiendo la lisis completa de las células. Finalmente se leyó la absorbancia a una longitud de onda de 595 nm utilizando un espectrofotómetro (Synergy, Biotek).

Se emplearon además otros dos métodos para evaluar la viabilidad celular que no requieren ningún proceso de aspiración ni lavado de las células, como son el ensayo del Alamar blue y el ensayo de la lactato deshidrogenasa (LDH). El ensayo del Alamar blue o ensayo de la resazurina se basa en la determinación de la actividad metabólica celular. Para ello el compuesto se añade directamente al medio de cultivo permitiendo así el seguimiento de la viabilidad celular durante el tiempo de tratamiento. Como se esquematiza en la Figura 12, el indicador redox resazurina (7-hidroxi-3H-fenoxazin-3-ona-10-óxido) que es color azul no fluorescente, no tóxico para las células y permeable a través de las membranas celulares, es convertido en resorufina por las células metabólicamente activas, el cual es de un color rosa fluorescente (Riss et al. 2004).

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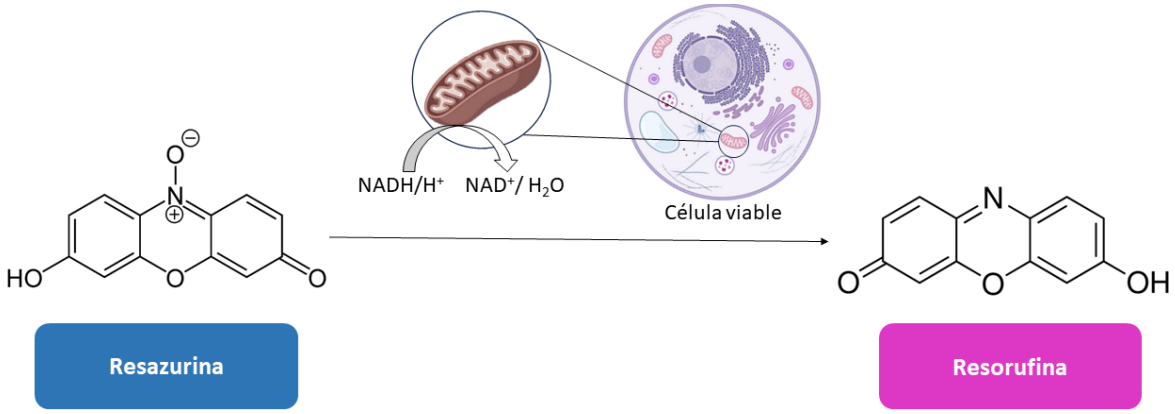


Figura 12. Esquema que muestra la estructura química de la resazurina (azul) y de la resorufina (rosa) y el proceso de reducción de la resazurina en células metabólicamente activas.

Antes de realizar los ensayos, se determinó el número de células apropiado para que la señal no se saturase. Tras la exposición de las células a los compuestos a estudiar, estas se incuban con un 10% de Alamar Blue en el medio de cultivo. Durante todo el periodo de incubación de las células con el compuesto, se realizaron mediciones tanto colorimétricas como fluorimétricas a lo largo del tiempo. En los estudios llevados a cabo en esta tesis doctoral se determinó la fluorescencia emitida a las 4, 8, 24 y 48 h a longitudes de onda de excitación/emisión de 530/590 nm con un lector de fluorescencia (Synergy, Biotek).

Otro de los ensayos empleados en esta tesis doctoral para determinar la viabilidad y muerte celular fue el ensayo de lactato deshidrogenasa. La LDH es una enzima citoplasmática que se encuentra en todas las células y es liberada al sobrenadante del cultivo celular cuando la membrana plasmática se daña. Como se representa en la Figura 13, la LDH liberada al medio convierte el lactato en piruvato al reducir el NAD^+ a NADH. En segundo lugar, la diaforasa en presencia de NADH reduce la sal de tetrazolio del ensayo (denominada INT) produciendo formazán de color rojo. La cantidad final de formazán es directamente proporcional a la cantidad de LDH liberada al medio (Kumar et al. 2018). Finalmente se leyó la absorbancia emitida utilizando un espectrofotómetro (Synergy, Biotek) a una longitud de onda de 490 nm, a la cual se le resta el valor de la absorbancia a 680 nm para obtener un valor final.

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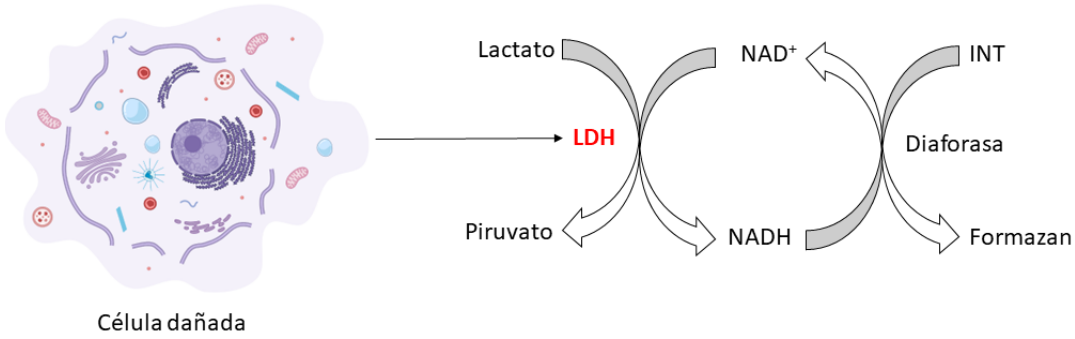


Figura 13. Representación esquemática del proceso de conversión del lactato a piruvato y reacción para medir la LDH liberada al medio por las células dañadas.

Como control positivo de máxima liberación de LDH se empleó el reactivo proporcionado en el kit de ensayo CyQUANT LDH Cytotoxicity Assay (Sigma), como control de liberación espontánea de LDH por las células se añade un 10% de agua al medio de cultivo.

3.3 ELECTROFISIOLOGÍA

La electrofisiología es una técnica para estudiar los efectos de cualquier compuesto sobre la función de cualquier canal de la membrana celular, y especialmente de los que son dependientes de voltaje. Esta técnica permite medir las propiedades biofísicas de los canales iónicos en escalas de milisegundos cuantificando los cambios eléctricos y el movimiento de iones a través de los canales. En esta tesis doctoral se empleó la electrofisiología para medir los efectos de los diferentes compuestos sobre los canales de sodio y de cloro dependientes de voltaje de la membrana celular.

En todas las células existe una diferencia de potencial transmembrana constante denominada potencial de membrana en reposo generado como consecuencia de que el interior de la célula contiene más cargas negativas que el exterior. La diferencia de potencial se debe a la existencia de gradientes de concentración iónica a ambos lados de la membrana y a la diferencia de la permeabilidad de la membrana celular a los distintos iones. El movimiento de cargas eléctricas a través de la membrana plasmática es un fenómeno que obedece a la ley de Ohm. La ley de Ohm establece que: "La intensidad de corriente en un circuito es directamente proporcional a la tensión aplicada a ella e inversamente proporcional a la resistencia del propio circuito". Por lo tanto, la relación entre la intensidad de corriente (I) medida en amperios (A), el potencial (V) medido en voltios y la resistencia eléctrica (R) medida en ohmios (Ω), resulta en que I=V/R.

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Para llevar a cabo la técnica de patch-clamp es necesario un equipo especial que se representa en la Figura 14. El equipo está rodeado de una caja de Faraday para evitar el ruido eléctrico y se encuentra sobre una mesa anti vibratoria regulada por una botella de aire comprimido cuya función es evitar vibraciones. Inicialmente, las células empleadas en cada experimento se cultivan en placas de 12 pocillos sobre cubreobjetos de 18 mm. Cuando éstas alcanzan una confluencia de aproximadamente el 80 % se mantuvieron en un incubador a 30 ºC para aumentar la expresión de los canales de sodio dependientes de voltaje antes de realizar los experimentos. El cubreobjetos con células se coloca en el interior de una cámara de registro, la cual se rellena para realizar los experimentos con tampón Locke´s como solución extracelular. En el interior de una micropipeta de vidrio con una punta estrecha que se fabrica a partir de microcapilares de borosilicato (1,5 mm de diámetro externo) se colocó el electrodo de registro junto con la solución intracelular compuesta en mM por 120 CsF, 15 NaCl, 10 EGTA, 10 HEPES con pH ajustado a 7,2.

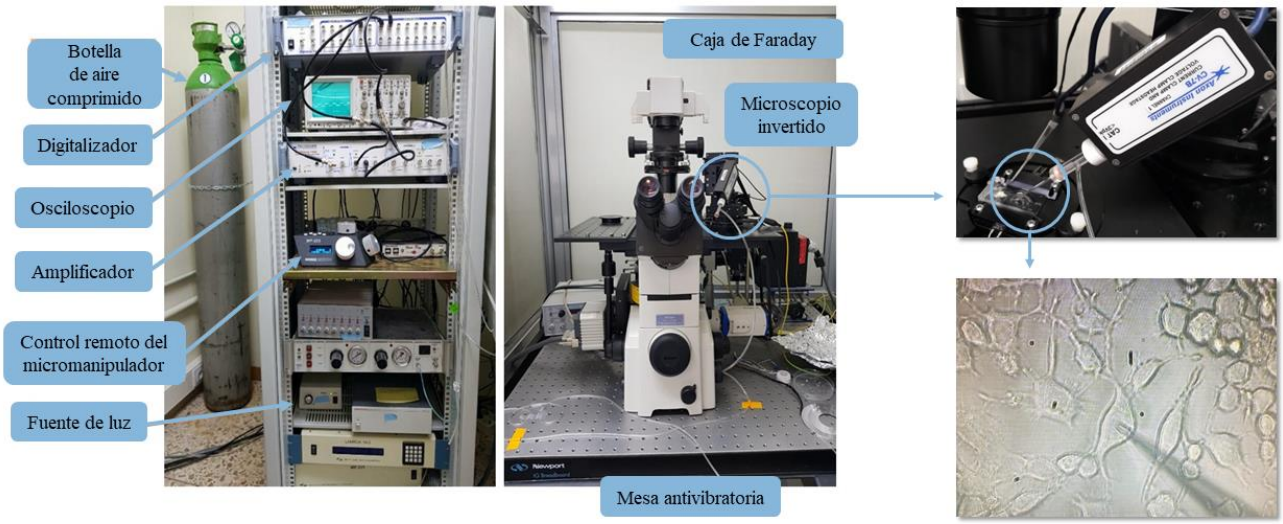


Figura 14. Equipamiento para realizar los registros electrofisiológicos.

El estudio de la actividad de los canales iónicos se realizó en la configuración de célula entera en modo de fijación de voltaje lo que permite registrar y analizar las corrientes que pasa a través de los canales de toda la membrana celular. Para obtener la configuración de célula entera, la micropipeta de vidrio, pulida con calor, que contiene el electrodo se aproxima a la superficie de una célula y se aplica presión negativa para formar un sello hermético de gran resistencia (del orden de 5-10 gigaohmios (GΩ)) entre la membrana plasmática de la célula y la punta de

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la pipeta. Este sello mantiene la célula unida a la punta de la pipeta manteniendo la integridad de la membrana como se esquematiza en la Figura 15. Aplicando de nuevo presión negativa se rompe una zona de la membrana, de modo que la solución intracelular electrolítica que contiene la micropipeta queda en contacto con el citosol y se establece una continuidad entre la pipeta y el interior de la célula teniendo un acceso de baja resistencia a toda la célula, lo que permite controlar el voltaje de la misma y registrar todas las corrientes que pasan a través de los canales iónicos de la membrana. Estos registros de corriente se transmiten hacia un amplificador de voltaje, en este caso el Multiclamp 700B de Axon instruments que amplifica la señal obtenida y la transmite a un digitalizador (Digidata 1440, Axon Instruments) el cual convierte la señal analógica en digital para que esta pueda ser leída e interpretada. La señal es filtrada mediante un filtro Bessel a 10 kHz y las resistencias fueron compensadas al menos en un 70% siempre que fue posible con un circuito de compensación interna.

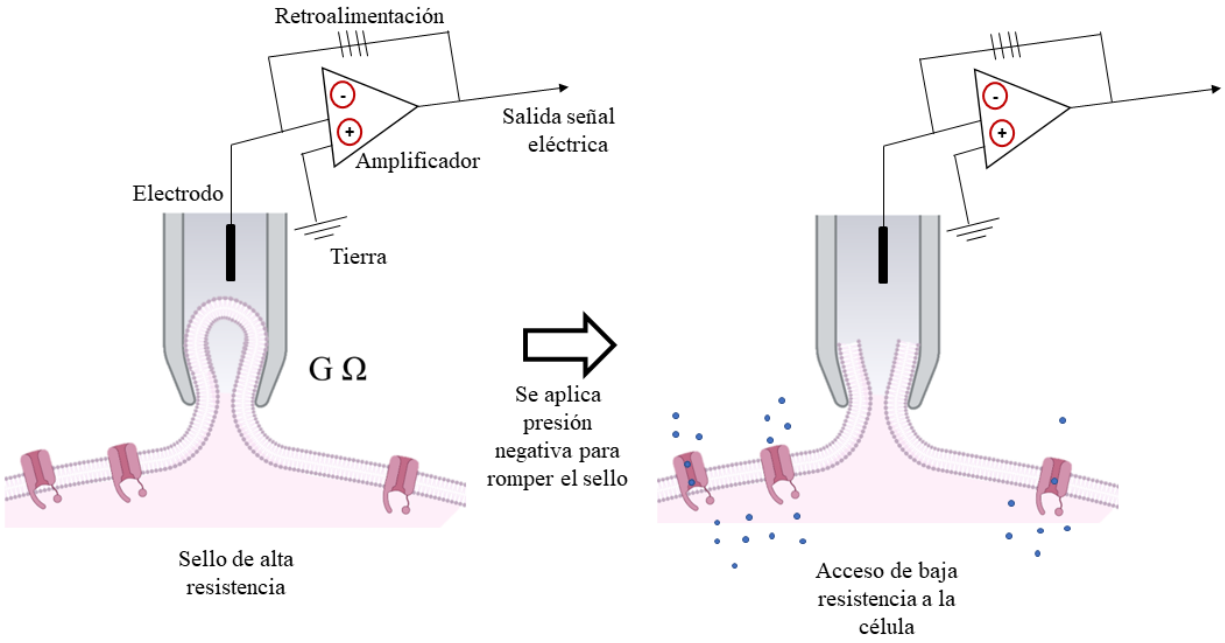


Figura 15. Representación de la técnica de patch-clamp en configuración célula entera. Establecimiento de un sello de alta resistencia entre la membrana celular y la micropipeta de vidrio que contiene un electrodo. Cuando se aplica presión negativa se rompe parte de la membrana celular permitiendo el acceso al registro de todas las corrientes que fluyen a través de los canales. Estas corrientes se registran mediante el electrodo que está en el interior de la pipeta conectado a un amplificador.

Para medir las corrientes de sodio dependientes de voltaje se ha fijado el voltaje a −55 mV para alcanzar la configuración de célula entera. tras la cual, se esperaron 5 minutos para permitir el equilibrio entre la disolución intracelular del electrodo y el contenido citoplasmático. A

continuación, se iniciaron los registros siguiendo un protocolo que aplica un prepulso de −90 mV seguido de 17 pulsos de 50 ms de duración desde −80 a +80 mV (en incrementos de 10 mV) para el registro de las corrientes de activación. En el caso del registro de las corrientes de inactivación rápida del canal de sodio el protocolo consistió en la aplicación de 10 prepulsos de 300 ms de duración entre −100 y 0 mV en incrementos de 10 mV y el posterior registro de la corriente a 0 mv. En cuanto al registro de las corrientes de cloro, el protocolo empleado fue la aplicación de un prepulso de −50 mV seguido de 20 pulsos de 400 ms de duración desde −100 hasta +100 mV con incrementos de 20 mV por cada paso.

3.4 INMUNOCITOQUÍMICA Y ANÁLISIS MICROSCÓPICO

La técnica de inmunocitoquímica se ha empleado en esta tesis doctoral para realizar un marcaje del citoesqueleto de actina con el fin de evaluar los efectos de ciguatoxinas en la integridad del citoesqueleto celular. Esta técnica se empleó en la línea celular N2a previamente cultivadas sobre cubreobjetos de 18 mm de diámetro. Las células se lavaron en tampón fosfato salino (PBS) que contenía en mM: 137 NaCl, 8,2 Na_2HPO_4, 1,5 KH_2PO_4, y 3,2 KCl. A continuación, se fijaron con paraformaldehido al 4 % y sacarosa al 4 % disueltos en PBS durante 20 minutos a temperatura ambiente. Tras un lavado con PBS, la reactividad inespecífica se bloqueó con albúmina sérica bovina (BSA) al 5% y las células se permeabilizaron con 0.1% Tritón X-100. Posteriormente, se añadió el anticuerpo monoclonal primario de ratón anti-actina (1:200 Millipore) en una solución de PBS con 2.5 % de BSA y 0.1 % de Tritón X-100 y las células se incubaron con el anticuerpo primario durante toda la noche a 4 ºC. A continuación, se realizaron tres lavados para eliminar el anticuerpo primario y las células se incubaron protegidas de la luz durante 1 hora a temperatura ambiente con el anticuerpo secundario fluorescente anti-ratón (Alexa Fluor 488, Thermofisher en una dilución 1:500). Una vez transcurrido este tiempo, se realizaron tres lavados con PBS, los cubreobjetos se montaron sobre portaobjetos con líquido de montaje (PBS/glicerol 4:1 v/v) y se sellaron con esmalte de uñas. Los resultados se visualizaron con un microscopio láser confocal (Nikon, Mellville, NY).

3.5 ESTUDIOS DE TOXICIDAD *IN VIVO*

Los ensayos *in vivo* que se llevaron a cabo en esta tesis doctoral para obtener los resultados de toxicidad aguda y subcrónica de compuestos involucrados en la ciguatera se desarrollaron acorde con la normativa de experimentación animal siguiendo el principio de las 3R (reducción, refinamiento y remplazo). Todos los procedimientos *in vivo* se realizaron de conformidad con la legislación europea relativa a la protección de los animales empleados en experimentación

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(Directiva 2010/63/UE), la legislación nacional recogida en el Real Decreto 53/2013 (BOE 2013) y autonómica reflejada en el Decreto 296/2008 (DOG 2008). Los procedimientos experimentales fueron aprobados por el Comité Institucional de Ética en Experimentación Animal de la Universidad de Santiago de Compostela y autorizados en los procedimientos número 011/14, autorizado el 14 de agosto de 2014 (MR110250) AE/LU-002, autorizado el 1 de marzo de 2014 (A12X00509, renovado el 1 de marzo de 2019). Para el planteamiento de los protocolos utilizados para evaluar la toxicidad *in vivo* se siguieron las directrices internacionales de la Organización para la Cooperación y el Desarrollo Económico (OCDE) que recogen las normas recomendadas para evaluar la toxicidad de compuestos químicos. Los estudios de toxicidad aguda se han realizado siguiendo el protocolo Up-and-Down recogido en el Test 425 (OECD 2022b) y los estudios de toxicidad subcrónica basados en el Test 407 (OECD 2008).

Durante la realización de los estudios toxicológicos agudos, a los ratones se les administró una única dosis de toxina por vía oral mediante sonda gástrica en la que la toxina se diluye en 200 µl de solución salina fisiológica, o bien una única administración intraperitoneal de toxina empleando una aguja de 25G.

Grupos de animales empleados para evaluar la toxicidad aguda de compuestos implicados en la ciguatera:

- MTX1 administración intraperitoneal única a 200, 400, 800, 1000, 1200, 1600 y 3200 ng/kg de peso corporal.
- MTX1 a dosis de 800 ng/kg MTX1 administración única por sonda.
- CTX3C a 330 y 1050 ng/kg administración única por sonda.

Tras la administración de la toxina los animales se observaron continuamente durante las primeras 2 horas y periódicamente hasta el sacrificio 96 horas después de la administración de la toxina.

En el caso de la determinación de la toxicidad subcrónica, las toxinas se administraron diariamente durante 28 días, comenzando con la dosis más baja e iniciando el siguiente período de dosificación después de terminar el primer mes de tratamiento.

Los grupos de animales empleados para evaluar la toxicidad subcrónica oral de los compuestos implicados en la intoxicación por ciguatera fueron:

- CTX3C a dosis de 10, 32, 102 ng/kg administrados por sonda cada 24 h.

- Gambierona a dosis de 172, 550 y 1760 ng/kg administrados por sonda cada 24 h.
- MTX1 a dosis de 800, 2560 y 5000 ng/kg administrados por sonda cada 24 h.
- MTX3 a dosis de 550 y 1760 ng/kg administrados por sonda cada 24 h.

En este caso además se realizaron controles de peso el primer día del tratamiento (día 0) y semanalmente a partir de entonces y simultáneamente se registró el consumo de alimento. Durante todos los estudios los animales tuvieron libre acceso a comida y agua. Al final del tratamiento, para ampliar la información obtenida los animales se mantuvieron en jaulas metabólicas tras la administración de la toxina, tanto en el tratamiento agudo como subcrónico, durante 24 horas antes del sacrificio. Estas jaulas permiten la monitorización de la cantidad de comida y agua ingerida, así como separar las heces y la orina producidas para tomar muestras. Tras el sacrificio se realizó una necropsia con toma de muestras de corazón, pulmón, hígado, riñón, bazo, estómago, intestino delgado, intestino grueso y cerebro las cuales se procesaron para realizar un análisis histológico.

Los animales que durante el desarrollo de los estudios mostraron signos de dolor o sufrimiento, así como los animales moribundos se sacrificaron humanitariamente en una cámara de CO_2 adecuada a su tamaño y se consideraron en los resultados de los estudios de toxicidad. Inmediatamente después del sacrificio se extrajo la sangre directamente de la cavidad ventricular del corazón y se trasfirió de inmediato a microtubos con heparina (2.5 U. I./ml). Las muestras de sangre se enviaron al laboratorio del Hospital Veterinario Universitario Rof Codina donde se procesaron y se realizaron las determinaciones bioquímicas correspondientes.

Los parámetros sanguíneos que se analizaron fueron:

- Niveles de electrolitos (Cl^-, Na^+, K^+)
- Enzima alanina aminotransferasa (ALT)
- Enzima aspartato aminotransferasa (AST)
- Enzima lactato deshidrogenasa (LDH)
- Enzima creatina cinasa (CK)
- Niveles de glucosa
- Niveles de colesterol

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4 . RESULTADOS

A continuación, se recogen los resultados obtenidos durante esta tesis doctoral que han sido publicados en diversas revistas internacionales. Los resultados obtenidos se agrupan en dos bloques y se presentan a continuación:

TRABAJOS PUBLICADOS

- In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins
- Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels.
- *In vivo* subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity.
- Mouse N2a neuroblastoma assay: Uncertainties and comparison with alternative cell-based assays for ciguatoxin detection.
- Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins.
- Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C in human voltage-gated $Na_v1.6$ sodium channels.

BLOQUE I: BIOTOXINAS MARINAS IMPLICADAS EN LA INTOXICACIÓN POR CIGUATERA. EVALUACIÓN IN VIVO E IN VITRO PARA ESTABLECER LOS FACTORES DE EQUIVALENCIA TÓXICA

En esta sección se recogen los resultados obtenidos después de la evaluación de los datos obtenidos tanto *in vivo* como *in vitro* sobre las biotoxinas marinas implicadas en la intoxicación por ciguatera. En esta parte se han obtenido 4 publicaciones que se describen a continuación.

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Publicación

In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins

Resumen

Las ciguatoxinas causan intoxicación por ciguatera, siendo esta la intoxicación alimentaria humana más común relacionada con toxinas marinas. Las ciguatoxinas del pacífico son los análogos de CTX más abundantes y estudiados. Sin embargo, la creciente distribución de los análogos caribeños y los limitados datos disponibles sobre sus efectos biológicos plantearon la necesidad de reevaluar sus potencias puesto que, durante décadas, las directrices establecidas por las agencias reguladoras han asumido que la CTX del caribe era diez veces menos potente que la CTX del pacífico. En el estudio presentado en esta tesis, se analizan los distintos efectos de análogos pacíficos y caribeños de las ciguatoxinas usando células N2a (el método utilizado internacionalmente para detectar ciguatoxinas) que evidenciaron una citotoxicidad similar de los análogos pacíficos y caribeños. Por otra parte, se analizó el efecto de los distintos análogos sobre canales de sodio dependientes de voltaje mediante estudios electrofisiológicos que a su vez demostraron una potencia similar de los análogos pacíficos y caribeños. Este trabajo se completó con simulaciones *in silico* que demostraron que existe una unión diferente de las ciguatoxinas caribeñas y pacíficas a los canales de sodio dependientes de voltaje puesto que, los análogos pacíficos se unen con el anillo A de su estructura química hacia la parte intracelular del canal mientras que los análogos caribeños se unen con su anillo A orientado hacia la parte extracelular del canal. Los resultados obtenidos en este trabajo permitieron sugerir que la potencia de los análogos caribeños y pacíficos de la ciguatoxina era similar en base a sus efectos sobre el voltaje de activación de los canales de sodio.

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ORIGINAL PAPER

In Silico Simulations and Functional Cell Studies Evidence Similar Potency and Distinct Binding of Pacific and Caribbean Ciguatoxins

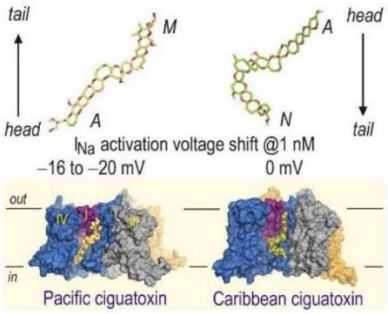
Sandra Raposo-García[1] · David Castro[2] · Emilio Lence[3] · Pablo Estévez[2] · José Manuel Leão[2] · Concepción González-Bello[3] · Ana Gago-Martínez[2] · M. Carmen Louzao[1] · Carmen Vale[1] · Luis M. Botana[1]

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Abstract

Ciguatoxins (CTX) cause ciguatera poisoning, which is the most common reported human food poisoning related to natural marine toxins. Pacific ciguatoxins are the most abundant and studied CTX analogues; however, the growing distribution of Caribbean analogues and the limited data available on their biological effects make necessary to re-evaluate their relative potency. For decades, the guidelines established by regulatory agencies have assumed that the potency of the Caribbean CTXs were tenfold lower than the Pacific CTXs. We present here an integrated study involving Neuro-2a cells (the method used worldwide to test ciguatoxins), electrophysiological assays, and in silico simulations that evidence the similar cytotoxicity of Caribbean and Pacific ciguatoxins and their asymmetry binding within sodium channels. The binding mode of the toxins was first explored by molecular docking using the GOLD program and the resulting binary complexes were further studied by Molecular Dynamics simulation studies using the molecular mechanics force field AMBER. The simulation studies explain their distinct impact on the activation potential of the channel as experimentally observed and provide a detailed picture of the effects caused by these toxins on an atomic scale.

Graphical Abstract



Keywords Ciguatoxins · Sodium channels · Molecular modeling · Ciguatera poisoning · Toxicity

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Abbreviations

CTX	Ciguatoxin
CP	Ciguatera poisoning
EFSA	European food safety authority
FAO	Food and agriculture organization
FDA	Food and drug administration
IC_{50}	Half-maximal inhibitory concentration
MD	Molecular dynamics
PM	Pore module

TEF	Toxicity equivalency factor
VGSC	Voltage-gated sodium channels
VSM	Voltage-sensing module
WHO	World health organization

Introduction

Ciguatoxins (CTX) are marine biotoxins produced by benthic dinoflagellates of the genera *Gambierdiscus* and *Fukuyoa* (Gomez et al. 2015; Holmes and Lewis 1994). On the base of their chemical structures, four different ciguatoxin groups were described: Caribbean CTXs (C-CTX1), Indian CTXs (I-CTX1), and Pacific ciguatoxins, which are divided into two different groups, CTX3C and CTX4A and their derivatives as summarized by the FAO/WHO (Food and Agriculture Organization and the World Health Organization) report (FAO 2020). Among these groups, Pacific ciguatoxins analogues (P-CTXs) are the most widely distributed, studied, and evaluated. The chemical structures of Pacific and Caribbean ciguatoxins are represented in Fig. 1.

Regarding C-CTXs, the studies performed until now have shown that the most commonly found analogues are the Caribbean Ciguatoxin-1 and -2, named as C-CTX1 and C-CTX2 (Estevez et al. 2019b). Ciguatoxin-producing dinoflagellates were initially found in Indo-pacific oceans and in the Caribbean Sea reviewed by Shmukler and Nikishin (Shmukler and Nikishin 2017); however, during the last decades, as a possible result of climate change (Ramilo et al. 2021; Xu et al. 2016) and anthropogenic activities, including the globalization of trade, these microalgal organisms have spread to European coasts (Botana 2016; FAO 2020; Otero et al. 2010), and this fact rises the need to fully revaluate the potency of Caribbean and Pacific ciguatoxin analogues.

CTXs cause in humans the syndrome named ciguatera poisoning (CP) due to the ingestion of high levels of the toxin accumulated by carnivorous fish in their musculature, liver, and viscera after predating herbivorous fish, reviewed by Friedman et al. (2017) (Díaz-Asencio et al. 2019; FAO 2020; Friedman et al. 2017). Nowadays, more than 400 fish species are associated with CP in humans, with barracuda, red snapper, grouper, amberjack, sea bass, surgeonfish, and

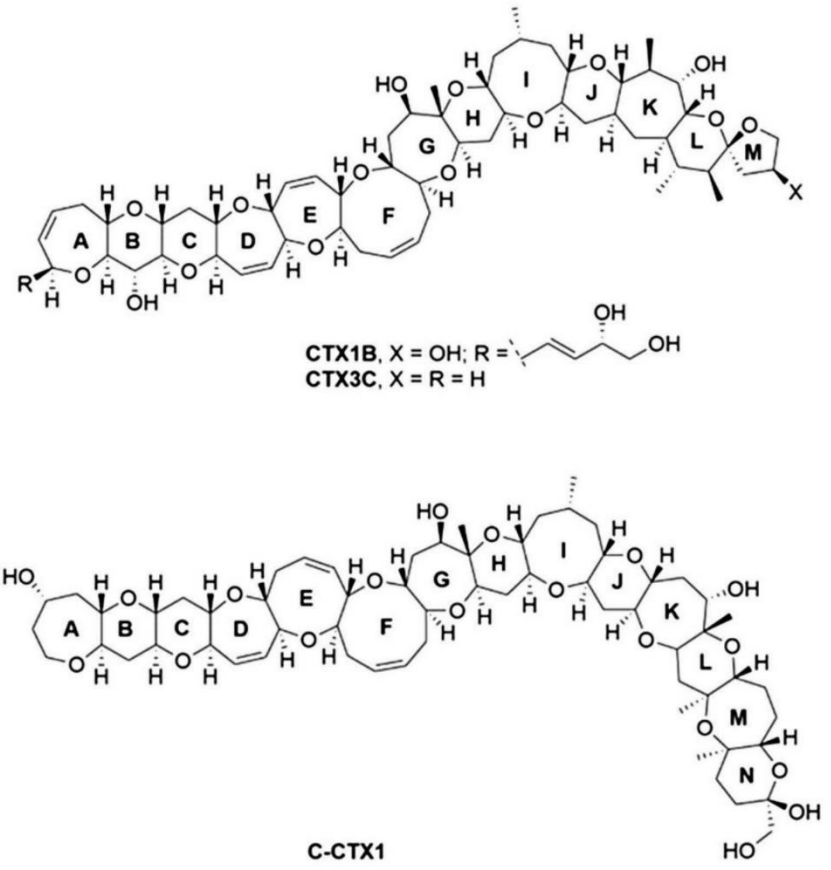


Fig. 1 Chemical structure and ring denotation of the principal Pacific and Caribbean ciguatoxins

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moray eel as the main responsible of these intoxications (FAO 2020; Pérez-Arellano et al. 2005). Moreover, recently, other species of marine invertebrates such as bivalves, echinoderms, and gastropods were also reported as additional vectors of CP (FAO 2020; Rhodes et al. 2020; Silva et al. 2015). CP is characterized by cold allodynia, gastrointestinal, neurological, and cardiovascular symptoms, starting from 30 min after the ingestion of contaminated fish to 24/48 h and persist for weeks and even months (Friedman et al. 2007). Each year up to 500,000 CP cases might occur globally reviewed by Dickey (2008) (Díaz-Asencio et al. 2019; Dickey 2008; Inserra et al. 2017).

In the last years CP has expanded to new regions such as Canary Islands, Madeira Islands, Selvagens archipelagos, New Zealand, and the Mediterranean Sea (Costa et al. 2018; EFSA 2010; Otero et al. 2010; Tudó et al. 2020). In 2004, CP was first confirmed after consumption of flesh from lesser amberjack (*Seriola rivoliana*) from the Canary Islands, a non-endemic region for CP (Pérez-Arellano et al. 2005) and later on associated with the presence of Caribbean ciguatoxins in Madeira and Canary Islands (Boada et al. 2010; Estevez et al. 2019c, 2020; Otero et al. 2010; Sanchez-Henao et al. 2020). Thus CP is a notifiable disease in the Canary Islands since the year 2015 (Sanchez-Henao et al. 2019) and the current situation in Europe points to an increasing expansion of CTXs (Canals et al. 2021; Diogène et al. 2021); in fact, human intoxications from imported fish have been recently described in Germany (Loeffler et al. 2022). The lack of information regarding the relative toxicity of Pacific and Caribbean ciguatoxins makes it difficult to establish their potency, a remarkable fact since their increasing appearance and distribution raises global public concerns regarding human health.

In vivo studies carried out in the 90s evidenced a half lethal dose (LD_{50}) for intraperitoneal P-CTX of 0.25 μg kg^{-1} body weight (Vernoux and Lewis 1997) and 3.6 μg kg^{-1} and 1 μg kg^{-1}, respectively, for C-CTX1 and C-CTX2 (Lewis et al. 1999, 1991). However, the mouse bioassay is currently no longer considered an appropriate method to detect ciguatoxins in fishery products due to poor specificity and ethical concerns (EFSA 2010). In the European Union (EU), seafood products which may contain ciguatoxins are not allowed to be placed on the market (EU 2019). In contrast, the United States Food and Drug Administration (FDA) has established a guidance level for Caribbean CTXs of 0.1 μg kg^{-1} C-CTX1 equivalents and 0.01 μg kg^{-1} for P-CTX1B (FAO 2020; FDA 2011) based on their intraperitoneal toxicity in mice which implies that Pacific ciguatoxins are tenfold more potent than their Caribbean congeners (Lewis et al. 1991; Vernoux and Lewis 1997).

Nowadays there are several drawbacks which hinder the establishment of the real potency of these emerging marine toxins: (i) the data obtained so far are uncertain because the presence of CTXs in fish tissues is difficult to detect and quantify (Caillaud et al. 2010), (ii) absence of animal toxicity reports for these compounds, and (iii) CTX doses related with clinical symptoms of CP in humans are not well defined. These factors are aggravated by the lack of certified reference standards for the CTX group of toxins (EFSA 2010). Finally, lethality as reported with the mouse bioassay is not a useful value to quantify CTX potency since these toxins cause complex non-lethal symptoms and CP due to their effect on voltage-gated sodium channels (VGSC) specifically the domain IV of site 5 (FAO 2020). Although the effect of ciguatoxins is primarily on voltage-gated sodium channels (Catterall et al. 1992), these compounds can also alter potassium-gated voltage channels functioning (Hidalgo et al. 2002). The levels of C-CTX allowed in food are 1.8 μg kg^{-1} in inedible fish, 0.9 to < 1.8 μg kg^{-1} edible in small quantities, and edible with less than 0.9 μg kg^{-1} (Lewis et al. 1999).

The spread worldwide and the scarce reliable information about the biological effects of these emergent toxins in the same cellular model led to several international organizations such as FAO, WHO, and EFSA to highlight the need for a full re-evaluation of the toxicity and relative potencies of these toxins (EFSA 2010; FAO 2020). This work was designed to fully evaluate and compare the cytotoxicity of Pacific and Caribbean ciguatoxin analogues, as well as their effects on the functional activity of sodium channels. Besides, to achieve a better understanding of the experimental results, docking and molecular dynamics (MD) simulations have been used to explore, in atomic detail, the plausible binding mode of these toxins to the α subunit of VGSC, as well as to get an insight of the conformational changes promoted by these compounds. The molecular modeling results on Na_v channels presented here suggest that both analogues bind to sodium channels in an opposite conformation but have similar toxicity as evaluated using internationally adopted methods to detect these toxins (Caillaud et al. 2010; Castro et al. 2020).

Experimental Section

Modeling and Simulation Studies

Building of the Three-Dimensional Model of $Na_v1.6$ Channel in the Unbound Form

The three-dimensional structure of $Na_v1.6$ channel was created using the AlphaFold method (Jumper et al. 2021) (UniProt accession code Q9UQD0) and further validated by Molecular Dynamics simulation studies. To this end, the protein was immersed in a cube of ~ 80,000 TIP3P water molecules and neutralized by addition of sodium ions (3) using

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the molecular mechanics force field ff14SB of AMBER 17 (Case et al. 2017). The residues 1–108, 427–735, and 1778–1980, which correspond to long and unfolded loops not involved in the membrane part of the channel, were deleted for the simulation. Computation of the protonation state of titratable groups at pH 7.0 was carried out using the H^{++} Web server (Gordon et al. 2005). As a result of this analysis, all histidine residues were considered in their neutral forms except H1678. The neutral histidine residues were protonated in ε position except H920 and H1354, for which the δ position was selected. Disulfide bridges were used between the following pairs of cysteine residues: C281/C333, C324/C339, C906/C912, C944/C953, C1356/C1376, and C1721/C1736. The minimization of the protein and MD simulation of the resulting minimized structure were carried out following our previously described protocol (Vazquez-Ucha et al. 2021) and 200-ns simulations were performed. The cpptraj module in AMBER 17 was used to analyze the trajectories and to calculate the root-mean-square deviation of atomic positions (*rmsd*) of the protein during the simulation (Roe and Cheatham 2013). The molecular graphics program PyMOL (DeLano 2002) was employed for visualization and depicting protein structures.

Building of the Three-Dimensional Models of *$Na_v1.6$@ciguatoxin* Binary Complexes

(1) Ligand preparation: The geometries of ciguatoxins CTX1B, CTX3C, and C-CTX1 were minimized using a restricted Hartree–Fock (RHF) method and a 6-31G(d) basis set, as implemented in the ab initio program Gaussian 09 (Frisch et al. 2009). Partial charges were derived by quantum mechanical calculations using Gaussian 09, as implemented in the R.E.D. Server (version 3.0) (Vanquelef et al. 2011), according to the RESP model (Cornell et al. 1995). The missing bonded and non-bonded parameters were assigned, by analogy or through interpolation, from those already present in the AMBER database (GAFF) (Wang et al. 2006, 2004).

(2) Docking studies: The binding mode of ciguatoxins CTX1B, CTX3C, and C-CTX1 were first explored by docking using the program GOLD 2020.2.0 (Jones et al. 1997) and the coordinates of $Na_v1.6$ channel in the unbound form. The snapshot after 200 ns of simulation was selected. The previously minimized ciguatoxin geometries were used as MOL2 files. Each ligand was docked in 25 independent genetic algorithm (GA) runs, and for each of these, a maximum number of 100,000 GA operations were performed on a single population of 50 individuals. Operator weights for crossover, mutation, and migration in the entry box were used as default parameters (95, 95, and 10, respectively) as well as the hydrogen bonding (4.0 Å) and van der Waals (2.5 Å) parameters. The position of M388 was used to define the active site, and the radius was set to 30 Å. The GOLD scoring function was used.

MD Simulations Studies of *$Na_v1.6$@ciguatoxin* Binary Complexes

(1) Minimization: Ligand coordinates obtained by docking were employed as starting point for MD simulations of the corresponding binary complexes. The complexes immersed in a cube of TIP3P water molecules and sodium ions were minimized in four stages: (1) initial minimization of the ligands (1000 steps, first half using steepest descent and the rest using conjugate gradient); steps (2), (3), and (4) were performed as steps (a), (b), and (c) in the minimization of the unbound form. A positional restraint force of 50 kcal mol^{-1} $Å^{-2}$ was applied to those unminimized atoms during the first three stages (1–3).

(2) Simulations: MD simulations of the $Na_v1.6$@ciguatoxin binary complexes were performed as indicated for the unbound protein form (see above). For $Na_v1.6$@CTX1B and $Na_v1.6$@C-CTX1 complexes, simulations of 200 ns were carried out, while for $Na_v1.6$@CTX3C complex, 100-ns simulation was performed.

Binding Free Energies Calculations

The binding free energy for CTX1B, CTX3C, and C-CTX1 was calculated by the MM/PBSA approach implemented in Amber Tools 17 (Miller et al. 2012) ante-MMPBSA.py module was used to create topology files for the complex, enzyme, and ligands and binding free energies were calculated with the MMPBSA.py module. A single trajectory approach was used to calculate binding free energies considering only the last 100 ns of the 200-ns MD trajectories. For the 100-ns MD trajectories, the last 80 ns were considered. The Poisson–Boltzmann (PB) and Generalized Born (GB) implicit solvation models were employed. Both models provided similar results.

Toxins and Drugs Used

For the N2a-MTT assay and patch-clamp recordings, C-CTX1-purified solution (25.7 ng/mL) and C-CTX1 Fish Tissue Reference Material (FTRM) (0.284 ± 0.019 ng/g) were purified from an amberjack (*Seriola spp.*) from the Canary Islands (Spain) and were prepared at University of Vigo, in the framework of the project EuroCigua.

For the LC–MS/MS analyses CTX1B, 52-*epi*-54-deoxyCTX1B, 54 deoxyCTX1B, CTX3C, CTX4A, and CTX4B reference materials were kindly provided by Professor Takeshi Yasumoto (Japan Food Research Laboratories, Japan).

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C-CTX1 pure standard was kindly provided by Dr. Robert W. Dickey (University of Texas, USA) via Dr. Ronald Manger (Fred Hutchinson Cancer Research Center, Seattle, USA). CTX1B standard solution (4488 ng/mL) was kindly provided by Professor Takeshi Yasumoto (Japan Food Research Laboratories, Japan). CTX3C standard solution (100 ng) was purchased from FUJIFILM Wako Chemicals Europe GmBH (Neuss, Germany).

The stocks standard solutions C-CTX1, CTX1B, and CTX3C used for N2a-MTT assay were diluted in methanol HPLC grade (MeOH, Merck KGaA, Darmstadt, Germany) until a concentration of 10 pg/µL and were stored in dry conditions at − 20 °C.

For electrophysiological recordings Pacific ciguatoxins, CTX3C as well as CTX1B, were purchased from Wako (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 µM (corresponding to 10,230 ng/mL of CTX3C and 11,113 ng/mL of CTX1B), respectively. For experiments, 1-µM solutions (1023 ng/mL CTX3C and 1111.3 ng/mL CTX1B) were prepared in Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose, pH 7.4. The maximum solvent concentration used as a control was 0.1% DMSO and had no effect on voltage-gated sodium current amplitude. Similarly, C-CTX1 was dissolved in DMSO at a final concentration of 1 µM (1141.4 ng/mL C-CTX1) and dilutions were performed in Locke's buffer, from an original solution of 25.7 ng/mL.

Sample Pretreatment

C-CTX1 FTRM was extracted and purified following the conditions described by Estevez et al. (Estevez et al. 2019a). Briefly, Fish tissue (200 g) was extracted twice with acetone (2 × 600 mL) using an Ultraturrax® homogenizer. Acetone layers were combined (estimated volume of 1200 mL) and evaporated to an aqueous residue using a multievaporator Syncore Polivap set at 45 °C. The aqueous residue (estimated volume 150 mL) was extracted twice with diethyl ether (2 × 200 mL). The combined organic layers (400 mL) were evaporated to a solid residue using a multievaporator Syncore Polivap set at 45 °C. The solid residue was dissolved in 90% methanol (60 mL) and defatted with twice the volume of *n*-hexane (120 mL). The methanol residue was evaporated to dryness under reduced pressure and submitted to the purification step.

The purification step was carried out using Solid-Phase Extraction (SPE) cartridges. An equivalent of 15 g of fish tissue was loaded in each SPE cartridge. The solid residue from the extraction was dissolved in ethyl acetate (26 mL) and aliquots of 2 mL were loaded in a Florisil SPE cartridge (J. T. Baker, 500 mg) previously conditioned with ethyl acetate (3 mL). The cartridge was washed with ethyl acetate (3 mL) and C-CTX1 was eluted with ethyl acetate:methanol 9:1 (v:v) (5 mL). The toxic eluate was evaporated to dryness under a N2 stream at 40 °C. The solid residue was reconstituted in 60% MeOH (26 mL) and aliquots of 2 mL were loaded in a C18 SPE cartridge (SUPELCLEAN, Supelco, 500 mg) previously conditioned with 60% MeOH (3 mL). The cartridge was washed with 60% MeOH (3 mL) and C-CTX1 was eluted with 90% MeOH (5 mL). The eluates from C18 SPE were combined and evaporated to dryness being filtered through 0.22-µm PVDF filter (Syringe Driver filter Unit, Millex®-CV 0.22 µm, 13 mm).

LC–MS/MS Analysis

LC–MS/MS analyses were performed following the conditions proposed by Estevez et al. (Estevez et al. 2019a) using an Agilent 1290 Infinity LC system coupled to an Agilent 6495 triple quad iFunnel (Agilent Technologies, Waldbronn, Germany).

CTXs were separated using a C18 column (Poroshell 120-EC-C18, 3.0 × 50 mm, 2.7 µm, Agilent USA). The column temperature was set at 40 °C and the mobile phases for the chromatographic separation were (A) 0.1% formic acid and 5 mM of ammonium formate in water and (B) MeOH. The gradient started at 78% of B with a linear gradient to 88% of B in 10 min, holding 5 min and increasing to 100% of B until 18 min to wash the column. The gradient returned to 78% of B at 18 min and the column was equilibrated during 4 min prior to the next injection. The flow rate was set at 0.4 mL/min and the injection volume was 1 µL.

The mass spectrometer operated in positive ionization mode (ESI^+) monitoring by dynamic Multiple Reaction Monitoring (dMRM) the sodium adduct $[M+Na]^+$ of the different CTXs as precursor and product ion at a collision energy (CE) of 40 eV. Source and interface parameters were as the following: drying gas flow, 15 L/min, drying gas temperature 290 °C, sheath gas flow 12 L/min, sheath gas temperature 400 °C, nebulizer pressure 50 psi, fragmentor potential 380 V, nozzle voltage 300 V, and capillary voltage 5000 V.

The following toxins with reference material available were monitored (Table 1): CTX1B, C-CTX1, 52-*epi*-54-deoxyCTX1B, 54-deoxyCTX1B, CTX3C, CTX4A, and CTX4B.

Human Cell Cultures ($hNa_v1.6$ HEK cells)

Human embryonic kidney cell line (HEK293) stably expressing the human $Na_v1.6$ alpha subunit of the sodium channels were kindly provided by Dr Andrew Powell (GlaxoSmithKline R&D, Stevenage, UK) cultured in DMEM/F12 medium supplemented with Glutamax, MEM non-essential

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Table 1 MRM transitions of the different CTXs monitored by LC–MS/MS

Toxin	Precursor ion $[M+Na]^+$ (*m/z*)	Product ion $[M+Na]^+$ (*m/z*)	Fragmentor (V)	CE (eV)	CAV (eV)
CTX1B	1133.6	1133.6	380	40	4
C-CTX1	1163.7	1163.7	380	40	4
52-*epi*-54-deoxyCTX1B	1117.6	1117.6	380	40	4
54-deoxyCTX1B	1117.6	1117.6	380	40	4
CTX3C	1045.6	1045.6	380	40	4
CTX4A	1083.6	1083.6	380	40	4
CTX4B	1083.6	1083.6	380	40	4

amino acids solution (Gibco, 1% w/v), and 10% fetal bovine serum as previously described (Boente-Juncal et al. 2021). Geneticin (Gibco) was added at a final concentration of 0.4 mg/mL to increase the expression of sodium channels. Cells were seeded at a density of 30,000 cell/mL and maintained at 37 °C in a 5% CO_2/95% O_2 incubator until the cultures reached an 80% of confluence and placed at 30 °C for 24–48 h before electrophysiological measurements (Burbidge et al. 2002).

N2a-MTT Assay

Cytotoxicity assays were carried out using two different batches of Neuro-2a (N2a) cell line (ATCC® CCL-131), purchased from the American Type Culture Collection (LGC standards S.L.U., Barcelona, Spain) and numbered as 63649750 and 70005648. Base culture medium Roswell Park Memorial Institute (RPMI-1640, R8758, Sigma, Irvine, UK) was supplemented with 1% of 100 mM sodium pyruvate (S8636, Sigma, Irvine, UK), 1% of 200 mM L-Glutamine (G7513, Sigma, Irvine, UK), 1% of Penicillin–Streptomycin formed by 5,000 units and 5 mg mL^{-1}, respectively, (P4458, Sigma, St. Louis, MO, USA), and 10% or 5% v/v of fetal bovine serum (FBS, F2442, Sigma, St. Louis, MO, USA) to obtain an adequate complete growth media (RPMI-10 and RPMI-5, respectively). For passing or seeding, cells were unattached with trypsin EDTA solution (1x) (Sigma-Aldrich, St. Louis, MO, USA). Other reagents for N2a-MTT assay include phosphate-buffered saline (PBS), veratridine hydrochloride, ouabain octahydrate, DMSO, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), all of them purchased from Sigma-Aldrich (St. Louis, MO, USA).

N2a cells were maintained in T75 flask (Corning, NY, USA) with 30 mL of RPMI-10 at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured using a split 1:5 regarding initial cell culture every 48 h. The experiments were done for three different cell passages per batch: passages 214/215/224 and 203/205/220 were selected in batch 63649750 and 70005648, respectively.

For the cytotoxicity assay, methanol extracts of CTXs standards (C-CTX1, CTX3C, and CTX1B) were evaluated using the N2a-MTT assay protocol previously described by Castro et al., (2020). Briefly, 40,000 cells were seeded per well into 96-well assay plates (Corning, NY, USA) in 0.2 mL of RPMI-5 and left to grow for 24 h at 37 °C under a humidified atmosphere enriched with 5% CO_2. Then, cells were sensitized with a mixture 10:1 (v/v) of Ouabain/Veratridine solution (O/V). A good sensitivity for detection of CTX-like compounds was reached for a concentration of O/V which provokes a reduction of 20% in cell viability respect to non-sensitized cells.

Ten serial dilutions were prepared from the CTXs stock standard solutions in RPMI-C, and sensitized cells were exposed 16 h to each concentration level, ranging from 0.00019 to 0.019 nM for C-CTX1, 0.0002 to 0.02 nM for CTX1B, and 0.00021 to 0.021 nM for CTX3C, in replicates of four wells. After this time, cell viability was estimated from absorbance due to formazan produced by the colorimetric MTT assay (Mosmann 1983). Absorbance readings were obtained with a multi-well scanning spectrophotometer (Thermo Fisher Scientific, Ratastie, Finland) using 530 nm as testing wavelength and 630 nm as reference wavelength. Absorbance data were expressed as percentage of cell viability relative to control. Resulting values were fitted to a sigmoidal four-parameter logistic function with hill slope (4PL). From these dose–response curves, inhibitory concentrations (IC_{50}), standard concentration required to cause a reduction of 50% in cell viability expressed in nM, were calculated with the SigmaPlot v.12.0 software.

Electrophysiological Recordings

Whole-cell voltage clamp recordings, achieved by gentle mechanical suction of the membrane patch, were performed in the HEK293 cell line expressing the human $Na_v1.6$ alpha subunit as previously described (Burbidge et al. 2002). Recording electrodes were fabricated from borosilicate glass microcapillaries (outer diameter, 1.5 mm), and the resistance was 5–10 MΩ. The progress of seal formation was evaluated by monitoring the

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decrease in membrane resistance. Series resistance was compensated by about 70% with the internal compensation circuitry of the CV-7B current clamp and voltage clamp headstage. Cells were maintained at a holding potential (V_{hold}) of − 55 mV and data were recorded after the membrane resistance has stabilized. Voltage-gated sodium currents were recorded using standard whole-cell voltage clamp recordings at room temperature with a Multiclamp 700B amplifier and digitalized with the Digidata 1440A data acquisition system (both from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz. Leak currents were subtracted from active currents by the P/N leak protocol provided by the pClamp10 software. After establishing the whole-cell configuration, cells were allowed to stabilize for 5 min before current recording protocols were initiated to ensure adequate equilibration between the internal pipette solution and the cell cytoplasm. Offline analysis was performed with the pClamp10 software (Axon Instruments). Intracellular electrode solution contained (in mM): 120 CsF, 15 NaCl, 10 EGTA, and 10 HEPES (pH 7.25 adjusted with CsOH), while the extracellular solution was Locke's buffer. To obtain the concentration–response data each ciguatoxin was directly added to the recording chamber and in all the experiments the effect of CTXs on VGSC was evaluated 5 min after bath application of each toxin concentration or the corresponding amount of solvent. To record sodium currents (I_{Na}) and the activation of voltage-gated sodium channels voltage steps from − 80 to + 80 mV (10 mV increments, 17 steps and 0.15 s duration) were applied. Data analysis was performed using GraphPad Prism 5. All data are expressed as means ± SEM of n determinations. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P values < 0.05 were considered statistically significant.

Results

In the present work the binding of Caribbean and Pacific ciguatoxins to sodium channels was studied by an integrated approach that demonstrated similar toxic activity at the cellular level but different interactions with the protein that cause distinct effects on the activation voltage of sodium channels.

Molecular Modeling Studies

The binding mode of CTX1B, CTX3C, and C-CTX1 with the α subunit of the human $Na_v1.6$ channel was investigated in silico. As no three-dimensional structure of this protein was available, a homology model was constructed using the AlphaFold method (Jumper et al. 2021) and further validated by Molecular Dynamics (MD) simulation studies. To this end, the protein was immersed in a truncated cube of TIP3P water molecules and neutralized by addition of sodium ions using the molecular mechanics force field AMBER 17 (Case et al. 2017). The minimization of the protein and the MD simulation (200 ns) of the resulting three-dimensional structure was carried following our previously reported protocol (Vazquez-Ucha et al. 2021). The reported model in the unbound form is stable as no significant changes were observed during the whole simulation. The latter is easily visualized by the analysis of the root-mean-standard deviation (rmsd) of the whole-protein backbone, which showed relatively low values considering that it is a protein of about 2000 residues (Fig. S1).

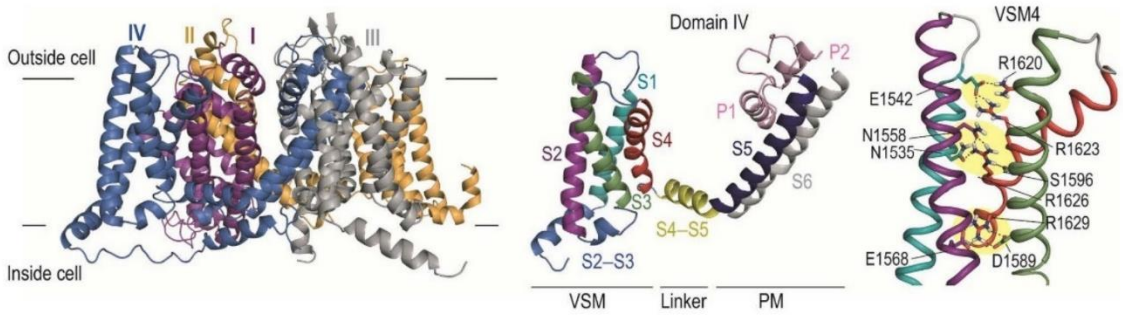


Fig. 2 Overall and detailed views of the three-dimensional model of the α subunit of the human $Na_v1.6$ channel in the unbound form obtained by MD simulation studies. Snapshot taken after 200 ns of simulation is given. The four domains of the channel (I–IV) are shown in purple, orange, gray, and blue, respectively. Detailed views of domain IV, as well as the main hydrogen-bonding interactions (black-dashed lines and yellow shadow) of the inner part of the voltage-sensing module 4 (VSM4) are provided. Relevant side-chain residues are shown and labeled

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As shown in Fig. 2, the α subunit of the human $Na_v1.6$ channel consists of four homologous but not identical transmembrane domains (I–IV), each one composed by six transmembrane segments (S1–S6) that form (i) a voltage-sensing module (VSM) containing segments S1–S4; (ii) a pore module (PM) containing segments S5 and S6 and a P-loop that connects them; and (iii) the helical hairpin S4–S5 that connects both modules that seem to act as an elbow in the electromechanical motion of the VSM (Wisedchaisri et al. 2019). Four positive gating charges (arginine residues) that are exquisitely distributed along S4 with their side chains pointing toward segments S1–S3 seem to modulate the VSM function through polar interactions. Thus, these positively charged residues, which are stabilized on the same face of S4 through diverse hydrogen-bonding and electrostatic interactions with the side-chain residues of helices S1–S3, undergo conformational changes as a response to the depolarization and repolarization of the membrane and would trigger long-distance motions in PM to open and close the pore (Ahern et al. 2016; Catterall et al. 2017).

Once the model of the unbound form was validated, the binding modes of Pacific ciguatoxins CTX1B and CTX3C and Caribbean ciguatoxin C-CTX1 were studied by docking using the program GOLD 2020.2.0 (Jones et al. 1997) and the coordinates of $Na_v1.6$ channel in the unbound form taken after 200 ns of simulation. To define the docking region, it was considered that (i) as brevetoxins, CTXs bind to the S5 segment of domain IV of the channel (Cestele and Catterall 2000; FAO 2020) and (ii) the alanine scanning mutagenesis studies reported by (Konoki et al. 2019) on $Na_v1.2$ channel showed that changes in the central site of segment S5, specifically residues I1654, G1655, L1656, L1657, and F1659, reduce the affinity of brevetoxin by two–threefold against the wild-type. This region of S5, which is conserved in the $Na_v1.6$ channel, was therefore selected for binding. To assess the reliability of the GOLD-proposed binding mode, the highest score solutions for each CTX obtained by docking were further analyzed by MD simulation studies following a similar protocol as for the unbound form. Following the same protocol, the binding mode of brevetoxin BTX-3 was also explored for comparison.

The outcomes of these simulation studies showed that all CTXs have in common the following features (Fig. 3): (i) as brevetoxin, these compounds bind to the cleft created by segment S5 of domain IV, segment S6 of domain I, and the P-loop (P1) that connects segments S5 and S6 of domain IV (Fig. S2). The latter binding involves about half of the cyclic polyether skeleton, specifically moieties F–M for CTX1B and CTX3C and moieties A–I for C-CTX1. All CTXs showed to be very stable within the pocket as no significant changes were observed during the whole simulation (Fig. S3), either for the ligand (Fig. S4) or the protein backbone (Fig. S5); (ii) all CTXs interact by hydrogen bonding with at least one protein residue. CTX1B and C-CTX1 interact with the ε-amino group of K385 (S6, domain I) through the hydroxyl group in rings M and A, respectively, while for CTX3C this polar contact occurs between the main carbonyl group in G1584 and the NH group in W1584 and its hydroxyl group (Fig. S6). C-CTX1 establishes additional hydrogen-bonding interactions with residues N1485 and T1482, which are located on the helical hairpin close to segment S5; and (iii) all CTXs lie on the protein cleft thanks to numerous lipophilic interactions, mainly between the axial hydrogen atoms and methyl groups of the linkage between rings and the side-chain residues within the cleft. The latter interactions occur in great extent through both faces of the ciguatoxins polycyclic skeleton. Full details of the residues involved are provided in the supporting information (Table S1).

While no significant differences on the overall binding mode of the two Pacific ciguatoxins were observed, except that CTX3C burrows deeper into the cleft than CTX1B as it lacks substituents on the terminal rings A and M (Fig. 3A, B), the same does not occur for the Caribbean ciguatoxin (Fig. 3C). Thus, the most relevant binding differences among these CTXs are as follows: (i) while Pacific ciguatoxins CTX1B and CTX3C bind to the α subunit of the human $Na_v1.6$ channel with the ring M pointing toward its extracellular part and ring A toward the cytoplasm [head(IN) to tail(OUT)], C-CTX1 binds in the reverse manner, thus with ring A toward the extracellular part of the channel [tail(IN) to head(OUT)] and (ii) the overall extended conformation of ciguatoxins CTX1B and CTX3C causes binding of moieties A–E of the cyclic polyether skeleton to the interface formed by segment S3 and helical hairpin S4–S5; the curved shape of the Caribbean ciguatoxin C-CTX1 avoids the interaction with the latter site of the channel and facilitates further contacts between its moiety J–N with segment S5 as well as adjacent sites near the cytoplasm.

Comparison of the conformation of domain IV of the channel upon CTXs binding with the obtained in the unbound form highlighted relevant differences in the relative arrangement of VSM4 and the helical hairpin S4–S5 in all complexes (Fig. 4). Since the Pacific ciguatoxins CTX1B and CTX3C gave similar results, only the outcomes of ciguatoxins CTX1B and C-CTX1 are presented herein but additional information is given in the supporting information (Fig. S7). For the $Na_v1.6$@CTX1B complex, the binding of a fragment of the ciguatoxin skeleton in the interface between segment S3 and linker S4–S5 causes an extended arrangement of the latter relative to segment S5, which implies a loss of the elbow conformation observed in the free form as well as a reduction in the overall plasticity of this region of the protein (Fig. 4A, B).

In addition, the protein site in segment S4 closest to the linker S4–S5 becomes less folded and segments S2 and S3

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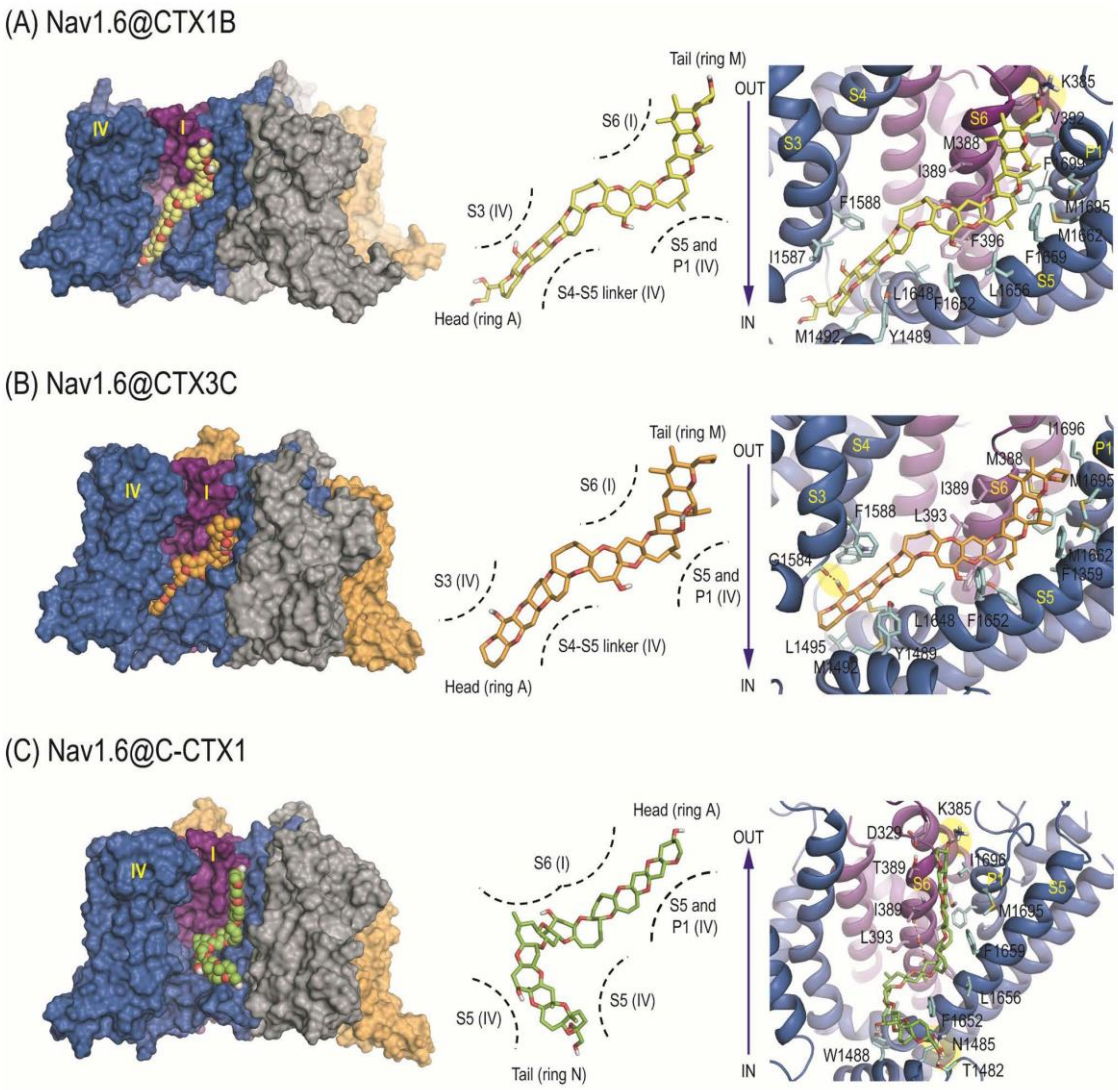


Fig. 3 Overall and detailed views of the three-dimensional models of the α subunit of the human $Na_v1.6$ channel in complex with CTX1B (**A**), CTX3C (**B**), and C-CTX1 (**C**) obtained by MD simulation studies. Snapshots taken after 200 ns of simulation are given. Domains I–IV are shown in purple, orange, gray, and blue, respectively. In the surface representation, the ciguatoxins are highlighted in spheres. Detailed views of the main contacts between ciguatoxins and the transmembrane segments of the protein are provided. Relevant side-chain residues are shown and labeled. Hydrogen-bonding interactions are provided as dashed lines (red) and with a yellow shadow. Note how both Pacific ciguatoxins bind in a similar manner (head to tail), interacting with the transmembrane segments of domains I and IV: segments S5 (including the P-loops that connect S5 and S6, IV) and S3 (IV), the S4–S5 linker that connect VSM4 with the PM4, and segment S6 (I). On the contrary, C-CTX1 binds with ring A pointing toward the extracellular part of the channel (tail to head) and the interaction with the VSM4 (S3 and S4) is weaker than for Pacific ciguatoxins

move away from the PM4 domain. Helices S3 and S4, and somewhat less pronounced helix S2, twist mainly at their C-terminal part, which is closest to the cytoplasm. As a result of all these conformational changes, the position of the central gating charge in S4, R1626, is less controlled since the stabilizing partners, the side chains of residues N1558 (S2) and S1596 (S3), move away from its guanidinium group (Fig. 4D, E). Thus, while for the unbound form,

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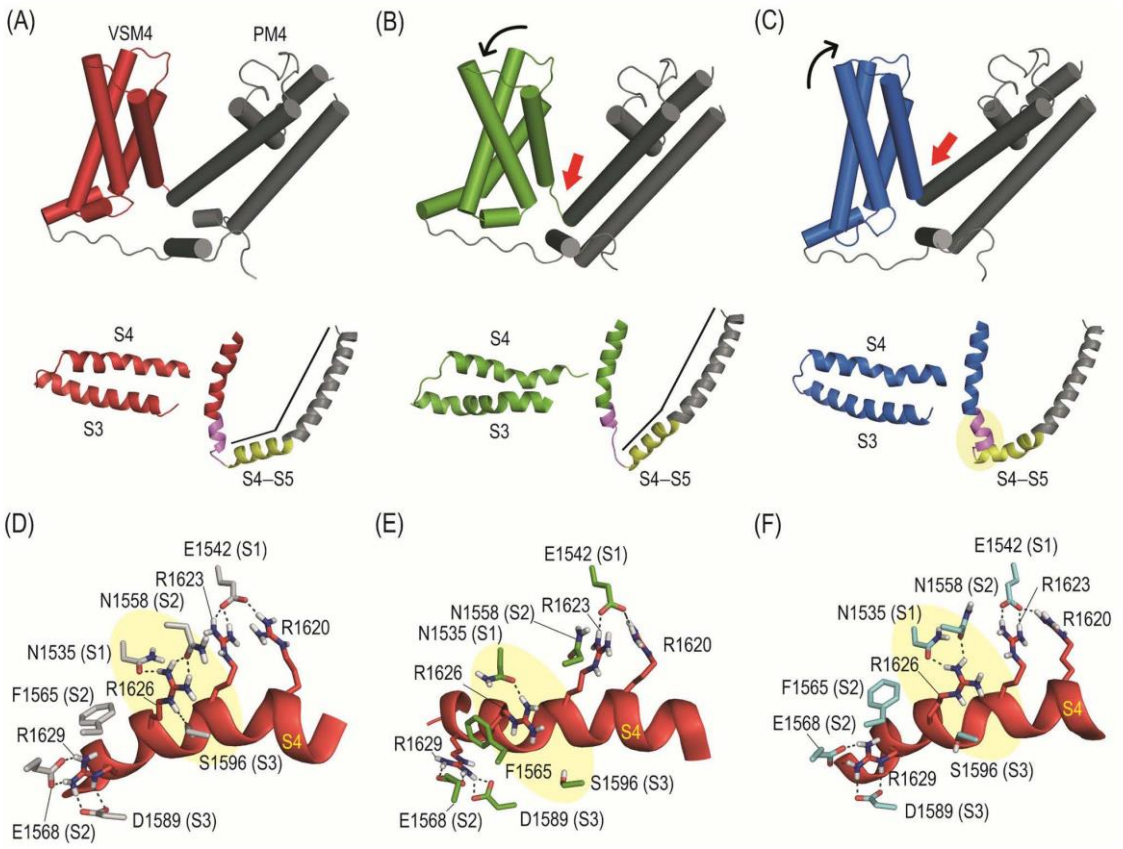


Fig. 4 **A–C** Comparison of the three-dimensional arrangement of domain IV of the human $Na_v1.6$ channel in the unbound form (**A**) and upon ciguatoxins CTX1B (Pacific, **B**) and C-CTX1 (Caribbean, **C**) binding. Snapshots taken after 200 ns of simulation and close views of relevant parts of the VSM are given. Note how upon CTX1B binding segment S3 (upper part) is displaced away from PM4 resulting in a new overall arrangement of VSM4, while C-CTX1 binding mainly promotes the folding of the bottom part of segment S4, which is located closer to the linker S4–S5. Note how segments S3 and S4 are twisted in the $Na_v1.6$@CTX1B complex. **D–F** Detailed view of the internal polar contacts of the arginine residues located in S4 in the unbound form (**D**) and the corresponding complexes with CTX1B (**E**) and C-CTX1 (**F**). Note how upon binding of CTX1B significant changes in the hydrogen-bonding interactions of residue R1626 (highlighted with a yellow shadow) are observed. In addition, a new arrangement for residue F1565, which is located on top of the guanidinium group of R1626 establishing a strong cation–π interaction, is also identified

the interaction between R1626 and N1558 was observed during ~ 70% of the simulation with an average distance of 2.9 Å, no contact was observed in the $Na_v1.6$@CTX1B complex (Fig. S8). A similar trend was identified for residue S1596, which reduces its interaction with R1626 by fourfold in the complex. Remarkably, a significant change is also observed in the disposition of the phenyl group in F1565, which turns to establish a strong cation–π interaction with the guanidinium group of R1626 (Figs. 4E and S9). On the other hand, the impact of C-CTX1 binding on the global arrangement of VSM4 and its relative positioning to PM4 proved to be distinct than the obtained for CTX1B (Fig. 4C, F). For the $Na_v1.6$@C-CTX1 complex, the results revealed that (i) a greater folding of the C-terminal part of segment S4, which is shifted toward the linker S4–S5, is observed; (ii) the layered arrangement of the protein region composed by linker S4–S5 and segment S5 is maintained; (iii) no twisting of the helices S2–S4 are observed; (iv) no significant changes in the interactions of residue R1626 as well as motion on the F1565 side chain are identified; and (v) the helical arrangement of linker S2–S3 is lost. As a result of these conformational changes, VSM4 tilts toward segment S5 in its intracellular site.

Despite the differences identified in the binding of the Pacific and Caribbean ciguatoxins with the channel cleft of domain IV, the calculated binding free energies

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of these ciguatoxins in the corresponding $Na_v1.6$@CTX complex proved to be similar as experimentally observed, with energy values of -63.5 ± 0.2 kcal mol^{-1} and -61.9 ± 0.2 kcal mol^{-1} for CTX1B and C-CTX1, respectively. These calculations were carried out using the MM/PBSA approach in explicit water (generalized Born, GB) and employing frames every 0.1 ns over the last 100 ns of MD simulations as implemented in Amber (Miller et al. 2012).

Functional Consequences of the Different Binding of Pacific and Caribbean ciguatoxins to Human Sodium Channels

C-CTX1 purified solution and C-CTX1 FTRM (Castro et al. 2022) used for the evaluation of the toxicity in functional assays were first analyzed by LC–MS/MS to confirm the presence of C-CTX1 by following the conditions described previously (Estevez et al. 2019a). C-CTX1 was confirmed in the reference material by comparison with authentic C-CTX1. Retention time and ion transition of C-CTX1 selecting the sodium adduct as precursor and product ion *m/z* 1163.7 $[M+Na]^+$/*m/z* 1163.7 $[M+Na]^+$ were consistent with the detected in authentic C-CTX1 (Fig. S10).

The quantitation of C-CTX1 was performed with CTX1B applying a correction factor to convert the CTX1B equivalents to C-CTX1 equivalents. CTX1B calibration curve ranged from 0.450 ng mL^{-1} to 44.660 ng mL^{-1}. A C-CTX1 correction factor ([C-CTX1] = 0.5 [CTX1B]) was obtained by interpolating C-CTX1 pure standard in the CTX1B calibration curve. Therefore, C-CTX1 in the sample was quantified in CTX1B equivalents using the calibration curve. CTX1B eq. were transformed to C-CTX1 eq. with the correction factor previously obtained (Fig. S11). CTX3C and CTX1B, at concentrations between 0.0001 nM and 10 nM shifted the activation voltage of human sodium channels in the negative direction (Fig. 5A). In the absence of toxin the voltage activation of sodium channels occurred approximately at − 30 mV. By addition of increasing concentrations of P-CTX analogues (Fig. S11), one-way ANOVA followed by Dunnett's test did not show statistically significant differences between the activation voltage of $Na_v1.6$ in control conditions and in the presence of 0.0001 nM, 0.001 nM, 0.01 nM, and 0.1 nM neither for CTX1B nor CTX3C (Fig. 5A). The effects on the activation voltage of $Na_v1.6$ caused by CTX3C and CTX1B were observed at concentrations of 1 nM and higher (Fig. 5A). The sharp negative shift in the activation voltage of sodium channels reached 26 mV after addition of 5 nM CTX1B or 10 nM CTX3C in both cases (Fig. S11). However, for the Caribbean analogue C-CTX1 (Fig. 5A), the activation voltage of sodium channels in control cells was -33.1 ± 1.7 mV ($n = 13$) and -30.0 ± 5.7 mV ($n = 3$) in the presence of 1 nM C-CTX.

Although the effects of the Pacific ciguatoxin analogues on VGSC have been studied previously and these compounds have shown to cause a concentration-dependent decrease in sodium current amplitude (Inserra et al. 2017; Perez et al. 2011; Stevens et al. 2011; Strachan et al. 1999; Yamaoka et al. 2004), the activity of the Caribbean analogues on sodium channels has not been tested so far. In this study the potency of CTX3C and CTX1B on VGSC was re-evaluated and compared, for the first time, with the effect of the Caribbean analogue C-CTX1 in human sodium channels.

Regarding CTX1B in the absence of toxin, the mean peak current was -1965 ± 276 pA ($n = 14$) and it decreased in a concentration-dependent manner in the presence of the different concentrations of CTX1B added to the recording chamber. Thus, peak sodium current was -1205 ± 505 pA after bath application of 0.0001-nM CTX1B ($n = 3$), -1151 ± 414 pA for 0.001 nM ($n = 3$), -1395 ± 308 pA for 0.01 nM ($n = 6$), -869 ± 306 pA for 0.1 nM ($n = 8$), -375 ± 144 pA for 1 nM ($n = 5$), -393 ± 199 pA for 5 nM ($n = 5$), and -217 ± 67 pA for 10 nM ($n = 3$). The normalized peak sodium currents amplitudes in the absence and presence of the different CTX1B concentrations are summarized in Fig. 5B and representative sodium traces in control conditions or in the presence of 1 nM CTX1B are shown in the inset.

In the same context, the response of sodium channels to CTX3C was also evaluated (Fig. 5C). In control cells the peak sodium current was -1506 ± 197 pA ($n = 13$) and it decreased in a concentration-dependent manner in the presence of the different concentrations of CTX3C added to the recording chamber. Thus, peak sodium current was -1022 ± 65 pA after bath application of 0.0001 nM ($n = 5$), -872 ± 87 pA for 0.001 nM ($n = 5$), -967 ± 175 pA for 0.01 nM ($n = 4$), -933 ± 212 pA for 0.1 nM ($n = 7$), -407 ± 131 pA for 1 nM ($n = 3$), -475 ± 184 pA for 5 nM ($n = 3$), and -525 ± 210 pA for 10 nM ($n = 3$). The normalized peak sodium currents in the absence and presence of the different CTX3C concentrations and the representative sodium traces in control conditions or in the presence of 1 nM CTX3C are shown in Fig. 5C.

The evaluation of the effect of C-CTX1 on VGSC was performed at concentrations of 0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, and 1 nM, since the matrix effect did not allow the use of higher toxin concentrations because cell seals became unstable. In this case, C-CTX1 caused a decrease of the peak sodium current similar to that caused by the Pacific analogues, and this effect was concentration dependent. In $Na_v1.6$ human sodium channels, in the absence of toxin the maximum peak sodium current amplitude was -1916 ± 306 pA ($n = 13$). The addition of increasing C-CTX1 concentrations caused a progressive concentration-dependent decrease in the maximum peak sodium current intensity. Thus as shown in Fig. 5D, after bath application of

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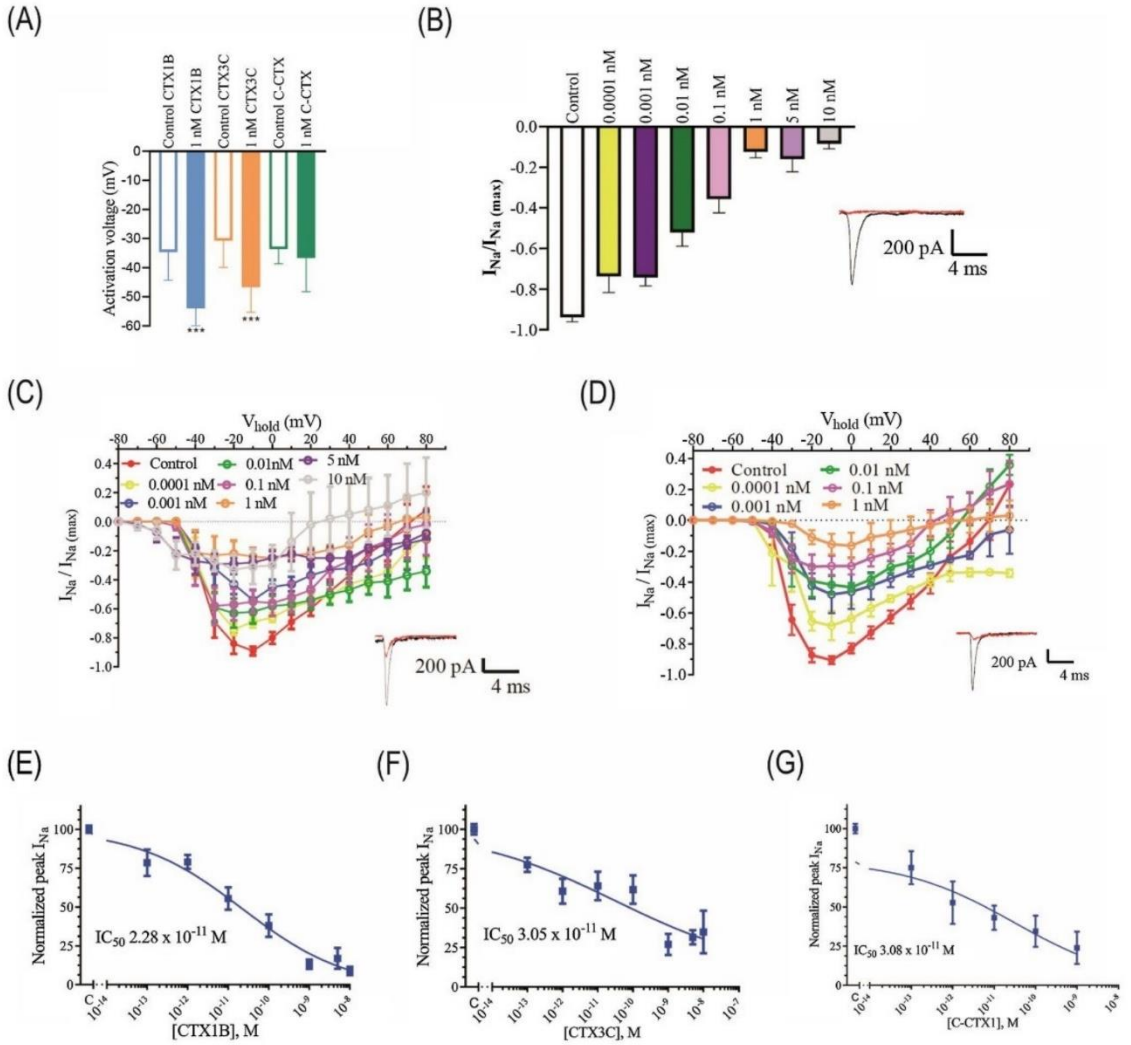


Fig. 5 **A** Effects of Pacific and Caribbean ciguatoxins on the activation voltage of sodium channels. **B–D** Current–voltage relationship for the effect of different concentrations of ciguatoxins CTX1B (**B**), CTX3C (**C**), and C-CTX1 (**D**) on sodium currents and representative sodium currents traces elicited at − 20 mV in the absence of toxin (black) and in the presence of 1 nM of ciguatoxin (red) are shown in the inset. **E–G** Concentration–response graphs indicating the effect of CTX1B (**E**), CTX3C (**F**), and C-CTX1 (**G**) on the normalized peak inward sodium currents at − 10 mV and the corresponding IC_{50} obtained in each condition

0.0001-nM C-CTX1 I_{Na} was − 1434 ± 89 (n = 3), − 969 ± 71 pA for 0.001 nM (n = 3), − 886 ± 409 pA for 0.01 nM (n = 5), − 705 ± 260 pA for 0.1 nM (n = 5), and − 438 ± 93 pA for 1 nM (n = 3). Normalized peak sodium currents intensities in the absence and presence of the different C-CTX1 concentrations and representative sodium traces in control conditions and in the presence of 1 nM C-CTX1 are shown in Fig. 5D.

The percent inhibition of the peak inward sodium currents by different concentrations of CTX were used to obtain a concentration–response curve (Fig. 5E-G). Nonlinear fit of the data yielded an estimated IC_{50} for CTX1B of 2.28×10^{-11} M (95% confidence interval (CI) from 1.47×10^{-12} M to 3.54×10^{-11} M) (Fig. 5E), while the IC_{50} inhibition of peak inward sodium current for CTX3C was 3.05×10^{-11} M (95% CI from 6.47×10^{-13} to 2.1×10^{-9}) (Fig. 5F) and 3.08×10^{-11} M for the Caribbean analogue

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Table 2 Effects of the three ciguatoxin analogues on the activation voltage of VGSC, their IC_{50}, and relative potency taking into account their effect on $Na_v1.6$ intensity

Analogue	IC_{50} I_{Na} inhibition (M)	Relative potency	Na_v activation voltage at 1 nM toxin (mV)	Activation voltage shift (mV)
Control CTX1B			− 34.62 ± 2.7 (n = 13)	
CTX1B	2.28×10^{-11}	1.00	− 54.00 ± 4.0 (n = 5)	− 20
Control CTX3C			− 30.71 ± 2.4 (n = 14)	
CTX3C	3.05×10^{-11}	0.74	− 46.67 ± 2.9 (n = 9)	− 16
Control C-CTX1			− 33.64 ± 1.5 (n = 11)	
C-CTX1	3.08×10^{-11}	0.74	− 33.33 ± 8.8 (n = 3)	0
Control fish extract			− 34.12 ± 1.230 (n = 17)	
Fish extract with C-CTX1	1.7×10^{-11}	1.3	− 33.33 ± 3.333 (n = 3)	0

C-CTX1 (95% CI from 5.68×10^{-14} to 1.6×10^{-8} M) (Fig. 5G).

The effects of the three ciguatoxin analogues on sodium channels are summarized in Table 2 and demonstrate that the relative potency of these compounds on sodium channel intensity is similar but they have different effects on voltage channel activation.

Effect of the Samples Extracted from Ciguatera Fish Caught in Canary Island on VGSC

As for the purified toxins, bath application of fish tissue extract (*Seriola spp.*) containing different concentrations of C-CTX1 decreased the peak sodium current in a concentration-dependent manner (Fig. S12). These decreases were slightly lower than the effects observed when cells were exposed to the same concentrations of purified C-CTX1. Figure S12A shows the normalized peak sodium currents in the absence and in the presence of fish extract containing different C-CTX1 concentrations. The percentage inhibition of peak inward sodium currents by different concentrations of fish tissue extract was used to obtain a concentration–response curve (Fig. S12B). Non-linear fit of the data yielded an estimated IC_{50} inhibition of peak inward sodium current for the fish extract containing C-CTX1 of 1.7×10^{-11} M (95% CI from 1.07×10^{-12} to 2.7×10^{-10} M). In this case, as with pure C-CTX1 the shift in the activation voltage was not observed.

N2a-MTT Cytotoxicity Assay

C-CTX1, CTX1B, and CTX3C toxic potency were evaluated under the same conditions in six different days and for two cell batches Dose–response curves were obtained for three analogues and toxic potency was determined from IC_{50} values (Fig. 6).

For batch 63649750, CTX1B was the most toxic compound, with an $IC_{50} = 1.42 \pm 0.14$ pM, while C-CTX1 and CTX3C showed approximately the same toxicity, with values for IC_{50} of 1.98 ± 0.42 pM and 2.07 ± 0.55 pM, respectively (Table 3). For batch number 70005648, CTX1B yielded an $IC_{50} = 1.14 \pm 0.29$ pM, while the IC_{50} values for C-CTX1 and CTX3C were 1.81 ± 0.36 pM and 1.61 ± 0.43, respectively (Table 3). No statistically significant differences were observed between the IC_{50} of C-CTX1, CTX1B, or CTX3C (Student's t test; $p = 0.612$, 0.311, and $p = 0.217$), respectively. Previous studies showed similar toxicity increases, as follows: C-CTX1 ≤ CTX3C < CTX1B (Castro et al. 2020). Toxic potencies of C-CTX1 and CTX3C are estimated using the formula IC_{50} (CTX1B)/IC_{50} (C-CTX1 or CTX3C) (Table 3). The three CTXs analogues showed a similar *in vitro* toxicity, and C-CTX1 and CTX3C were only about 0.3-fold less toxic than CTX1B.

Discussion

CP is an emerging food safety hazard. Many studies have been performed aiming to determine the toxicity and the relative potencies for all the CTXs analogues. However, the lack of suitable certified reference materials and the limited amounts of contaminated product available made these tasks a real challenge. So far, most of these studies were performed using P-CTX analogues because they are the most abundant ones; however, CTX group of toxins isolated in the Caribbean and in the Indian Oceans are emerging and spreading worldwide. So far, some agencies as the FDA considered to have different toxic potencies for Pacific and Caribbean CTXs, therefore caution should be taken in using reference materials or tests developed from Pacific ciguatoxins standards (EFSA 2010).

In the decade of the 90s the first comparison between the toxicity of Pacific and Caribbean CTXs was performed using the mouse bioassay (Lewis et al. 1999; Vernoux and Lewis 1997) and the results provided showed a tenfold higher toxicity for Pacific ciguatoxin versus the Caribbean congeners. The mouse intraperitoneal LD_{50} obtained were 0.25, 2.3, and 0.9 μg kg^{-1} for P-CTX1, P-CTX 2, and P-CTX

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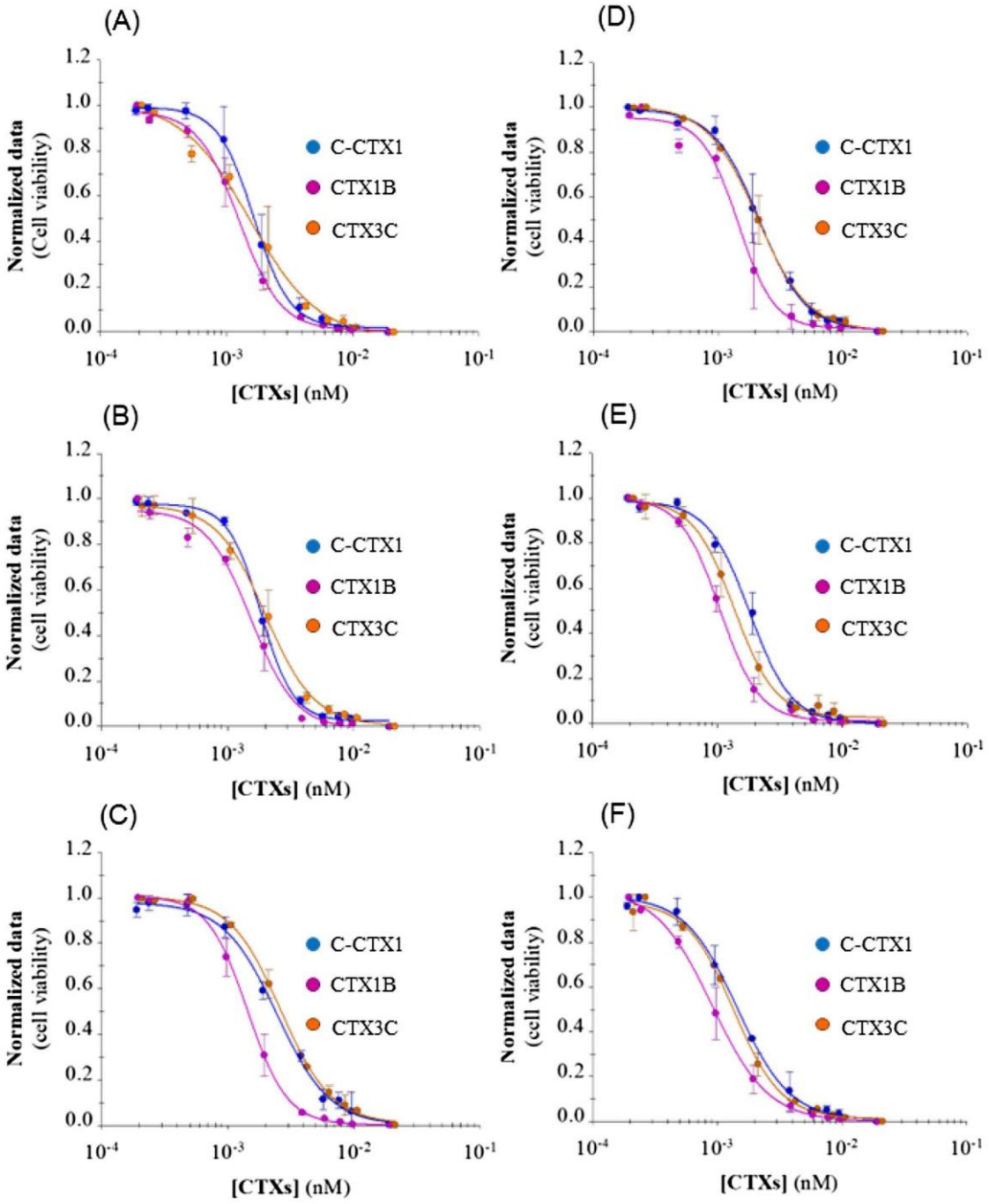


Fig. 6 Dose–response curves of three CTXs standards: C-CTX1, CTX3C, and CTX1B in N2a cells obtained after 16 h exposure to ouabain and veratridine. Left, concentration-response curves from batch 63649750 at passages number 214 (**A**), 215 (**B**), and 224 (**C**). Right concentration-response curves from batch 70005648 at passages number 203 (**D**), 205 (**E**), and 220 (**F**)

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Table 3 Toxic activity (IC_{50} values), slope values, and relative toxic potency (IC_{50} ratio) in different days for the ciguatoxins analogues C-CTX1, CTX1B, and CTX3C

Cell passage (#)	C-CTX1			CTX1B			CTX3C		
	IC_{50} (pM)	Slope	Relative potency	IC_{50} (pM)	Slope	Relative potency	IC_{50} (pM)	Slope	Relative potency
BATCH (#): 63649750									
214	1.65	− 3.1	0.77	1.27	− 2.6	1	1.55	− 1.6	0.82
215	1.84	− 3.4	0.84	1.55	− 2.5	1	2.02	− 2.3	0.76
224	2.45	− 2.1	0.59	1.44	− 2.8	1	2.64	− 2.2	0.54
BATCH (#): 70005648									
203	2.17	− 2.4	0.68	1.47	− 3.0	1	2.10	− 2.2	0.70
205	1.81	− 2.6	0.58	1.05	− 2.8	1	1.36	− 2.6	0.77
220	1.45	− 2.1	0.63	0.92	− 1.9	1	1.36	− 2.3	0.67

3, respectively (Lewis et al. 1991) and 3.6 and 1 μg kg^{-1}, for C-CTX1 and C-CTX2 after pH variations. These data were considered by the FDA to establish the current legislation regarding CTX limits in fishery products, assuming that Pacific ciguatoxins and C-CTX1 are the prevalent toxins. These initial studies on the different toxic potencies of Pacific and Caribbean ciguatoxin analogues must be interpreted with caution since the first report on the toxic effect of Caribbean ciguatoxin analogues was performed after the isolation and purification of toxic compounds from thawed flesh fish from the horse-eye jack (*Caranx Lantus*) caught in the French West Indies and autoclaving fish tissues for 30–40 min at 120 °C (Vernoux and Lewis 1997). This procedure was not evaluated to demonstrate that it did not affect the chemical integrity or efficacy of the toxic compounds. It is noteworthy that although, in this paper the authors claimed to follow the same procedure as that followed previously to isolate Pacific ciguatoxins (Lewis and Sellin 1992; Lewis et al. 1991), in the latter case fish viscera was cooked at 70 °C instead of 120 °C. Although ciguatoxins are considered thermostable, their stability is unclear, as there is evidence of interconversion in storage between C-CTX1 and C-CTX2 (Estevez et al. 2019a; Pottier et al. 2001). These valuable initial data were adopted by the expert meeting of FAO to publish their opinion on ciguatera poisoning (FAO 2020) as well as by the FDA to establish the current legislation regarding CTX limits in fishery products (FDA 2011). However, the low availability of purified Caribbean ciguatoxins has prevented further comparison of the toxic potency of the Caribbean and Pacific congeners. Nowadays, several Pacific ciguatoxins with purity higher than 99% are commercially available and were used in this study; however, no available materials for Caribbean ciguatoxins are on the market.

Until now, toxicity equivalency factors (TEFs) and IC_{50} for ciguatoxins analogues were calculated using mainly *in vitro* cytotoxicity assays on neuroblastoma cells. Currently, *in vivo* experiments based on the mouse bioassay are considered of poor specificity besides the experimental ethical concerns generated by the use of animals (EFSA 2010). The EFSA contaminants panel in the food chain established TEF values for the CTX group of toxins based on their acute intraperitoneal LD_{50} in mice: CTX1 = 1, CTX3C = 0.2, and C-CTX-1 = 0.1. These TEFs should be applied to express individual analogues identified with quantitative detection methods as CTX1B equivalents. But ciguatoxins are a special food safety problem because their toxicity is not associated to lethality but to a chronic syndrome, ciguatera, that may last intermittently several months and shows more than 150 different clinical symptoms (FAO 2020). Therefore, the lethal dose is well above the threshold damage, and lower concentrations should be considered to understand the toxic ciguatera syndrome.

After Lewis and Vernoux *in vivo* research (Lewis et al. 1991; Vernoux and Lewis 1997), other studies were carried out to evaluate the differences in the potency of the different CTXs congeners by the N2a bioassay but in different conditions; thus, the results obtained until date were not clear, varying from a similar toxicity levels for the 3 main analogues (Dechraoui et al. 2007) to a higher toxicity of CTX3C (Loeffler et al. 2021) or even obtaining a higher toxicity of the Caribbean analogue compared to the Pacific one (Dickey 2008).

In the present work we re-evaluated the potency of two Pacific analogues CTX1B and CTX3C as well as the effect of the Caribbean analogue C-CTX1, using N2a bioassay and confirming the results by evaluating the direct effect of the analogues on VGSC. These effects were obtained using the patch-clamp technique, which is the most sensitive assay available so far to study the changes that occur on ion channels. Moreover, these results were confirmed and compared in other laboratories with the cytotoxic results of the same analogues obtained using the most commonly employed *in vitro* technique to evaluate the CTXs cytotoxicity, the Neuro-2a MTT assay, as well as in silico evaluation assays. Previous studies have reported the effect of Caribbean ciguatoxin analogues in cultured cells and in VGSC using electrophysiology methods (Hogg et al. 1998, 2002);

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Table 4 Comparison of the relative toxicity of CTX1B, CTX3C, and C-CTX1 using *in vitro* techniques with the relative toxicity accepted by authorities

Analogue	I_{Na} inhibition	N2a (B-63649750)		N2a (B-70005648)		EFSA
		Mean	SD	Mean	SD	
CTX1B	1.00	1.00	–	1.00	–	1.00
CTX3C	0.74	0.71	0.14	0.71	0.05	0.2
C-CTX1	0.74	0.73	0.13	0.63	0.05	0.1

however, as far as we know this is the first study analyzing the effect of Caribbean and Pacific ciguatoxins in the same model and using three independent parameters. Besides, in this report the differences in the binding of the CTXs congeners to sodium channels were studied by MD simulation studies, suggesting that the binding of Pacific and Caribbean CTX to sodium channels alter the function of the pore. The results obtained clearly show that the three CTXs analogues caused a similar toxicity, but strikingly CTX3C and C-CTX1 exhibited the same potency, a fact that is at odds with the previously published data (EFSA 2010; Lewis et al. 1991; Vernoux and Lewis 1997). CTX1B had the highest cytotoxic effect and the greatest potency to inhibit sodium currents, followed by CTX3C and C-CTX1 which were very similar on their *in vitro* effects. The higher potency obtained by the fish tissue extract may be due to the matrix effect, since as previously described (Castro et al. 2020) matrix effects can lead to changes in the cytotoxic effect of CTXs depending on the total lipid content of the fish specimens. In this study, the shift in the voltage activation of sodium currents, commonly caused by CTXs, was evaluated and Pacific analogues significantly shifted the voltage activation of sodium channels to more hyperpolarising potentials at concentrations of 1 nM and higher. Again, this observation is in accordance with the previously described effects of ciguatoxins on sodium channels (Inserra et al. 2017; Strachan et al. 1999; Yamaoka et al. 2004). However, this fact could not be corroborated for the Caribbean analogue C-CTX1 nor the fish extract since, in this case, lower concentrations were used (from 0.0001 nM to 1 nM), due to the low availability of compounds. However, at 1 nM, C-CTX1 did not modify the activation potential of sodium channels. The MD simulation studies performed with Pacific ciguatoxins suggested that the shift in the activation voltage of sodium channels caused by these toxins would be due to their interaction with the interface between VSM4 (segment S3) and the linker S4–S5 that connect the latter with segment S5. This binding, which proved to be similar for CTX1B and CTX3C, seems to promote relevant conformational changes in segments S2–S4, altering the ability of the positively charged residues (specifically R1626) to achieve the optimal conformation for establishing polar interactions with the counterion residues inside VSM. As a result of all these changes, the capacity of VSM to perform the required electromechanical motion as a response to the depolarization/repolarization of the membrane would be modified. These findings would agree with the role of the linker S4–S5 in the established "sliding helix" mechanism of voltage sensing reported by (Wisedchaisri et al. 2019) as the elbow that slide helix S4 through the channel for controlling the motion of the gated residues (arginines) for voltage sensor functions.

On the other hand, the simulation studies carried out with the $Na_v1.6$@C-CTX1 complex suggested that there is no direct interaction between the Caribbean ciguatoxin and this hinge zone of the channel, as well as no significant changes on the polar interactions network of the positively gated residues. This finding would also justify the experimentally observed lack of shifting on the voltage activation of the channel caused by this toxin. In this case, C-CTX1 seems to reduce the plasticity of the C-terminal part of helix S4, which is connected to the linker S4–S5, which would also compromise the intrinsic sliding of S4 for modulating the channel activity as for the Pacific ciguatoxins. Globally, despite the differences found in the binding mode of both types of ciguatoxins, either in its binding orientation in the channel $Na_v1.6$ (head to tail and vice versa) or the specific binding pocket of part of its cyclic polyether skeleton, both CTXs seem to freeze the intrinsic motion of the VSM for activity, which would also trigger long-distance conformational changes in PM.

The combined information provided in this work matches, from different approaches and technologies, the current observations regarding the toxicity of CTXs: (i) The calculated binding free energies of -63.5 ± 0.2 kcal mol^{-1} and -61.9 ± 0.2 kcal mol^{-1} for CTX1B and C-CTX1 explain the long-term effects of these toxins, as these energies are consistent with high-affinity ligands (high K_{on} and low K_{off}); (ii) the fact that the binding orientation of both types (head to tail and vice versa) generate different modes of voltage sensor activation explains why C-CTX do not modify the activation shift, and this is related to the different observed toxic profiles of P-CTX vs C-CTX. P-CTX is associated to more intense neurological symptoms, while C-CTX is associated to gastrointestinal effects (probably of neurological origin) and later peripheral nervous symptoms (Boucaud-Maitre et al. 2018); and (iii) the activation shift, also observed for other low toxicity molecules, such as gambierol, gambierone, or MTX3 (Raposo-Garcia et al. 2022), contributes to neuronal firing (observed specially in P-CTX toxicity), but

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not to mice lethality, that seems to be mainly associated to peak currents, as evidenced by data shown in this paper.

In view of these data, a full re-evaluation of the relative potencies of the CTXs-group of toxins should be pursued using purified compounds and the most sensitive techniques available to guarantee food safety and thus protect human and animal health. Given the TEF results shown in Table 4 that we have obtained by independent studies with patch clamp in human cells and with mouse neuroblastoma, the current legal requirements that assign a tenfold lower potency for Caribbean ciguatoxins do need to be re-evaluated. As shown in Table 4, the mean functional relative potency of the ciguatoxin congeners used in this study was 1 for CTX1B, 0.73 ± 0.036 for CTX3C, and 0.71 ± 0.032 for C-CTX1, summarizing that the potencies of Pacific and Caribbean ciguatoxins are quite similar.

One of the main concerns of this study, which is the same for any other study that uses *Caribbean ciguatoxins*, is the lack of a reference calibrant. Although the quantitation of C-CTX was done against an NMR-quantitated P-CTX, still clearly there is an urgent need of these certified toxins to eliminate quantitative uncertainties.

Conclusion

The results obtained in this study demonstrate that the Pacific analogue CTX1B is the most potent compound of this group, while CTX3C and C-CTX1 caused the same effect confirmed by the cytotoxicity assay and similar effects on the amplitude of sodium currents evaluated by electrophysiological recordings. Both in the cytotoxicity test and in the decrease on sodium current intensity the effect of both analogues CTX3C and C-CTX1 was only 0.3 times lower than that of CTX1B. These data are contrary to what is stated by EFSA and the American FDA, which established a relative potency of C-CTX1 10 times lower than Pacific analogues. Therefore, this study highlights the need to re-evaluate the relative toxicities and potencies of ciguatoxins to establish safety limits for the presence of these emergent toxins in commercialized fish. In addition, docking analyses and simulation studies have demonstrated that the distinct binding mode of ciguatoxins with the channel $Na_v1.6$ (head to tail and vice versa) justify their different impacts on the activation potential of sodium channels and confirm that this parameter will be more suitable to detect ciguatoxins in suspicious fish samples.

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Data Availability Enquiries about data availability should be directed to the authors.

Declarations

Conflict of Interest The authors declare no financial or commercial conflict of interest.

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Supporting Information for:

In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins

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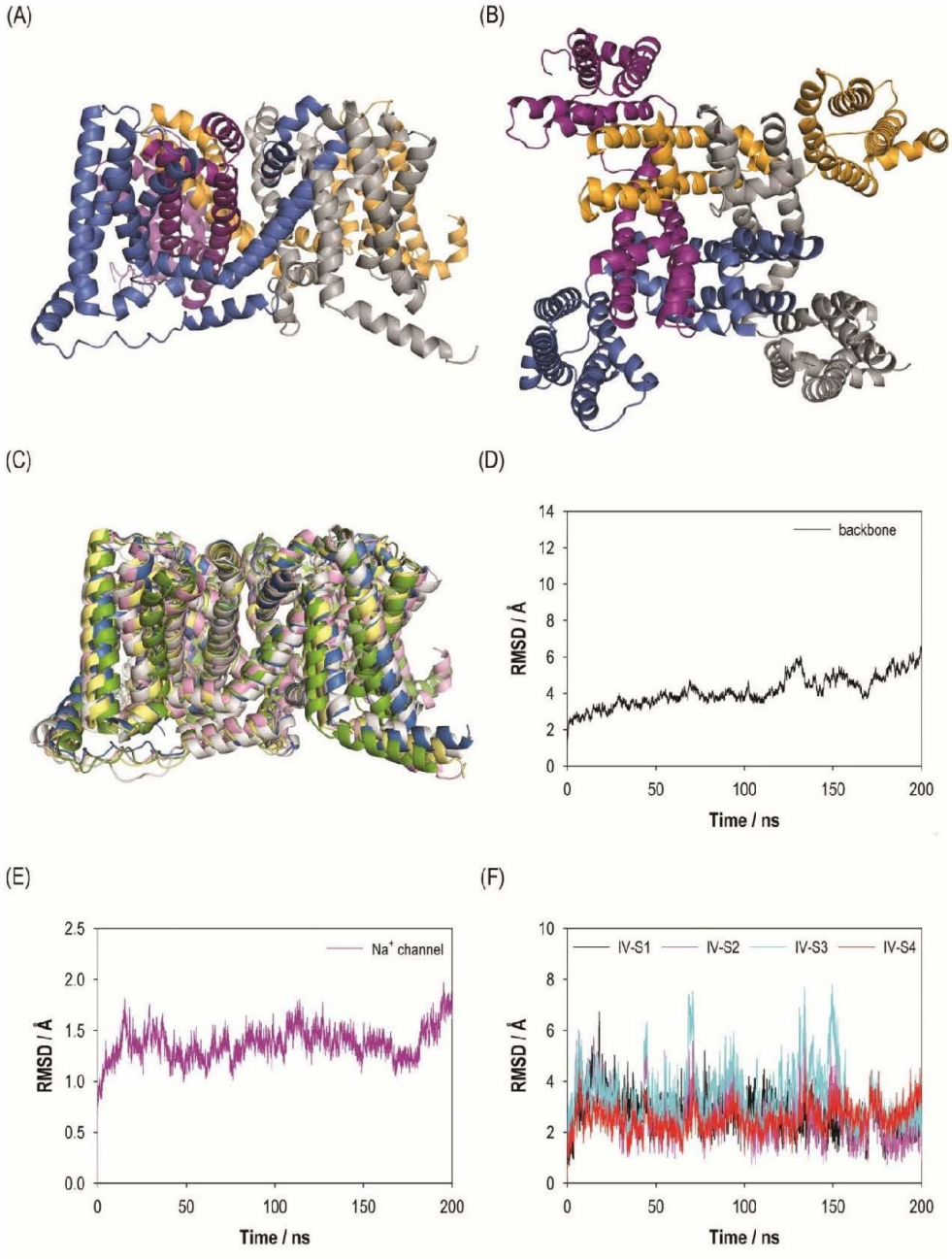


Figure S1. Side (A) and extracellular (B) view of the transmembrane domain of the $Na_v1.6$ channel after 200 ns of MD simulation. (C) Overlapping of different snapshots obtained from the MD simulation. (D–F) RMSD plots for the protein backbone (Cα, C, O and N atoms) calculated from the MD simulation: (D) whole protein (black line), (E) Pore module (α-helices S5 and S6, purple line) and (F) α-helices S1 (black), S2 (pink), S3 (cyan) and S4 (red).

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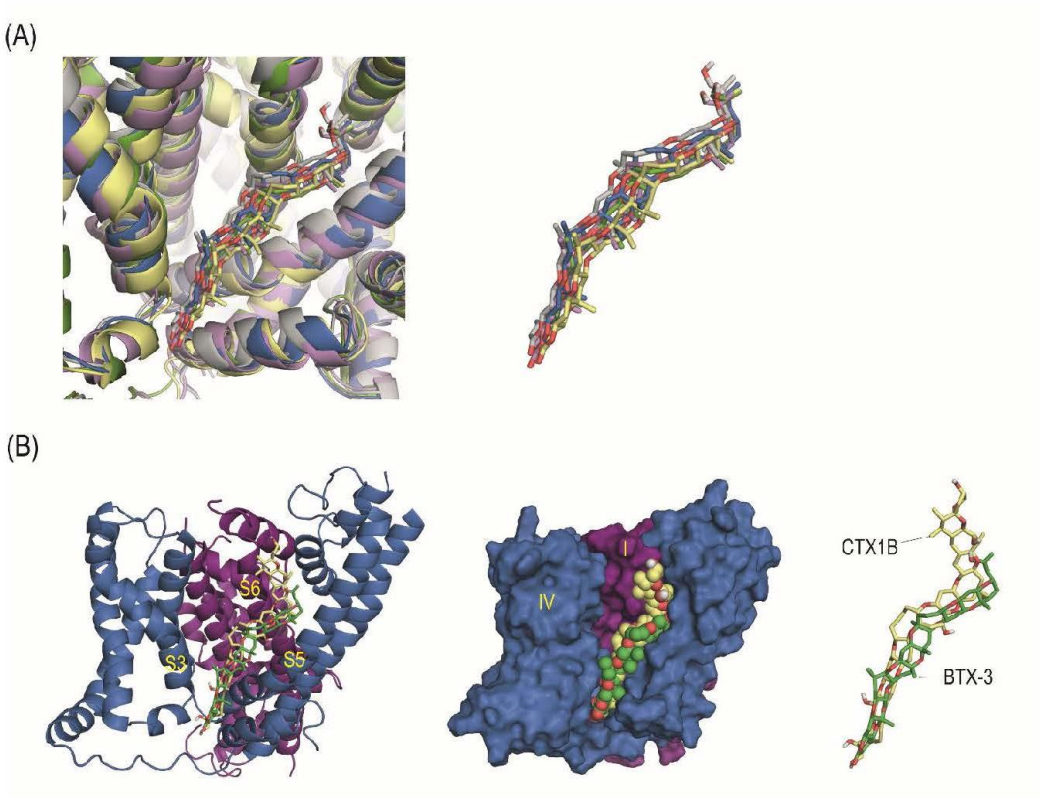


Figure S2. (A) Overlapping of different snapshots obtained from the MD simulation of $Na_v1.6$ in complex with brevetoxin BTX-3. Note the high stability of the brevetoxin in the pocket as no relevant modifications are observed. (B) Comparison of the binding mode of toxins CTX1B and BTX-3 in the $Na_v1.6$ channel. Two representations of the protein (cartoon and surface) and ligands (sticks and spheres) are provided. An overlapping of both toxins is also given. Only domains I and IV are shown. Note how both toxins bind to the cleft created by segments S5 and S6 of domain IV.

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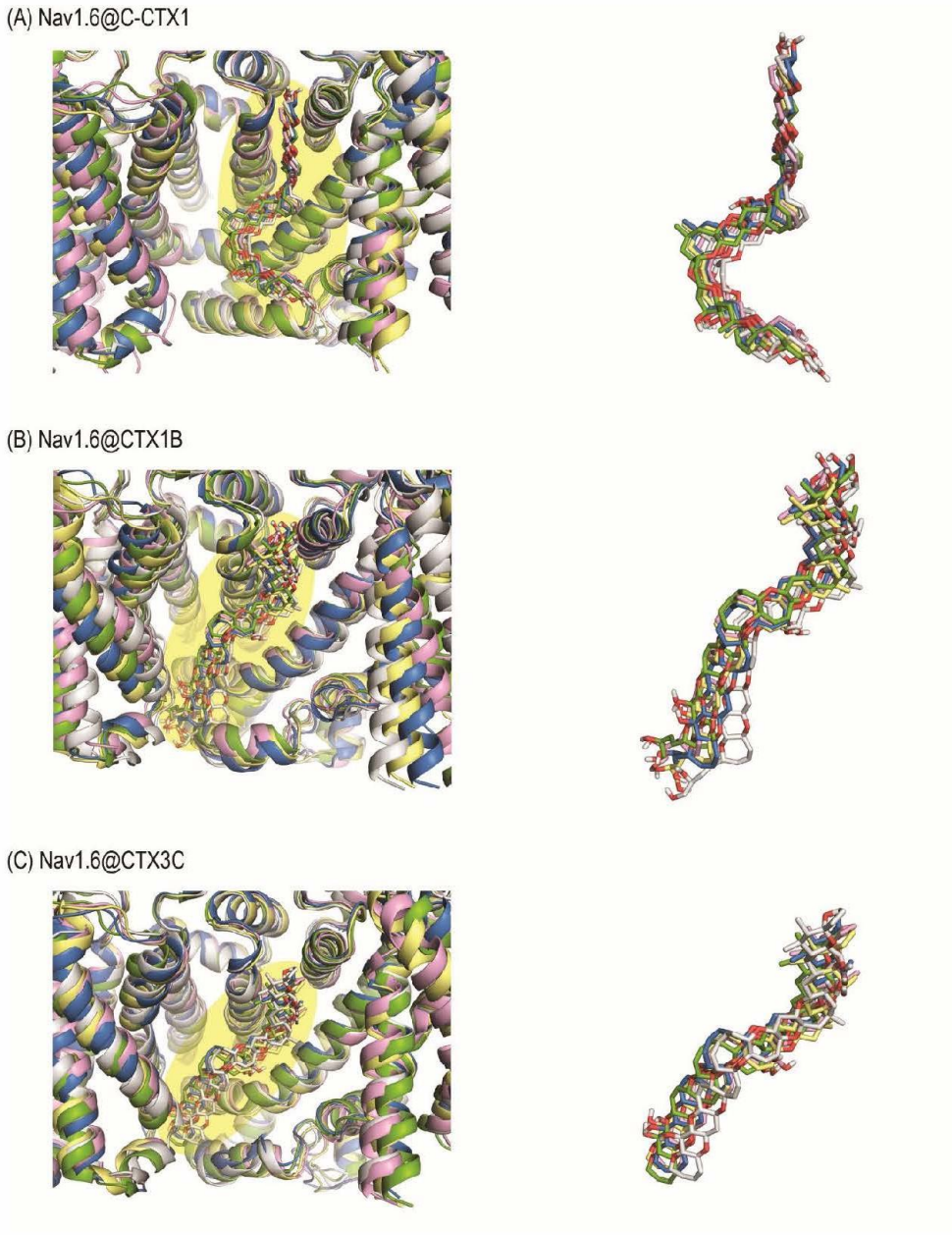


Figure S3. Overlapping of different snapshots obtained from the MD simulation of the complexes $Na_v1.6$@C-CTX1 (A), $Na_v1.6$@CTX1 (B) and $Na_v1.6$@CTX3C (C). For clarity a yellow shadow highlights the position of the ciguatoxin in the complex, with only the ligand displayed in the right side of each panel.

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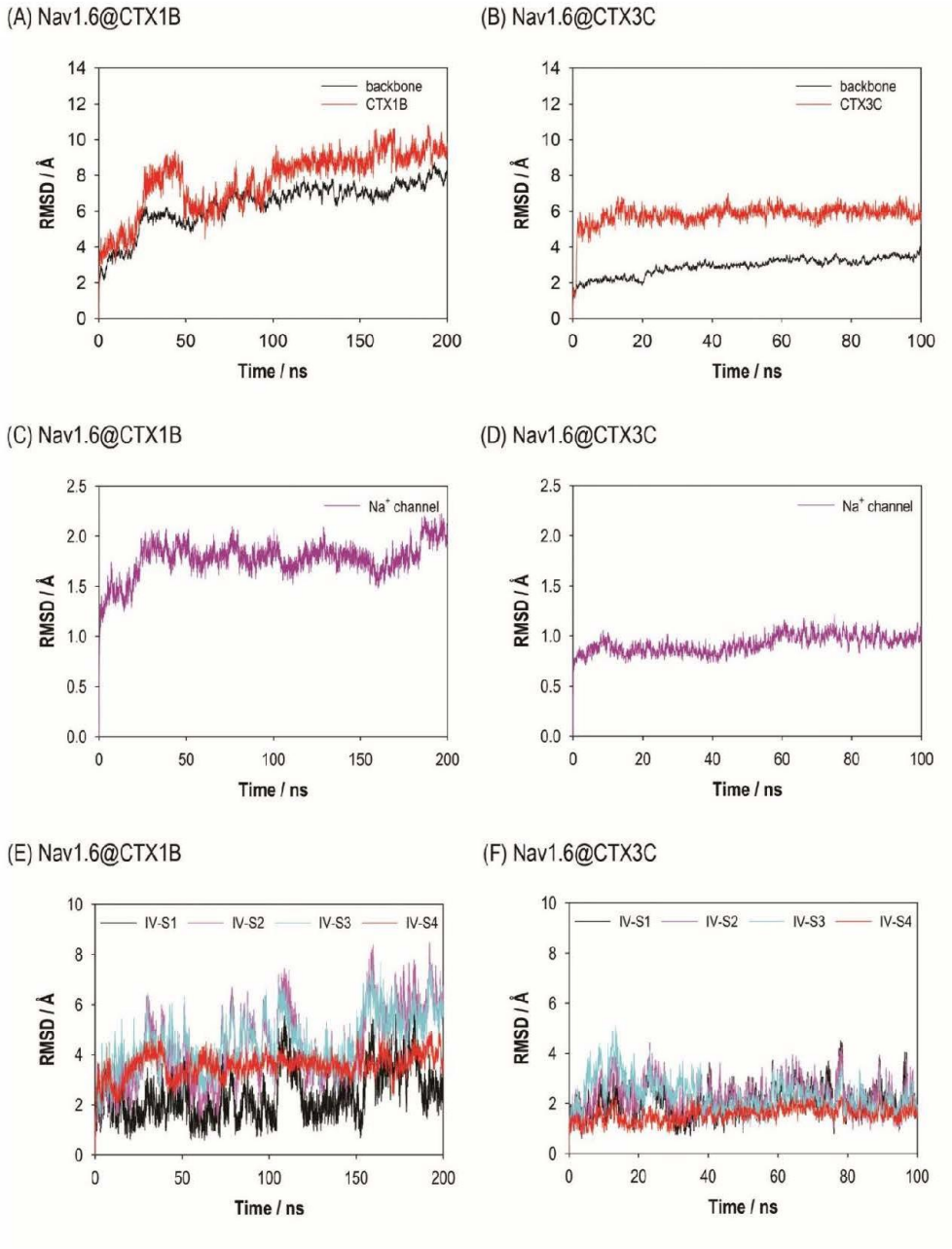


Figure S4. RMSD plots for the protein backbone (Cα, C, O and N atoms) and ciguatoxins calculated from MD simulations of the $Na_v1.6$@CTX1B (A, C and E) and $Na_v1.6$@CTX3C (B, D and F) complexes. (A, B) backbone (black lines) and ciguatoxins (heavy atoms, red lines); (C, D) pore module (α-helices S5 and S6, purple lines); and (E, F) α-helices S1 (black), S2 (pink), S3 (cyan), S4 (red) of domain IV.

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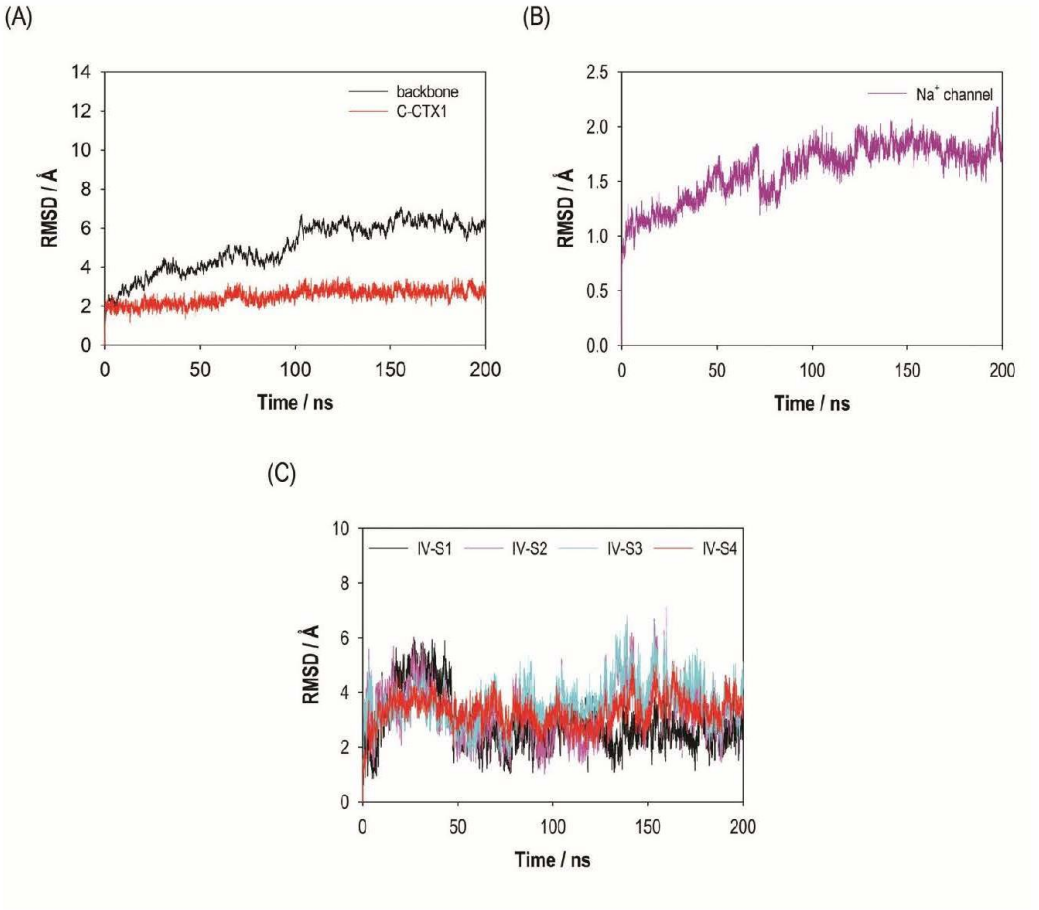


Figure S5. RMSD plots for the protein backbone (Cα, C, O and N atoms) and C-CTX1 calculated from MD simulation of the Nav1.6@C-CTX1 complex: (A) backbone (black line) and C-CTX1 (heavy atoms, red lines); (B) pore module (α-helices S5 and S6, purple lines); and (C) α-helices S1 (black), S2 (pink), S3 (cyan), S4 (red) of domain IV.

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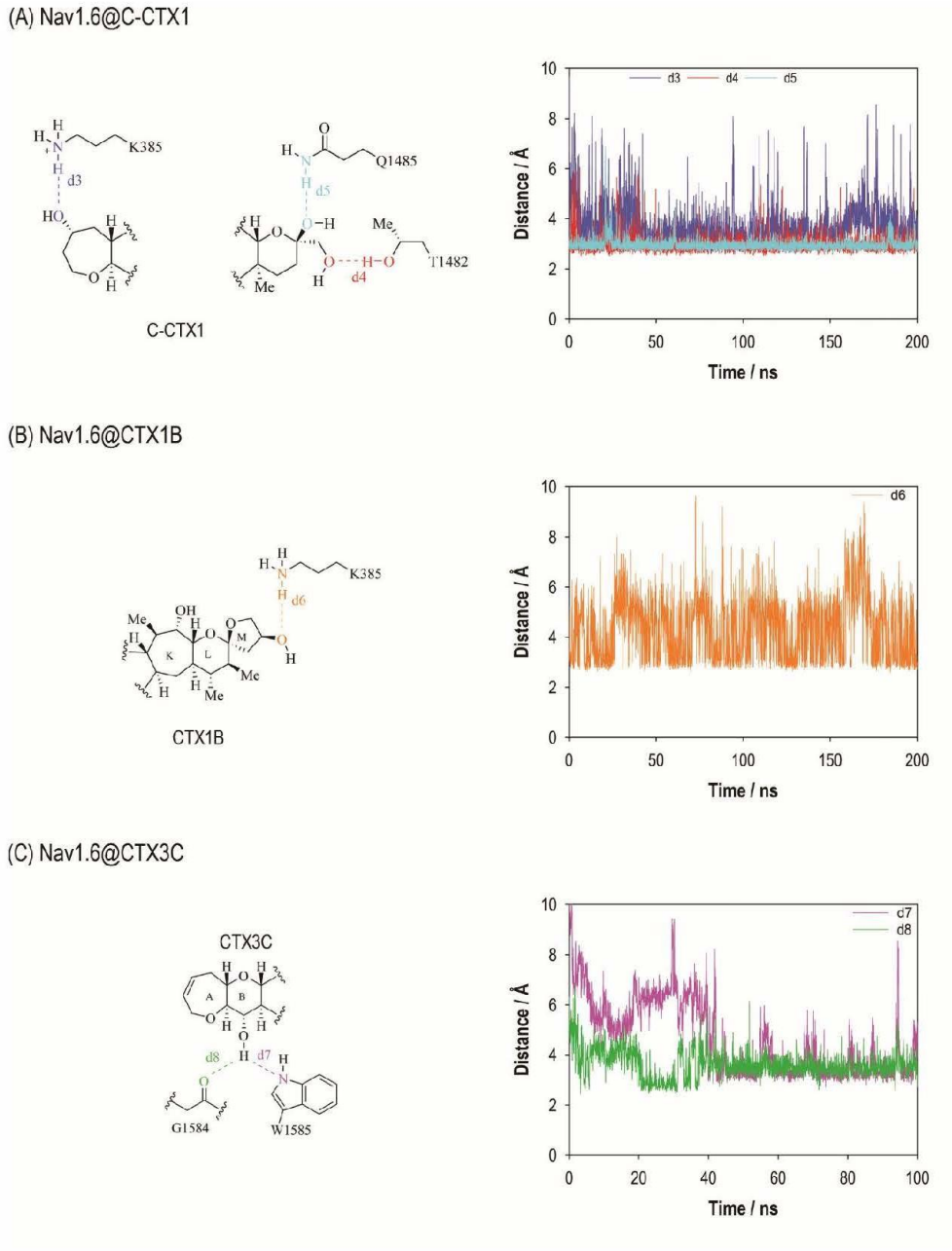


Figure S6. Variation of the distance of relevant hydrogen-bonding interactions identified in MD simulations of $Na_v1.6$@ciguatoxin complexes between the ciguatoxins and different residues of the protein: (A) Nav1.6@C-CTX1 with K385 (d3, blue), T1482 (d4, red) and Q1485 (d5, cyan); (B) Nav1.6@CTX1B with K385 (d6, orange); and (C) Nav1.6@CTX3C (d7, pink). Schematic representations of the contacts studied are shown on left side of each panel.

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Table S1. Main contact interactions of CTX3C, CTX1B and C-CTX1 with the α subunit of human $Na_v1.6$ channel in the $Na_v1.6$@CTX complexes obtained by MD simulation studies

Residue	Segment or protein region (domain)	CTX3C	CTX1B	C-CTX1
D328	S6 (I)			L
K385	S6 (I)		HB + L	HB + L
T386	S6 (I)	L	L	L
M388	S6 (I)	L	L	L
I389	S6 (I)	L	L	L
V392	S6 (I)	L	L	L
L393	S6 (I)	L	L	L
F895	S6 (I)			L
T1482	Loop connecting S1 (IV) and S6 (III)			HB
E1484	Loop connecting S1 (IV) and S6 (III)			L
Q1485	Loop connecting S1 (IV) and S6 (III)			HB + L
Y1488	Helical hairpin connecting S1 (IV) and S6 (III)	L		L
Y1489	Helical hairpin connecting S1 (IV) and S6 (III)		L	
M1492	S5 (IV)	L	L	
K1494	Helical hairpin connecting S1 (IV) and S6 (III)	L		
L1495	S5 (IV)	L	L	
G1584	S2–S3	HB	L	
W1585	S3 (IV)	HB + L		
F1588	S5 (IV)	L		
M1645	S5 (IV)	L	L	
L1648	S5 (IV)	L	L	L
P1649	S5 (IV)			L
F1652	S5 (IV)	L	L	L
G1655	S5 (IV)	L	L	L
L1656	S5 (IV)			L
F1659	S5 (IV)	L	L	L
M1662	S5 (IV)		L	L
G1692	S5 (IV)		L	L
M1695	S5 (IV)	L	L	L
I1696	S5 (IV)	L	L	L
Y1154	S5 (IV)	L		

HB = hydrogen-bonding interaction; L = lipophilic interaction

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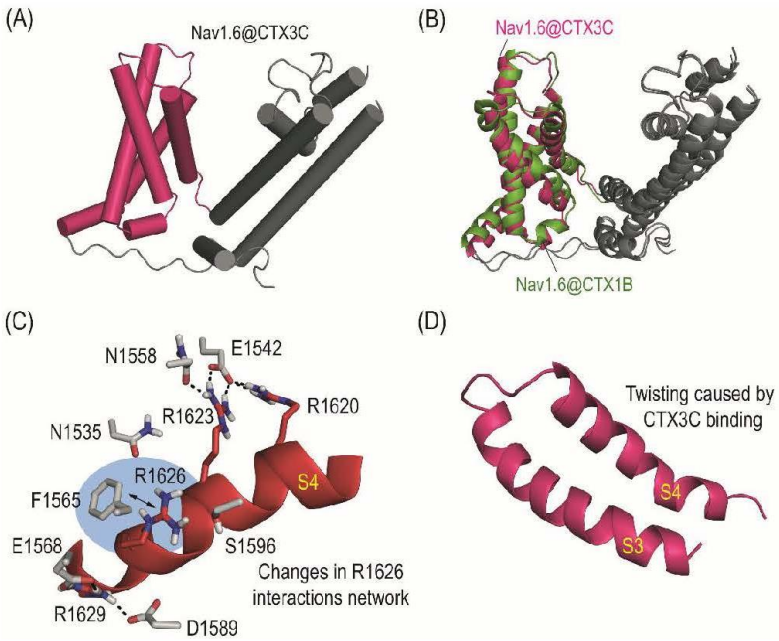


Figure S7. (A) Three-dimensional arrangement of domain IV in the human $Na_v1.6$ channel in complex with Pacific ciguatoxin CTX3C. Snapshot taken after 100 ns of simulation is given. (B) Comparison of domain IV in the $Na_v1.6$@CTX1B and $Na_v1.6$@CTX3C binary complexes. Note how both Pacific ciguatoxins cause a similar arrangement of domain IV. (C) Detailed view of the internal polar contacts of the arginine residues located in S4 in the $Na_v1.6$@CTX3C binary complex. Note how significant changes in the hydrogen-bonding interactions of residue R1626 (highlighted with a blue shadow) are observed. In addition, a new arrangement for residue F1565, which is located on top of the guanidium group of R1626 stablishing a strong cation-π interaction, is also observed. (D) Detailed view of helices S3 and S4 of domain IV in the in the $Na_v1.6$@CTX3C binary complex. Note how the twisting effect caused by the toxin on these helices.

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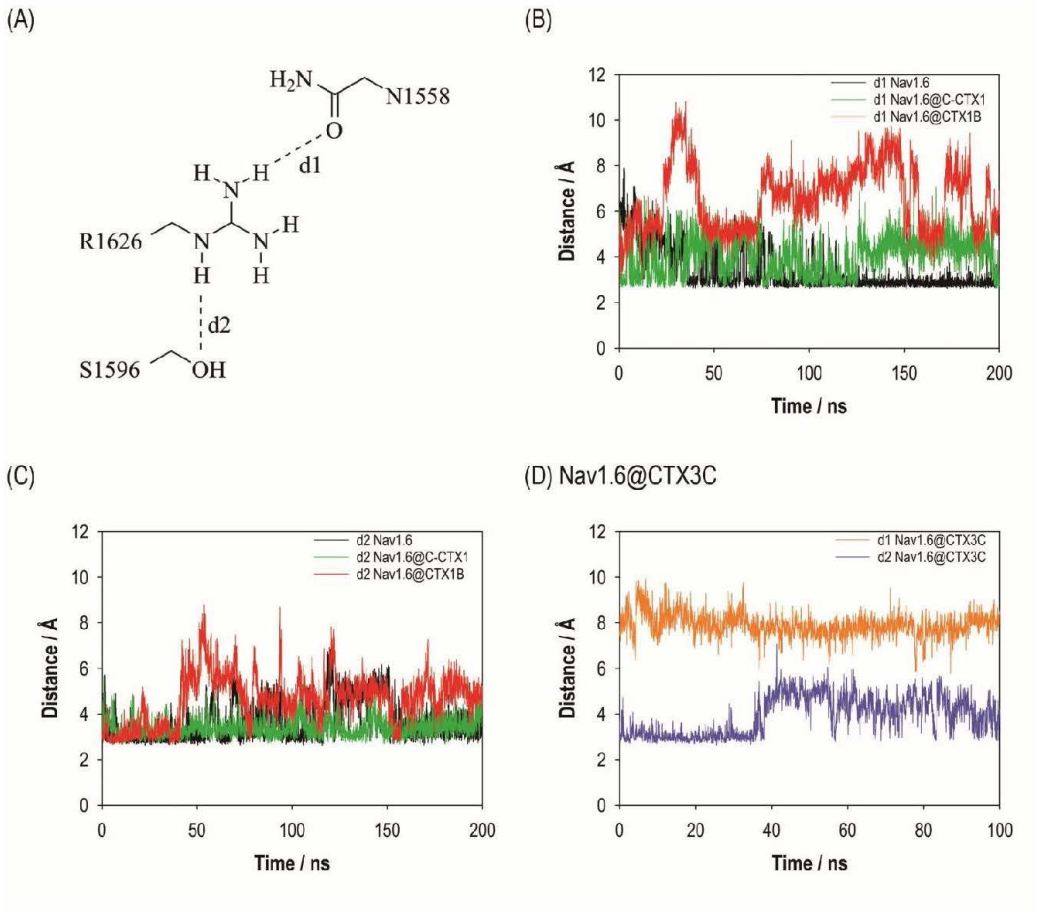


Figure S8. (A) Variation of the distance between residues R1626 with N1558 (d1) and S1596 (d2) calculated from the MD simulations of Nav1.6 (B, C, black lines), and Nav1.6@C-CTX1 (B, C, green lines), Nav1.6@CTX1 (B, C, red lines) and Nav1.6@CTX3C (D, orange and blue lines, respectively) complexes.

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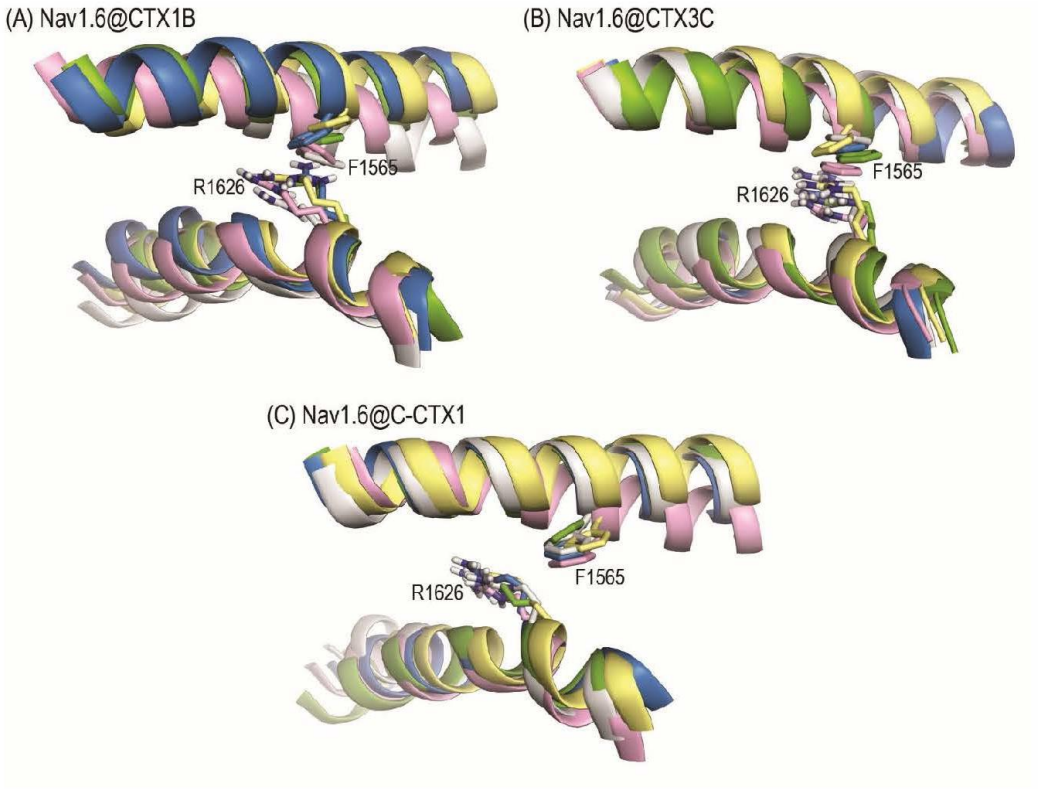


Figure S9. Overlapping of different snapshots obtained from the MD simulation of the Nav1.6@CTX1 (A), Nav1.6@CTX3C (B) and Nav1.6@C-CTX1 (C) complexes, showing the side-chains of F1565 and R1626. Note how both residues are in close and almost parallel disposition in the complexes with CTX1B and CTX3C, while for C-CTX1 no interaction between them is observed.

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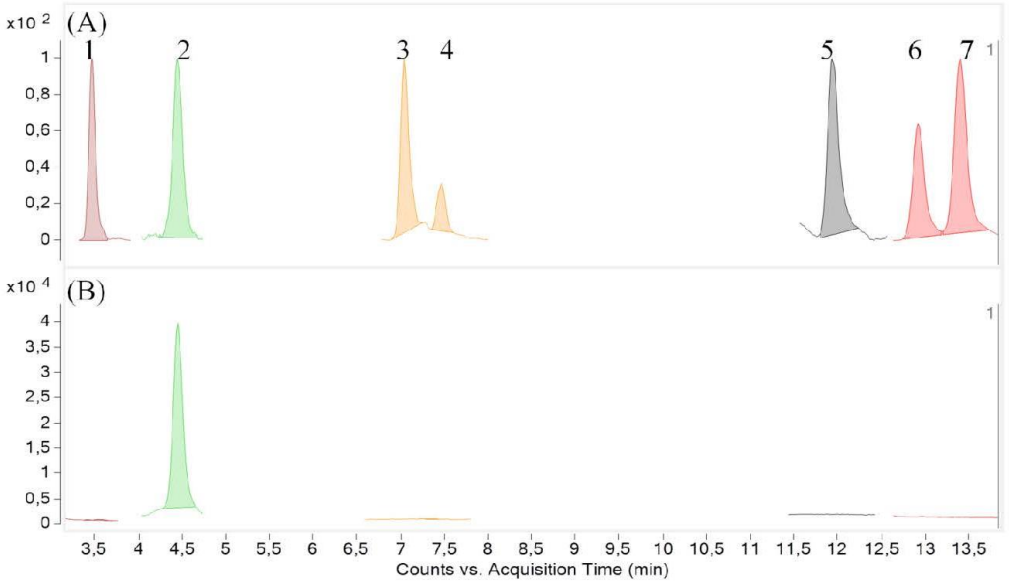


Figure S10: LC-MS/MS chromatogram of: (**A**) Standard mixture containing CTX1B (1), C-CTX1 (2), 52-*epi*-54-deoxy-CTX1B (3), 54 deoxy-CTX1B (4), CTX3C (5), CTX4A (6), and CTX4B (7); (**B**) C-CTX1 purified solution (25.7 ng/mL) isolated from amberjack (*Seriola* spp.).

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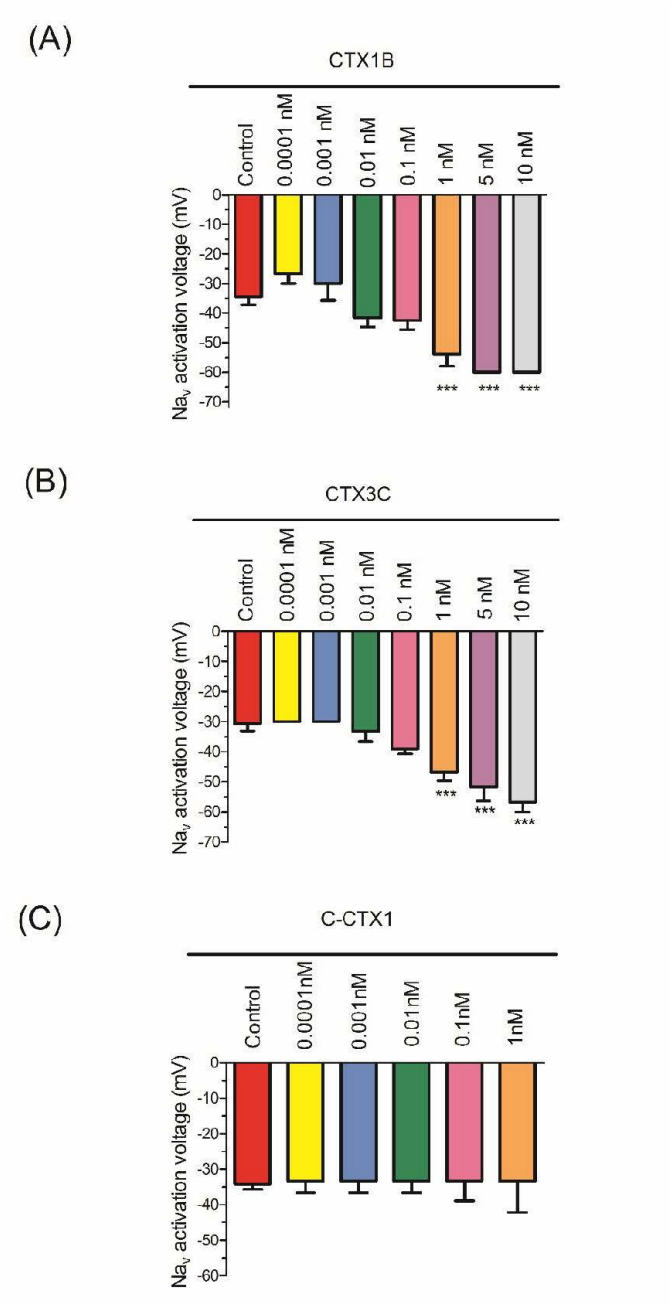


Figure S11. Action of Pacific CTX1B (A), CTX3C (B) and Caribbean C-CTX1 (C) on the activation voltage of sodium channels.

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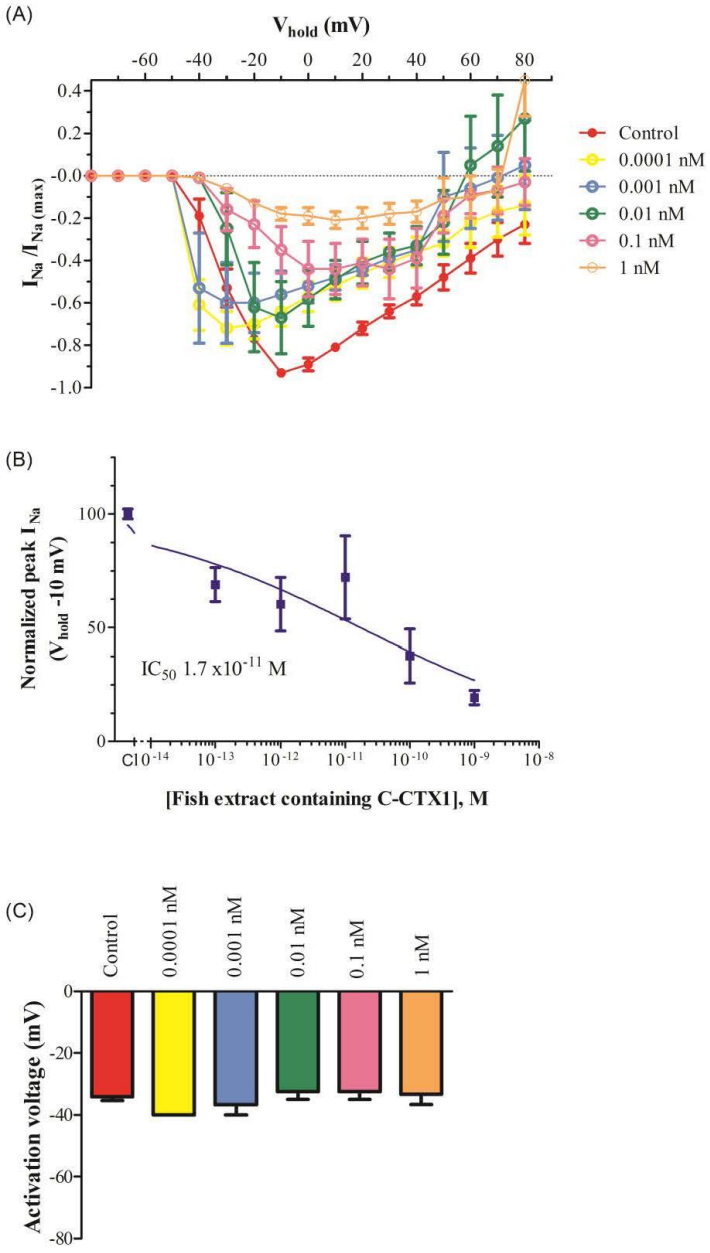


Figure S12. I-V relationship showing the matrix effects of fish extracts on sodium channels (A) and the corresponding non linear fit (B) together with the absence of effects on the activation voltage of sodium channels. Data are mean ± sem of three to five independent experiments.

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Información de la publicación

In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins

Sandra Raposo-Garcia[1], David Castro[2], Emilio Lence[3], Pablo Estévez[2], José Manuel Leão[2], Concepción González-Bello[3], Ana Gago-Martínez[2], M.Carmen Louzao[1], Carmen Vale[1] and Luis M. Botana[1]

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Publicación

Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels.

Resumen

Las ciguatoxinas son producidas por dinoflagelados del género *Gambierdiscus* y *Fukuyoa* y responsables de la intoxicación humana por ciguatera. Dada la creciente expansión de las ciguatoxinas en aguas europeas, surgió la necesidad de establecer factores de equivalencia toxica para los diferentes compuestos que pueden contribuir a esta intoxicación. Por ello, en este trabajo se han analizado por primera vez los efectos de seis compuestos que pueden estar implicados en la ciguatera, teniendo en cuenta su efecto sobre el voltaje de activación de los canales de sodio dependientes de voltaje puesto que en el trabajo anterior se demostró que este parámetro era el que mejor definía la potencia de este grupo de toxinas. Los resultados presentados en la siguiente publicación demostraron que la potencia de estos compuestos sobre los canales de sodio dependientes de voltaje era, por orden decreciente, CTX1B, CTX3B, CTX4A, gambierol, gambierona y MTX3. Además, los datos corroboraron que el voltaje de activación de los canales de sodio era más sensible para detectar ciguatoxinas que su efecto sobre la amplitud de la corriente de sodio. Los resultados obtenidos fueron publicados en el siguiente artículo en el año 2022.

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Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels

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ABSTRACT

Ciguatoxins (CTXs) which are produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* and share a ladder-shaped polyether structure, are causative compounds of one of the most frequent foodborne illness disease known as ciguatera fish poisoning (CFP). CFP was initially found in tropical and subtropical areas but nowadays the dinoflagellates producers of ciguatoxins had spread to European coasts. Therefore, this raises the need of establishing toxicity equivalency factors for the different compounds that can contribute to ciguatera fish poisoning, since biological methods have been replaced by analytical techniques. Thus, in this work, the effects of six compounds causative of ciguatera, on their main target, the human voltage-gated sodium channels have been analyzed for the first time. The results presented here led to the conclusion that the order of potency was CTX1B, CTX3B, CTX4A, gambierol, gambierone and MTX3. Furthermore, the data indicate that the activation voltage of sodium channels is more sensitive to detect ciguatoxins than their effect on the peak sodium current amplitude.

1. Introduction

Ciguatera fish poisoning (CFP) is a human foodborne illness caused by the ingestion of marine fish contaminated with the lipid soluble marine toxins ciguatoxins (CTXs) which are produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa* and share a ladder-shaped polyether structure (Yasumoto et al., 1977). Traditionally, CFP was thought to affect mainly tropical and subtropical regions, however, nowadays CFP is a public health concern worldwide mainly due to the global warming and the expansion of international tourism and trade of fishery products (Chinain et al., 2021; Katikou, 2021). CFP is characterized by gastrointestinal, cardiovascular, and neurological disorders in humans, affecting between 50000 and 500000 people per year (Friedman et al., 2007, 2017a). It is the most frequent foodborne illness related to fish consumption. Although rarely fatal, the duration, severity and number of ciguatera symptoms depend on the quantity of ciguatoxin ingested which is also related to the type of fish and the ocean in which the fish was caught (Nicholson and Lewis, 2006). In addition, the fish part consumed is also important due to differences in toxin accumulation among the fish edible tissues. In this sense, recently, it was demonstrated that the tissue surrounding the eyeball had CTXs levels two to four times higher than those of the flesh (Oshiro et al., 2021a). Reef fish as barracuda (*Sphyraenidae*), amberjack (*Seriola*), grouper (*Serranidae*), snapper (*Lutjanidae*) or parrotfish (*Scaridae* spp.) are known to potentially accumulate these toxins (Chinain et al., 2019; Chinain et al., 2021; FDA, 2021a). Besides fish, other marine invertebrates including gastropods, bivalves and echinoderms have been reported to contain CTXs and represent another important source of

Abbreviations: CFP, ciguatera fish poisoning; CI, Confidence interval; CTXs, ciguatoxins; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; HEK, Human embrionic kidney; I_{Cl}, chloride current amplitude; I_{Na}, sodium current amplitude; MBA, mouse bioassay; MTX3, maitotoxin 3; Pacific CTXs, P-CTX; TEF, toxicity equivalency factor; V_{hold}, holding potential; VGSC, voltage gated sodium channels.

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poisoning to seafood consumers (WHO, 2020).

So far, four types of ciguatoxins have been described according to their carbon skeleton and origin, the Pacific CTXs (P-CTX) from the Pacific Ocean are classified in two types P-CTX I (also known as CTX4A type) and II (also known as CTX3C type), the Caribbean-CTX (C-CTX) found in Caribbean Sea and Indian-CTX (I-CTX) in the Indian Ocean (WHO, 2020). All these ciguatoxin members share their main target which is the site 5 of the α-subunit of the voltage gated sodium channels (VGSC) causing a negative shift in the voltage dependence of the channel activation as well as a concentration–dependent decrease in the maximum inward sodium current that results in an increase in cell excitability (Lombet et al., 1987; Pearn, 2001).

In addition to ciguatoxins, species of *Gambierdiscus* and *Fukuyoa* produce other secondary metabolites with a ladder-shaped polyether structure as gambieric acids, gambierol or gambierone. It is currently unclear whether any of the other compounds produced by these microalgal species play a role in CFP intoxication events due to the lack of information regarding their effects. Although there are studies on the acute toxicity of these compounds administered by the intraperitoneal route (Murray et al., 2021), the reference to estimate the relative potency of CTXs and its related compounds in these studies is the death of the mice when the main problem of CFP is not death but neurological symptoms which can last up to years (Pearn, 2001). While the effects of gambierol and CTXs analogues CTX3C and CTX1B on VGSC have been widely studied (Martin et al., 2014, 2015; Nicholson and Lewis, 2006; Stevens et al., 2011) there is a significant lack of information regarding the effects produced by the rest of the CTXs analogues. In addition, there is a complete absence of data regarding the effects caused by CTXs-related compounds as gambierone or maitotoxin 3 (MTX3 or 44-methylgambierone) on the voltage dependent channels. However, it is clear that sharing a similar polycyclic ether backbone structure is not enough to have similar cellular effects (Cagide et al., 2011). In this regard, there is scarce information on the effects of newly identified ciguatoxin-like structures. Thus, gambierone, a compound which has been isolated from a culture of *Gambierdiscus belizeanus* (Rodriguez et al., 2015), showed an *in vitro* potency 5000 times lower than that of CTX3C. Although these studies performed using gambierone showed that 100 μM gambierone did not caused any effect on the maximum peak sodium current (Rodriguez et al., 2015), these data were obtained using automatic patch-clamp, and revealed that gambierone had a much lower effect on VGSC than the CTX3C analogue. Even though these effects are in agreement with the *in vivo* toxicity of gambierone (Murray et al., 2021), some questions about the interpretation of the *in vitro* electrophysiological comparison of CTX3C and gambierone potencies remain open since the sodium channel activation was interpreted as the peak sodium current inactivation (Rodriguez et al., 2015). Further studies had demonstrated that gambierone altered cellular function at concentrations in the nanomolar range (Boente-Juncal et al., 2019).

Notwithstanding that there are limits and official controls in the European legislation for the most frequent marine biotoxins, CTXs are not yet regulated in Europe, and the current European legislation establishes that the presence of CTXs in fishery products is not allowed (Regulation, 2004). However, the prevalency of CFP in other areas has forced the American Food and Drug Administration (FDA) to implement a regulatory limit for the levels of CTXs in fishery products and set recommended limits of ciguatoxins that represent the point at which the agency could take legal action that could include removing product from market. Although the ciguatoxin levels set by FDA are not always suitable for critical limits the presence of ≥0.1 μg/kg of Caribbean ciguatoxin-1 (C-CTX-1) equivalents and ≥0.01 μg/kg pacific ciguatoxin-1 (P-CTX-1) equivalents in finfish, primarily reef fish could cause fish withdrawal from trade (FDA, 2021b).

The lack of information regarding the relative potencies of the ciguatoxin-related compounds and the worldwide spread of these toxins (Chinain et al., 2021; Darius et al., 2018; Estevez et al., 2021; Habibi et al., 2021; Silva et al., 2015) together with the probably incoming need to set regulatory limits for the presence of CTXs-like compounds in aquatic food products (EFSA, 2010) requires a detailed analysis of the toxic effect of these toxins. In this study, for the first time, the effect of CTX1B, CTX4A (both of them belonging to the group I of Pacific ciguatoxins) and CTX3B (included in the group II of Pacific ciguatoxins) on their main target, the VGSC, were evaluated. Besides, the effects of gambierol, gambierone and maitotoxin-3 on the same channels were also analyzed in order to elucidate their relative potencies and provide data that will allow authorities to establish safety limits for CTXs analogues in flesh fish if needed. Since there is a limited commercially availability of ciguatoxin analogues, animal toxicity data and doses related with clinical symptoms of CFP in humans are nor well defined (EFSA, 2010). The lack of certified reference standards and reference materials for the CTX-group of toxins difficult these tasks (EFSA, 2010). Therefore, the *in vitro* effects of these compounds were analyzed in the present work.

2. Material and methods

2.1. Toxins and drugs used

Ciguatoxins analogues CTX4A and CTX3B were purchased from the Laboratoire des Biotoxines Marines of Institut Louis Malarde (French Polynesia, Tahiti). CTX1B was obtained from Wako (Fujifilm, Wako chemicals Europe Gmbh). All the products contained 100 μg of purified compounds and stock solutions were prepared in DMSO (Dimethyl sulfoxide) at a final concentration of 0.94 μM for CTX4A, 0.98 μM for CTX3B and 1 μM for CTX1B. If necessary, subsequent dilutions were performed in Locke's buffer containing in mM: 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4). The maximum solvent concentration used as a control was 1% DMSO and had no effect on the voltage-gated sodium current amplitude. Gambierone was purchased from CIFGA (Lugo, Spain), the stock solution was 157.4 μM and was diluted to 10 μM in DMSO. MTX3 was also supplied from CIFGA with a stock solution of 164.4 μM and working solutions were prepared in DMSO with a final concentration of 10 μM. Gambierol was synthesized by Dr H Fuwa (Fuwa et al., 2002) and working solutions of 10 μM were prepared in Locke's buffer from a stock solution of 5.7 mM in DMSO. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Human cell cultures

Human embryonic kidney cell line (HEK293) expressing the human $Na_v1.6$ alpha subunit of the sodium channels was kindly provided, under a material transfer agreement, by Dr Andrew Powell (GlaxoSmithKline R&D, Stevenage, U.K.). Cells were cultured in DMEM/F12 medium supplemented with glutamax, MEM nonessential amino acids solution (Gibco, 1% w/v), 10% foetal bovine serum and 0.4 mg/mL Geneticin (G418, Gibco). Cells were grown in a 95% O_2/5% CO_2 atmosphere at 37 °C and with 95% humidity, replacing the medium every 2–3 days. One or two days before electrophysiological recordings, the cells were placed at 30 °C to improve the sodium channel expression as indicated by the provider.

2.3. Electrophysiology

Cells seeded on glass coverslips, were placed in a recording chamber with 0.5 mL extracellular solution containing (in mM): 119 NaCl, 5.9 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 22.8 $NaHCO_3$, and 0.1% glucose (pH was adjusted to 7.4 prior to use). Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter) and had resistances ranging from 5 to 10 MΩ and filled with an intracellular pipette solution that contained (in mM): 140 CsF, 10 EGTA, 10 HEPES, 5 NaCl, 2 $MgCl_2$, pH adjusted to 7.3. Cells were maintained at a holding potential (V_{hold}) of −55 mV. Experiments were initiated 5–10 min after

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establishing the whole-cell configuration to ensure adequate equilibration between the internal pipet solution and the cell interior. Voltage-gated sodium currents were recorded in HEK293 $Na_v1.6$ cells using the whole-cell voltage-clamp mode, at room temperature (20–24 °C) in a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) and the Digidata 1440A data acquisition system (from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz and analyzed offline, using the pClamp 10 software (Axon Instruments). Compensation circuitry was used to reduce the series resistance error by at least 70%. The effect of CTXs on sodium channels was evaluated after 5 min exposure of the cells to the toxin. To record the activation of voltage-gated sodium currents (I_{Na}), maintaining the holding potential at −55 mV, voltage steps from −80 to +80 mV (10 mV increments) were applied. The voltage dependence of inactivation was determined by applying conditioning pulses from −100 to 0 mV in 10 mV increments, prior to a test pulse to 0 mV. The amplitude of chloride currents (I_{Cl}) was recorded by application of a voltage step protocol from −100 to + 100 mV with 20 mV step increase and 400 ms duration.

2.4. Statistical analysis

Data analysis was performed using GraphPad Prism 5. All data are expressed as means ± SEM of *n* determinations. Statistical comparison was by ANOVA followed by post hoc Dunnett's tests. The p values ≤ 0.05 were considered statistically significant.

3. Results

In this work the effects of several compounds potentially involved in CFP, on their main cellular target, the voltage gated sodium channels were evaluated. The chemical structures of the compounds analyzed in this work are shown in Fig. 1.

3.1. Effect of CTX1B on voltage-gated sodium channels

The most potent CTXs analogue known to date is the Pacific CTX1B (Ledreux and Ramsdell, 2013; Lewis et al., 1991; Yogi et al., 2014), therefore, in this study the effect of CTX1B on the size of sodium currents was firstly evaluated. As shown in Fig. 2A, CTX1B at concentrations of 0.001 nM and higher caused a concentration-dependent decrease in sodium current amplitude. Non-linear fit of the data shown in Fig. 2B yielded an estimated IC_{50} of 2.28×10^{-11} M (95% confidence interval (CI) from 1.47×10^{-12} M to 3.45×10^{-10} M). Moreover, CTX1B caused a negative shift in the activation voltage of sodium currents from −34.6 ± 2.68 mV in control conditions to −54 ± 4 mV ($p < 0.0001$) in the presence of 1 nM CTX1B, and to −60 ± 0 mV after bath application of 5 nM CTX1B, similar to the effect caused by 10 nM CTX1B as shown in Fig. 2C.

3.2. Effects of CTX4A and CTX3B on voltage-gated sodium channels

As previously generally described for ciguatoxins (Ikehara et al., 2017; Schlumberger et al., 2010) a significant effect of CTX4A decreasing the maximum peak inward sodium current was observed and represented in Fig. 3A as the normalized peak sodium currents in the absence and presence of the different CTX4A concentrations. In control conditions, the peak sodium current was −1364 ± 174 pA (n = 14), and after bath application of the highest CTX concentration studied, 10 nM CTX4A, the maximum peak inward sodium current decreased up to −625 ± 97 pA (n = 5). The percent inhibition of the peak inward sodium currents by different concentrations of this toxin was used to obtain a concentration–response curve shown in Fig. 3B. Nonlinear fit of the data shown in Fig. 3B yielded an estimated IC_{50} for the CTX4A inhibition of the peak inward sodium current of 1.35×10^{-8} M (95% CI from 6.69×10^{-10} M to 2.7×10^{-7} M), noteworthy, at the concentration of 10 nM CTX4A inhibited peak sodium currents only by 50%. Besides, the evaluation of the effect of different concentrations of CTX4A on the activation potential of the sodium channels demonstrated that CTX4A produced a significant negative shift in the activation voltage of sodium channels, at concentrations of 10 nM as shown in Fig. 3C. In control conditions, the sodium channel currents activated at −37.0 ± 3.0 mV (n = 14) while the voltage activation was shifted to −53.3 ± 3.3 mV in the presence of 10 nM CTX4A (n = 3; $p < 0.05$ vs control conditions) as shown in Table 2. The evaluation of the effect of this toxin on the fast inactivation voltage of sodium channels did not show statistically significant changes at any of the concentration used (Supplementary Fig. 1).

In the same context, the effect of CTX3B on sodium currents was also evaluated. Fig. 4A represents the normalized peak sodium currents in the absence and presence of the different CTX3B concentrations. In control cells, the peak sodium current was −2041 ± 242 pA (n = 8) and decreased in a concentration-dependent manner in the presence of the different concentrations of CTX3B added to the recording chamber. Thus, after addition of 10 nM CTX3B, the maximum peak inward sodium current decreased by about 90% reaching a value of −225 ± 85 pA (n =

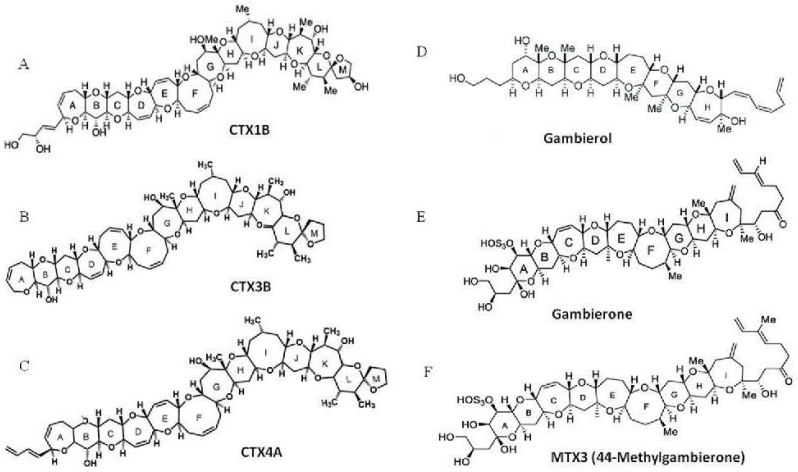


Fig. 1. Chemical structure of ciguatoxins and ciguatoxin-related compounds.

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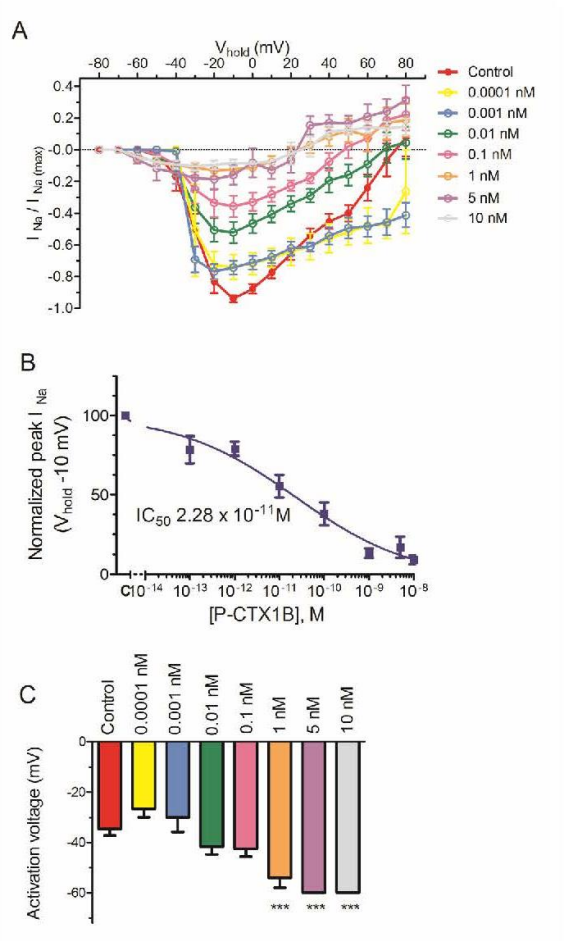


Fig. 2. Effect of CTX1B on the maximum peak sodium current amplitude. A. Current-voltage relationship for the effect of different concentrations of CTX1B on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX1B on the inhibition of peak sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different concentrations of CTX1B.

3). The percent inhibition of the sodium current amplitude by different concentrations of this toxin was used to obtain a concentration–response curve, as shown in Fig. 4B. Nonlinear fit of the data yielded an estimated IC_{50} for the CTX3B inhibition of peak inward sodium current of 8.10×10^{-11} M (95% CI from 1.06×10^{-11} to 6.17×10^{-10} M). The negative shift in the voltage activation of the sodium channels elicited by addition of different concentrations of CTX3B to the recording chamber was statistically significant at concentrations of 5 nM CTX3B and higher. Thus, the activation voltage of sodium channels was shifted from -38 ± 1.2 mV ($n = 8$) in control conditions to -50 ± 1.2 mV ($n = 3$; $p < 0.05$ vs control conditions) for 5 nM CTX3C and -56.6 ± 6.67 mV ($n = 3$; $p < 0.001$ vs control conditions) after bath application of 10 nM CTX3B as shown in Fig. 4C. As well as for CTX4A, the addition of increasing concentrations of CTX3B did not cause any statistically significant change in the fast inactivation of the sodium channels (Supplementary Fig. 2).

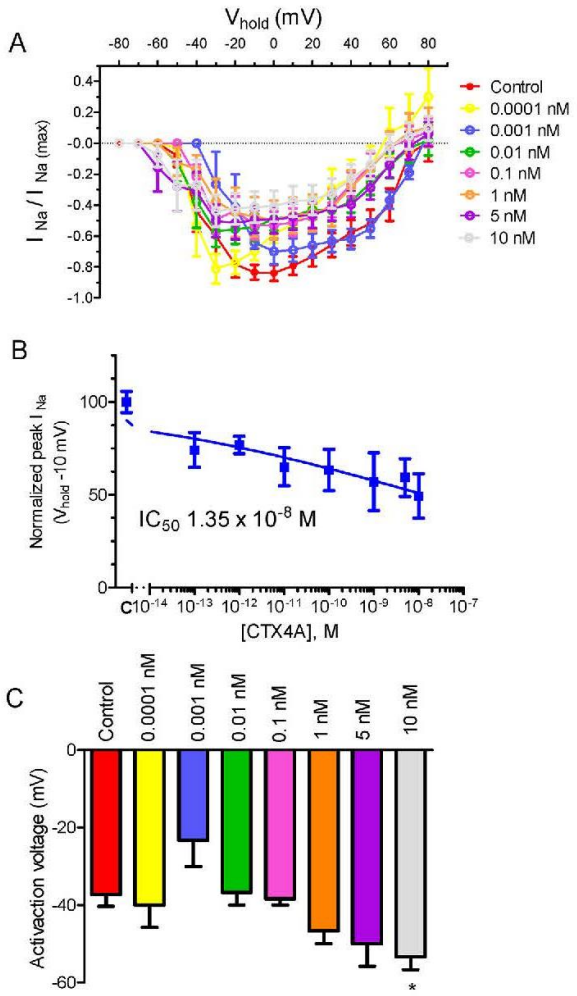


Fig. 3. Effect of CTX4A on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of CTX4A on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX4A on the inhibition of peak inward sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different concentrations of CTX4A.

3.3. *Effect of gambierol on voltage-gated sodium channels*

Previous studies had shown that the main target of gambierol were the voltage gated potassium channels (Kopljar et al., 2009; Rubiolo et al., 2015). However, it has been demonstrated that gambierol, at micromolar concentrations, also acts on the site 5 of the VGSC (Cagide et al., 2011; Inoue et al., 2003; Louzao et al., 2006), as a partial agonist, thus, the gambierol concentrations employed to evaluate the effect of these compound were higher than those used for ciguatoxins. However, electrophysiological techniques allowed us to evaluate the direct effect of gambierol on the sodium channels at concentrations lower than those used in previous studies (Cagide et al., 2011; Louzao et al., 2006). The evaluation of the effect of gambierol on VGSC was performed at toxin concentrations of 100 nM, 200 nM, 300 nM and 500 nM. In this case, none of the toxin concentrations employed elicited any change in the maximum peak inward sodium current as shown in Fig. 5A, which represents the normalized peak sodium currents in the absence and

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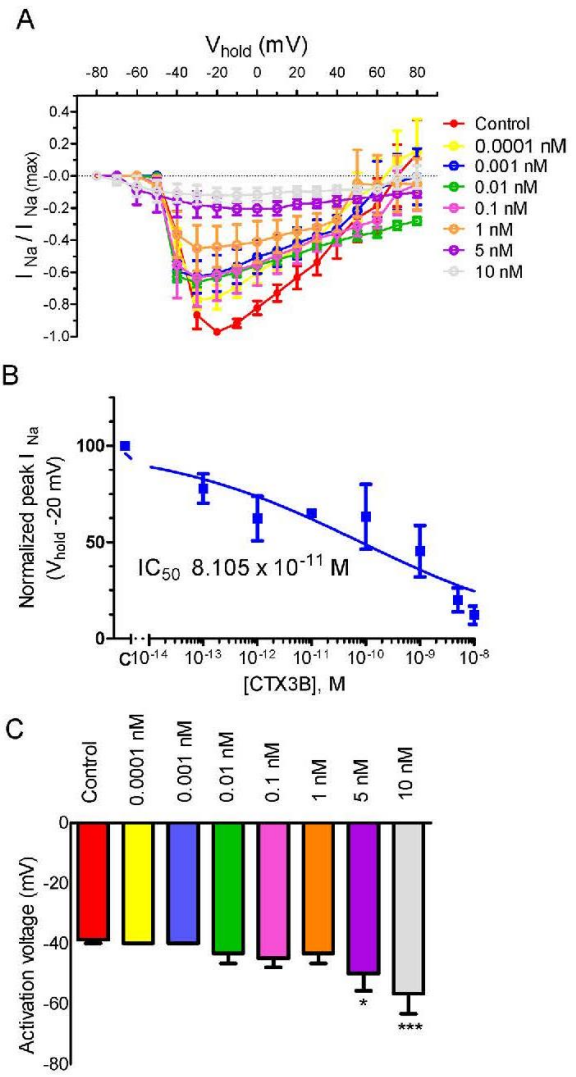


Fig. 4. Effect of CTX3B on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of CTX3B on the peak amplitude of sodium currents B. Concentration-response graph indicating the effect of different concentrations of CTX3B on the normalized amplitude of the inward sodium currents. C. Activation voltage of sodium currents in absence of toxin, and after bath application of different CTX3B concentrations.

presence of the different toxin concentrations that reached −2053 ± 433 pA (n = 10) in control conditions and −2325 ± 1173 pA (n = 3) in the presence of 500 nM gambierol. However, the analysis of the activation voltage of sodium channels showed that concentrations of 100 nM gambierol and higher led to a statistically significant shift in the voltage activation of sodium channels. As shown in Fig. 5B, in control conditions the VGSC activated at −31.0 ± 2.3 mV (n = 10) and concentrations of 100 nM gambierol, which did not cause any effect in the maximum peak inward sodium current, elicited a hyperpolarizing shift in the activation voltage of sodium channels, thus in the presence of 100 nM gambierol the channels opened at −46.6 ± 3.3 mV (n = 3; $p < 0.05$ vs control conditions). No statistically significant changes were observed in the fast inactivation of the VGSC by addition of different concentrations of gambierol (Supplementary Fig. 3).

3.4. Effect of MTX3 on voltage-gated sodium channels

MTX3 is a homologue of gambierone with very similar reported biological activity at nanomolar concentrations regarding its effect on cell viability and cytosolic calcium concentration (Boente-Juncal et al., 2019; Rodriguez et al., 2015). The lack of information regarding the effect of MTX3 on sodium channels led us to study in detail its effects on VGSC, in order to compare its biological activity with that of other compounds involved in CFP. The evaluation of the effect of MTX3 on VGSC was performed at concentrations of 100 nM, 200 nM, 500 nM and 1 μM. None of the toxin concentrations studied elicited any change in the maximum peak inward sodium current as represented in Fig. 6A, thus the peak sodium currents in the absence and presence of the different toxin concentrations had an amplitude of −1152 ± 299 pA (n = 10) in control conditions and −1044 ± 222 pA (n = 9) in the presence of 1 μM MTX3. Despite no decrease in the maximum peak sodium current was observed, as shown in Fig. 6B and 500 nM MTX3 elicited a significant shift in the voltage activation of sodium channels hyperpolarizing their activation voltage from −40.0 ± 3.3 mV (n = 10) in control conditions to −50.0 ± 5.0 mV (n = 3; $p < 0.01$) in the presence of the toxin. No statistically significant changes were observed in the fast inactivation of the channels by addition of different concentrations of MTX3.

3.5. Effect of gambierone on voltage-gated sodium channels and chloride channels

Gambierone is a CTXs-related compound that affects VGSC and cytosolic calcium (Boente-Juncal et al., 2019; Rodriguez et al., 2015). No further studies regarding its effects were performed until date. Bath application of the highest gambierone concentration studied, 1 μM, did not cause any effect on the maximum peak sodium current, as shown in Fig. 7A where normalized sodium current amplitude in the absence and presence of the different toxin concentrations are represented. However, the addition of 200 nM gambierone elicited a statistically significant change in the voltage activation of the channel using one-way ANOVA followed by Dunnett's test. In control conditions, the VGSC activated at −40.0 ± 1.8 mV (n = 8) while the channels activated at −53.0 ± 3.3 mV (n = 3; $p < 0.05$) after bath application of 200 nM gambierone as represented in Fig. 7B. As well as for the previous toxins, no significant effects on the fast inactivation of voltage gated sodium channels were observed (Supplementary Fig. 4).

In addition, we evaluated for the first time the effect of gambierone on chloride currents. As previously shown, in HEK 293 cells, in the same conditions, recorded output currents correspond to chloride currents (Boente-Juncal et al., 2021). Bath application of 200 nM gambierone elicited a decrease in the mean maximum I_{Cl} from 2823 ± 667 pA (n = 3) in control conditions to 1104 ± 181 pA (n = 3) in the presence of gambierone as shown in Fig. 8. These values yielded statistically significant differences between the chloride current elicited after bath application of gambierone to the cells compared with control conditions after a voltage step of 100 mV ($p < 0.05$ vs control) using one-way ANOVA followed by Dunnett's test.

3.6. Comparison of the relative potency of six ciguatoxin analogues on voltage-gated sodium channels

Taking as a reference the IC_{50} value for the inhibition of peak inward sodium currents obtained for CTX1B using the same technique and the same procedure, the relative potency of all the ciguatoxin analogues employed in this work was calculated, obtaining the data represented in Table 1 to clarify the similarities and differences in the biological activity of these compounds. This table shows that while ciguatoxins decreased the peak sodium current amplitude, neither gambierol, nor gambierone or MTX3 affected sodium current size. The effect of the

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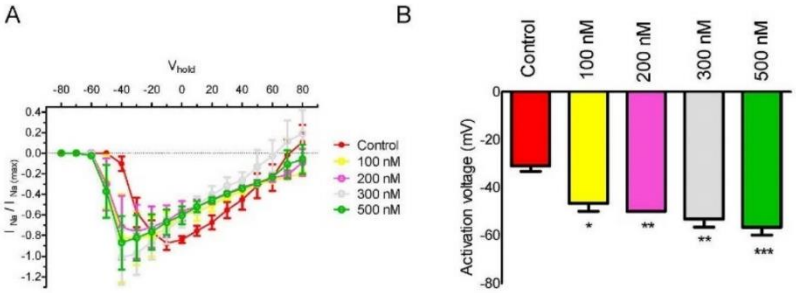


Fig. 5. Effect of gambierol on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of gambierol on peak inward sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different gambierol concentrations.

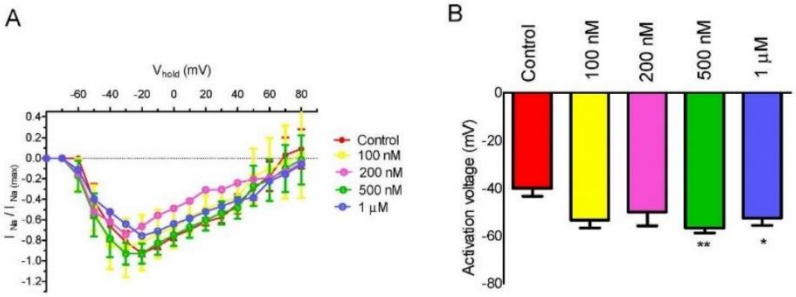


Fig. 6. Effect of MTX3 on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of MTX3 on sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different MTX3 concentrations.

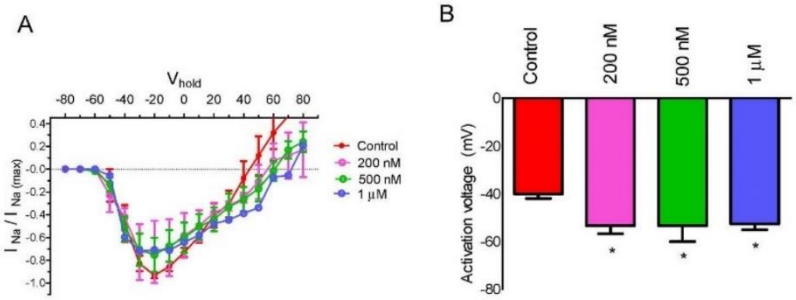


Fig. 7. Effect of gambierone on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of different concentrations of gambierone on sodium currents. B. Voltage activation of sodium currents in absence of toxin, and after application of different gambierone concentrations.

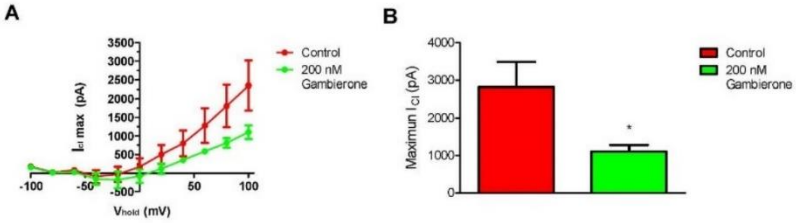


Fig. 8. Characterization of the activation properties of chloride currents evoked from HEK293 cells. A. I–V curve for the activation of chloride currents in the absence and presence of 200 nM gambierone showing that gambierone significantly decreased I_{Cl}. Currents were obtained 5 min after bath application of solvent or 200 nM gambierone. B. Bar graphs showing the mean I_{Cl} amplitude in absence or in presence of gambierone at 200 nM. Data are mean ± SEM of three different cells.

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Table 1
Relative potency of CTXs analogues to inhibit peak inward sodium currents.

Ciguatoxin analogue	IC_{50} value	Relative potency
CTX1B	2.28×10^{-11} M	1
CTX3B	8.105×10^{-11} M	0.28
CTX4A	1.35×10^{-8} M	0.0016
Gambierol	No effect	
Gambierone	No effect	
MTX3	No effect	

Table 2
Concentration of CTXs and CTXs analogues necessary to cause a significative shift in the voltage activation of the sodium channels and its relationship to their effect on the activation voltage of sodium channels.

Ciguatoxin analogue	Activation voltage	Toxin Concentration	TEF	EFSA TEFs (EFSA, 2010)
CTX1B	−34 mV to −54 mV	1 nM	1	1
CTX3B	−38.7 mV to −50 mV	5 nM	0.28	0.3
CTX4A	−37 mV to −53 mV	10 nM	0.1	0.1
Gambierol	−31 mV to −46 mV	100 nM	0.01	No data
Gambierone	−40 mV to −53 mV	200 nM	0.005	No data
MTX3	−40 mV to −56 mV	500 nM	0.002	No data

different CTX-related compounds on sodium current amplitude is summarized in Table 1.

However, under our assay conditions, it is noteworthy that the activation potential of sodium channels was more sensitive to detect ciguatoxin related compounds than the inhibition of peak sodium currents. Thus, taking into account that all the compounds included in this work shifted the activation potential of voltage gated sodium currents by about 15 mV as shown in Table 2, the following TEFs can be proposed for this group of toxins. As represented in Table 2, the TEFs for the ciguatoxin-related compounds are compared with the TEFs proposed 10 years ago by EFSA (EFSA, 2010).

4. Discussion

Toxicity equivalency factors are useful tools to evaluate the risk that chemicals represent for human health and are commonly applied to many environmental chemicals including marine biotoxins (WHO, 2020). Taking into account the EFSA reports on marine toxins highlighting the need of studies for their toxicity (EFSA, 2010), the present work was undertaken to evaluate the effect of these compounds on their main cellular target, the voltage sensitive sodium channels.

CFP is an emergent food safety hazard in which, besides ciguatoxin analogues, other related compounds as gambierol, gambierone and gambieric acids are involved (Chinain et al., 2021; Katikou, 2021). Most of the effects caused by this group of toxins are explained by their binding at the site 5 of VGSC, activating the channel at resting membrane potentials and causing an increase in the membrane excitability, leading to spontaneous and repetitive action potentials in excitable cells (Vale et al., 2015). However, so far, it was assumed that not all the CTXs analogues had the same ability to cause these effects, because their action on voltage gated channels has been assessed in different cell lines and with different methodologies (Boente-Juncal et al., 2019; Cagide et al., 2011; Cuypers et al., 2008; Ghiaroni et al., 2005; Murray et al., 2021; Rodriguez et al., 2015; Rubiolo et al., 2015). The results concluded that the potency of the CTXs analogues was different, depending among many other reasons on their chemical structure, their polarity and their oxidation state (Lewis et al., 1991). The relative potencies of CTXs analogues and related compounds have so far been determined by the mouse bioassay (MBA), which implies intraperitoneal administration of the toxins and led to the establishment of a LD_{50} for each compound. These data allowed regulatory authorities to recommend possible TEFs for some of these toxins (EFSA, 2010; WHO, 2020). However, the main problem of CFP are the chronic symptoms, especially neurological disturbances, which can last up to years (Pearn, 2001). Hence, the present work was undertaken to study the effect of each compound on VGSC analysing their effect on the amplitude of the sodium currents and also on their activation voltage. In this study, for the first time, the effect of CTX1B, CTX4A and CTX3B, three of the Pacific ciguatoxins analogues been commonly found in fish samples was compared (Oshiro et al., 2021b; Roue et al., 2020; Yogi et al., 2014), taking the relative potency of the most potent analogue, CTX1B, as the reference value. Our data showed that the relative potency of CTX3B based on its effect on VGSC, was 0.28 while the relative potency of CTX4A was much lower, 0.0016 when the peak sodium current was analyzed. Furthermore, gambierol, gambierone and MTX3 had no effect on the size of the sodium currents. Besides their effect on peak sodium amplitude, the concentration needed to cause a significant negative shift in the activation voltage of the sodium channels was evaluated (5 nM for CTX3B and 10 nM for CTX4A). The results presented here allowed to conclude that CTX3B was more potent than CTX4A. This fact is important considering that CTX3B appears frequently in flesh fish samples that also contain CTX3C (Oshiro et al., 2021b), being both quite potent analogues in their effects on VGSC, although CTX3C had less effect than CTX1B. These results are in consonance with previous data reporting that CTX4A was more than 50-fold less potent at activating Na_v than other CTXs analogues and was four times more effective on potassium channels than CTX1B (Schlumberger et al., 2010). In this study the order of potency found for the negative shift in sodium channel activation between CTX1B and CTX4A differed only 10 times.

Gambierol is a polyether secondary metabolite produced by *Gambierdiscus toxicus*. Its main target are the voltage-gated potassium channels (Cuypers et al., 2008; Ghiaroni et al., 2005; Konoki et al., 2015; Rubiolo et al., 2015). In addition, gambierol has been demonstrated to activate sodium channels at higher concentrations, acting on the same site of the sodium channel as CTXs (Cagide et al., 2011; Inoue et al., 2003; Louzao et al., 2006). However, all the previous studies regarding the effect of gambierol on VGSC used very high toxin concentrations (0.5 μM and higher). The use of electrophysiological techniques allowed to demonstrate that the toxin had a direct effect on VGSC promoting activation of the channels at resting membrane potentials at much lower concentrations (100 nM), without affecting the maximum peak sodium currents. The same effect was observed for gambierone at nanomolar concentrations although previous studies had reported a negative shift of the voltage-dependent activation curve at 100 μM (Rodriguez et al., 2015), using automatic patch clamp. In this work, bath application of gambierone at concentrations as low as 200 nM shifted the activation voltage-gated sodium channels by 15 mV in the negative direction. Although the relative potency of gambierol and gambierone to inhibit I_{Na} was much lower than CTXs, both compounds, may have a greater implication in ciguatera poisoning than previously thought because they promote neuronal excitation. In addition, gambierone elicited a decrease in chloride currents amplitude. Therefore, future studies must be undertaken to evaluate the effect of the ciguatoxin-related compounds on chloride homeostasis since there is broad experience showing that intraneuronal ionic balance and, indeed, the alterations in the intracellular concentration of chloride and sodium ions leads to an alteration in neuronal function (Friedman et al., 2017b), which is one of the most persistent sequelae of ciguatera (Pearn, 2001). The negative shift in the activation voltage of sodium channels allowed also to quantify the potency of MTX3, a compound whose biological activity has been reported to be very similar to the effect of gambierone, characterized by the lack of cytotoxicity at nanomolar concentrations and a small effect on the cytosolic calcium concentration (Boente-Juncal et al.,

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2019). However, these results clearly show differences between the activity of both compounds on VGSC, highlighting that despite none of them elicited a decrease on the maximum sodium peak inward current, 200 nM gambierone also caused the activation of sodium currents at hyperpolarized membrane potential while 200 nM MTX3 did not cause any effect, but the hyperpolarization in the voltage-activation of sodium channels elicited by MTX3 was observed at concentrations of 500 nM.

The shift to negative potential of the response observed with all the compounds suggest that, to a certain degree, all ciguatoxin analogues contribute to the neuronal firing observed in ciguatera intoxications. Under certain conditions, an external sensory stimuli might trigger a ciguatera crisis, and this effect on the negative potential shift could be behind them. Thus, the data presented here suggest that the fact that we may obtain two different sets of TEF, depending on the electrochemical parameter to consider, implies that there is an urgent need to harmonize the criteria to set the toxicological response that defines TEF. This is especially relevant when the TEF value is linked to a regulatory toxin level, as it is the case in the EU for certain phycotoxins. Further studies should be pursued to be able to establish the relative potency of the different CTXs analogues in order to being able to know the dangers when they appear in combination even at low concentration and in limits below those established by the legislation.

5. Conclusion

The activation voltage of sodium channels allows to detect low potent ciguatoxin-related compounds and this effect is more representative than the effect elicited by these compounds on peak sodium current amplitude.

The toxicity equivalency factors for six ciguatoxin-related compounds are CTX1B (1) > CTX3B (0.28) > CTX4A (0.1) ≫>gambierol (0.01) > gambierone (0.005) > MTX3 (0.002).

CRediT authorship contribution statement

Sandra Raposo-Garcia: Writing – original draft, Data curation, analyzing data and, Formal analysis, All authors have read and agreed to the published the version of the manuscript. **M. Carmen Louzao:** Conceptualization, and, Funding acquisition, All authors have read and agreed to the published the version of the manuscript. **Haruhiko Fuwa:** supply of gambierol under a material transfer agreement. All authors have read and agreed to the published the version of the manuscript. **Makoto Sasaki:** supply of gambierol under a material transfer agreement. All authors have read and agreed to the published the version of the manuscript. **Carmen Vale:** Writing – original draft, Data curation, analyzing data and, Formal analysis, All authors have read and agreed to the published the version of the manuscript. **Luis Botana:** Conceptualization, and, Funding acquisition, All authors have read and agreed to the published the version of the manuscript. **Haruiko Fuwa** and **Makoto Sasaki:** synthesis of gambierol.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2022.112812.

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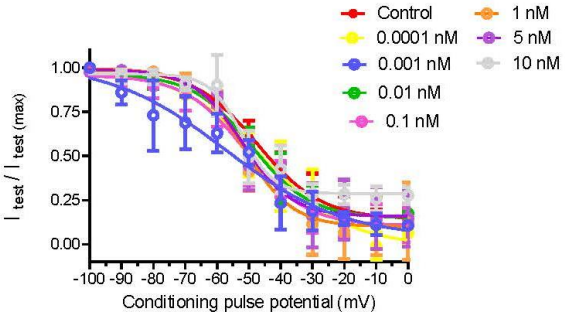
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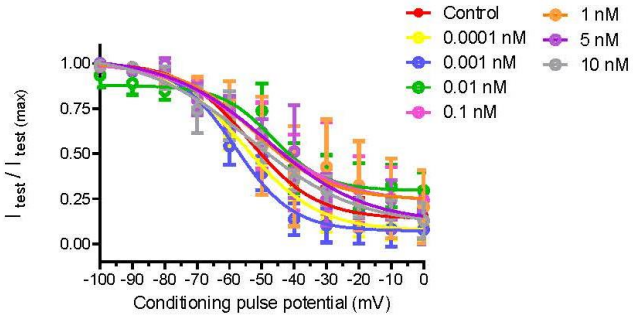
Supplementary material

Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels

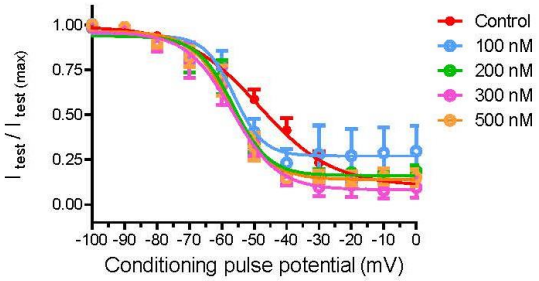
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Supplementary figure 1: Effect of different concentrations of CTX4A on hNa_v 1.6 fast inactivation.

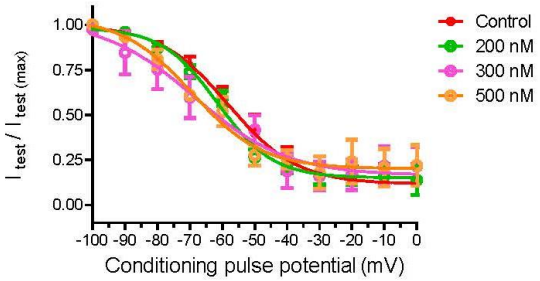


Supplementary figure 2: Effect of different concentrations of CTX3B on hNa_v 1.6 fast inactivation.



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Supplementary figure 3: Effect of different concentrations of gambierol on hNa_v 1.6 fast inactivation.



Supplementary figure 4: Effect of different concentrations of gambierone on hNa_v 1.6 fast inactivation.

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Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels.

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Publicación

***In vivo* subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity.**

Resumen

Dentro del grupo de las toxinas producidas por dinoflagelados de los géneros *Gambierdiscus* y *Fukuyoa* en este trabajo se estudian las maitotoxinas (MTX1 y MTX3), ciguatoxinas (CTX3C) y análogos (gambierona). Todos estos compuestos participan en la intoxicación por ciguatera por lo que era importante evaluar sus efectos *in vivo* debido a la necesidad planteada por la Autoridad Europea de Seguridad Alimentaria con el fin de completar los datos toxicológicos disponibles para el grupo de compuestos de las ciguatoxinas. En primer lugar, en este trabajo se estudiaron los efectos *in vivo* utilizando pautas adoptadas internacionalmente después de una única dosis vía oral o intraperitoneal. Se evaluó la toxicidad aguda después de la administración intraperitoneal de la maitotoxina 1 y la toxicidad aguda por vía oral de una única dosis de ciguatoxina del Pacífico CTX3C y de la maitotoxina 1, que no afectaron a la supervivencia de los ratones después de un periodo de 96 h. Por lo tanto, a continuación, se administraron dosis orales repetidas de gambierona, CTX3C, maitotoxina 3 y maitotoxina 1. Ninguno de estos compuestos mostró efectos sobre la supervivencia de los animales en un periodo de 28 días. Por lo tanto, este estudio demostró la necesidad de reevaluar la toxicidad de estos compuestos por vía oral ya que hasta el momento la maitotoxina 1 se consideraba una de las toxinas más potentes con una dosis letal 50 por vía intraperitoneal de 50 ng/kg, pero que fue realizada sin identificar la pureza química ni describir los procedimientos del bioensayo en ratón empleados en 1993. Los resultados obtenidos en este estudio se muestran a continuación.

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BIOLOGICS

In vivo subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity

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Abstract
Ciguatoxins are marine compounds that share a ladder-shaped polyether structure produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa*, and include maitotoxins (MTX1 and MTX3), ciguatoxins (CTX3C) and analogues (gambierone), components of one of the most frequent human foodborne illness diseases known as ciguatera fish poisoning. This disease was previously found primarily in tropical and subtropical areas but nowadays, the dinoflagellates producers of ciguatoxins had spread to European coasts. One decade ago, the European Food Safety Authority has raised the need to complete the toxicological available data for the ciguatoxin group of compounds. Thus, in this work, the in vivo effects of ciguatoxin-related compounds have been investigated using internationally adopted guidelines for the testing of chemicals. Intraperitoneal acute toxicity was tested for maitotoxin 1 at doses between 200 and 3200 ng/kg and the acute oral toxicity of Pacific Ciguatoxin CTX3C at 330 and 1050 ng/kg and maitotoxin 1 at 800 ng/kg were also evaluated showing not effects on mice survival after a 96 h observation period. Therefore, for the following experiments the oral subchronic doses were between 172 and 1760 ng/kg for gambierone, 10 and 102 ng/kg for Pacific Ciguatoxin CTX3C, 550 and 1760 ng/kg for maitotoxin 3 and 800, 2560 and 5000 ng/kg for maitotoxin 1. The results presented here raise the need to reevaluate the in vivo activity of these agents. Although the intraperitoneal lethal dose of maitotoxin 1 is assumed to be 50 ng/kg, without chemical purity identifications and description of the bioassay procedures, in this work, an intraperitoneal lethal dose of 1107 ng/kg was obtained. Therefore, the data presented here highlight the need to use a common procedure and certified reference material to clearly establish the levels of these environmental contaminants in food.

Keywords Maitotoxin 1 · Ciguatoxin · Gambierone · Maitotoxin 3 (MTX3) or 44-methylgambierone · Ciguatera fish poisoning · In vivo · Acute toxicity · Subchronic toxicity

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Abbreviations

CFP	Ciguatera fish poisoning
CTX	Ciguatoxins
HAB	Harmful algal bloom
MTX	Maitotoxin
LD_{50}	Lethal dose 50
i.p.	Intraperitoneal
bw	Body weight
H&E	Haematoxilin-eosin
FDA	American Food and Drug Administration
EFSA	European Food Safety Authority

Introduction

Gambierdiscus and *Fukuyoa* species are marine dinoflagellates producers of toxins causative of a widespread human illness known as Ciguatera Poisoning (CP) which includes carnivorous fish that contribute with the higher incidence to Ciguatera Fish Poisoning (CFP) and toxic compounds accumulated by marine invertebrates that also accumulate ciguatoxins but the incidence is minor (Chinain et al. 2021). This syndrome comprises gastrointestinal, neurological, and cardiovascular symptoms (Lehane and Lewis 2000). Blooms of these dinoflagellates have expanded worldwide reaching even European coasts (Canals et al. 2021; de Haro et al. 2021; Katikou 2021; Murray et al. 2021). The presence of *Gambierdiscus* species (Canals et al. 2021; Katikou 2021), the related toxins (Tudó et al. 2020) and CFP intoxications have been repetitively identified in Europe during the last decades (Canals et al. 2021; Katikou 2021), especially in the Canary Islands (Canals et al. 2021; Perez-Arellano et al. 2005), Madeira (Otero et al. 2010) and France (de Haro et al. 2021). CFP is one of the most common forms of seafood poisoning, caused by the consumption of fish contaminated with ciguatoxins (CTXs) produced by microalgae of the genus *Gambierdiscus* (Bagnis et al. 1979) and *Fukuyoa* (Gomez et al. 2015). CTXs enter into the food chain when herbivorous fish consume dinoflagellates and bioaccumulate toxins along the food chain until the compounds reach the apex of the trophic pyramid where humans experience full effects of its bioaccumulation (Goater et al. 2011). Global incidence rates of CFP may arise up to 50,000 people annually, although under-diagnosis and under-reporting occur. Distribution of CFP is boosted paralleling a worldwide increase in harmful algae bloom (HAB) events, international trade expansion and anthropogenic global warming (Botana 2016).

Marine dinoflagellates of the genus *Gambierdiscus* produce several bioactive polyether compounds such as maitotoxins (MTXs), ciguatoxins, gambierol (Lewis 2001; Pearn 2001; Watters 1995; Yasumoto 2001) and the newly identified ladder-shapped polyethers gambierone (Rodriguez et al. 2015) and MTX3 also named 44-methylgambierone (Boente-Juncal et al. 2019a). The implications of all of these compounds in CFP have been suggested by several authors and lead to gastrointestinal, cardiovascular and neurological symptoms in humans that vary among patients and include paresthesia, generalized pruritus, painful dysesthesias, allodynia, ataxia and hyperalgesia (Botana 2016; Murray et al. 2021, 2020; WHO 2020). Traditionally CFP was typically found only in tropical and subtropical areas but during the last decade has expanded to other regions including Europe (Canals et al. 2021; de Haro et al. 2003; EFSA 2010; Katikou 2021), a fact that raises the need to establish regulations for the levels of these food contaminants.

Besides ciguatoxins, which can cause long-term neurological sequela in humans as a consequence of their permanent activation of voltage-gated sodium channels (Benoit et al. 1986; Hidalgo et al. 2002; Martin et al. 2015a, b; Molgo et al. 1990), the structure of an additional ciguatoxin-congener, named MTX3, has been recently elucidated (Boente-Juncal et al. 2019a). Initial studies on the in vitro biological activity of MTX3described an effect similar to that of the synthetic ciguatoxin CTX3C although of much lower potency (Boente-Juncal et al. 2019a) and with a LD_{50} in vivo between 20 and 38 mg/kg when administered by the intraperitoneal (i.p.) route (Murray et al. 2020) and of 3.8 mg/kg when algal extracts containing MTX3 were administered intraperitoneally after extraction from 4.776×10^6 cells of *G. belizeanus* CCMP401 (Kohli et al. 2015). Gambierone is another compound thought to be associated with CFP, since it is routinely found in dinoflagellates producers of ciguatoxins (Rodriguez et al. 2015) that has a ciguatoxin-like activity but low i.p. potency, with a LD_{50} of 2.4 mg/kg after i.p administration of the compound purified from *G. cheloniae* CAWD232 monoclonal cultures (Murray et al. 2021). Regarding MTX1, this compound was historically assumed to be one of the more toxic natural compounds with an i.p. LD_{50} in mice of 50 ng/kg, described initially after the structure elucidation of this toxin (Murata et al. 1993) but i.p. administration to mice of *Gambierdicus australes* samples, containing a combination of MTX1 and 44-methylgambierone, led to a LD_{50} between 200 and 1060 ng/kg (Munday et al. 2017), while in the same report administration of the algal extracts by gavage gave a LD_{50} between 20,000 and 84,200 ng/kg. The discrepancies among these values prompted us to further evaluate the toxicity of the ciguatera-related compounds following internationally adopted guidelines for the testing of chemicals (OECD 2008a; b).

With the aim of ultimately set the oral subchronic toxicity of the ciguatoxin-related compounds involved in CFP, first, the acute i.p. toxicity of MTX1 as well as an approach to the acute oral toxicity of MTX1 and CTX3C were carried out to establish the posterior doses to be administered by gavage during a 28-day period (OECD 2008a) and thus determine

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the oral toxicity of the compounds evaluated. The data presented evaluated the daily subchronic exposure of mice to different toxins responsible of CFP following internationally adopted guidelines and blood and urine biochemistry parameters. Moreover, the acute i.p. toxicity of MTX1 was about 20 times lower than that previously described, even using a 96-h observation period. A fact that is consistent with the observation of no lethality of the toxin even at daily oral doses of 5000 ng/kg.

Materials and methods

Toxins and drugs used

MTX1 and CTX3C both purity higher than 99%, were obtained from Wako (Fujifilm Wako Pure Chemical Corporation, Japan) and dissolved at a concentration of 10 µM in dimethyl sulfoxide (DMSO). Immediately before administration, MTX1 was diluted in physiological buffer solution, to a concentration of 5 µM to obtain the series of tested doses. Working solutions of CTX3C from a stock solution of 1 µM were also prepared. Gambierone was purchased from CIFGA S.A. (Lugo, Spain), the stock solution was 157.4 µM and was diluted to 10 µM in DMSO. MTX3 was also supplied from CIFGA with a stock solution of 164.4 µM and working solutions were prepared in DMSO at a final concentration of 10 µM. Control animals received the same amount of physiological saline solution that contained the corresponding amount of toxin solvent, always lower than 1% (v/v) either for the oral route (0.9% at the highest MTX1 dose) or for the i.p. administration (0.84%).

In vivo experimental procedure

In vivo studies were performed with Swiss male and female mice weighting of body weight (bw) ranging from 22 to 26 g and 4 weeks old at the beginning of the experiments. All in vivo procedures were performed in compliance to the European legislation (EU directive 2010/63/EU) and according to the Spanish legislation (Real Decreto 53/2013). The principles were approved by the Institutional Animal Care Committee of the Universidad de Santiago de Compostela under the procedure number 011/14, authorized on August 14th 2014 (MR110250) and under procedure number 06/19/LU-002, authorized on March 1st, 2019 (A12X00509).

Before administration, the stocks of CTX3C, gambierone, MTX1 and MTX3 were diluted in 0.9% saline solution in a final volume of 200 µl. For acute studies, animals received either a single oral dose of toxin by gavage or, in the case of MTX1, a single i.p. injection. The same procedure was followed for control animals. The i.p. doses for MTX1 were 200, 400, 800, 1000, 1200, 1600 and 3200 ng/kg body weight while the oral acute dose was 800 ng/kg bw for MTX1 and 330 and 1050 ng/kg bw for CTX3C. No acute studies were performed for MTX3 and gambierone since previous works highlighted their much lower toxicity (Murray et al. 2020, 2021). After toxin administration, mice were observed continuously for the first 2 h and periodically afterwards until sacrifice of surviving animals at the end of the treatments. During the procedures, moribund animals or mice obviously in pain or presenting signs of severe agony were humanely killed and considered in the interpretation of the test results as animals that died during the experiment.

For the repeated oral dosing, toxins were administered in three separate treatments and animal groups, each lasting 28 days, starting at the lower dose and initiating the next dosage period after finishing the first month treatment. To create toxin groups and control groups, animals were randomly selected, marked to allow identification, and kept in their cages for 5 days to acclimatize before the beginning of the experiments, as required by the current protocols (OECD 2008b). In the oral treatments CTX3C at doses of 10, 32, 102 ng/kg bw was administrated by gavage every 24 h. Gambierone administration to mice included doses of 172, 550 and 1760 ng/kg bw while MTX1 was fed to mice at doses of 800, 2560 and 5000 ng/kg bw. Finally, MTX3 was daily administrated to mice at doses of 550 and 1760 ng/kg bw. During the 28-day treatment period, animals were housed in rooms with controlled temperature, humidity, and a restricted photoperiod at the animal facilities of the School of Veterinary Medicine of the University of Santiago de Compostela (Code: AE-LU-002). The toxins doses and the routes of administration used in each case are summarized in Table 1.

For the repeated oral treatment, control group and toxin-treated mice groups were weighted the first day of the treatment (day 0) and weekly thereafter. Food consumption was also recorded at days 7, 14, 21 and 28 from the beginning

Table 1 Toxin doses and administration routes evaluated in this work

Toxin doses (ng/kg)	MTX1	CTX3X	Gambierone	MTX3
Acute i.p	200, 400, 800, 1000, 1200, 1600, 3200			
Acute oral	800	330, 1050		
28-day oral subchronic	800, 2560, 5000	10, 32,102	172, 550, 1760	550, 1760

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of the treatments. Euthanasia was performed on day 28, and 24 h before sacrifice animals were individually placed in metabolic cages to monitor and obtain samples of urine and feces production during this period. For all the treatments animals were euthanized in a CO_2 chamber and directly, after death confirmation by cervical dislocation, blood and tissue samples were collected. Mice were subjected to a detailed necropsy including observation of thoracic organs (heart and lung), abdominal organs (liver, kidneys, spleen, stomach, duodenum, rectum) and brain.

Evaluation of enzyme and electrolyte levels in plasma of control and toxin-treated animals

Blood was obtained directly from the ventricle of euthanized mice as previously described (Boente-Juncal et al. 2020) and conserved in microtubes containing lithium heparin (2.5 UI/ml) until the end of the necropsy and tissue collection (30 min). Afterwards, each blood sample was mixed for 30 s and centrifugated for 90 s at 15,800 rpm/12000×*g* (IDEXX Stat Spin, IDEXX Europe B.V., Hoofddorp, The Netherlands). The computer controlled biochemical analyzer IDEXX Catalyst Dx was used for the analysis of plasma parameters. In most of the samples, 300 µL of plasma were used to evaluate electrolyte levels (Cl^-, Na^+ and K^+), alanine transaminase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH), and creatine kinase (CK). Initially, all the markers were evaluated in undiluted samples, but when the plasma level of the marker was above the reference range of the analyzer, an automatic dilution with physiological saline was performed. Samples with altered visual properties that could alter biochemical parameters (high hemolysis, icterus or lipidemia) were not further processed.

Urine analysis

Parameters as urine color, turbidity, protein, glucose, ketones, blood hemoglobin, bilirubin, and urobilinogen were analyzed using an IDEXX VetLab UA reflectance photometer, which reads and evaluates IDEXX UA Strips. Urine specific gravity was measured with a refractometer. Because for some mice a small amount of blood or urine were obtained, it was not possible to measure all the parameters for each animal.

Preparation of samples for light microscopy

After sacrifice, necropsy and macroscopic examination of the different organs, samples were prepared for light microscopy. Tissue samples were fixed by immersion in buffered Bouin's solution (75% picric acid, 20% formaldehyde, 5% acetic acid) for 24 h at 4 °C, transferred to 70% alcohol and embedded in paraffin wax according to standard laboratory procedures. Tissue samples were cut into sections of 3 µm thickness and mounted into slides. Sections were stained with haematoxylin–eosin (H&E) for structural assessment of the tissues and examined under a light microscope. Digitalized images were obtained with an Olympus microscope digital camera DP74 coupled to an Olympus AX70 microscope.

Statistical analysis

Data analysis was performed using GraphPad Prism 5. One-way ANOVA followed by Bonferoni *t* test or Student's *t* test were used to assess significant differences between control and SPX-treated neurons. All values are expressed as mean ± SEM of at least three independent experiments.

Results

In this in vivo study, an acute and repeated oral dosing evaluation of the effect of several compounds involved in ciguatera fish poisoning was performed. First, the acute toxicity of the most representative analogues (MTX1 and CTX3C) was evaluated using a single approach to elucidate the optimum doses that could give consistent results for the 28-day oral approach. Although MTX1 was so far considered one of the most toxic compounds known to date, with a reported initial i.p. LD_{50} in mice of 50 ng/kg (Murata et al. 1993), a much higher LD_{50} (approximately 0.2 mg/kg) for algal extracts containing a mixture of MTX1 and MTX3 was reported after a 96-h observation period (Munday et al. 2017). Therefore, this study was initiated following the same approach, using first MTX1 due to its assumed high toxicity by the i.p. route (Murata et al. 1993).

Acute i.p. toxicity of MTX1

To optimize the dose of MTX1 needed to establish the optimum quantities of toxin to evaluate its oral toxicity, an initial approach was performed evaluating the effect of the i.p. injection of MTX1. The effects of i.p. administration doses of MTX1, ranging from 200 to 3200 ng/kg, after a 96-h observation period and the corresponding survival times for each animal are summarized in Table 2. Non-linear fit of the mortality of animals after i.p. administration of increasing MTX1 (Fig. 1A) allowed to calculate an estimated i.p. LD_{50} for MTX1 of 1107 ng/kg [95% confidence interval (CI) from 1075 to 1140 ng/kg, $R^2 = 0.9981$]. A scatter plot showing the relative survival times for each mouse at the different MTX1 doses are shown in Fig. 1B.

The symptoms shown by the animals after acute i.p. administration of the different MTX1 doses are summarized in Table 3. Most of the animals showed lethargy and piloerection even with low doses of i.p. MTX1 (200 and

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Table 2 Mortality induced by single i.p. administration of MTX1 to mice and survival times corresponding with each treatment

Dose (ng/kg)	Mice number	Dead	Survival time (h)	Mortality %
Control	4	0	96	0
MTX1				
200	3	0	96	0
400	5	0	96	0
800	10	2	3.5, 34, 96, 96, 96, 96, 96, 96, 96, 96	20
1000	7	3	24, 48, 72, 96, 96, 96,96	42.8
1200	7	4	24, 24, 48, 72, 96,96,96	57.1
1600	5	4	4, 16, 24, 24, 96	80
3200	4	4	2.5, 5, 6, 32	100

400 ng/kg). Stretching and Straub´s tail reaction (dorsiflexion of the tail orientated vertical or curling over the mouse produced by contraction of the sacrococcygenus muscle, (Supplementary Figs. 1A and B) were observed in mice after administration of the toxin at 400 ng/kg and higher, an effect whose intensity and duration was dependent on the toxin dose. At higher doses of MTX1, ataxia and kyphosis were also observed. Furthermore, MTX1 at 800 ng/kg and higher caused dyspnea that led to a profound abdominal breathing that was evident in most animals. The dyspnea was justified after opening the thoracic cavity of the moribund animals, that revealed accumulation of blood and internal bleeding. Also, an intense cyanosis of the tail in five of the animals was observed after the first 48 h of i.p. administration of the toxin. Dyspnea was observed in all the animals treated with the highest MTX1 dose.

As shown in Fig. 2, single i.p. administration of MTX1 increased several blood enzyme levels in respect to control animals although most of the parameters were still within the normal physiological range. Figure 2 summarizes the biochemical levels of the different parameters in control and MTX1-treated mice. Although ALT levels were increased in some of the animals treated with MTX1, the differences between control and toxin-treated animals did not reach statistically significant differences at any of the toxin doses employed (Fig. 2A). Blood enzyme levels were not analyzed in mice treated with MTX1 at doses of 1600 and 3200 ng/kg due to the sudden death of the animals after the first continuous 2 h of the observation period. Similarly for the blood AST levels shown in Fig. 2B, i.p. administration of MTX1 (Fig. 2B) In addition, no statistically significant changes were observed neither for LDH nor CK parameters (Fig. 2C and D, respectively).

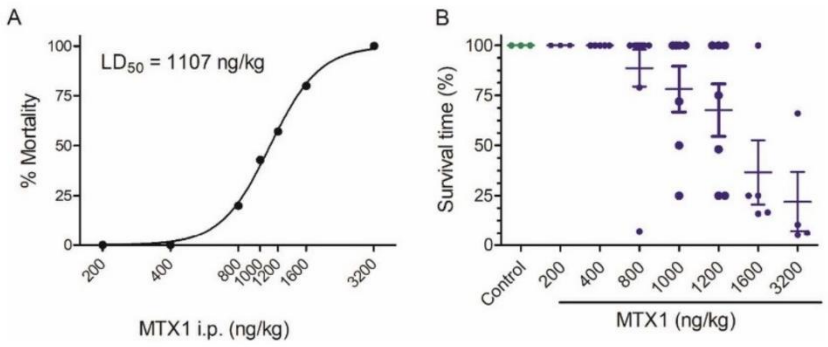


Fig. 1 Intraperitoneal toxicity of MTX1 after a 96-h observation period. **A** Lethality of MTX1 in Swiss female mice after injection of different toxin doses. **B** Scatter plot graph presenting the percentage of survival time for each mouse. The survival results are expressed as means ± SEM of the data obtained from three to ten animals after an observation period of 96 h

Table 3 Main symptoms elicited in mice after acute i.p. administration of MTX1 and number of animals with symptoms at each MTX1 dose

I.p. MTX dose (ng/kg)	200	400	800	1000	1200	1600	3200
Lethargy	1/3	5/5	10/10	7/7	7/7	5/5	4/4
Piloerection	1/3	2/5	7/10	7/7	7/7	5/5	4/4
Stretching	2/3	5/5	5/10	7/7	7/7	5/5	4/4
Straub tail reaction	0/3	2/5	5/10	5/7	7/7	5/5	4/4
Ataxia	0/3	2/5	7/10	5/7	7/7	5/5	4/4
Kyphosis	0/3	0/5	2/10	5/7	5/7	5/5	0/4
Dyspnea	0/3	0/5	4/10	5/7	5/7	3/5	4/4
Cyanosis	0/3	0/5	1/10	2/7	2/7	2/5	0/4
Abdominal swelling	0/3	0/5	5/10	7/7	7/7	5/5	4/4

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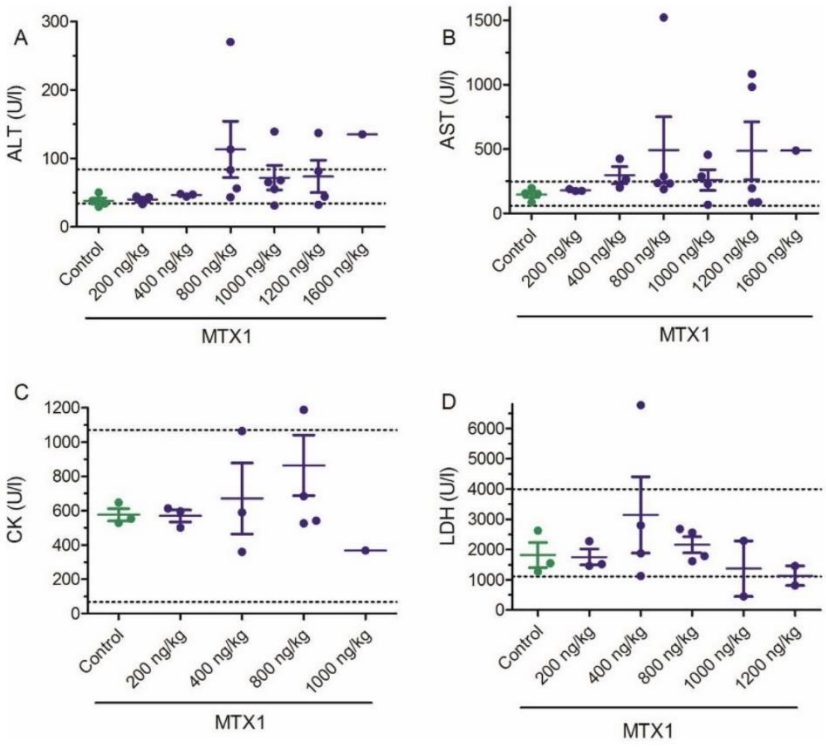


Fig. 2 Scatter plot graphs showing the effects of different i.p. doses of MTX1 (200, 400, 800, 1000, 1200 and 1600 ng/kg), on blood levels of ALT, AST, CK and LDH. Data are expressed as mean ± SEM from at least two animals. The physiological ranges for the blood level of each enzyme are indicated by the dotted lines

Simultaneously, the effect of the i.p. administration of MTX1 on blood electrolyte levels was evaluated as shown in Fig. 3, but no statistically significant differences on blood ion levels were found between control animals and animals treated intraperitoneally with different doses of MTX1.

After euthanasia, animals were subjected to macroscopic observation on necropsy. As shown in Supplementary Fig. 2, macroscopic alterations were present in animals treated at doses of 1000 ng/kg MTX1 and higher. All animals treated with these doses of MTX1 had the stomach and small intestines empty of solid contents only having feces in the final sections of the large intestine. It is noteworthy to remark that despite abdominal swelling, all treated animals had a remarkable weigh loss (between 1 and 3.6 g). Thus, abdominal distension was observed in the gastrointestinal tract especially in the stomach, small intestine and caecum due to gas accumulation in MTX-treated animals. Additionally, two animals treated with MTX1 at doses of 1200 ng/kg presented several haemorrhagic zones in the stomach observed macroscopically as shown in Supplementary Fig. 3.

Images of different tissues (20×) stained with H&E for structural assessment under light microscopy are represented in Fig. 4. As shown in the corresponding images, histological examination of the heart of control and MTX mice dosed with 3200 ng/kg of MTX1 (Figs. 4A and B, respectively) that survived for 32 h did not show any evidence of structural alterations. In both cases, normal cylindrical-shaped cardiac myocytes with elongated nuclei, were observed. Similarly, liver sections (Fig. 4C and D) of control and MTX1-treated mice showed an eosinophilic cytoplasm with large spherical and central nuclei, which corresponds to a normal morphology. The kidney sections from control animals and from MTX1-treated mice (Fig. 4E and F) did not reveal any apparent histological alterations by light microscopy. Small intestine of control and MTX1-treated mice (Fig. 4G and H) did not show any apparent changes in the arrangement of plicae around the lumen or in the surrounding musculature. Large intestine samples were also stained and observed but had unaltered basic structure formed by the mucosa and the submucosa layer, the smooth muscle layers, and the serosa layer both in control and MTX1-treated mice (data not shown). Since this mouse only survived 32 h, samples of the same tissues of mice dosed intraperitoneally with 800 ng/kg of MTX1 that survived the 96-h observation period are also shown in Supplementary Fig. 4.

Acute oral evaluation of the toxicity of CTX3C and MTX1

After confirming that a single i.p. dose of 800 ng/kg of MTX1 did not elicit drastic changes in blood parameters,

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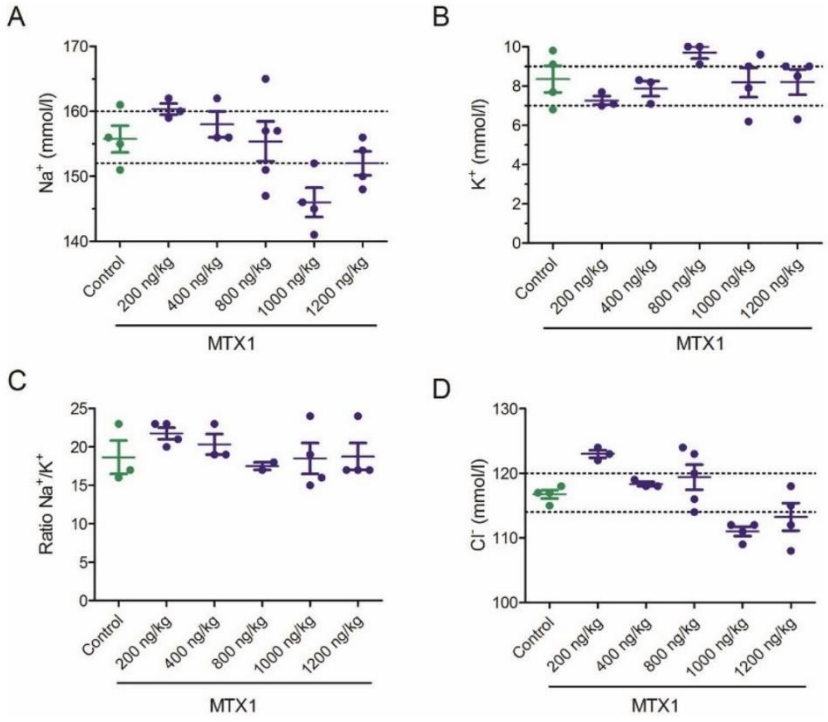


Fig. 3 Scatter plot graphs showing the effects of different i.p. doses of MTX1 on blood electrolyte levels. Data are expressed as mean ± SEM. The respective minimum and maximum reference values of blood electrolyte levels are indicated by the dotted lines

initially the same toxin dose was evaluated by the oral route administrating the toxin by gavage and comparing the effect of MTX1 with the effects elicited by CTX3C. Since the cellular effects of MTX1 are much more pronounced than those of CTX3C (Boente-Juncal et al. 2019a), to establish the toxin doses needed for the subchronic toxicity approach, a single dose of 800 ng/kg of MTX1 was orally administrated to three animals. Additionally, CTX3C at doses of 330 and 1050 ng/kg was evaluated in the acute toxicity study to establish a reference range prior to start the repeated oral exposure of mice to the toxins since the initial acute LD_{50} dose proposed for CTX3C was approximately 1.5 µg/kg (Hirama et al. 2001). As indicated in Table 4, neither MTX1 nor CTX3C caused death of the animals after the 96-h observation period.

No significant effects in blood enzyme levels of ALT, AST, CK and LDH nor in blood electrolyte levels were observed in mice treated with a single oral dose of CTX3C, as shown in Figs. 5 and 6, respectively. Although apparently CTX3C caused a dose-dependent decrease in LDH values the data did not reach statistically significance ($p = 0.0752$; $df = 4$; $t = 2.389$) between control animals and those treated with 1050 ng/kg CTX3C.

In the same animals, the effect of oral acute exposure of mice to CTX3C at concentrations of 330 ng/kg and 1050 ng/kg on food intake, feces and urine production during a 24-h period before euthanasia was measured and is summarized in Table 5 and supplementary Fig. 5. In the case of CTX3C oral administration of the toxin at the dose of 1050 ng/kg decreased urine production in 24 h by about 85% suggesting that either activation or inhibition of voltage-gated sodium channels can alter renal function as previously described for the sodium channel blocker tetrodotoxin (Boente-Juncal et al. 2019b).

Repeated dose effects in mice of the oral administration of toxins involved in ciguatera

Based on the previous results for the oral acute toxicity of MTX1 and CTX3C in mice, the effect of 28-day repeated oral doses of these compounds was explored during a 28-day exposure period. First, since previous studies have shown that intermittent administration of P-CTX1 at 100 ng/kg caused CFP symptoms after 15 days, repeated dosing (Terao et al. 1992), an initial dose of 10 ng/kg CTX3C was chosen. Further doses were increased by the 3.2 rate as indicated in the OECD guidelines for the testing of chemicals (OECD 2008a; b). Therefore, at the end of the study, three independent groups of mice were feed daily by gavage as summarized in Table 1. The effects of the 28-days oral administration of the different toxin doses in mice survival time and mortality rate are summarized in Table 6. As shown in this table

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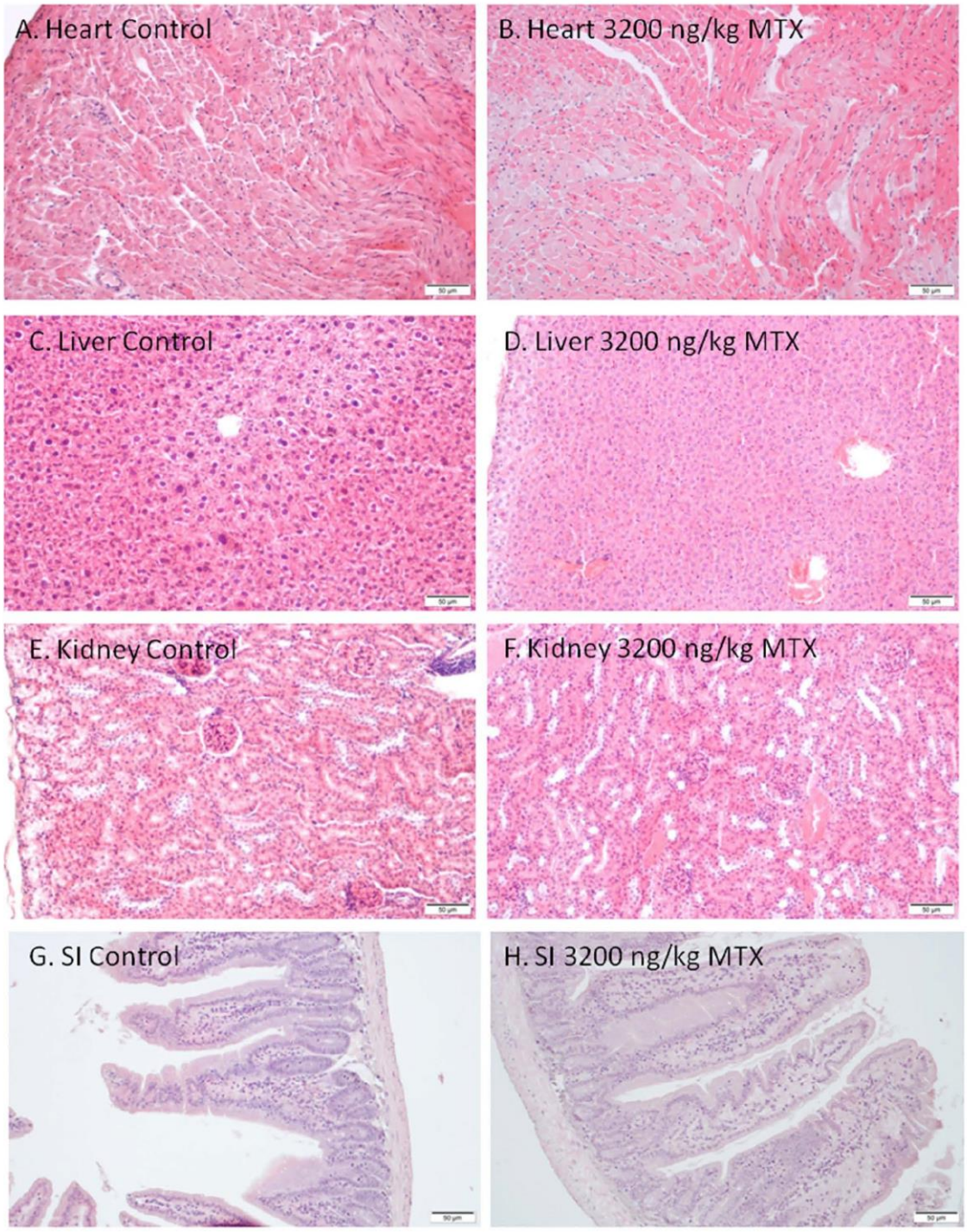


Fig. 4 Light microscopy images of heart, liver, kidney and small intestine of a control mouse (**A**, **C**, **E**, **G**) and an animal dosed intraperitoneally with 3200 ng/kg of MTX1 that survived 32 h (**B**, **D**, **F**, **H**)

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Table 4 No mortality was induced by single gavage administration of CTX3C or MTX1 to Swiss female mice during the 96-h observation period

Dose (ng/kg)	Mice number	Dead	Survival time (h)	Mortality %
Control	3	0	96	0
CTX3C				
330	3	0	96	0
1050	3	0	96	0
MTX1				
800	3	0	96	0

MTX1 elicited a dose dependent death, but no toxicity was observed for CTX3C, gambierone or MTX3 at the doses employed. The lowest oral dose of MTX1 was 800 ng/kg since acute oral administration of this toxin dose only elicited piloerection and some degree of immobility during the first 24-h observation period, symptoms that reversed completely after 48 h of feeding the mice by gavage with a single MTX1 dose. During the 28-day treatment period, no changes in body weight in any of the toxin-treated animal groups, were observed as summarized in Supplementary Table 1.

As well as in the acute i.p. studies, the enzyme and electrolyte disturbances in the plasma of control and animals treated with toxins for 28 days were analysed and summarized in Fig. 7. None of the toxins caused significative alterations in the hepatic parameters of AST, and ALT (Fig. 7A and B, respectively). Similarly, CK remained within physiological values in all animals treated with toxins except in one of the animals treated with 172 ng/kg of gambierone (Fig. 7C). Moreover, none of the compounds altered the plasma levels of LDH at any of the doses evaluated, and only statistically significant increases in LDH levels were found in mice treated with 5000 ng/kg of MTX1 (Fig. 7D), but the blood LDH levels in MTX1-treated animals were within the physiological range (1.105–3.993 U/L) in all samples.

The effects of the repeated oral administration of different doses of CTX3C, gambierone, MTX3 and MTX1 on blood electrolyte levels were also evaluated as shown in Fig. 8. Although none of the toxins affected blood sodium levels (Fig. 8A), in mice fed orally with doses of 10, 32 and 100 ng/kg of CTX3C statistically significant increases in blood potassium levels were found at doses of 100 ng/kg ($df=8$; $t=3.277$; $p=0.011$). As shown in Fig. 8B, the mean K^+ blood values in control animals were 7.24 ± 0.266 mmol/L ($n=7$) while in mice fed orally with CTX3C at 100 ng/kg, potassium levels were 8.87 ± 0.44 mmol/L (n = 3). Hence, due to the increase in blood potassium concentration, the ratio Na^+/K^+ decreased (Fig. 8C). Regarding the oral treatment with 172, 550 and 1700 ng/kg of gambierone, also statistically significant disturbances in potassium levels as well

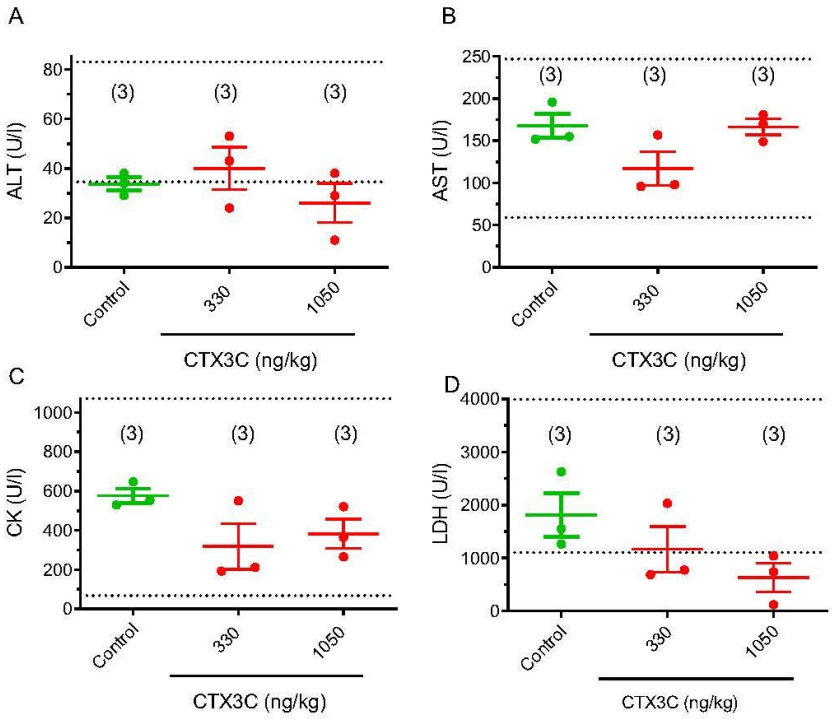


Fig. 5 Scatter plot graphs showing the effects of 330 and 1050 ng/kg CTX3C administered by gavage on the blood levels of ALT (**A**), AST (**B**), CK (**C**), and LDH (**D**) after an observation period of 96 h. Data are expressed as means ± SEM from three determinations. The respective minimum and maximum reference values of blood enzyme levels are indicated by the dotted lines

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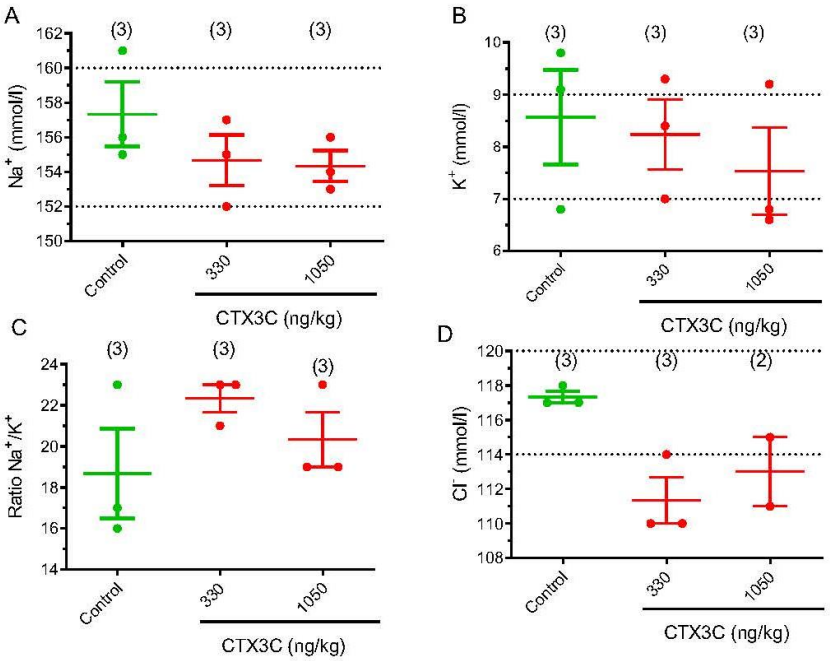


Fig. 6 Scatter plot representation of the blood levels of sodium (**A**), potassium (**B**), ratio sodium/potassium (**C**) and chloride (**D**) in control Swiss female mice and in mice dosed by gavage with CTX3C at 330 and 1050 ng/kg bw after an observation period of 96 h. Data are expressed as means ± SEM The dotted line indicates the maximum and minimum physiological reference value for each parameter. Values are expressed as mean ± SEM from three determinations except for determination of Cl^- in animals treated with 1050 ng/kg of CTX3C since the size of the sample was not enough in one of the treated animals

Table 5 Results obtained after a single oral administration of CTX3C at doses of 330 ng/kg and 1050 ng/kg regarding food consumption, feces, and urine production for 24 h

Group/analyzed parameters (24 h in metabolic cages $n=3$)	Control	CTX3C 330 ng/kg	CTX3C 1050 ng/kg
Food consumption (g)	3.78 ± 0.42	4.7 ± 0.6	3.7 ± 0.4
Feces (g)	1.57 ± 0.12	1.2 ± 0.3	1.3 ± 0.2
Urine (ml)	4.1 ± 1.44	1.5 ± 0.2*	0.6 ± 0.1**

as alterations in the Na^+/K^+ ratio were observed (Fig. 8). Thus, mean K^+ blood values after the 28 days oral dosing of mice with gambierone at the lowest concentrations evaluated (172 ng/kg) were 9.03 ± 0.48 mmol/L ($n=3$; $df=8$; $t=3.505$; $p=0.008$) and 8.05 ± 0.65 mmol/L ($n=3$; $df=8$; $t=2.796$; $p=0.0234$) at the dose of 1700 ng/kg. No alterations in blood electrolyte levels were found in mice treated with MTX3 or with MTX1.

As for the acute i.p. treatment, after euthanasia, samples of heart, liver, kidney, and small intestine of control and 800 ng/kg MTX1-treated mice were studied. Representative light microscopy images of H&E staining in heart sections of control and MTX1-treated mice did not show any evidence of structural alterations in the heart (Fig. 9A and B). In both cases, normal cardiac myocytes, which are cylindrical in shape and have an elongated nucleus, were observed. Stained liver samples are shown in Fig. 9C and D. In contrast to liver sections from treated mice, in which hepatocytes showed an eosinophilic cytoplasm and a round nucleus (Fig. 9D), hepatocytes in the livers of control mice showed a more vacuolated (non-stained) cytoplasm (Fig. 9C) that could represent cellular swelling, lipid accumulation, or glycogen stores. These differences may be due to the feeding status of the animal at the time of death. No evident histological alterations in the kidney sections from mice treated with 800 ng/kg MTX1 were observed (Fig. 9E and F). Finally, small intestine showed unaltered mucosa in both control and treated animals and no apparent histological alterations (Fig. 9G and H). Both intestines showed a normal basic structure formed by the mucosa and the submucosa layer, the smooth muscle layers, and the serosa layer.

Effects of 28-day repeated oral administration of ciguatoxin-related compounds on food consumption, feces, and urine parameters

As shown in Table 7, none of the ciguatera toxins evaluated over the 28-day treatment period altered body weight or feces production, however urine production in mice treated with different doses of the toxins decreased as summarized

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Table 6 Mice mortality and survival times observed after daily dosing of Swiss female mice with CTX3C, gambierone, MTX3, or MTX1 during a 28-day period

Dose (ng/kg)	Mice number	Death	Survival time (days)	Mortality %
Control	7	0	28	0
CTX3C				
10	3	0	28	0
32	3	0	28	0
102	3	0	28	0
Gambierone				
172	3	0	28	0
550	3	0	28	0
1760	3	0	28	0
MTX3				
550	2	0	28	0
1760	3	0	28	0
MTX1				
800	3	0	28	0
2560	3	2	5,17,28	66
5000	5	5	1,6,10,14,15	100

in the same table. Therefore, urine parameters were evaluated after free catch collection of the samples during the 24-h period before euthanasia. Urine parameters analyzed in control and treated mice included color, clarity, specific gravity, protein, glucose, ketones, blood/hemoglobin, bilirubin, and urobilinogen are summarized in Supplementary Table 2.

Interestingly, mice treated with CTX3C, at doses of 32 and 100 ng/kg had a slight decrease in urine production during the 24-h period after the last toxin dose with a urine volume of 1 ± 0.02 mL and 1.1 ± 0.5 mL, respectively ($n = 3$) versus the amount of urine collected from control animals during the same period which was 2.9 ± 0.9 mL in seven untreated animals, although these results did not reach statistically significant differences. Remarkably, urine color darkened after increasing the CTX3C dose, changing from pale yellow in control mice to dark yellow in mice treated with 100 ng/kg of CTX3C. Urine turbidity was also higher in mice treated with CTX3C at doses of 32 and 100 ng/kg. Besides, urine-specific gravity increased in animals treated with the highest toxin doses, in agreement with the increment in urine glucose levels. Furthermore, moderate urobilinogenuria was also present in the animals treated with the highest doses of CTX3C. Similarly, mice treated with gambierone at the dose of 1760 ng/kg had a remarkable decrease in urine production during the 24-h period after the last toxin dose and reached 1.3 ± 0.2 mL ($n = 3$). Urine color was darker, and turbidity and urine-specific gravity increased in gambierone-treated mice. Furthermore, there was a steep increase in the urine urobilinogen levels even at the lowest gambierone dose (170 ng/kg). The same effects were found in mice treated with MTX1, although in this case a smaller number of samples were obtained since one animal died overnight during the 24-h observation period.

Discussion

Ciguatoxins are marine biotoxins involved in CFP, however other compounds produced by the marine phytoplankton also play an important role in this intoxication. So far, the available in vivo toxicity studies with the ciguatoxins and their related compounds causative of CFP, were mostly acute toxicity studies administering the toxins by the i.p. route (Hirama et al. 2001; Lewis and Hoy 1993; Murray et al. 2021; Vernoux and Lewis 1997; WHO 2020). Regarding the toxins belonging to the ciguatoxin group, the most potent analogue was P-CTX1B, with a mean i.p. LD_{50} of 0.32 µg/kg (WHO 2020) after i.p. administration of the toxin and the more potent analogue at the cellular level (Raposo-Garcia et al. 2022). Based on the available reports, CTX3C acute i.p. toxicity has also been established for mice at 1.6 µg/kg bw (Hirama et al. 2001; WHO 2020). However, in vivo toxicological data for many compounds presumably involved in CFP are still lacking due to their scarce commercial availability and the large number of existing analogues, a fact that led EFSA to indicate the need to perform more in vivo toxicity studies to settle the levels of these toxins allowed in food (EFSA 2010). The acute in vivo toxicity data for CTXs obtained so far indicated that the i.p. and oral routes had a similar LD_{50}, suggesting a very high oral uptake of CTX (Bottein et al. 2011). Regarding gambierone, its i.p. acute toxicity in mice has been recently studied in a 14-day i.p. exposure establishing an acute LD_{50} of 2.4 mg/kg bw (Murray et al. 2021), while the LD_{50} for MTX3 in the same conditions was between 20 and 38 mg/kg, therefore, indicating a tenfold higher toxicity of gambierone versus MTX3 (Murray et al. 2020).

So far, the in vivo toxicity data for MTX1 is controversial since some reports had shown an acute LD_{50} by the i.p. route of 0.05 µg/kg, (Murata et al. 1993), however other reports using maitotoxin extracted from *Gambierdiscus toxicus* confirmed that i.p. injection of 200 and 400 ng/kg of MTX1 lead to ascites and gastrointestinal alterations without causing animal death (Terao et al. 1988). In contrast, a much lower toxicity of MTX1 in algal extracts has been recently described (Munday et al. 2017). Noteworthy, algal extracts containing only MTX1 and MTX3 were 100 times more toxic by the i.p. than by the oral route (Munday et al. 2017). Therefore, the in vivo data presented in this work that lead to obtain an i.p. LD_{50} for MTX1 of 1107 ng/kg remarks the need to reevaluate the effect of these compounds following internationality adopted guidelines. A summary of the wide

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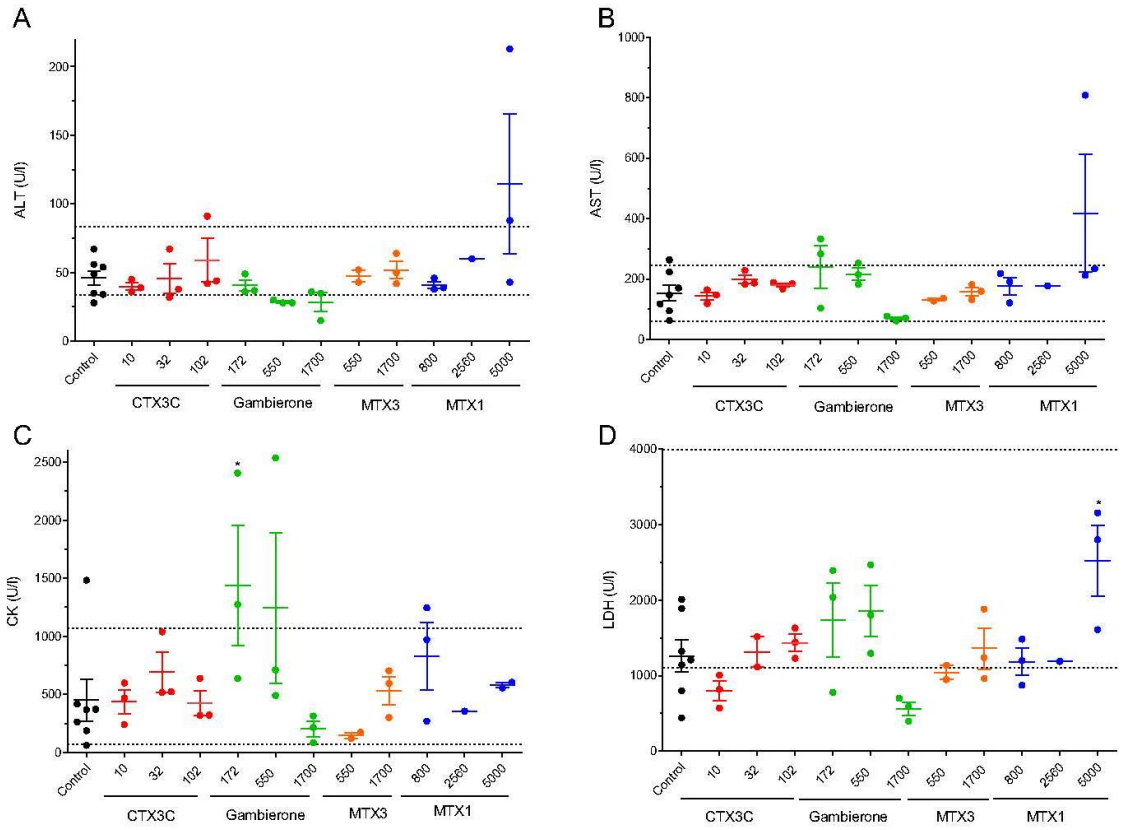


Fig. 7 Scatter plot graphs representing blood levels of alanine transaminase (ALT) (**A**), aspartate transaminase (AST) (**B**), creatin kinase (CK) (**C**), and lactate dehydrogenase (LDH) (**D**) in control Swiss female mice and in animals dosed daily by gavage during a 28-day period with CTX3C, gambierone, MTX3 or MTX1 at different doses. *$p < 0.05$ versus control mice. The respective minimum and maximum reference blood values for each parameter are marked by the dotted lines

range of LD_{50} for MTX1 proposed by different authors is reflected in Table 7 as extracted from (Munday 2014). One of the problems associated with establishing a reliable LD_{50} for MTX1 is the ambiguity of the toxicity terms previously reported (Munday 2014). Some of the early values reported with algal extracts and toxic fractions do not provide information about purity and presence of other toxic contaminants, hence these values should not be taken into account (Table 8).

To date, no additional reports on the chronic toxicity of any of these toxins have been published despite the worldwide increasing incidence of CFP. Nowadays it is known than more of 400 fish species can contain toxins involved in ciguatera (Canals et al. 2021) and ciguatera fish poisoning is considered an emerging food borne illness worldwide (WHO 2020) representing both environmental and public health challenges (Loeffler et al. 2021). During the last decade, the incidence of ciguatera in European coasts has increased progressively with ciguatoxins or their vectors been reported in France, Portugal and Spain (Bresnan et al. 2021; Canals et al. 2021; Costa et al. 2018; de Haro et al. 2021; Rossignoli et al. 2020). In a recent report from EFSA (Canals et al. 2021), both autochthonous and imported fish have been involved in ciguatera fish poisoning in Spain, Germany, France, Austria, Portugal and Switzerland, a fact that reveals the need to establish regulatory levels for ciguatera as well as to include these toxins on the surveillance controls of marine toxins (Katikou 2021).

The lack of data on the chronic oral toxicity of all of the compounds causative of CFP as well as the opinions of EFSA on the need to evaluate the toxicity of ciguatoxin-related compounds (Canals et al. 2021; EFSA 2010) prompted us to study their in vivo effects. Indeed the American Food and Drug Administration (FDA) has implemented a maximum limit of 0.01 mg/kg fish of P-CTX1 or 0.1 mg/kg fish of C-CTX1 before taken legal action to remove fish

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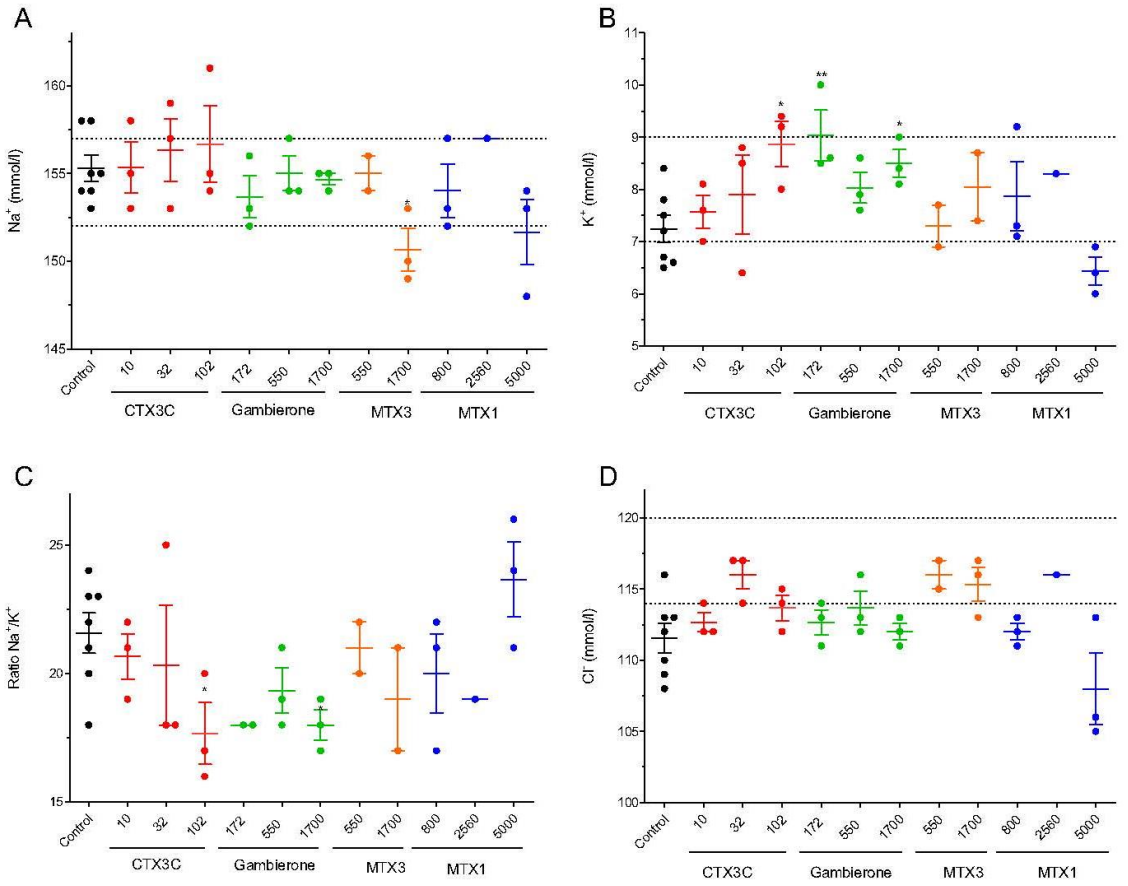


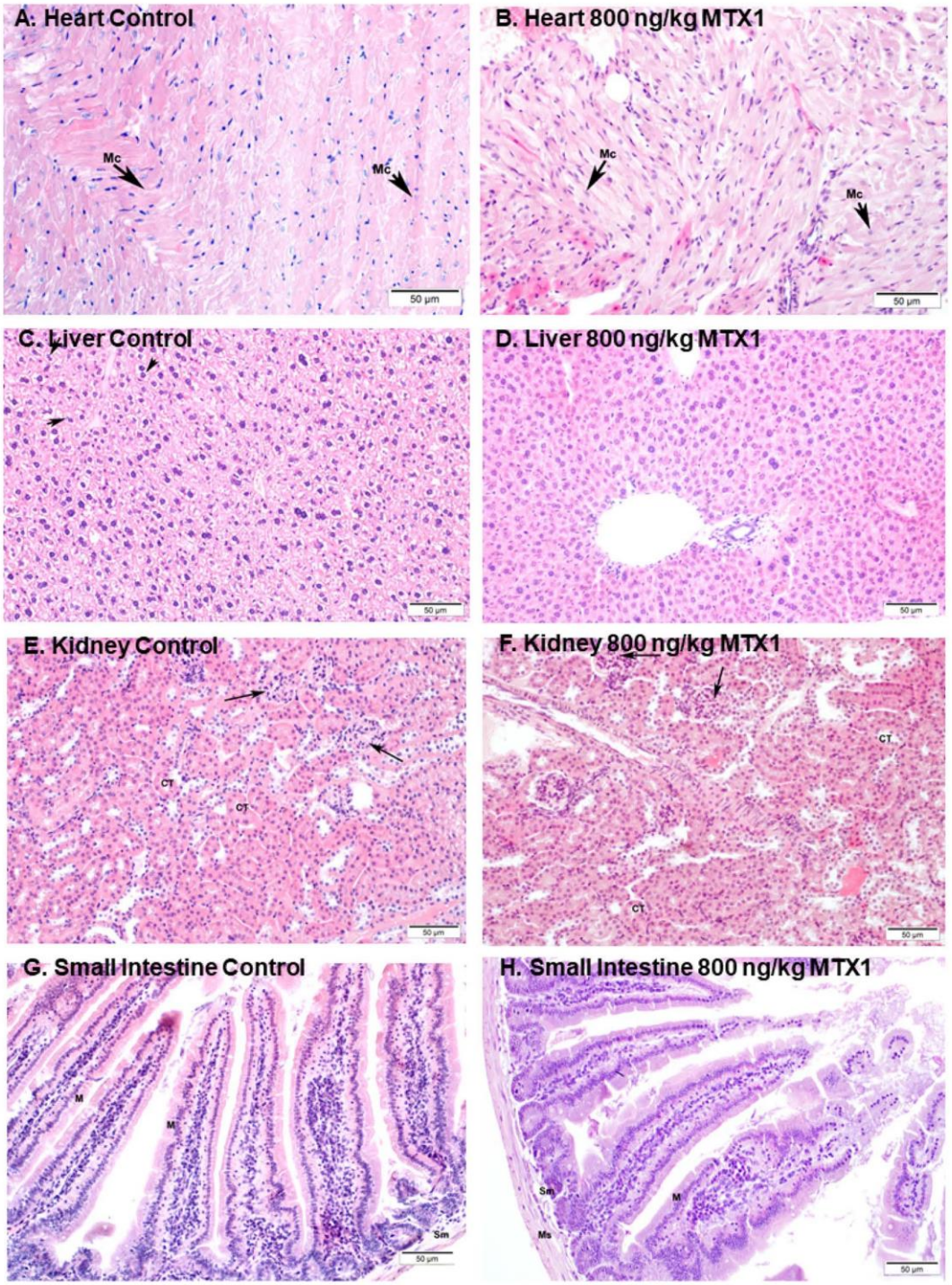
Fig. 8 Scatter plot representation of the blood levels of sodium (Na^+) (**A**), potassium (K^+) (**B**), ratio sodium/potassium (Na^+/K^+) (**C**) and chloride (Cl^-) (**D**) in control Swiss female mice and in mice dosed daily by gavage, over a 28-day period, with CTX3C, gambierone, MTX3, MTX1. $*p<0.05$; $**p<0.01$ versus control mice. The respective minimum and maximum reference values of blood electrolyte levels are indicated by the dotted lines

from the market (FDA 2021). Usually ciguatera fish poisoning has been associated with the ingestion of fishes such us tuna, red snapper, mackerel, barracuda, warsaw grouper, hotfish and amberjack (WHO 2020). While not regulatory levels in fishery products for the compounds belonging to the ciguatoxin group have been yet adopted in the European Union, the current legislation does not allow fish containing ciguatoxins to be commercialized (EC 2019) (EC 2019; Friedman et al. 2008). In our study, daily oral administration of CTX3C (10–102 ng/kg) to mice during a month altered urine parameters but did not cause observable symptoms or lethality.

Furthermore, acute exposure of mice to MTX1 or CTX3C by the i.p. route revealed a NOAEL for MTX1 below 200 ng/kg after a 96-h observation period while for the acute oral administration of MTX1 no symptoms or biochemical alterations were observed at doses of 800 ng/kg thus confirming the lower effect of MTX1 by the oral route, as previously described (Munday et al. 2017). For CTX3C, no symptoms or deaths were observed at the higher dose evaluated, 1050 ng/kg of CTX3C, a fact that is in agreement with its i.p. LD_{50} ranging from 1200 to 2500 ng/kg (Dechraoui et al. 1999; Yogi et al. 2014).

To complete the data available as well as to obtain an approach to the repeated oral exposure toxicity of MTX1 and CTX3C daily dosage of mice with the toxins was performed. In this context, the data presented in this report constitute the first approach for the evaluation of their toxicity after daily ciguatoxin ingestion. The results presented in this work indicated that CTX3C at doses of 102 ng/kg and gambierone administered daily at 1700 ng/kg did not cause animal death but altered blood potassium levels. While for MTX1 doses

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◂**Fig. 9** Representative haematoxylin–eosin stained sections of control mice (**A**, **C**, **E** and **G**) and animals treated daily for 28 days with MTX1 at the dose of 800 ng/kg (**B**, **D**, **F** and **H**). Sections of the myocardium ventricle of control (**A**) and treated mouse (**B**) showing normal cardiomyocites (Mc). Sections of the liver parenchyma from a control mouse (**C**), and from a mouse treated with 800 ng/kg MTX1 (**D**). Compared to hepatocytes from MTX-treated animals more cytoplasmic vacuoles (non-stained spaces, indicated by arrows) were present in the hepatocytes of control mice. Kidney sections of a control mouse (**E**) and a mouse treated with 800 ng/kg MTX (**F**). Renal corpuscles (arrows) and convoluted tubules (CT) are shown. Microscope micrographs of the small intestine, from control mice (**G**) and mice treated with MTX at 800 ng/kg (**H**). *M* mucosa layer, *Sm* submucosa layer, *Ms* muscular layer. Microscope magnifications of 20×

of 800 ng/kg, for 28 days did not cause animal death nor any macroscopic organ alterations at necropsy, however doses of 2560 ng/kg and 5000 ng/kg of the toxin caused animal death and difficulty to obtain samples for biochemical analysis. These difficulties in obtaining samples for biochemical analysis due to the animal treatment, sudden dead and the poor condition of some of the animals treated before sacrifice do not allow these parameters to be compared.

The approach to obtain data about the acute i.p. toxicity of MTX1 yielded an estimated LD_{50} of 1107 ng/kg after a 96-h observation period, a result that should be taken into account in view of the huge range of LD_{50} doses reported so far for MTX1 which vary from 50 to 200,000 ng/kg (Munday et al. 2017; Murata et al. 1994; Reyes et al. 2014; Yokoyama et al. 1988). In agreement with previous studies on the i.p. toxicity of MTX1 (Murata et al. 1993), the lowest i.p. dose employed in this work, 0.2 µg/kg, already caused lethargy, piloerection (1/3 mice), and stretching (two out of three animals), however, at this dose, neither alterations in ALT, AST, CK and LDH parameters nor in the electrolyte balance were observed. When mice were fed with a single MTX1 dose of 800 ng/kg no mortality was observed and animals showed only transitory minor symptoms like lethargy and piloerection, but not biochemical alterations in blood parameters were detected. In the case of a single oral dose of CTX3C of either 330 or 1050 ng/kg, animals did not have symptoms or alterations in the blood parameters during the 96-h observation period. In the case of MTX1, significant macroscopic alterations in the digestive tract produced by i.p. administration of the toxin at doses from 1000 ng/kg and higher is in accordance with the increase in mortality rate. However, lower doses studied did not elicited neither death nor macroscopic alterations in animals which indicates that the toxicity of MTX1 is lower than that previously described (Murata et al. 1993).

Important in vivo disturbances by the CTX-related compounds employed in this work occurred during the repeated oral administration of these compounds. We demonstrated here that, after daily feeding of mice with MTX1 for 28 days, at a dose of 800 ng/kg only affected urine parameters. However, daily oral dosage of mice with MTX1 at 5000 ng/kg led to a mortality rate of 100% with survival times between 24 h and 15 days highlighting the need to pursue detailed studies about the chronic effects of these compounds.

The changes in urine parameters caused by ciguatoxins may suggest that the glomerular filtration barrier could be damaged as previously described for other sodium channel modulators (Boente-Juncal et al. 2020). These data are in agreement with the persistent oliguria described previously in rats after single i.p. or oral administration of P-CTX1 at 2600 ng/kg (Bottein et al. 2011). A fact that could arise from the demonstrated presence of active ciguatoxins in urine and feces during 4 days after exposure (Bottein et al. 2011) attributed to a poor elimination of the toxin in urine or to the reabsorption of the toxin in the urinary system. The fact that the same effect was caused by CTX3C at doses of 330 and 1050 ng/kg remarks the need to revaluate the toxicity of the ciguatoxin group of compounds since the effect of CTX3C was evident at doses similar to that used for P-CTX1. In mice, no quantitative assessment of the CTXs bioavailability has been described, however, conducted LD_{50} studies (i.p. and oral) using semi purified toxins (> 85 percent) on male Institute of Cancer Research (ICR) mice (4 weeks old), suggested a very high oral uptake with identical LD_{50} values and almost similar signs of intoxications for the two routes of administration (Ito et al. 1996; Lehane and Lewis 2000; Lewis and Hoy 1993; Lewis et al. 1993).

Altogether, the new findings described in the present study together with the increase of CFP-reported cases in Europe, raise the need to perform additional studies on the chronic oral in vivo toxicity of this marine group of toxins to prevent human illness and accurately regulate the food levels of these emerging marine toxins.

Conclusion

The toxicity of ciguatoxin-related compounds needs to be reevaluated since there is a wide range of lethal doses in the literature for all these compounds.

The toxicity of MTX1 historically considered one of the most toxic natural compounds need to be reevaluated since this work evidence that much higher MTX1 doses did not elicit neither animal dead nor organs alterations.

CTX3C, Gambierone and MTX3 did not cause death after a 28-day oral administration period.

The results presented here evidence the need to evaluate the toxicity of environmental pollutants using reference materials and harmonized validated protocols.

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Table 7 Food consumption, feces and urine production during the 24 h prior to sacrifice, in control mice and in animals treated orally with different doses of CTX3C, gambierone, MTX3 and MTX1 during 28 days

Control ($n=7$)			
Food consumption (g)	5.3 ± 0.7		
Feces (g)	1.7 ± 0.3		
Urine (ml)	2.9 ± 0.9		
CTX3C ($n=3$)	10 ng/kg	32 ng/kg	100 ng/kg
Food consumption (g)	3.9 ± 0.6	5 ± 0.9	4.7 ± 0.2
Feces (g)	1.6 ± 0.2	1.4 ± 0.4	1.3 ± 0.1
Urine (ml)	1.8 ± 0.4	1 ± 0.02	1.1 ± 0.5
Gambierone ($n=3$)	172 ng/kg	500 ng/kg	1760 ng/kg
Food consumption (g)	2.9 ± 1.2	5.4 ± 0.6	5.2 ± 0.3
Feces (g)	1 ± 0.5	1.4 ± 0.4	1.6 ± 0.2
Urine (ml)	4.5 ± 1.6	1.3 ± 0.7	1.3 ± 0.2
MTX3	500 ng/kg ($n=2$)	1760 ng/kg ($n=3$)	
Food consumption (g)	3.8 ± 0.4	3.5 ± 0.3	
Feces (g)	1.2 ± 0.4	0.8 ± 1.4	
Urine (ml)	0.5 ± 0.3	2.2 ± 1.5	
MTX1	800 ng/kg ($n=3$)	2560 ng/kg ($n=3$; 1 surviving mouse)	5000 ($n=5$), no surviving animals
Food consumption (g)	5.8 ± 0.8	5.2	
Feces (g)	1.5 ± 0.1	1.5	
Urine (ml)	1 ± 0.3	0.2	

Values are expressed as mean ± SEM

Table 8 Diversity in the range of lethality doses described for purified MTX1 and algal extracts containing the toxic fraction

Route of administration/toxin origin	LD_{50} (ng/kg)	Animal model	References
I.p	50	mice	Murata et al. (1993)
I.p	130	mice	Yokoyama et al. (1988)
I.p	150	mice	Munday et al. (2017), Reyes et al. (2014)
I.p. (algal extracts)	160–1260	mice	Munday et al. (2017), Reyes et al. (2014)
I.p. (toxic fraction)	200,000	mice	Legrand and Bagnis (1984)
Oral (algal extracts)	16,000–100,000	mice	Munday et al. (2017)

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Author contributions SR, CC, AB-J, AC and CV writing original draft, analyzing data, funding and formal analysis. LMB and MCL conceptualization and funding. MC light microscopy. MRV and MC biochemical and urine analysis. All authors have read and agreed to the published the version of the manuscript.

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Declarations

Conflict of interest The authors declare that they have not known competing financial interest or personal relationships that could have appeared to influence the study.

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In vivo *effects of ciguatoxin-related compounds, reevaluation of the toxicity of maitotoxin 1*

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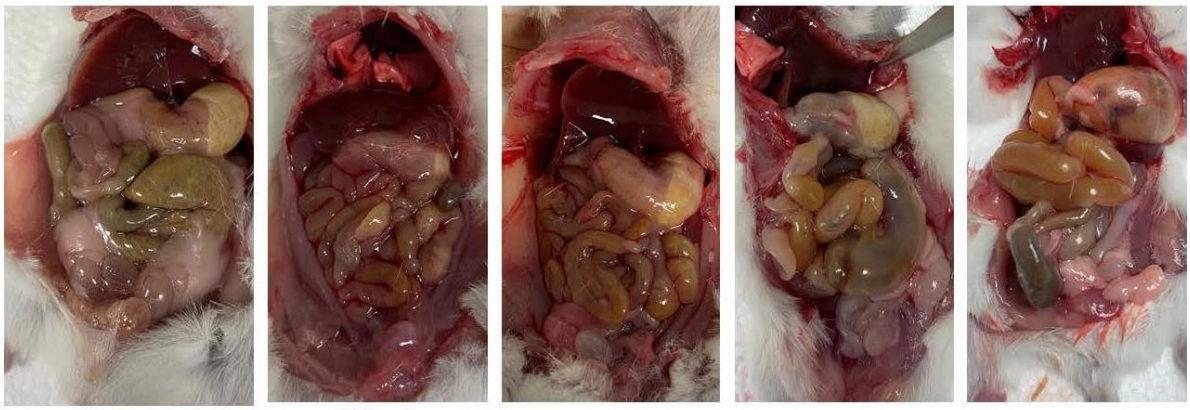
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Supplementary Figure 1. Straub´s tail reaction after i.p. administration of different doses of MTX1 (A and B). Subcutaneous edema was obvious in a mouse treated with 1,000 ng/kg of MTX1 before (C) and after (D) euthanasia.



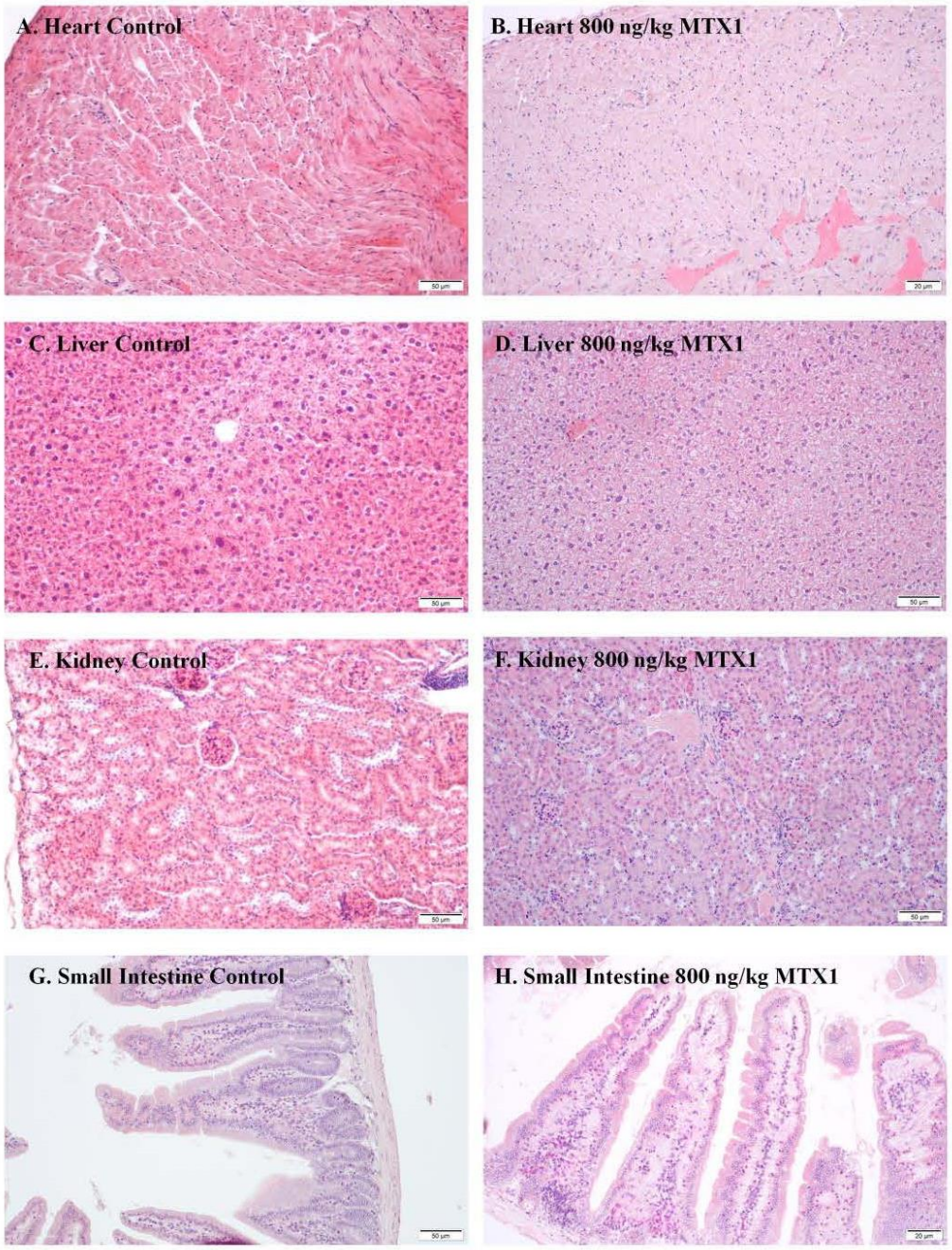
Supplementary Figure 2. Representative image of the abdominal tract of control mice and animals treated intraperitoneally with different MTX1 doses.

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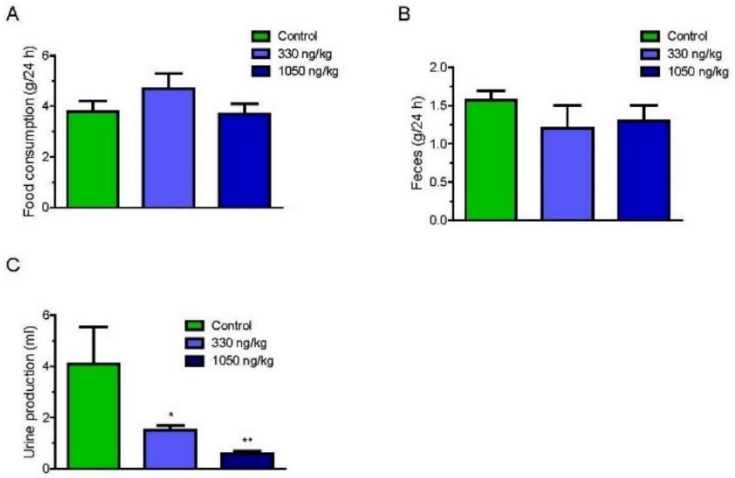
Supplementary Figure 3. Haemorrhagic zones present in the stomach of animal treated with 1,200 ng/Kg of MTX1. As shown in the right these animals have the intestines empty of solid content and the caecum full of gas.

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Supplementary Figure 4. Representative hematoxylin–eosin (H&E) staining of different organs of control mice (A, C, E, G) and mice treated with 800 ng/kg MTX1 in a single ip administration after a 96-hour observation period (B, D, F, H). Scale bar is 50 µm

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Supplementary Figure 5. Metabolic cage parameters after acute intraperitoneal injection of CTX3C. A. Food consumption. B. Feces production. C. Urine production. *p < 0.05, **p < 0.01 vs control values. Data are expressed as mean ± SEM from 3 animals.

Supplementary Table 1. Repercussion of repeated oral administration of control diluent, CTX3C, gambierone, MTX3 and MTX1 on body weight for the 28-day treatment period. Values are expressed as mean ± SEM and the number of animals is represented in parenthesis.

	Day 0	Day 7	Day 14	Day 21	Day 28
Body weight (g)	**(n = 7)**	**(n = 7)**	**(n = 7)**	**(n = 7)**	**(n = 7)**
Control	23.1 ± 1	24.6 ± 0.8	26.7 ± 0.6	27.7 ± 0.7	27.6 ± 0.7
CTX3C	**(n = 3)**	**(n = 3)**	**(n = 3)**	**(n = 3)**	**(n = 3)**
10 ng/kg	26.5 ± 0.7	27.5 ± 0.4	27.6 ± 0.5	28.1 ± 1	28.40 ± 1.3
32 ng/ kg	24.4 ± 0.7	25.8 ± 0.7	27.2 ± 1.3	28.3 ± 1.2	29.3 ± 1.4
102 ng/ kg	26.3 ± 0.3	26.8 ± 1	26.9 ± 0.9	26.2 ± 0.74	26.1 ± 1.4
Gambierone	**(n = 3)**	**(n = 3)**	**(n = 3)**	**(n = 3)**	**(n = 3)**
172 ng/kg	25.9 ± 0.4	26.6 ± 0.4	27.2 ± 0.2	27.9 ± 0.3	28.4 ± 1.4
550 ng/kg	22.6 ± 1.1	23.8 ± 1.7	25.5 ± 2.2	26.8 ± 2.6	28.3 ± 2.8
1760 ng/kg	26.4 ± 0.8	26.3 ± 0.9	25.8 ± 0.8	26.1 ± 0.4	25.7 ± 1.1
MTX3					
550 ng/kg	24.5 ± 1.5 (n = 2)	24.2 ± 1.2 (n = 2)	25 ± 1.9 (n = 2)	25.1 ± 2.1 (n = 2)	25.2 ± 2.1 (n = 2)
1760 ng/kg	24.7 ± 1.3 (n = 3)	26.1 ± 1.6 (n = 3)	26.8 ± 1.3 (n = 3)	27.6 ± 1.7 (n = 3)	27.7 ± 1.5 (n = 3)
MTX1					
800 ng/kg	26 ± 1.7 (n = 3)	26.4 ± 1.5 (n = 3)	27.5 ± 1.8 (n = 3)	28.6 ± 1.7 (n = 3)	29.4 ± 2.2 (n = 3)
2560 ng/kg	24.2 ± 0.7 (n = 3)	26.5 ± 0.3 (n = 3)	25.8 ± 1.6 (n = 2)	22.8 (n = 1)	23.4 (n = 1)
5000 ng/kg	23.1 ± 1.3 (n = 5)	22.4 ± 2 (n = 3)	21.3 ± 2 (n = 3)		

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Supplementary Table 2. Analytical results of urine after repeated oral administration of control diluent and CTX3C, gambierone, MTX1 and MTX3 to mice for 28 days. In the urinalysis parameters as urine color, turbidity, specific gravity, urine protein, glucose, ketones, blood/hemoglobin, bilirubin, and urobilinogen were considered. Results are expressed as mean ± SEM and the number of animals appeared in parenthesis. * n = 3 values except for maitotoxin-1 treated animals that died during the treatment.

Parameters	(7) Control	(3) 10 ng/kg CTX3C	(3) 32 ng/kg CTX3C	(3) 102 ng/kg CTX3C	(2) 170 ng/kg Gambierone	(3) 500 ng/kg Gambierone	(3) 1760 ng/kg Gambierone	(3) 500 ng/kg MTX3	(3) 1760 ng/kg MTX3	(3) 800 ng/kg MTX1	(1) 2600 ng/kg MTX1
Colour	(5) Pale Yellow (1) Amber (1) Dark Yellow	(2) Pale Yellow (1) Dark Yellow	(1) Amber (2) Dark Yellow	(3) Dark Yellow	(2) Pale Yellow	(1) Pale Yellow (2) Dark Yellow	(3) Dark Yellow	(2) Amber	(1) Amber (2) Dark Yellow	(3) Pale Yellow	(1) Dark Yellow
Turbididy	(4) Clear (2) Slightly cloudy (1) Cloudy	(1) Clear (2) Slightly cloudy	(1) Clear (2) Cloudy	(3) Cloudy	(2) Clear	(1) Slightly (2) Cloudy	(3) Cloudy	(1) Cloudy (1) Very cloudy	(3) Very cloudy	(3) Cloudy	(1) Cloudy
Specific Gravity	(1) 1020 (1) 1030 (1) 1032	(1) 1046 (1) 1050 (1) >1050	(3) >1050	(3) >1050	(1) 1020 (1) 1036	(1) 1050 (2) > 1050	(3) > 1050	(1) > 1050 (1) 1044	(1) 1016 (2) > 1050	(3) > 1050	(1) > 1050

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	(3) >1050										
Urine protein (g/l)	0.3 ± 0.3 (3) 0 (3) Trace (1) 1	0 ± 0 (1) 0.3 (2) Trace	1 (2) Trace (1) 1	0.3 ± 0 (1) Trace (3) 0.3	2.1 ± 1.5 (1) 0.3 (1) 1 (1) 5	0.65 ± 0.35 (1) 0.3 (1) 1	:0.5 ± 0.2 (2) 0.3 (1) 1	0.3 ± 0 (2) 0.3	0.2 ± 0.1 (2) 0.3 (1) 0	0.3 ± 0 (1) Trace (2) 0.3	(1) 1
Glucose (mmol/l)	0.9 ± 0.6 (5) 0 (2) 3	1 ± 1 (2) 0 (1) 3	5.8 ± 2.8 (4) 3 (1) 17	3 ± 0 (4) 3	0 ± 0 (2) 3	0 ± 0 (2) 0	0 ± 0 (3) 0	1.5 ± 1.5 (1) 0 (1) 3	1 ± 1 (1) 0 (2) 3	1 ± 1 (1) 0 (2) 3	(1) 0
Ketones (mmol/l)	0.3 ± 0.2 (3) 0 (3) 1.5	0.3 ± 0.3 (4) 0 (1) 1.5	1.2 ± 0.3 (1) 0 (4) 1.5	0.4 ± 0.4 (3) 0 (1) 1.5	0.8 ± 0.8 (1) 0 (1) 1.5	0 ± 0 (2) 0	1 ± 0.5 (1) 0 (2) 1.5	0 ± 0 (2) 0	1 ± 0.5 (1) 0 (2) 1.5	0.5 ± 0.5 (2) 0 (1) 1.5	(1) 5
Blood/Haemoglobin (ery/µl)	0 ± 0 (7) 0	0 ± 0 (5) 0	12 ± 9.7 (3) 0 (1) 10 (1) 50	2.5 ± 2.5 (3) 0 (1) 10	0 ± 0 (2) 0	5 ± 5 (1) 0 (1) 10	0 ± 0 (3) 0	10 ± 10 (1) 0 (1) 10	8.3 ± 8.3 (2) 0 (1) 25	0 ± 0 (3) 0	(1) 0
Bilirubin (µmol/l)	24 ± 14 (4) 0	10.2 ± 4.2 (2) 0	43.4 ± 6.6*	25.3 ± 8.3 (3) 17	17 ± 0 (2) 17	50 ± 0* (2) 50	11.3 ± 5.7 (1) 0	8.5 ± 8.5 (1) 0	17 ± 0 (3) 17	83 ± 16.7 (1) 50	(1) 50

11

	(1) 17 (1) 50 (1) 100	(3) 17	(1) 17 (4) 50	(1) 50			(2) 17	(1) 17		(2) 100	
Urobilinogen (µmol/l)	0 ± 0 (7) 0	10.2 ± 4.2 (2) 0 (3) 17	6.8 ± 4.2 (3) 0 (2) 17	0 ± 0 (4) 0	8.5 ± 8.5 (1) 0 (1) 17	8.5 ± 8.5 (1) 0 (1) 17	11.3 ± 5.7 (1) 0 (2) 17	8.5 ± 8.5 (1) 0 (1) 17	0 (3) 0	5.7 ± 5.7 (2) 0 (1) 17	(1) 70

In vivo subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity.

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Publicación

Mouse N2a neuroblastoma assay: Uncertainties and comparison with alternative cell-based assays for ciguatoxin detection.

Resumen

La creciente expansión de la intoxicación por ciguatera hizo que aumentara la necesidad de desarrollar un método confiable y rápido para la detección de ciguatoxinas con el objetivo de garantizar la seguridad alimentaria. El ensayo de citotoxicidad en células N2a sensibilizadas con ouabaína y veratridina (O/V) se utiliza habitualmente para la detección de ciguatoxinas, sin embargo, este método aún no se ha estandarizado. En este estudio se demostró la inexistencia de corrientes de sodio dependientes de voltaje en las células N2a además de que la mezcla de ouabaina y veratridina provocaba pérdida de adherencia celular afectando a los datos obtenidos mediante el ensayo del MTT, y confirmó la ausencia de efectos tóxicos causados por la CTX sola utilizando métodos que no requerían eliminación del medio, como los ensayos de lactato deshidrogenasa (LDH) y el Alamar blue. Se evaluaron diferentes líneas celulares como alternativas y los resultados obtenidos mostraron que el neuroblastoma humano SH-SY5Y no era apto para la detección de CTX por su mayor sensibilidad a O/V y a la ausencia de corrientes de sodio dependientes de voltaje. No obstante, en la línea celular HEK293 transfectada con la subunidad α1.6 de los canales de sodio dependientes de voltaje se obtuvo citotoxicidad solo en presencia de ciguatoxinas sin necesidad de sensibilización con O/V a concentraciones de 0,1 nM CTX3C (102,3 µg/ml). En esta misma línea la sensibilización de las células con O/V permitió detectar la presencia de la ciguatoxina mediante el método clásico de citotoxicidad MTT en concentraciones tan bajas como 0,0001 nM CTX3C (102,3 ng/ml), lo que permitió proponer una línea celular alternativa para la detección de compuestos que actúan sobre canales de sodio dependientes de voltaje.

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Mouse N2a Neuroblastoma Assay: Uncertainties and Comparison with Alternative Cell-Based Assays for Ciguatoxin Detection

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Abstract: The growing concern about ciguatera fish poisoning (CF) due to the expansion of the microorganisms producing ciguatoxins (CTXs) increased the need to develop a reliable and fast method for ciguatoxin detection to guarantee food safety. Cytotoxicity assay on the N2a cells sensitized with ouabain (O) and veratridine (V) is routinely used in ciguatoxin detection; however, this method has not been standardized yet. This study demonstrated the low availability of sodium channels in the N2a cells, the great O/V damage to the cells and the cell detachment when the cell viability is evaluated by the classical cytotoxicity assay and confirmed the absence of toxic effects caused by CTXs alone when using the methods that do not require medium removal such as lactate dehydrogenase (LDH) and Alamar blue assays. Different cell lines were evaluated as alternatives, such as human neuroblastoma, which was not suitable for the CTX detection due to the greater sensitivity to O/V and low availability of sodium channels. However, the HEK293 Nav cell line expressing the α1.6 subunit of sodium channels was sensitive to the ciguatoxin without the sensitization with O/V due to its expression of sodium channels. In the case of sensitizing the cells with O/V, it was possible to detect the presence of the ciguatoxin by the classical cytotoxicity MTT method at concentrations as low as 0.0001 nM CTX3C, providing an alternative cell line for the detection of compounds that act on the sodium channels.

Keywords: ciguatoxins; sodium channels; MTT; ciguatera fish poisoning; toxicity; LDH; Alamar blue

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1. Introduction

Ciguatera fish poisoning (CF) is the most commonly reported foodborne illness related to the consumption of fish containing marine algal neurotoxins known as ciguatoxins. These toxins act by modulating voltage-gated sodium channels (VGSCs), specifically, they alter their functionality by acting on site 5 of their alpha subunit, making the channels open at resting cell membrane potentials [1]. CTXs are characterized by causing a negative shift in the activation voltage of sodium channels in a concentration-dependent manner reported in different in vitro models [2–4].

The most common symptoms of CF are gastrointestinal, such as nausea, vomiting, diarrhoea and abdominal pain. Cardiovascular disturbances, mainly bradycardia and hypotension, are reported after CTX consumption. Neurological symptoms such as paraesthesia of the lips and extremities, cold allodynia, ataxia and painful dysesthesias are also frequent [5]. Neurological sequelae derived from the consumption of fish containing CTXs may last from weeks to up to years. All CTX analogues play a critical role in the CF syndrome since, under some conditions, external sensory stimuli might trigger a ciguatera crisis due to the negative shift in the activation voltage of sodium channels [4]. It is often difficult to relate CF intoxication to the consumption of fish contaminated with ciguatoxins due to the wide variety and poor specificity of the ciguatera symptoms. The real number of

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ciguatera poisoning cases can reach up to 50,000–500,000 per year [6], but underreporting and misdiagnosis of the real CF cases were not contemplated in any study. There is no specific effective treatment either for acute or for chronic ciguatera poisoning. Supportive medical management of CF clinical effects is the available tool to fight against the CF manifestations [7].

CTX compounds are produced by benthic dinoflagellate species from the *Gambierdiscus* and *Fukuyoa* genera and enter the marine food chain through herbivorous fish and invertebrates [7]. CTXs were classified according to their geographical origin as Pacific ciguatoxins (P-CTX) identified in the Pacific Ocean, Caribbean ciguatoxins (C-CTX) first isolated in the Caribbean Sea and Indian ciguatoxins (I-CTX) found in the Indian Ocean. So far, P-CTXs have been the most studied compounds as a consequence of their abundance and distribution and were recently divided into two separate groups based on their chemical structure, the CTX3C group and the CTX4A group [7]. Among CTXs, P-CTX1 was considered the most potent analogue, and the recommended safety limit for CTXs in fish for human consumption has been set at 0.01 ng P-CTX-1 toxin equivalent/g of fish tissue by the United States Food and Drug Administration [8], a fact that has been recently refuted by the observation that the Caribbean analogue had similar potency [9], raising the need for the development of fast and sensitive methods to detect low levels of these compounds in food.

To monitor the levels of CTXs in fish flesh prior to sale and to decrease the probability of CTX consumption, many methods have been developed [10–12], but the complexity and variability of such toxins have made the development of a reliable method to routinely monitor CF with high specificity and sensitivity a real challenge. The current methodology available for CTX detection is very wide and diverse, from in vivo tests, not used due to the low availability of the compounds and ethical concerns, to cell-based assays, receptor-binding assays, liquid chromatography coupled with mass spectrometry (LC/MS) or immunoassays [13–17]. Currently, the most widely used methods to quantify CTXs are the cell-based assays because they are cheap, fast and easy to develop. Formerly, mouse bioassay (MBA) was the most commonly used method to evaluate the relative potency of CTXs as well as to detect their presence in food [18–20] despite its multiple disadvantages and ethical concerns [6]. However, the use of the MBA as a method to detect CTXs and therefore to evaluate their potency has the drawback such as that the data obtained are derived from establishing dose/survival-time relationships, and the end point of the assay was the death of the tested animal. Thus, death was considered as an indicator of CTX toxicity, while the main effect of CF is not the death but the symptoms [7,21].

Among the in vitro methods to detect CTXs in food, cell viability is commonly measured in the Neuro2a cell line from mouse by using the MTT (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan) assay [22]. However, these marine toxins have no cytotoxic effect in non-excitable cells, such as undifferentiated neuroblastoma cells, and their detection requires the addition of the sodium/potassium (Na^+/K^+) ATPase pump blocker ouabain (O), together with the sodium channel activator veratridine (V) increasing the intracellular sodium load. Under this O/V treatment, the cytotoxic effect of CTXs was potentiated allowing for the detection of the presence of these toxins in samples [22]. However, despite being used at low concentrations, ouabain and veratridine have their own toxic effect on cells [12,23]. The N2a cell bioassay has not been validated at an international level yet because there is a high degree of variability in the literature regarding the conditions including the number of cells, amount of O/V, medium components and cell density [12]. Therefore, a large number of variations of this method have been developed over the years trying to improve its sensitivity and specificity [10,24].

Other detection methods for agents acting on the sodium channels have been developed and include the fluorometric detection of membrane depolarization as the indicator of the CTX presence [25] or the use of neuroblastoma cell lines such as SH-SY5Y [23] considered more appropriate due to their human origin. Moreover, the O/V treatment was demonstrated to cause damage in cells at concentrations from 100 to 10 μM O/V [12],

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but some studies were developed using O/V concentrations as high as 140/14 μM O/V [26], 220/22 μM O/V [24], 250/25 μM O/V [27–29] or even 500/50 μM O/V [30] or 1000/50 μM O/V [22]. Therefore, there is an increasing need to reevaluate the current methodology to detect ciguatoxins since they are emerging toxins in Europe whose detection at low levels in food is the key to protect the health of consumers. Thus, the present paper aims to compare different methods and different cell lines to improve the detection of CTXs, trying to clarify which method is more effective for this purpose.

2. Results

A wide range of experiments were developed to reevaluate the current cell-based assays available to detect the presence of ciguatoxins in food using three different cell lines. The experiments involved the routinely used mouse neuroblastoma N2a, the human neuroblastoma cell line SH-SY5Y that was purposed to be more suitable to mimic the toxicity in humans caused by CTXs [23] and the human HEK293 $Na_v1.6$ cell line due to its overexpression of the human sodium channels. With the aim to determine the most suitable method to assess the toxicity of ciguatoxins, electrophysiological recordings and different methods to determine cell viability were studied.

2.1. Action of CTXs in the N2a Cell Line

Although the use of the mouse neuroblastoma N2a cell line to detect the presence of ciguatoxins has been routinely employed over the years [10,11,24,26,29,31,32], there is a lack of consensus on the development of a method for detecting agents acting on the sodium channels.

2.1.1. Electrophysiological Recordings of Sodium Currents in N2a Cells

The sodium channel activity in the N2a cells was evaluated, and the results obtained were in agreement with previous studies showing low expression and availability of sodium channels in the N2a cell line [33–35]. The recording of the maximum sodium currents (I_{Na}) in the undifferentiated N2a cells hardly reached −300 pA as shown in Figure 1A, demonstrating low sodium channel activity. In view of these results, the cells were exposed for 24 h to 10 μM forskolin, a molecule that elevate the intracellular concentration of the second messenger, cyclic adenosine monophosphate (cAMP), which plays a crucial role in cell differentiation [36–42]. Moreover, the activation of PKA by forskolin is known to increase the intracellular trafficking of several membrane-bound proteins (glucose transporter GLUT4, chloride channel CFTR, $K_v1.1$ K^+ channel and $Na_v1.5$ Na^+ channel) [43–46]. As indicated in Figure 1B, the current–voltage curve (I-V) showed an increase in the sodium current amplitude at all the recording potentials in the N2a cells treated for 24 h with forskolin. Thus, the maximum peak sodium current at −10 mV was −285 pA ± 50 pA in the control cells ($n = 5$) and −1706 ± 453 pA ($n = 5$) in the N2a cells pretreated with 10 μM forskolin for 24 h, demonstrating the low natural expression of sodium channels in the undifferentiated N2a cells.

Since the N2a cell exposure to O/V is justified as a way to increase the sensitivity and specificity of the assay to compounds acting on VGSCs [24,32], electrophysiological recordings in the N2a cells were performed after the 24 h exposure of the cells to 100/10 μM O/V. As shown in Figure 1C, the cell exposure to 100/10 μM O/V for 24 h did not increase the intensity of the sodium channel currents, and further addition of 0.1 nM CTX3C to the recording chamber did not modify the sodium channel currents, demonstrating that the O/V cell treatment did not allow for the detection of CTX3C due to its direct effect on the sodium channels. Additionally, after the 24 h cell exposure to 100/10 μM O/V, an output potassium current was registered. The diminution of the current evoked after the addition of the known potassium channel blocker 4-aminopyridine at 15 mM, as shown in Figure 1D, confirmed the increase in the potassium current. This effect increasing outward potassium currents was, as expected, due to the effect elicited in the Na^+/K pump by ouabain [47].

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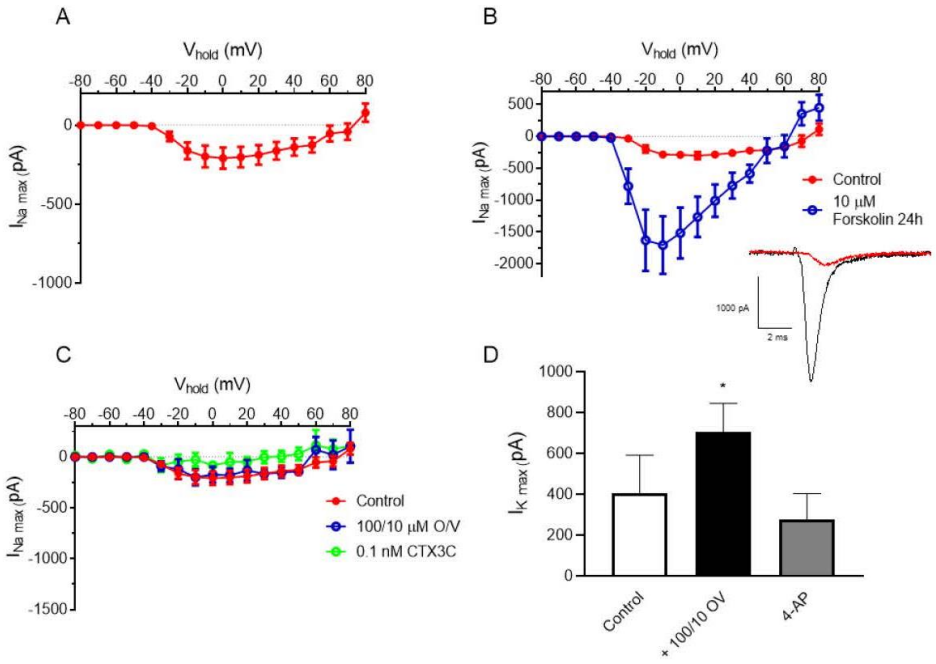


Figure 1. Sodium and potassium currents in N2a cells: (**A**) current–voltage relationship of sodium current amplitude in the N2a cell line without treatment with forskolin; (**B**) current–voltage relationship of sodium currents in N2a cells treated with 10 µM forskolin; (**C**) neither 100/10 µM O/V nor 0.1 nM CTX3C modified the current–voltage relationship for sodium currents in N2a cells (non-forskolin-treated); (**D**) increase in potassium currents after the addition of 100/10 µM O/V corroborated by the blockade with 15 mM 4-aminopyridine. Data are expressed as mean ± SEM (* $p \leq 0.05$).

2.1.2. Evaluation of the Effect of CTX3C Using the MTT Assay on the N2a Cell Line

The N2a cell viability was tested in the presence of O/V at concentrations ranging from 60/6 to 340/34 µM, since these proportions are usually employed in the literature for the screening of ciguatoxins. As shown in Figure 2A, the 24 h cell exposure to O/V elicited a concentration-dependent decrease in cell viability. These data indicated a cytotoxic effect of O/V in the cells, even at concentrations suggested not to have destructive effects in the N2a cells [12] since the combination of O/V at 100/10 µM elicited a decrease in cell viability from 100 ± 0.7% in the control conditions to 84.6 ± 1.8% ($n = 3$; $p < 0.01$) in the presence of O/V. To discriminate the independent cytotoxic effect produced in the cells by ouabain and veratridine, their cytotoxicity was evaluated separately. As illustrated in Figure 2B, veratridine did not cause alterations in the N2a cell viability after the 24 h exposure to concentrations from 6 to 34 µM. However, as shown in Figure 2C, the 24 h exposure of the N2a cell to ouabain concentrations of 220 µM and higher elicited a significant reduction in cell viability of 16.06 ± 2.41% ($n = 3$; $p < 0.01$) compared to the control conditions.

With these data, the IC_{50} values were calculated for the O and O/V conditions by fitting the data with a log (inhibitor) vs. normalized response model of cell viability (Supplementary Figure S1). The IC_{50} obtained for ouabain alone was 591.9 µM (confidence interval (CI): 430.2 to 814.5 µM) while the IC_{50} obtained for ouabain when combined with veratridine was 216.8 µM (CI: 205 to 229.2 µM) as shown in Supplementary Figure S1B based on the ouabain concentrations.

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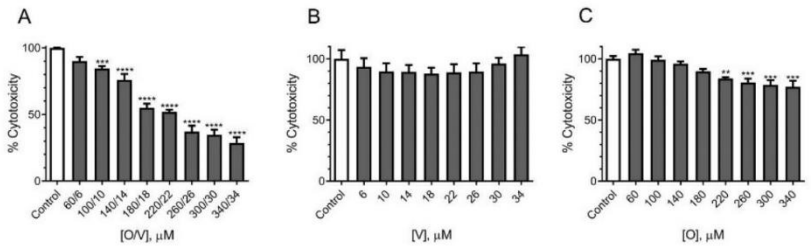


Figure 2. Cell viability of N2a cells assessed by the MTT test after 24 h exposure to the different combinations of O/V, veratridine alone and ouabain alone: (**A**) different O/V concentrations, (**B**) veratridine from 6 to 34 µM and (**C**) ouabain from 60 to 340 µM. Data are expressed as mean ± SEM of three independent replicates and as percentage of control cells ($n = 3$ in all the assays). Statistical differences were determined by ANOVA followed by Dunnet's test (** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$).

The cytotoxicity caused by ciguatoxins in the N2a cells was studied in the absence and the presence of O/V. As shown in Figure 3A, no cytotoxic effect was observed after the cell exposure only to CTX3C in the absence of the O/V treatment. As expected, a concentration-dependent cytotoxic effect was detected after the cell exposure to 100/10 µM O/V with increasing CTX3C concentrations, yielding an IC_{50} value of 1.7×10^{-12} M (95% confidence interval (CI) from 4.7×10^{-13} to 5.837×10^{-12}) by fitting the data with a log (inhibitor) vs. normalized response model, shown in Figure 3B. Figure 3C summarizes the cytotoxic effect of the 100/10 µM O/V treatment and further exposure to CTX3C (IC_{50} 6×10^{-13} M, 95% CI from 1.5×10^{-13} to 2.39×10^{-12} M); for this reason, the decrease in the cell viability due to the ciguatoxin was calculated as a function of the effect of the 100/10 µM O/V control value in Figure 3D. These data illustrate that the results obtained by performing the traditional MTT test on the sensitized N2a cells at our disposal were similar to those of different laboratories [9,32,48,49].

2.1.3. Evaluation of the Effect of CTX3C Using the LDH Test in the N2a Cell Line

The LDH assay was performed to directly evaluate cell death due to the cell membrane damage elicited by different O/V concentrations. As shown in Figure 4A, even the lowest O/V concentration of 60/6 µM increased the LDH liberation by about 30% after the 24 h treatment. With the aim to further explore which compound is responsible for the cell damage, the cells were exposed to V or O separately, and the results are illustrated in Figure 4B,C, respectively. The results obtained show that whilst veratridine did not cause any increase in the LDH release, ouabain at the highest concentrations slightly increased the LDH liberation. Once the impact of the O/V cell treatment in the LDH release was known, the cytotoxic effect of CTX3C was evaluated in the presence of a commonly used concentration of 100/10 µM O/V which is considered nondestructive [12]. As shown in Figure 4D, when the percentage of the LDH release was compared with the spontaneous release in the control cells, all toxin concentrations increased the LDH liberation in the cells treated with 100/10 µM O/V. However, as illustrated in Figure 4E, when the LDH release was calculated as a function of the LDH release in the cells treated with 100/10 µM O/V and further exposed to CTX3C, there was no significant increase in the LDH liberation, evidencing that the cytotoxicity observed was due to the O/V treatment. No additional cell damage elicited by CTX3C was observed apart from that caused by the 100/10 µM O/V, indicating that this method does not allow for the detection of the presence of CTXs.

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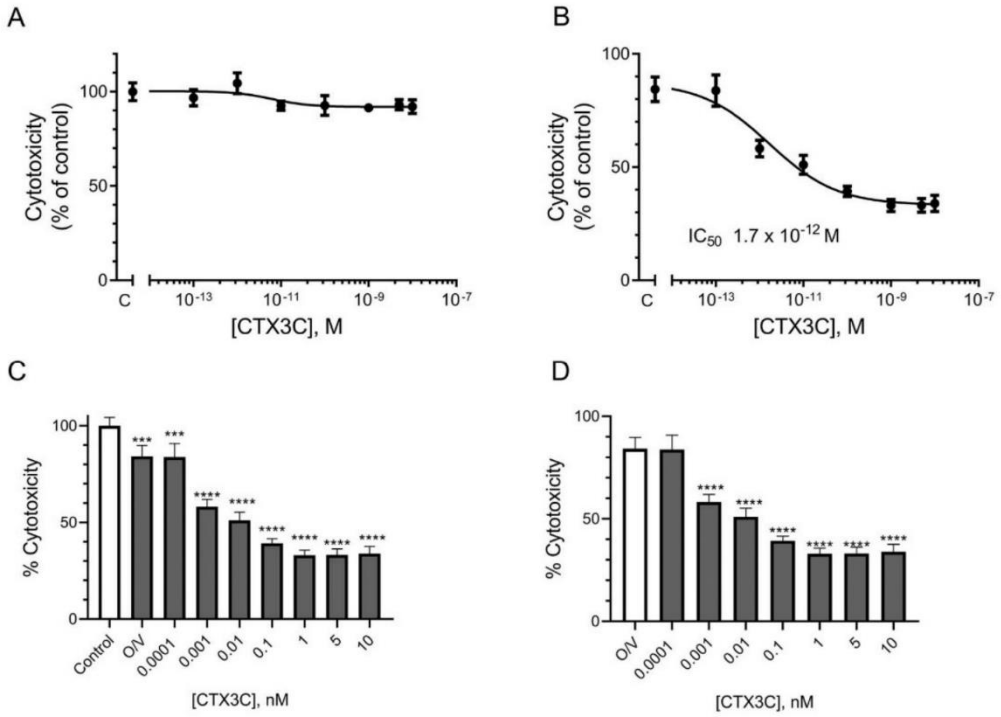


Figure 3. Cytotoxic effect of CTX3C assessed by the MTT method: (**A**) effect of CTX3C in N2a cells not exposed to O/V; (**B**) dose–response curve of N2a cells exposed to increasing concentrations of CTX3C in the presence of 100/10 µM O/V; (**C**) decrease in N2a cell viability after the exposure to 100/10 µM O/V and increasing CTX3C concentrations compared to control conditions; (**D**) decrease in N2a cell viability after the exposure to 100/10 µM O/V and increasing CTX3C concentrations compared to single 100/10 µM O/V treatment. (*** $p < 0.001$ and **** $p < 0.0001$).

Due to the lack of consensus about a standardized protocol for the cellular assay on the N2a cell line, additional studies were performed by changing the cell culture conditions, specifically the FBS concentration and the number of seeded cells which were adjusted according to the LDH and Alamar blue protocols, to assess whether these parameters would affect the results obtained. As shown in Supplementary Figure S2, no differences were observed neither in the MTT assay nor in the LDH test with different cell culture conditions (seeded with a 5% FBS and treated with a 2% FBS supplemental medium). We concluded that the FBS concentration had no influence on the results obtained in none of the studies as described for the MTT assay [12]. For the cells treated with the 100/10 µM O/V and ciguatoxin for 24 h and cultured in a 10% FBS, an IC_{50} value of 1.7×10^{-12} M was obtained, very similar to the IC_{50} value of 6.3×10^{-12} M obtained for the cells under the same conditions but seeded with the 5% FBS and treated with the 2% FBS.

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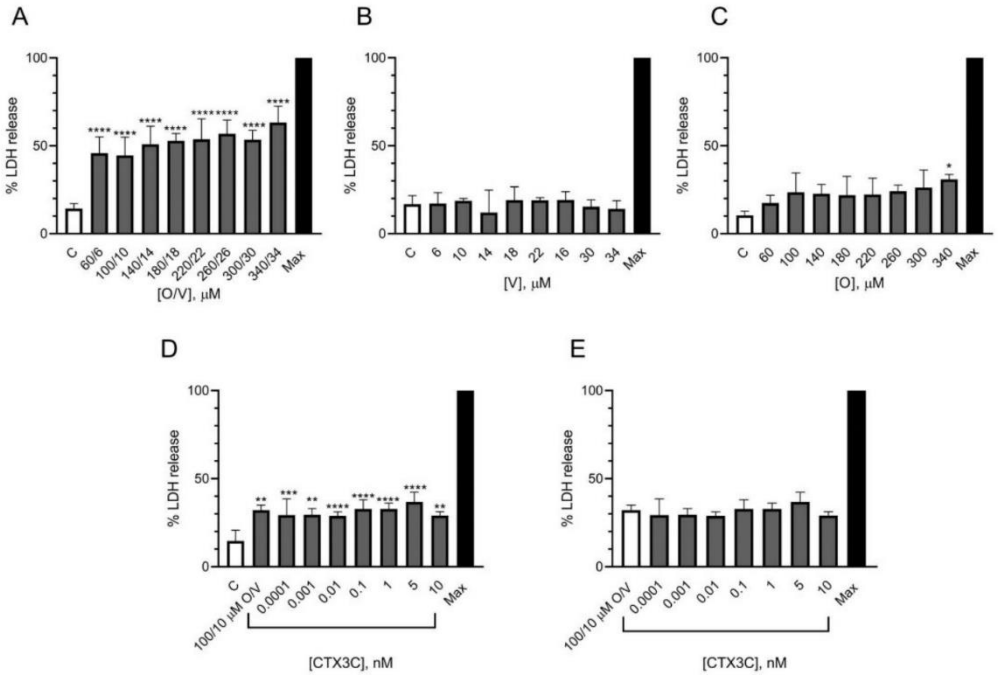


Figure 4. Cytotoxic effects of different O/V and CTX3C conditions after 24 h treatment in mouse neuroblastoma cells determined by the LDH assay: (**A**) cytotoxic effects of different O/V concentrations; (**B**) absence of cytotoxic effects of different V concentrations; (**C**) cytotoxic effects of different O concentrations; (**D**) cytotoxic effects of 100/10 µM O/V and different CTX3C concentrations compared to control conditions; (**E**) absence of cytotoxic effects of 100/10 µM O/V and different CTX3C concentrations compared to 100/10 µM O/V-treated cells. Data are mean ± SEM of four independent replicates. Statistical differences were determined by one-way ANOVA and with Dunnett's multiple comparison test (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$). Max represents the maximum LDH release obtained following the manufacture's instruction kit.

2.1.4. Alamar Blue

The Alamar blue method is widely used to determine cell viability after exposure to chemicals by a direct decrease in the fluorescence measurements over time in the culture plate with no need for medium removal. In this assay, the cells were exposed to 100/10 µM O/V and increasing CTX3C concentrations, and their viability was evaluated. Figure 5 illustrates the fluorescence measurements after the 4, 8 and 24 h cell treatment. The results obtained did not show any effect of CTX3C in the decrease in the cell viability after the 4, 8 or 24 h treatment.

2.1.5. Direct Effects of CTX3C and Ouabain/Veratridine in the N2a Actin Cytoskeleton

Since in the initial experiments to evaluate the effects of O/V and CTXs on cells led to cellular damage and detachment, and previous works reported alterations in cytoskeleton caused either by CTXs [50,51] or by ouabain in different cell lines [52–55], the next approach to investigate the real effects of the O/V and CTX on the cells was by immunocytochemistry experiments marking the actin cytoskeleton. As shown in Figure 6, the 24 h exposure of the N2a cells only to 1 nM CTX3C caused changes in the cytoskeleton, giving a diffuse image of actin distribution, more pronounced at higher CTX3C concentrations (10 nM CTX3C), corroborating the damage to the cell cytoskeleton caused by CTXs alone. However, the actin cytoskeleton damage in the cells exposed only to 100/10 µM O/V was not

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noticeable, but when the cells were exposed for 24 h to 100/10 µM O/V and 1 or 10 nM CTX3C, the cells displayed spherical morphology and higher damage. It also highlights a remarkable decrease in cellular density as well as complete disruption of the actin cytoskeleton organization.

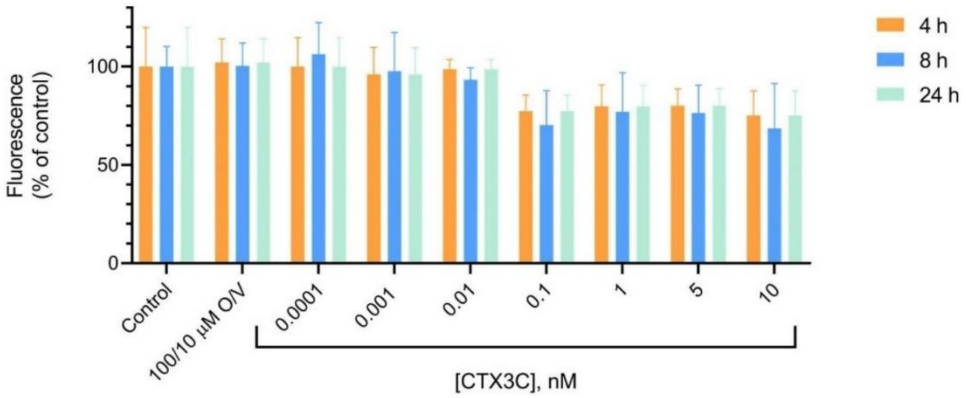


Figure 5. Alamar blue fluorescence at different times expressed as percentage of the control value, illustrating no significant decrease in fluorescence emitted by treated cells compared to control conditions. Statistical differences were determined by one-way ANOVA and with Dunnett's multiple comparison test. All the determinations are the mean ± SEM of 3 independent culture plates in triplicate wells.

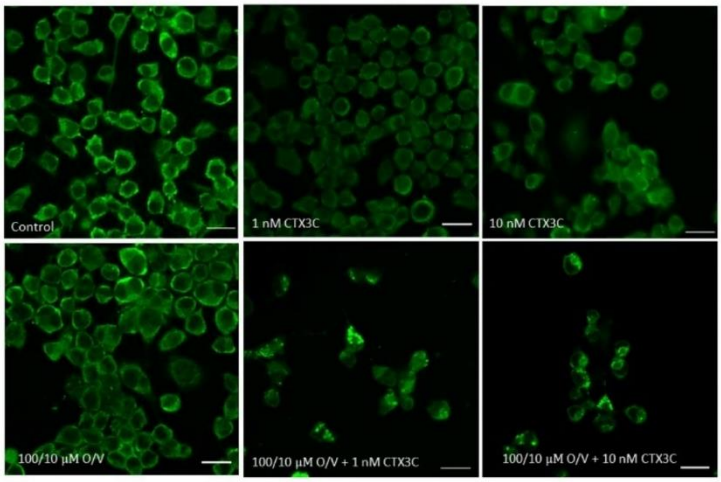


Figure 6. Representative confocal microscope images of the actin cytoskeleton stained in green in control N2a cells and in the same cells treated with either 1 or 10 nM CTX3C alone, 100/10 µM O/V or combinations of these with 1 or 10 nM CTX3C for 24 h in the culture medium. Scale bar is 20 µm.

2.2. Increasing N2a Sensitivity to Ciguatoxins

In view of the remarkable increase in the sodium current amplitude of sodium channels obtained after the N2a cell exposure to 10 µM forskolin, further experiments were developed to evaluate if these effects correlated with increased sensitivity of the N2a cells to ciguatoxins. Once it was proved that the 24 h cell exposure to 10 µM forskolin was not toxic to the cells (Supplementary Figure S3), the cytotoxicity elicited by the ciguatoxin

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in the cells exposed for 24 h to 10 μM forskolin was evaluated by the MTT assay. As shown in Figure 7A,B, in the cells treated for 24 h with forskolin and thereafter exposed to 100/10 μM O/V and increasing CTX3C concentrations, a concentration-dependent decrease in the cell viability was obtained, leading to an IC_{50} of 4.3×10^{-12} M (CI from 2.4×10^{-12} to 7.9×10^{-12}) by fitting the data with a log (inhibitor) vs. response (four parameters) model. Noteworthy, the cell viability decrease in this culture condition was higher than that in the non-forskolin-exposed cells, being 18.8 ± 2.4% at 10 nM CTX3C compared to 81.3 ± 5.8% observed in the presence of 100/10 μM O/V and compared to the decrease from 84.4 ± 5.5% in the control cells which were not pretreated to the 33.9 ± 3.6% viability in the presence of 10 nM CTX3C.

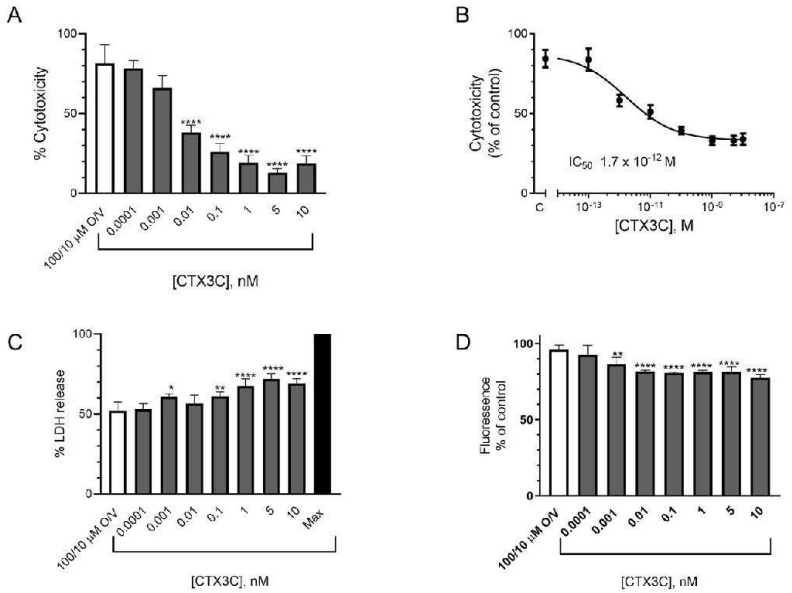


Figure 7. Effects of CTX3C on the viability of N2a cells pretreated for 24 h with 10 μM forskolin using different assays: (**A**) MTT assay; (**B**) concentration–response graph of the decrease in cell viability by increasing CTX3C concentrations as evaluated by the MTT assay; (**C**) cytotoxicity of CTX3C determined by the LDH assay; (**D**) cytotoxicity of CTX3C determined by the Alamar blue assay measured 24 h after Alamar addition. Statistical differences were determined by ANOVA followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$ **** $p < 0.0001$, significantly different from the control).

When the cytotoxicity of the CTX in the cells pretreated with 10 μM forskolin was evaluated by the LDH and Alamar blue assays, the CTX cytotoxic effect was significantly increased compared to the non-forskolin-pretreated cells. As shown in Figure 7C, a significant increase in the LDH release was detected at doses of 0.001 nM CTX3C and above, reaching the levels of 69.2 ± 1.4% ($p < 0.001$) at 10 nM CTX3C compared to 52.0 ± 2.8% at 100/10 μM O/V. The results obtained with the Alamar blue assay illustrated in Figure 7D were very similar. In this case, the cells pretreated with 10 μM forskolin after the 24 h cell exposure to 100/10 μM O/V and increasing CTX3C concentrations showed a decrease in their metabolic rate. Thus, in the control conditions (single exposure to 100/10 μM O/V), the percentage of the cell viability was 96.2 ± 1.4% ($n = 4$) decreasing up to 86.5 ± 2.2% ($n = 4$) ($p = 0.0025$) when the cells were exposed to CTX3C at concentrations of 0.001 nM and higher. The highest CTX3C concentration evaluated, 10 nM, decreased the cell viability up to 77.6 ± 1% ($n = 4$). Therefore, the Alamar blue assay also allows for the detection

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of the presence of these compounds at low concentrations in the N2a cell pretreated with 10 μM forskolin for 24 h.

Since the Alamar blue assay allows for the measurement of fluorescence over time, the fluorescence measured at 4, 6, 8 and 24 h after the cell treatment (Figure 8) indicated that in forskolin-pretreated cells, the decrease in cell viability elicited by ciguatoxins could be detected at incubation times as brief as 4 h. A significant decrease in the cell viability was detected at 0.1 nM CTX3C with a decrease in fluorescence up to 69.1 ± 3.1% ($n = 4$) compared to 100 ± 6.3% ($n = 4$; $p = 0.0008$) in the control conditions and 106 ± 1.8% ($n = 4$) in the 100/10 μM O/V. After 6 h, 0.01 nM CTX3C was showed a decrease in fluorescence up to 78.7 ± 2.8% ($n = 4$; $p = 0.0006$), while at 8 h, CTX3C at 0.001 nM also showed a decrease in fluorescence up to 86.1 ± 3.1% ($n = 4$; $p = 0.0009$); decreases were expressed in all cases with respect to the fluorescence obtained in the O/V-treated cells.

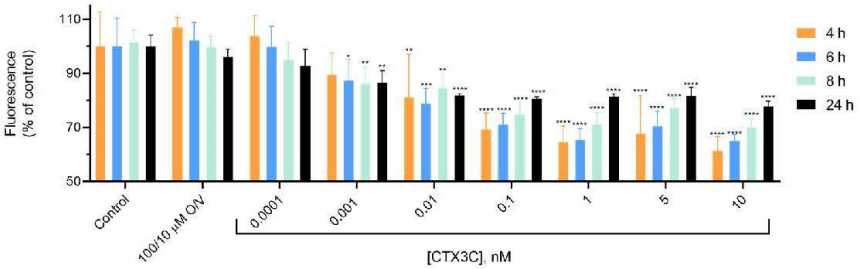


Figure 8. Alamar blue fluorescence at different times expressed as percentage of the fluorescence obtained in cells treated with 100/10 μM O/V after the addition of CTX3C at increasing concentrations. Statistical differences were determined by ANOVA test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, significantly different from the control).

2.3. Effect of CTX3C in the Human SH-SY5Y Neuroblastoma Cell Line

2.3.1. Voltage-Gated Sodium Channel Functionality

Electrophysiological experiments in the SH-SY5Y cells were performed, and the results obtained highlighted that the undifferentiated neuroblastoma cells had very small sodium currents as previously reported [56]. In the undifferentiated SH-SY5Y cells, the maximum peak inward sodium current hardly reached −267 ± 0.29 pA at −10 mV ($n = 3$) as shown in Supplementary Figure S4 (the red line); however, the incubation of the SH-SY5Y cells with 10 μM forskolin for 24 h triggered an increase in the amplitude of sodium channel currents up to −1524 ± 246 pA ($n = 6$) as shown in Supplementary Figure S4 (the blue line).

2.3.2. Evaluation of CT3C Cytotoxicity by MTT in Human Neuroblastoma Cells

Initially, the SH-SY5Y cell line was purposed as a potentially suitable cell model to evaluate CTX toxicity due to its human origin [23]. However, it has been previously reported that ouabain even at very low concentrations had a potent cytotoxic effect in this cell line [23]. With the aim to determine whether the human neuroblastoma cell line could be used with the MTT method for CTX detection, the toxicity of different O/V concentrations was analysed employing the same conditions as those described above for the N2a cell line. As shown in Figure 9A, the exposure of the SH-SY5Y cells to different O/V combinations decreased the cell viability by 36% at concentrations as low as 10/1 μM O/V and higher. When the O and V toxicity was assessed separately, as shown in Figure 9B, even the lowest O concentration employed, 60 μM, caused a decrease in the cell viability up to 60%. No significant decrease in the cell viability was observed when the cells were exposed to different V concentrations (Figure 9C). To mimic the same conditions as those in the previous experiments, the cells were coincubated at an O/V concentration of 100/10 μM. However, already at the proportion of 10/1 μM O/V, the cell viability decreased by 36%, a constant trend maintained after increasing the concentration ratios of O/V, which makes

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this method not suitable for the detection of ciguatoxins since considerable cell damage was elicited in the control cells, as summarized in Figure 9D. Since high V concentrations did not decrease the cell viability, 34 µM V was employed to enhance the CTX3C cytotoxicity with a compound that by itself does not cause the SH-SY5Y cell damage. The results obtained showed a significant decrease in the cell viability at CTX3C concentrations from 5 nM as shown in Figure 9E, indicating that this method does not allow for the detection of the presence of CTXs at lower concentrations.

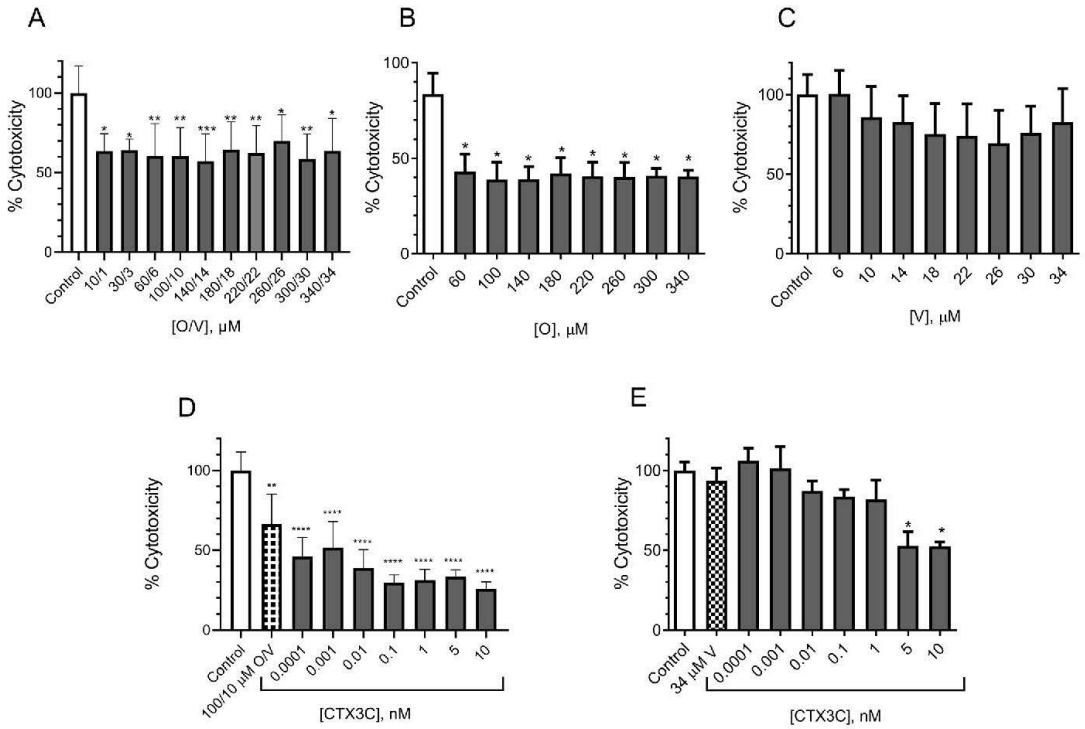


Figure 9. Cytotoxic effect measured by MTT assay on SH-SY5Y cells: (**A**) different O/V combinations; (**B**) different ouabain concentrations; (**C**) different veratridine concentrations; (**D**) O/V at 100/10 µM with different CTX3C concentrations; (**E**) veratridine at 34 µM with different CTX3C concentrations. Cell viability data are expressed as percentage of untreated control cells and the mean ± SEM of three independent experiments performed in triplicate (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ versus control (untreated cells)).

The high sensitivity of the SH-SY5Y cell line to the O/V treatments was also confirmed by the LDH assay. As summarized in Supplementary Figure S5, O/V concentrations of 100/10 µM caused cytotoxic effects in the cells. The results obtained indicate that the human SH-SY5Y neuroblastoma cell line is not a suitable cell model for CTX detection due to its very low sodium channel expression, high ouabain sensibility and high CTX3C detection levels when the cells are incubated with veratridine. In view of these results and the previous findings reported by Coccini [23], no further experiments were developed with this cell line.

2.4. Evaluation of the Effect of CTX3C in HEK293 Cells Expressing the Human $Na_v1.6$ Sodium Channel Alpha Subunit

The human HEK293 $Na_v1.6$ cell line is an embryonic kidney cell line stably expressing the $Na_v1.6$ subunit of sodium channels, which was used in previous reports to investigate

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the presence and potency of ciguatoxins as a function of their inhibition of the maximum peak inward sodium currents and the negative shift in their activation voltage [4,57,58]. Therefore, in the present work multiple studies were developed with this cell line to evaluate their accuracy as a new in vitro model to assess the toxicity of ciguatoxins.

The electrophysiological recordings in HEK293 (Figure 10) showed a maximum peak inward sodium current of −2896 ± 476 pA (n = 8) which decreased in a concentration-dependent manner after bath application of increasing CTX3C concentrations (from 0.000001 to 20 nM), indicating a significant decrease in the maximum peak intensity at concentrations from 0.001 nM, as shown in Figure 10B, attributable to their hyperpolarizing effect on the activation voltage of sodium channels and the consequent intracellular increase in sodium [4]. However, electrophysiological recordings cannot be used routinely due to their requirement of special equipment and qualified staff to be developed. Nevertheless, electrophysiological techniques are the gold standard to detect both the changes that occur in ion channels; thus, these recordings are the proof to demonstrate that the HEK293 Na_v cell line transfected with the alpha subunit of voltage-gated sodium channels is suitable for the detection of ciguatoxins.

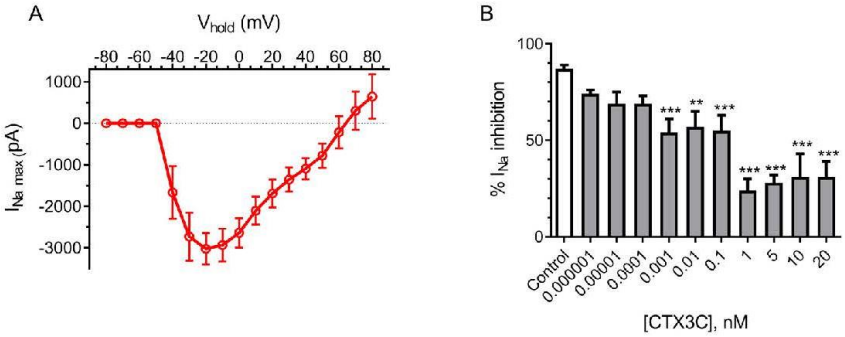


Figure 10. (**A**) Current–voltage relationship of sodium current amplitude in the HEK293 $Na_v1.6$ cell line. (**B**) Inhibition of the maximum peak inward sodium currents by bath application of different CTX3C concentrations. Data are expressed as mean ± SEM and as percentage of inhibition of the sodium current (** $p \leq 0.01$, *** $p \leq 0.001$).

MTT in HEK Cells Expressing the Human $Na_v1.6$ Sodium Channel Subunit

Due to the lack of data on the CTX toxicity in the HEK293 cell line evaluated by the routinely used MTT method, firstly, it was necessary to evaluate the sensibility of this cell line to O/V. As shown in Figure 11A, after the cell exposure to O/V concentrations from 60/6 to 340/34 µM, a significant cytotoxic effect was detected from 140/14 µM O/V, highlighting that, as well as for other cell lines, O/V caused the cell damage at these concentrations. Therefore, the in vitro toxicity of the ciguatoxin was tested directly on this line, exposing the cells only to the CTX and measuring the cell viability by the MTT assay. As shown in Figure 11B, the 24 h cell exposure to increasing CTX3C concentrations (from 0.0001 nM to 0.01 nM) did not cause any significant cytotoxic effect. However, when the cells were exposed to CTX3C concentrations of 0.1 nM and higher, the cell viability decreased up to 40% at 10 nM CTX3C, revealing that the HEK293 Nav cell line allows for the detection of the presence of CTXs at concentrations from 0.1 nM without the need to use any other compound to exacerbate the CTX toxicity. With the aim to obtain a lower detection limit and increase the sensitivity of the CTX detection using the HEK293 cell line, the cells were exposed to a nontoxic O/V concentration for this cell line of 100/10 µM and increasing CTX concentrations. Figure 11C shows the cytotoxic effect of the cell coexposure to 100/10 µM O/V and increasing CTX3C concentrations. In this case, even at the lowest CTX3C concentration tested (0.0001 nM), a significant cytotoxic effect on the cells was detected, highlighting the fact that this cell line allows for the detection of the presence of

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ciguatoxins at concentrations as low as 0.0001 nM with O/V added at a concentration of 100/10 μM demonstrated as nontoxic.

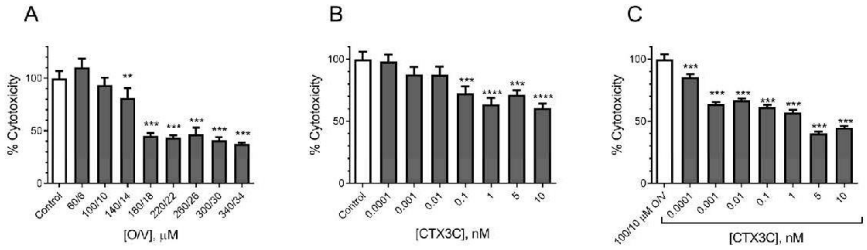


Figure 11. MTT assay on HEK $Na_v1.6$ cells: (**A**) effect of selected O/V concentrations; (**B**) cytotoxic effect of increasing CTX3C concentrations alone; (**C**) potentiated cytotoxic effect of different CTX3C concentrations with 100/10 μM O/V. Cell viability data are expressed as percentage of untreated control cells. Data are the mean ± SEM of three independent experiments (** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$).

The results obtained by electrophysiology and the MTT assay allows the HEK293 $Na_v1.6$ cell line to be purposed as a potential alternative in the detection of CTXs since the detection limit is the lowest reported so far together with the 100/10 μM O/V that does not cause any cell damage.

A comparison between different cell-based assay techniques in terms of their limit of quantification (LOQ) is summarized in Table 1, illustrating that MTT in the HEK293 Na_v cells sensitized with O/V had the lowest LOQ.

Table 1. Limits of quantification of ciguatoxin CTX3C by the different cell-based assay techniques in ciguatoxin detection as a function of the cell line employed.

METHOD	LOQ (M)
MTT in N2a sensitized with O/V	1×10^{-12}
LDH in N2a sensitized with O/V	No cytotoxic effect
Alamar blue in N2a sensitized with O/V	No cytotoxic effect
MTT in N2a sensitized with O/V and pretreated with 10 μM forskolin	1×10^{-11}
LDH in N2a sensitized with O/V and pretreated with 10 μM forskolin	1×10^{-12}
Alamar blue in N2a sensitized with O/V and pretreated with 10 μM forskolin	1×10^{-12}
MTT in HEK293 Na_v	1×10^{-10}
MTT in HEK293 Na_v sensitized with O/V	1×10^{-13}

3. Discussion

The search for a sensitive, reliable, fast and accessible method to detect the presence of ciguatoxins in samples has been a critical point over the years and has become more important in recent decades due to factors such as climate change which caused an expansion of the CTX-producing microalgae and the consequent expansion of CF to new regions [59]. Other factors such as the globalization of trade contributed to the increase in the cases of ciguatera poisoning intoxications in nonendemic regions [6]. Among all the methods used for CTX detection, the historically recognized and employed is the MTT assay on the N2a cell line [6]. However, this method has not been yet validated and established as an official reference method which makes it very difficult to compare the results obtained by different laboratories, since each uses its own assay conditions. Thus, many variations of this method using N2a as well as other different cell lines have been developed over the years with the aim to increase the sensitivity for CTX detection. In our work, the first

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limitation found when using the N2a cell line as a detection method for CTXs was their low sodium channel functionality as evidenced by the electrophysiological recordings and supported by previous data indicating the amplitude of sodium currents in this cell line ranging from −200 to −400 pA [33–35,60], underscoring the need for caution in the interpretation of cytotoxicity data derived from the use of neuroblastoma cells.

The lack of the cytotoxicity of CTX3C in the N2a cells treated with CTXs alone evaluated by the MTT method was previously reported [10,12,31,48], and the way to increase the cytotoxicity of ciguatoxins in this assay was the incubation of the cells at a nonstandardized concentration of O/V. The main problem is the cytotoxicity of the O/V treatments in the N2a cells which is also well documented [12,24]. Even so, different O/V concentrations as high as 500/50 µM O/V [30,61] were used when lower concentrations were proven to be highly toxic to cells [12]. Separate cell exposure to O and V also confirmed that V did not affect cell viability, a fact that could be explained since V acts on sodium channels, which are very scarce in neuroblastoma cells, being less toxic for the N2a cells than O, which acts on the Na^+/K^+ ATPase pump. In this study, an O/V concentration of 100/10 µM considered nontoxic was chosen. This O/V condition has been commonly used in recent assays with a generally accepted 20% reduction in cell viability to a dynamic range of 80% of cell viability [12,24,32,48]. Even though it has always been assumed that the N2a cells sensitized with O/V can detect the CTX effect on sodium channels assuming that the cytotoxicity observed by the MTT assay on N2a is due to the direct effect of ciguatoxins on the sodium channels; in this work, we demonstrated that the exposure of the N2a cells to 100/10 µM O/V for 24 h did not increase the sodium channel expression, not allowing for the detection of ciguatoxins due to their direct effect on the sodium channels, contrarily to what has been historically assumed and even patented [62]. Through the MTT assay under these conditions, the commonly used method was replicated obtaining similar cytotoxicity results for CTX3C as in previous studies [9,12,49] with a similar IC_{50}. However, when the cytotoxicity under the same assay conditions was evaluated using different methods, such as the LDH and Alamar blue assays, that did not require washing or medium removal, the results obtained were significantly different, not allowing for the detection of the CTX cytotoxicity at any of the studied concentrations. The immunocytochemistry images showed alterations in the actin cytoskeleton in the treated cells, according to previously reported results for ouabain [55] and ciguatoxins [51]. When the cells were exposed to a combination of these two compounds that cause alterations in the actin cytoskeleton, the damage was much more pronounced.

These data, together with the microscopic observations of the cell weakening and detachment after the treatment and the confocal microscopy images that confirmed the damage in the actin cytoskeleton as well as the electrophysiological recordings showing a very low rate of functional sodium channel expression, allow us to propose that the cytotoxic effects of CTX3C obtained by the MTT assay are not really quantifying the effects of ciguatoxins on their main target, the sodium channels. The N2a cells, although still viable but with the cytoskeleton damage, are not well adhered to the wells; therefore, the processes of washing, removal of the medium and incubation with shaking that the MTT method requires [11,63] make these still viable cells detach from the bottom of the wells, resulting in a false decrease in cell viability. This hypothesis is supported by the fact that alternative cytotoxicity detection methods, such as the Alamar blue or LDH assay, did not detect any additional decrease in the cell viability higher than that caused by the concentration of 100/10 µM O/V.

Although the main effect of forskolin is the activation of adenylate cyclase, the interaction of forskolin with sodium channels was reported as eliciting an increase in the sodium current amplitude in different cell lines [37,39,40]. Moreover, this molecule was reported as a promoter of neuroblastoma cell differentiation [36,42]. Therefore, the 24 h N2a cell exposure to 10 µM forskolin led to an increase in the N2a sodium current amplitude resulting in an increase in the CTX cytotoxicity under the same assay conditions as in both alternative methods studied, the LHD and Alamar blue assays. Moreover, in the MTT

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assay, despite obtaining a similar IC_{50} due to following the same assay procedure, higher sensibility was obtained in the forskolin-pretreated cells represented by a decrease in the cell viability up to 81.2 ± 2.3% (n = 4) with 10 nM CTX3C and 100/10 µM O/V compared to the nonpretreated cells where the cell viability under the same conditions decreased only up to 66.1 ± 3.6% (n = 4). Therefore, the data presented in this work demonstrate that the N2a cell pretreatment with 10 µM forskolin for 24 h enhances the N2a cell sensitivity to CTXs.

Since the N2a cell line is routinely used and recommended to monitor the presence of CTXs [6], all the results presented in this report led to the question whether this cell line was suitable for the detection and quantification of compounds whose main targets are the sodium channels, such as ciguatoxins. Since, as we demonstrate in this work, the direct effect of CTXs on the sodium channels is not being quantified, which has more serious consequences than the effects produced on the cytoskeleton, this leads to the under- or overestimation of the real CTX effect. Given the problems of the MTT assay on N2a, multiple alternative detection methods for voltage-gated sodium channel modulators have been investigated, involving diverse approaches, from the use of different cell lines [23] to the use of different assays based on the CTX ability to depolarize cell membrane [25] or receptor binding assays [28,64]. One of the alternatives proposed was the human neuroblastoma SH-SY5Y cell line [23], assuming that it would better mimic the effects caused by CTXs in the human nervous system. However, electrophysiological experiments corroborated the finding that the SH-SY5Y cell line, as well as N2a, did not show any functional sodium channels, which was in accordance with the previously reported results for the same cell line [56]. Despite being suggested as an alternative for CTX detection, the SH-SY5Y cell exposure to 10 nM O and 25 µM V decreased the cell viability by 25% compared to the control conditions, and the addition of 0.5 to 10 nM CTX3C only decreased the cell viability by 26 to 30% even at the highest CTX3C concentrations [23], indicating that this cell line allows neither for the detection of CTXs at low limits nor for their quantification. Similar results were obtained in our study where the decrease in the cell viability elicited by 100/10 µM O/V hardly increased even at the highest CTX3C concentrations, a fact that could be explained by the low sodium channel functionality in the undifferentiated SH-SY5Y cells. Therefore, the detection of ciguatoxins determined by the decrease in cell viability employing the O/V pretreatment may not be appropriate when using the human neuroblastoma SH-SY5Y cell line.

In contrast, in the HEK293 cells stably expressing the $Na_v1.6$ alpha subunit, CTX3C alone, at concentrations of 0.1 nM and higher, decreased the cell viability, reaching a 40% cell death at 10 nM CTX3C. This fact reveals that the HEK293 Nav cell line allows for the detection of ciguatoxins at concentrations from 0.1 nM CTX3C due to their true effect on the sodium channels without the need to use any other compound to exacerbate the CTX toxicity. This is a considerably low limit since no additional compounds are used to detect cytotoxicity by the MTT assay either in the SH-SY5Y or N2a cell lines. Moreover, when the MTT assay was performed in this cell line replicating the N2a conditions, the detection limit decreased up to 0.0001 nM CTX3C in the presence of O/V at a concentration of 100/10 µM demonstrated as nontoxic [12,49]. This fact reveals that the HEK293 Nav cell line constitutes an alternative for detecting the presence of CTXs at considerably low concentrations either with or without O/V.

4. Materials and Methods

4.1. Toxins and Drugs

Noncertified CTX3C was obtained from Wako (FUJIFILM Wako Germany) and dissolved at a concentration of 1 µM in DMSO. Serial toxin dilutions were performed in Locke's buffer solution containing (in mM) 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 5.6 glucose (pH 7.4). Forskolin was purchased from Sigma and dissolved in DMSO at a final concentration of 25 mM. Ouabain and veratridine were purchased from Thermofisher and dissolved in DMSO at a final concentration of 200 nM and 5 mM, respectively. MTT

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was purchased from Sigma, lactate dehydrogenase (LDH) and Alamar blue were both from Thermofisher.

4.2. Human and Mouse Cell Lines

Three different cell lines were employed and maintained at 37 °C in a humidified atmosphere with 5% CO_2 and 95% air, replacing the medium every 2–3 days.

4.2.1. N2a Mouse Neuroblastoma Cell Line

N2a cells were obtained from the American Type Culture Collection (CCL-131) at passage numbers from 180 to 200 and a seeding density of 25 × 103 cells per 200 µL and maintained in RPMI-1640 medium supplemented with 1% glutamine, 10% foetal bovine serum and 1% penicillin/streptomycin for cytotoxicity testing. The cells were seeded in 96-well plates or on glass coverslips in 12-well plates for electrophysiology testing at a density of 70 × 103 cells/well.

4.2.2. Human Neuroblastoma Cell Line SH-SY5Y

Human neuroblastoma cell line SH-SY5Y was obtained from the American Type Culture Collection (ATCC CRL-2266), cultured in DMEM/F12 medium (Invitrogen) at a 1:1 ratio supplemented with 10% foetal bovine serum and 1% GlutaMAX, both from Gibco, and antibiotics (100 UI/mL penicillin and 100 µg/mL streptomycin). The cells were subcultured in 25 cm^2 tissue culture flasks and seeded in 96-well microplates for experiments at a density of 12 × 10^3 cells per 200 µL of culture medium for cytotoxicity testing or on glass coverslips in 12-well plates for electrophysiology testing at 70 × 10^3 cells/mL.

4.2.3. Human Embryonic Kidney Cell Line (HEK293) Cell Culture

HEK293 human cells stably expressing the human $Na_v1.6$ alpha subunit of the sodium channels were kindly provided, under a material transfer agreement, by Dr. Andrew Powell (GlaxoSmithKline R&D, Stevenage, UK) at passage numbers from 33–34 to 40, cultured in DMEM/F12 medium supplemented with glutamax, MEM nonessential amino acid solution (Gibco, 1% *w*/*v*), 10% foetal bovine serum and 0.4 mg/mL geneticin (G418, Gibco) at a density of 30 × 10^3 cells per 200 µL for cytotoxicity assays. For electrophysiology, cells were seeded at a density of 70 × 10^3 cells/mL. Before testing, cells were kept at 30 °C for 24/48 h to improve the sodium channel expression [65].

4.3. Determination of Cell Viability

4.3.1. MTT Assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test was used to determine cell viability after treatments at different CTX3C concentrations (0.0001 nM, 0.001 nM, 0.01 nM, 0.1 nM, 1 nM, 5 nM and 10 nM) added to the culture medium for 24 h. DMSO at 10% was used as death control. After treatment, cells were incubated with MTT at a final concentration of 500 µg/mL in Locke's buffer containing 119 NaCl, 5.9 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 NaH_2PO_4, 22.8 $NaHCO_3$ and 0.1% glucose (pH was adjusted to 7.4 prior to use) for 50 min at 37 °C. After washing off excess MTT, cells were disaggregated with DMSO overnight, and cell viability was determined measuring the absorbance of the coloured formazan salt at 595 nm in a spectrophotometer plate reader.

4.3.2. Alamar Blue Assay

Cells were treated at the corresponding CTX3C concentrations, and at the end of the treatment, 10% *v*/*v* of alamarBlue® was added. Cells were incubated with the toxins and alamarBlue® for up to 48 h. The cytotoxicity was determined by fluorescence measurements using a FL600 microplate fluorescence reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) set at 530 nm (excitation) and 590 nm (emission). Cell viability was expressed as % of the control fluorescence emission.

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4.3.3. Lactate Dehydrogenase Assay

CyQUANT™ LDH cytotoxicity assay kit was used to analyse cell cytotoxicity, following manufacturer's instructions. Cells were cultured and treated for 24 h at the different CTX concentrations. After treatment, 50 µL of medium samples, by triplicate, was transferred to a 96-well flat-bottom plate, and LDH release was evaluated by reading the absorbance in a plate reader and subtracting the 680 nm absorbance value from the data obtained at 490 nm.

4.4. Immunocytochemistry

For immunocytochemistry control, 100/10 O/V- and CTX3C-treated N2a cells seeded on glass coverslips were washed three times with phosphate buffered saline (PBS, containing in mM 137 NaCl, 8.2 Na_2HPO_4, 1.5 KH_2PO_4 and 3.2 KCl) and fixed with 4% formaldehyde and 4% sucrose. Fixed cells were permeabilized with 0.1% triton X-100, and nonspecific fluorescence was blocked with 5% bovine serum albumin (BSA). To evaluate the 100/10 O/V and CTX3C effects on the cytoskeleton, cells were incubated with a monoclonal anti-actin mouse primary antibody (1:200, Millipore, Germany, diluted in PBS containing 2% BSA) overnight at 4 °C and washed three times with PBS. Afterward, cells were incubated with an anti-mouse fluorescent secondary antibody (Alexa Fluor 488, Thermofisher, at a 1:500 dilution) for 1 h at room temperature. Coverslips were washed three times with PBS and mounted in 50% glycerol/PBS, sealed with nail polish and analysed using a Nikon confocal laser scanning microscope (Nikon, Melville, NY, USA) with an ORCA-ER camera (Hamamatsu photonics, Shizuoka Prefecture, Japan).

4.5. Electrophysiology

Cells seeded on glass coverslips were placed in a recording chamber with 0.5 mL Locke's buffer as an extracellular solution. Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter, resistances from 5 to 10 MΩ) and filled with an intracellular pipette solution that contained (in mM) 140 CsF, 10 EGTA, 10 HEPES, 5 NaCl, 2 $MgCl_2$, pH adjusted to 7.3. Cells were maintained at a holding potential (V_{hold}) of −55 mV establishing the whole-cell configuration to ensure adequate equilibration between the internal pipet solution and the cell interior. Voltage-gated sodium currents were recorded at room temperature (20–24 °C) in a computer-controlled current and voltage clamp amplifier (Multiclamp 700B, Molecular Devices) and using the Digidata 1440A data acquisition system (from Axon Instruments, San Jose, CA, USA). Signals were sampled at 50 kHz after low-pass Bessel filtering at 10 kHz and analysed offline, using the pClamp 10 software (Axon Instruments). Compensation circuitry was used to reduce the series resistance error by at least 70%. The effect of CTXs on sodium channels was evaluated after 5 min exposure of the cells to the toxin. To record the activation of voltage-gated sodium currents (I_{Na}), voltage steps from −80 to +80 mV (10 mV increments) were applied.

4.6. Statistical Analysis

All data are expressed as means ± SEM of n determinations. Statistical comparison was by Student's *t*-test or ANOVA followed by Dunnet's test; *p*-values < 0.05 were considered statistically significant.

5. Conclusions

The N2a mouse neuroblastoma cell line currently used for the detection and quantification of ciguatoxins in samples does not have functional voltage-gated sodium channels. Therefore, the MTT method cannot detect the direct effect of the ciguatoxins on the sodium channels as historically assumed. It is proposed that what is being detected is a decrease in cell viability as a consequence of the methodology used. Due to the actin cytoskeleton damage caused by CTXs and the O/V treatment, cells, although viable, detach from the well and are removed. It was demonstrated that the 24 h exposure of neuroblastoma to 10 µM forskolin increases the expression of the sodium channels in the N2a cells; thus, this

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method could be used as an alternative for detecting the CTX compounds. Furthermore, the HEK293 cell line transfected with the 1.6 alpha subunit of the sodium channels was capable of detecting the presence of ciguatoxins even without the OV treatment. Additionally, in the case of using O/V to sensitize cells, 100/10 μM O/V was demonstrated not to be toxic for the HEK293 cells and to allow for the detection of ciguatoxins at lower concentrations compared to the rest of the methods.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md21110590/s1, Figure S1: Cytotoxic effects of O and O/V combinations in N2a cells; Figure S2: Cytotoxic effects of O/V and increasing CTX3C concentrations in N2a cells cultured in the presence of 5% FBS and treated with the different compounds in the presence of 2% FBS; Figure S3: No cytotoxic effects of 24 h incubation with 10 μM forskolin evaluated by MTT, LDH and Alamar Blue; Figure S4: Current-voltage relationship of sodium currents in SH-SY5Y cells. Figure S5: Cytotoxic effects in SH-SY5Y cells of different O/V concentrations after 24 h treatment.

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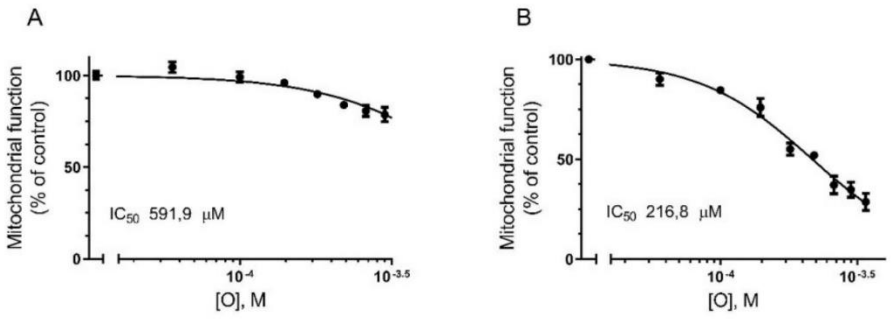


Figure S1: Cytotoxic effects of O and O/V combinations in N2a cells. A. Concentration response effect of 24 h exposure of cells to increasing O concentrations. B. Concentration response effect of 24 h exposure of cells to increasing O and V concentrations (60/6, 100/10, 140/14, 180/18, 220/22, 260/26, 300/30, 340/34 µM O/V).

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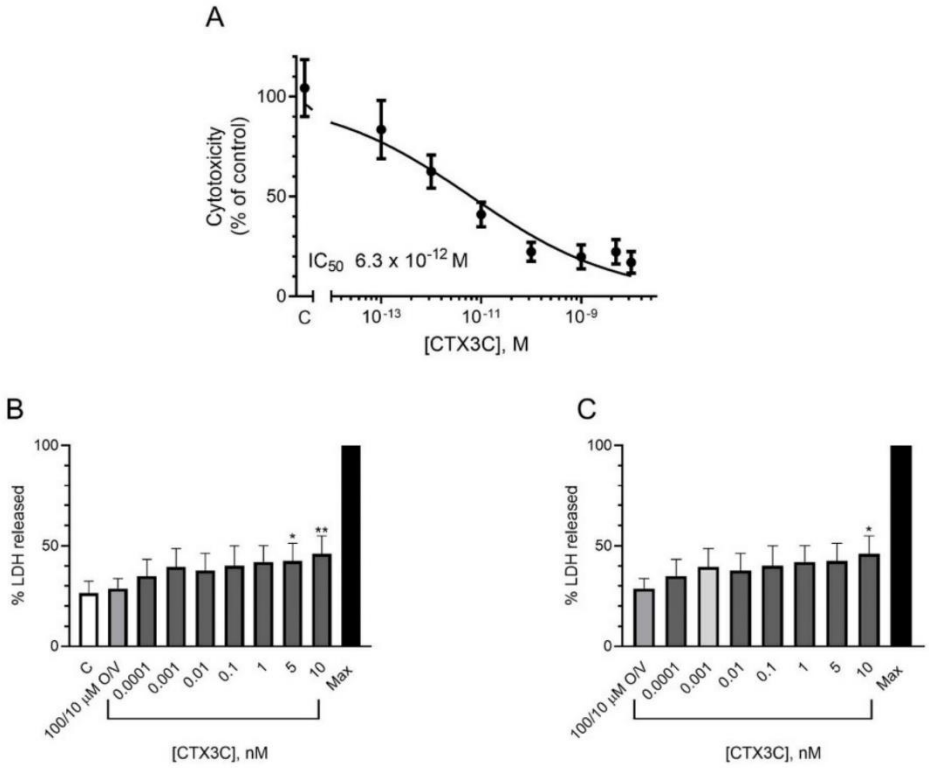


Figure S2: Cytotoxic effects of O/V and increasing CTX3C concentrations in N2a cells cultured in the presence of 5 % FBS and treated with the different compounds in the presence of 2 % FBSA. A. Concentration response effect of 24 h exposure of cells to increasing CTX3C concentrations and 100/10 µM O/V evaluated by the MTT assay. B. Absence of cytotoxic effects of O/V and increasing CTX3C concentrations in N2a cells evaluated by the LDH assay in contrast to control conditions. C. Absence of cytotoxic effects of O/V and increasing CTX3C concentrations in N2a cells evaluated by LDH assay in contrast to 100/10 µM O/V conditions. Cell viability data are expressed as percentage of untreated control cells, mean ± SEM of three independent experiments and cell. *$p \leq 0.05$. **$p \leq 0.01$.

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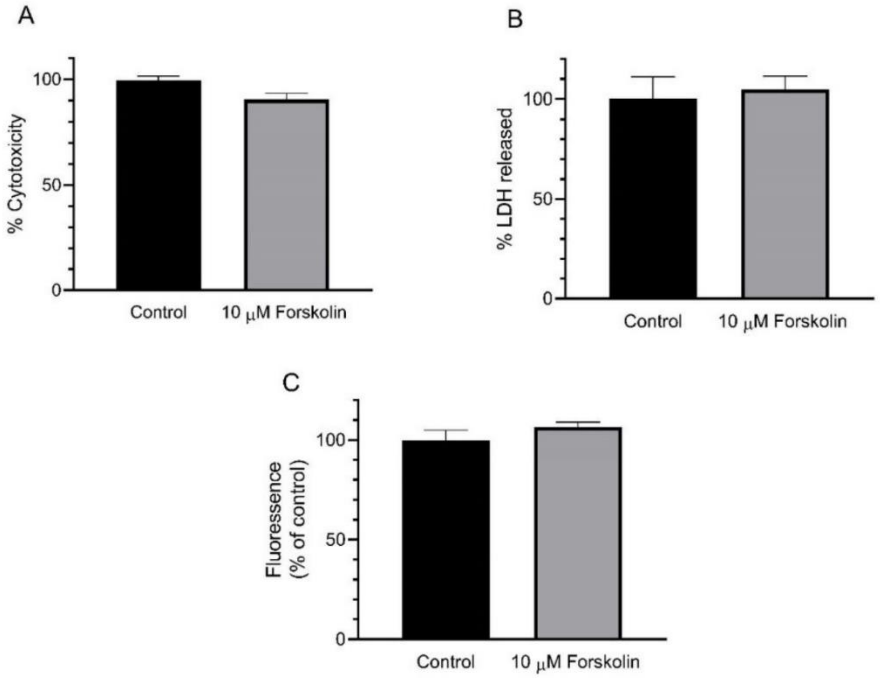


Figure S3: No cytotoxic effects of 24 h incubation with 10 μM forskolin evaluated by A. MTT assay. B. LDH assay. C. Alamar blue assay in the N2a cell line.

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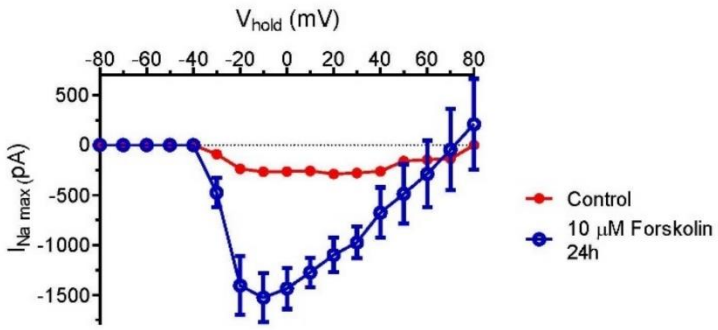


Figure S4: Current-voltage relationship of sodium currents in SH-SY5Y cells. Control cells (red) showed a maximum peak inward current of about –300 pA while cells seeded with 10 µM Forskolin and maintained 24 h showed an increase on maximum sodium current intensity up to -1500 pA.

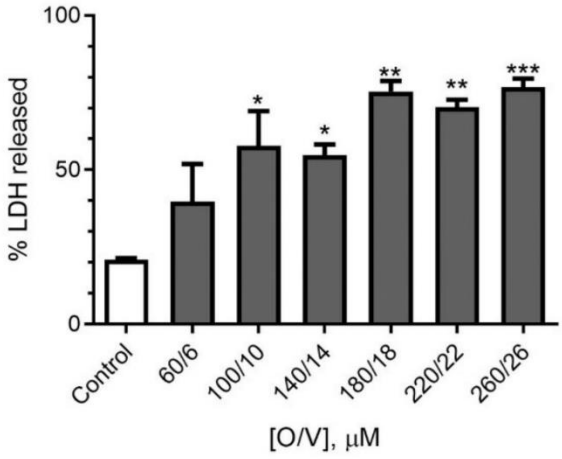


Figure S5: Cytotoxic effects in SH-SY5Y cells of different O/V concentrations after 24 h treatment.

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Mouse N2a neuroblastoma assay: Uncertainties and comparison with alternative cell-based assays for ciguatoxin detection.

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BLOQUE II: EFECTO SINÉRGICO DE CONTAMINANTES ALIMENTARIOS

En este segundo bloque se recogen los resultados obtenidos tras el estudio de los efectos combinados sobre canales de sodio dependientes de voltaje humanos de contaminantes alimentarios con la misma diana celular. Este tipo de estudios cobran cada vez más importancia dado que el consumo de una dieta equilibrada implica la exposición a múltiples residuos tóxicos. Por lo tanto, es necesario evaluar la toxicidad de estos compuestos en conjunto y establecer límites seguros que garanticen la seguridad de los consumidores. Los resultados obtenidos dieron lugar a dos publicaciones que se presentan a continuación.

Publicación

Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins

Resumen

Hasta el momento los niveles máximos de residuos en los alimentos se fijaron sin considerar los posibles efectos sinérgicos entre compuestos que comparten mecanismo de acción y diana celular, este es el caso de biotoxinas marinas emergentes como las ciguatoxinas y los compuestos piretroides, ampliamente utilizados en la agricultura. Como consecuencia de la presencia cada vez mayor de ciguatoxinas en las costas europeas y el creciente uso de productos fitosanitarios para el control de plagas alimentarias junto con la falta de datos y regulaciones que evalúen el efecto combinado de contaminantes ambientales con una misma diana molecular ocasionó la necesidad de analizar sus efectos *in vitro*. En este trabajo se investigaron los efectos de las ciguatoxinas y los piretroides en los canales de sodio dependientes de voltaje empleando células transfectadas con la subunidad α1.6 de origen humano. Los resultados presentados en este estudio indicaron que ambos tipos de compuestos tenían un marcado efecto sinérgico *in vitro*. Estos contaminantes alimentarios disminuyeron las corrientes máximas de entrada de sodio e hiperpolarizaron el voltaje de activación de los canales, efectos que se vieron potenciados por la presencia simultánea de ambos tóxicos. Este hecho puso de manifiesto la necesidad de reevaluar sus límites en los alimentos, así como su potencial toxicidad *in vivo*, considerando que actúan sobre la misma diana celular. Además, este trabajo sienta las bases celulares para seguir aplicando este tipo de estudios a otros contaminantes del agua y de los alimentos que puedan actuar de forma sinérgica y así implementar los límites regulatorios correspondientes teniendo en cuenta su presencia en una dieta saludable.

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Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins

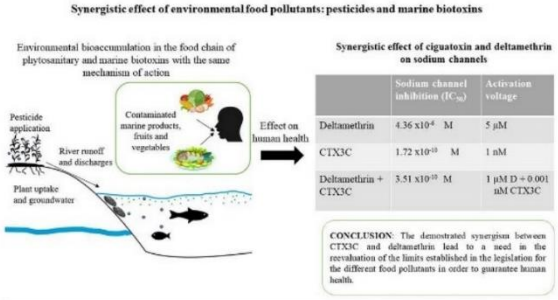
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HIGHLIGHTS

- Synergistic effects of a ciguatoxin, CTX3C and deltamethrin alters sodium channels.
- Deltamethrin increased 1000 times the effect of CTX3C.
- CTX3C increased 10 times the effect of deltamethrin.
- Food and water pollutants that share cellular targets require simultaneous regulations.

GRAPHICAL ABSTRACT



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ABSTRACT

Emerging marine biotoxins such as ciguatoxins and pyrethroid compounds, widely used in agriculture, are independently treated as environmental toxicants. Their maximum residue levels in food components are set without considering their possible synergistic effects as consequence of their interaction with the same cellular target. There is an absolute lack of data on the possible combined cellular effects that biological and chemical pollutants, may have. Nowadays, an increasing presence of ciguatoxins in European Coasts has been reported and these toxins can affect human health. Similarly, the increasing use of phytosanitary products for control of food plagues has raised exponentially during the last decades due to climate change. The lack of data and regulation evaluating the combined effect of environmental pollutants with the same molecular target led us to analyse their in vitro effects. In this work, the effects of ciguatoxins and pyrethroids in human sodium channels were investigated. The results presented in this study indicate that both types of compounds have a profound synergistic effect in voltage-dependent sodium channels. These food pollutants act by decreasing the maximum peak inward sodium currents and hyperpolarizing the sodium channels activation, effects that are boosted by the simultaneous presence of both compounds. A fact that highlights the need to re-evaluate their limits in feedstock as well as their potential in vivo toxicity considering that they act on the same cellular target. Moreover, this work sets the cellular basis to further apply this type of studies to other water and food pollutants that may act synergistically and thus implement the corresponding regulatory limits taking into account its presence in a healthy diet.

Abbreviations: ADI, acceptable daily intake; ARfD, acute reference dose; CFP, ciguatera fish poisoning; CTXs, ciguatoxins; CI, combination index; DMEM, Dulbecco's Modified Eagle Medium; DMSO, DiMethyl SulfOxide; EFSA, European Food Safety Authority; FAO, Food and agriculture organization; HEK cells, Human embryonic kidney cells; I_{Cl}, chloride current amplitude; I_{Na}, sodium current amplitude; MRL, Maximum Residue Level; V_{hold}, holding potential; VGSC, voltage gated sodium channels.

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1. Introduction

Voltage-gated sodium channels (VGSCs) are transmembrane proteins that conduct sodium ions across the membrane generating signals of communication between many different tissues, thus, they are considered to be one of the most important classes of ion channels due to their remarkable physiological role (Catterall et al., 2005) and are responsible for the transmission, generation, and propagation of action potentials in excitable cells. The different VGSC isoforms ($Na_v1.1$–$Na_v1.9$) are distributed differentially throughout the body, which correlates with their functional properties in the corresponding tissues (Catterall et al., 2005) and are the target of many natural and synthetic compounds that can affect human and animal health through food or water consumption (Morabito et al., 2018).

Among the environmental contaminants that can affect VGSC are marine biotoxins, such as ciguatoxins, and pyrethroid pesticides as deltamethrin (Gharaei et al., 2020; Paduraru et al., 2021; Petrovici et al., 2020; Radovanovic et al., 2021). These food pollutants have increased their presence during the last decades due to factors that include the increase in water temperature, demographic changes, pollution, and modifications in eating habits (Botana, 2016; Lu et al., 2019; Schreiner et al., 2016). Marine biotoxins as well as pesticides can accumulate into the food chain risking the health of consumers as established by the European Food Safety Authority, EFSA (EFSA, 2015; Loeffler et al., 2021). Pesticides can also affect human health by water contamination due to their widespread use and their persistence in the environment and enter both fresh and marine aquatic environments through atmospheric deposition, river runoff or municipal treatment discharges (Aznar-Alemany et al., 2017). Residues of pyrethroids have been found ubiquitously in the marine aquatic environment in many regions of the world (Li et al., 2017; Moschet et al., 2017; Schreiner et al., 2016; Stehle and Schulz, 2015; Van Metre et al., 2017) and also their presence in river waters has been reported (Lu et al., 2019; Maguire, 1992). Moreover, the presence of Caribbean and Pacific ciguatoxins has recently reached European Coasts (Ramos-Sosa et al., 2022).

Pesticides are synthetic compounds whose maximum residue limits (MRL) were regulated in the European legislation (EC Regulation 396/2005). The periodic samplings carried out by the competent authority (EFSA) indicate in their annual reports that, in food, residues are found in amounts higher than the MRL established in the legislation (EFSA, 2015). This situation is relevant because the use of some of them is prohibited in the European Union (EFSA, 2015; EFSA, 2020). They are dangerous compounds since numerous studies have shown the harmful effects of exposure to these chemical pollutants in zebrafish, mussels and crustaceans (Blahova et al., 2020; Pagano et al., 2020; Stara et al., 2019; Stara et al., 2020; Sula et al., 2020; Vajargah et al., 2021). One of the pesticides that acts on VGSC is deltamethrin, a type II synthetic pyrethroid that prolongs the opening state of VGSC and shifts the activation voltage of the channels to more negative potentials (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007; Field et al., 2017). Moreover deltamethrin decreases the open channel probability of voltage-gated chloride channels (Forshaw et al., 1993; Forshaw and Ray, 1990). Although initially pyrethroids were intended as a safe alternative to organophosphates and deltamethrin was considered to be less toxic to mammalian species than to insects (Bradberry et al., 2005; Ray and Fry, 2006), deltamethrin affects immune, reproductive and neuronal systems, skeletal muscles and tumour proliferation (Bradberry et al., 2005; Kaneko, 2011; Khalatbary et al., 2016; Prevarskaya et al., 2018; Sharma et al., 2014; Tewari et al., 2018; Tuzmen et al., 2008) and causes hepato- and nephrotoxic effects (Abdel-Daim and El-Ghoneimy, 2015; Gunduz et al., 2015). Thus, the chronic oral ingestion of deltamethrin-polluted water and food represents a source of poisoning to humans, even more if this compound is ingested in food that also may contain other VGSC modulators.

The other important group of food pollutants which act on VGSC are the marine biotoxins ciguatoxins, produced by dinoflagellates of the genus *Gambierdiscus* and *Fukuyoa*, that bind to site 5 of the alpha subunits of the sodium channels, keeping the protein in a permanently open state that leads to activate sodium conductance at normal cell resting membrane potentials (Hogg et al., 1998; Raposo-Garcia et al., 2022). Up to 500,000 cases of ciguatera fish poisoning (CFP) occur globally every year, becoming the most common foodborne illness related to fish consumption (Friedman et al., 2017). Ciguatoxins cause in human digestive, cardiovascular and neurological disorders which can last up to years and ciguatera is becoming an increasing risk in Europe (Loeffler et al., 2021). The available data detailed that in 2020 the 65 % of the fish samples analysed in Europe were positive for ciguatera (Diogène et al., 2021). Despite the increasing incidence of ciguatera in Europe, there are still no regulatory limits in the European legislation for ciguatoxins in food. However, the American Food and drug administration established a maximum level of ≥ 0.1 μg/kg for Caribbean ciguatoxin-1 (C-CTX-1) equivalents and ≥ 0.01 μg/kg for Pacific ciguatoxin-1 (P-CTX-1) equivalents (FDA, 2011).

The effects of these two groups of compounds on VGSC and on human health have been widely studied and reported separately (Bradberry et al., 2005; Chrustek et al., 2018; Dickey and Plakas, 2010; Solino and Costa, 2020), however, taking into account that the recommended daily intake of food includes products that may contain biotoxins (Costa et al., 2021; Solino and Costa, 2020) such as fish and molluscs two times a week and a daily intake of fruits and vegetables (https://www.who.int/news-room/fact-sheets/detail/healthy-diet) that may contain pollutant pesticides such as deltamethrin (EFSA, 2015), humans are exposed to a combination of all these pollutants.

So far, there is an absolute lack of data on the possible synergistic cellular effects that combinations of biological and chemical pollutants, that share the same mechanism of action, may have. Considering that, the effects of ciguatoxins and deltamethrin on human VGSC were analysed taking into account that evaluating these compounds separately and setting safety limits for them is not accurate as synergies could appear. Thus, obtaining these data represents a relevant fact to guarantee the health of consumers and avoid the appearance of chronic alterations such as endocrine (Kumar et al., 2020; Sirohi et al., 2021) or neurodegenerative diseases (Li et al., 2021) reported by the consumption of these VGSC modulators. Moreover, the consumption in the diet of toxic substances with the same mechanism of action can cause harmful effects even when the intake of these toxins is below the tolerable daily intake due to their possible summatory effects. The absence of studies and information on the possible cumulative biological activities of these compounds and the recent call of FAO for new toxicological data on these food pollutants (https://www.fao.org/agriculture/crops/thematic-sitemap/theme/pests/jmpr/en/) coupled with the fact that these compounds pose a food safety hazard since it has been previously demonstrated that deltamethrin is not significantly degraded during storage, nor is it destroyed in cooking (Dikshit, 2002; Kang and Lee, 2005; Lal and Dikshit, 2000). As well as ciguatoxins which are not degraded due to freeze or cooking (WHO, 2020), thus both are temperature-stable compounds. Therefore, the previous observations led us to develop this study with the aim to establish the initial cellular basis to predict the associated public health risks that can arise from the simultaneous presence of marine toxins and pesticides in food and thus lay the groundwork to develop, in the future, the corresponding regulations.

2. Materials and methods

2.1. Environmental pollutants

Pacific ciguatoxin CTX3C was purchased from Wako (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 μM. For experiments, 1 μM solutions were made in Locke's buffer containing (in mM): 154 NaCl, 5.6 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 5.6 glucose (pH 7.4). The maximum solvent concentration used as a control was 0.1 % DMSO and had no effect on the voltage-gated sodium current amplitude.

Deltamethrin was purchased from Merck (Germany) and dissolved in DMSO at a final concentration of 10 mM. For experiments, 100 μM solutions were prepared in Locke's buffer.

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2.2. Human cell cultures

Human embryonic kidney cell line (HEK293) stably expressing the human $Na_v1.6$ alpha subunit of the sodium channels was kindly provided, under a material transfer agreement, by Dr. Andrew Powell (GlaxoSmithKline R&D, Stevenage, U.K.). Cells were cultured in DMEM/F12 medium supplemented with glutamax, MEM non-essential amino acids solution (Gibco, 1 % *w/v*), 10 % foetal bovine serum and 0.4 mg/mL geneticin (G418, Gibco). Cell cultures were maintained at 37 °C in a 95 % O_2/5% CO_2 atmosphere and with 95 % humidity, replacing the medium every 2–3 days. About 24–48 h before electrophysiological recordings, cells were placed at 30 °C to improve sodium channel expression (Burbidge et al., 2002).

2.3. Electrophysiology

For electrophysiological recordings, cells were seeded on glass coverslips in 12 well plates at a density of 50.000 cel/mL and placed in a recording chamber with 0.5 mL of Locke's buffer as extracellular solution. Recording electrodes were fabricated with borosilicate glass microcapillaries (1.5 outer diameter) and had resistances ranging from 4 to 10 MΩ. Pipettes were filled with an intracellular solution that contained (in mM): 120 CsF, 10 EGTA, 10 HEPES, 15 NaCl, pH adjusted to 7.25. Cells were maintained at a holding potential (V_{hold}) of −55 mV and experiments were initiated 5 to 10 min after establishing the whole-cell configuration. Voltage-gated sodium currents were recorded at room temperature using the whole-cell voltage-clamp mode with a Multiclamp 700B amplifier and digitalized with the Digidata 1440A (both from Axon Instruments, California, U.S.A.). Signals were sampled at 50 kHz after low pass Bessel filtering at 10 kHz and analysed offline, using the pClamp10 software (Axon Instruments) and series resistance was compensated by at least 70 %. The effects of CTX3C and/or deltamethrin on sodium channels were evaluated after 5 min exposure of the cells to the compounds. To record voltage-gated sodium currents amplitude (I_{Na}) voltage steps from −80 to +80 mV (10 mV increments) were applied. The amplitude of chloride currents (I_{Cl}) was recorded by application of a voltage step protocol from −100 to +100 mV with 20 mV step increase and 400 ms duration.

2.4. Statistical analysis

Data analysis was performed using GraphPad Prism 5. All data are expressed as means ± SEM of *n* determinations. Statistical comparisons were performed using ANOVA followed by post hoc Dunnett's tests. *p* values ≤ 0.05 were considered statistically significant and IC_{50} values were determined by fitting the data with a log (inhibitor) vs normalized response model.

The combination index (CI) was calculated with the Chou & Talalay equation (Chou, 2010) following the formula:

$$Combination\ Index = \frac{D1}{(DX)1} + \frac{D2}{(DX)2}$$

where (DX)1 is the IC_{50} value of CTX3C alone, (DX)2 the IC_{50} value of deltamethrin alone and D1 and D2 are the IC_{50} values of CTX3C and

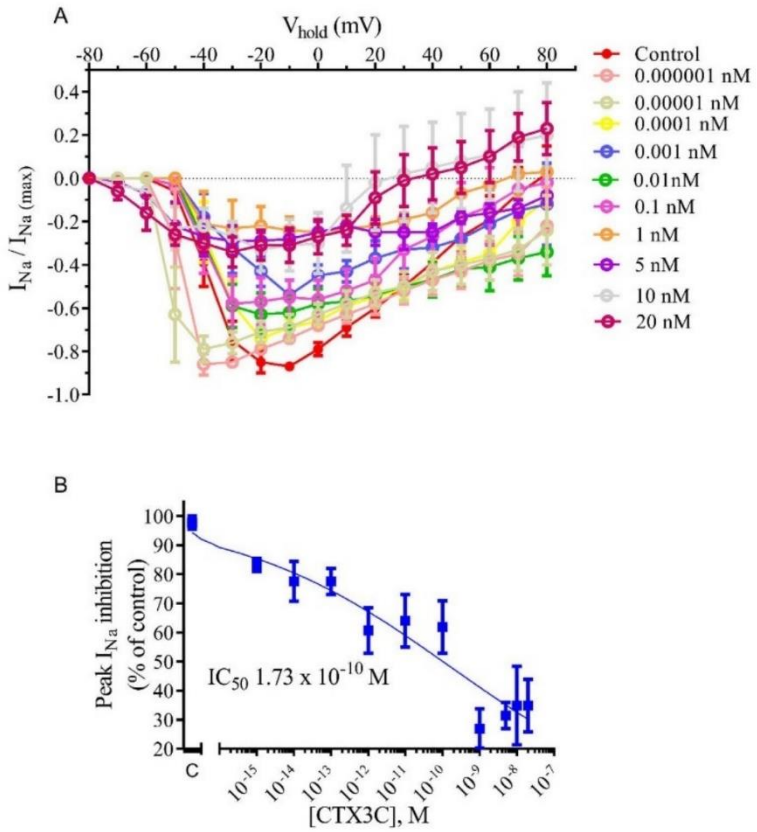


Fig. 1. Effect of CTX3C on sodium currents in HEK cells expressing the human $Na_v1.6$ alpha channel subunit. A. Current-voltage relationship for the effect of different concentrations of CTX3C on sodium current amplitude. B. Concentration-response graph indicating the effect of CTX3C on the inhibition of peak inward sodium currents.

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deltamethrin respectively in combination. According to this method, additivity is established if CI = 1, synergism if CI < 1 and antagonism if CI > 1.

3. Results

With the aim to explore the possible combined effect of two different classes of environmental food and water pollutants, ciguatoxins and the pesticide deltamethrin their effects on VGSC, separately and in combination, were assessed in human sodium channels.

3.1. Effect of CTX3C on voltage gated sodium channels

Although the effect of one of the most important CTXs analogue (CTX3C) on VGSC has been widely studied in several cellular models (Inserra et al., 2017; Martin et al., 2014; Martin et al., 2015a; Martin et al., 2015b; Raposo-Garcia et al., 2022) the possible synergistic effect between CTXs and the pesticide deltamethrin was investigated here for the first time. Fig. 1A represents the CTX3C intensity-voltage (I-V) curve for the normalized peak sodium currents in the absence and in the presence of different toxin concentrations. In control conditions, the peak sodium current at −10 mV was −1506 ± 197 pA (n = 13) decreasing in a concentration-dependent manner up to −525 ± 210 pA (n = 3) after bath application of 10 nM CTX3C. The percent inhibition of the peak inward sodium currents by different ciguatoxin concentrations was used to obtain the IC_{50} values shown in Fig. 1B. Nonlinear fit of these data yielded an estimated IC_{50} for the CTX3C inhibition of the peak inward sodium current of 1.73×10^{-10} M with a 95 % confidence interval ranging from 6.47×10^{-13} to 2.1×10^{-9} M (R^2 = 0.9164).

3.2. Effect of deltamethrin on voltage gated sodium channels

As previously reported for deltamethrin (Bothe and Lampert, 2021; Chinn and Narahashi, 1986) a significant decrease in peak inward sodium currents was observed (Fig. 2A). Thus, in control conditions, the peak sodium current was −3021 ± 378 pA (n = 11) and decreased up to −890 ± 238 pA (n = 3) after bath application of 10 μM deltamethrin. Higher deltamethrin concentrations almost completely blocked sodium entry into the cells. These data were used to obtain a concentration–

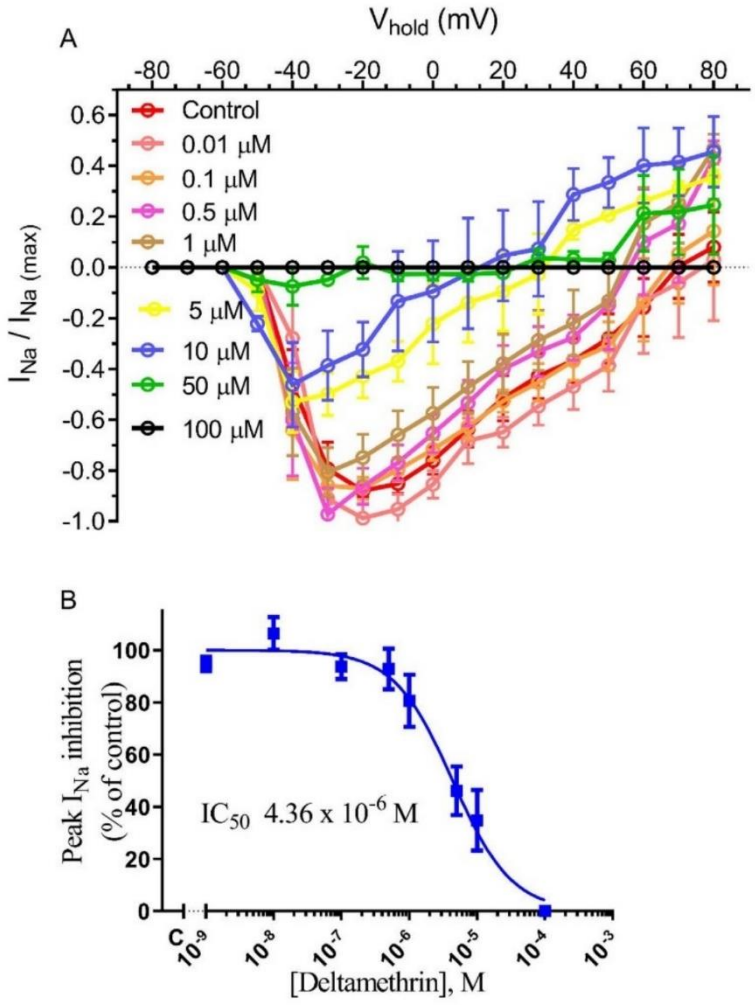


Fig. 2. Effect of deltamethrin on sodium currents in HEK cells expressing the $Na_v1.6$ alpha channel subunit. A. Current-voltage relationship for the effect of different concentrations of deltamethrin on sodium current amplitude. B. Concentration-response graph indicating the effect of deltamethrin on the inhibition of peak inward sodium currents.

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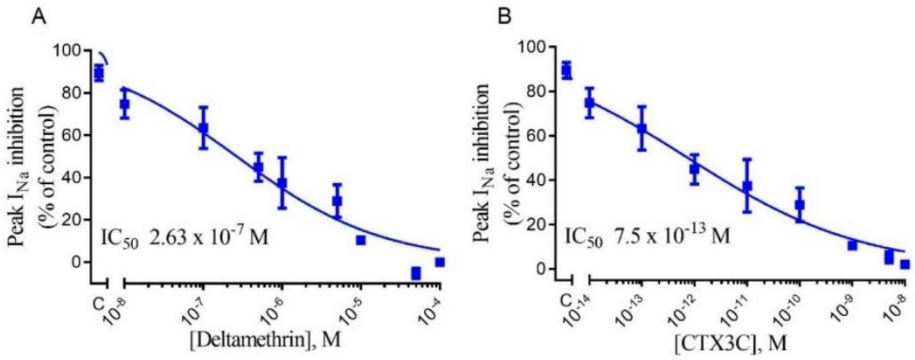


Fig. 3. Effect of increasing CTX3C and deltamethrin concentrations on sodium currents in HEK cells expressing the human $Na_v1.6$ alpha channel subunit. A. Concentration-response graph for the peak inhibition of sodium currents by different deltamethrin and CTX3C concentrations relative to the deltamethrin concentrations in the recording chamber B. Concentration-response graph for the peak inhibition of sodium currents by different deltamethrin and CTX3C concentrations relative to the CTX3C concentrations in the recording chamber.

response curve and calculate the corresponding IC_{50} for the inhibition of sodium current amplitude (Fig. 2B). In this case, an estimated IC_{50} of 4.36×10^{-6} M (95 % confidence interval from 3.15×10^{-6} M to 6.06×10^{-6} M, $R^2 = 0.9835$) for the deltamethrin inhibition of the peak inward sodium current was obtained. However, it is noteworthy to remark that 1 μM deltamethrin alone did not affect the activation voltage of sodium channels which was -36.4 ± 2.0 mV in control conditions ($n = 11$) and -42 ± 3.74 mV ($n = 5$) in the presence of 1 μM deltamethrin.

3.3. Evaluation of the combined effect of CTX3C and deltamethrin on voltage gated sodium channels

With the aim of further explore a possible synergistic effect of VGSC modulators, cells were exposed to a combination of ciguatoxin and deltamethrin. The concentrations evaluated were selected according to their IC_{50} value, obtained for each single compound separately. The addition of combined increasing concentrations of CTX3C (from 0.00001 nM to 5 nM) and deltamethrin (from 0.01 μM to 50 μM) elicited a concentration dependent decrease in sodium current amplitude. The percent inhibition of the peak sodium currents by the simultaneous presence of deltamethrin and CTX3C was used to obtain the concentration–response curves shown in Fig. 3. Nonlinear fit of these data yielded an estimated IC_{50} for CTX3C when combined with deltamethrin of 2.63×10^{-7} M (95 % confidence interval from 1.07×10^{-7} M to 6.45×10^{-7} M, $R^2 = 0.95$), represented in Fig. 3A, while the IC_{50} in the presence of deltamethrin and CTX3C was 7.5×10^{-13} M (95 % confidence interval from 3.43×10^{-13} M to 1.64×10^{-12} M, $R^2 = 0.98$) illustrated in Fig. 3B. With these values the combination index was determined as previously described (Chou, 2010) and yielded a value of 0.065. This value is lower than 1 which indicates that the effect of these two food pollutants in VGSC is synergistic, as illustrated in Table 1.

Table 1
Synergistic effects of ciguatoxin 3C and deltamethrin over the normalized sodium channel amplitude.

[CTX3C], (M)	[Deltamethrin], (M)	Mean normalized I_{Na} max	SEM
Control	Control	−0.83	0.03
$1e^{-14}$	$1e^{-8}$	−0.69	0.06
$1e^{-13}$	$1e^{-7}$	−0.59	0.09
$1e^{-12}$	$5e^{-7}$	−0.42	0.06
$1e^{-11}$	$1e^{-6}$	−0.35	0.11
$1e^{-10}$	$5e^{-6}$	−0.27	0.07
$1e^{-9}$	$10e^{-6}$	−0.1	0.01
$5e^{-9}$	$50e^{-6}$	−0.05	0.02
$1e^{-8}$	$100e^{-6}$	0	0.0001

3.4. Evaluation of the effect of a single low concentration of deltamethrin with increasing CTX3C concentration on voltage-gated sodium channels

With the aim to evaluate how a single low concentration of deltamethrin (1 μM) affects ciguatoxins action on sodium channels, cells were exposed to 1 μM deltamethrin and increasing CTX3C concentrations (from 0.0001 to 1 nM CTX3C). Fig. 4A represents the normalized peak sodium current amplitude in the absence and in the presence of 1 μM deltamethrin and different CTX3C concentrations. In this case 1 μM deltamethrin alone did not cause any significant effect on maximum current intensity but further addition of increasing CTX3C concentrations elicited the decrease of the peak sodium current from -2546 ± 172 pA ($n = 7$) to -522 ± 624 pA ($n = 3$) after bath application of 1 nM CTX3C. These data were used to obtain a concentration–response curve (Fig. 4B) that provided an estimated IC_{50} for the inhibition of the peak inward sodium current by 1 μM deltamethrin, in combination with increasing CTX3C concentrations of 3.51×10^{-10} M (95 % confidence interval from 1.46×10^{-10} M to 8.46×10^{-10} M; $R^2 = 0.9333$).

3.5. Effects of the combinations of environmental pollutants on the activation voltage of sodium channels

Since the activation voltage has been reported to be more sensitive to detect the effect of VGSC modulators (Raposo-Garcia et al., 2022) this parameter was evaluated in cells exposed to CTX3C, deltamethrin and a combination of both (Fig. 5). Bath application of increasing deltamethrin concentrations (from 0.01 to 50 μM) shifted the activation voltage from -36 ± 2.0 mV ($n = 11$) in control conditions to -46 ± 2.5 mV in the presence of 5 μM deltamethrin alone, as represented in Fig. 5A ($n = 5$; $p < 0.05$ vs control conditions). Fig. 5B represents the significant negative shift in the activation voltage of the sodium channels from -30 ± 2.4 mV ($n = 14$) in control conditions to -46 ± 2.8 mV ($n = 9$; $p < 0.001$ vs control conditions) in the presence of 1 nM CTX3C. The highest CTX3C concentration evaluated, 10 nM, elicited a hyperpolarization in the sodium channel activation voltage up to -56 ± 3.3 mV ($n = 6$; $p < 0.001$). In contrast, very low concentrations of ciguatoxin, 0.01 nM, combined with 1 μM deltamethrin, concentrations which separately did not cause changes in the activation voltage of the channels, elicited a negative shift in the activation voltage of the sodium channels from -31 ± 3 mV ($n = 16$) in control conditions to -45 ± 2.6 mV ($n = 8$; $p < 0.05$ vs control conditions) as shown in Fig. 5C. Higher concentration combinations also led to a hyperpolarizing change in the activation of the channel. Similarly, (Fig. 5D), deltamethrin at 1 μM did not cause any alteration in the activation voltage of sodium

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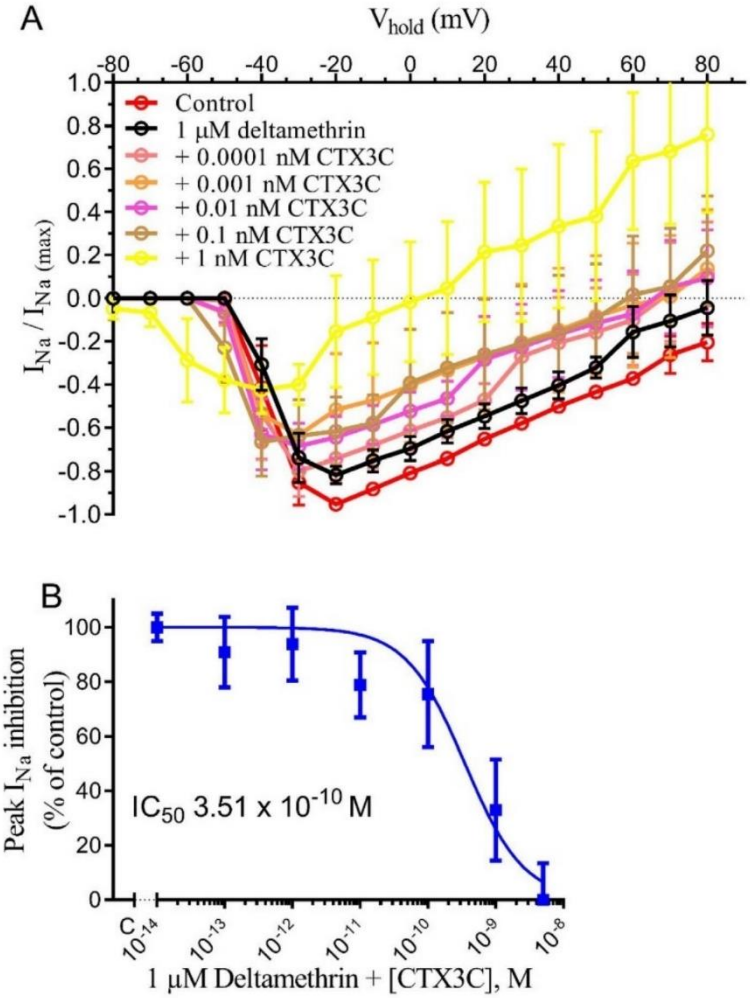


Fig. 4. Effect of 1 μM deltamethrin and different CTX3C concentrations on sodium currents in HEK $Na_v1.6$ cells. A. Current-voltage relationship for the effect of 1 μM deltamethrin and different CTX3C concentrations on sodium current amplitude. B. Concentration-response graph for the peak inhibition of sodium currents indicating the synergistic effect of deltamethrin and CTX3C.

channels, however its combination with different CTX3C concentrations caused a statistically significant negative shift in the opening of the channels at concentrations of 0.001 nM CTX3C and higher. Thus, bath application of 1 μM deltamethrin and 0.001 nM CTX3C shifted the activation voltage of sodium channels from -34 ± 2.0 mV ($n = 7$) in control conditions to -45 ± 2.9 mV ($n = 4$; $p < 0.05$ vs control conditions). The highest CTX3C concentration evaluated, 1 nM CTX3C in combination with 1 μM deltamethrin displaced the activation voltage of the channels from -34 ± 2.0 mV (n = 7) in control conditions to -53 ± 3.3 mV ($n = 3$; $p < 0.001$).

Altogether, these data indicate a combined effect of CTX3C and deltamethrin on sodium channels since bath application of 0.001 nM CTX3C alone did not cause any effect but combined with 1 μM deltamethrin hyperpolarized their activation voltage by about 20 mV.

3.6. Effect of deltamethrin on chloride channels

Previous studies have reported that deltamethrin decreased the open channel probability of voltage-gated chloride channels (Forshaw et al., 1993). Therefore, the effect of this pyrethroid on chloride current amplitude was recorded in the absence and 5 min after bath application of 1 μM deltamethrin. As shown in Supplementary Fig. 1A, current-voltage relationship shows that concentrations as low as 1 μM deltamethrin, which did not cause any significant effect on VGSC, increased the amplitude of voltage-gated chloride currents. This effect was statistically significant since the size of chloride currents increased from 651 ± 161 pA ($n = 12$) in control conditions to 1470 ± 321 pA ($n = 4$) in the presence of 1 μM deltamethrin ($p < 0.05$ vs control) as represented in Supplementary Fig. 1B. Representative recording traces of chloride currents in the absence (Supplementary Fig. 1C) and presence of 1 μM deltamethrin (Supplementary Fig. 1D) are shown.

4. Discussion

The separate effects and the risks for human health of food pollutants such as marine biotoxins and pyrethroids have been reported and the corresponding authorities have implemented legislation and opinions to control the presence of these compounds in food (Diogène et al., 2021; EFSA, 2015; EFSA, 2020; EFSA, 2010; FDA, 2011). However, the possibility of synergies

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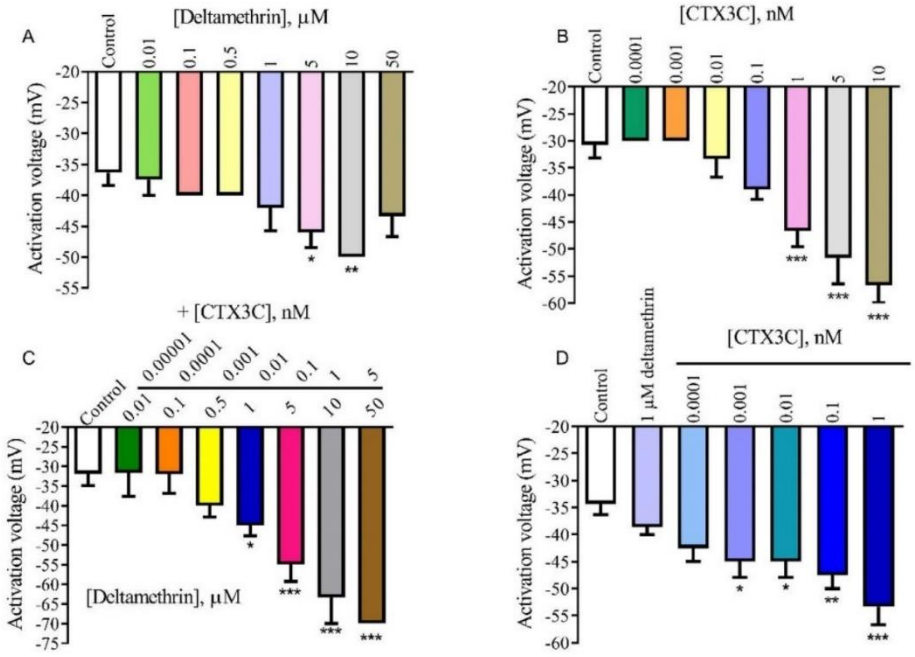


Fig. 5. Single and combined effect of 1 µM deltamethrin and different CTX3C concentrations on the activation voltage of sodium channels. A. Effect of different concentrations of deltamethrin alone in the activation voltage of sodium channels. B. Activation voltage of sodium channels after bath application of different CTX3C concentrations. C. Simultaneous presence of deltamethrin and CTX3C hyperpolarized the activation voltage of sodium channels. Bar graphs represents the different concentrations of deltamethrin in the recording chamber together with different ciguatoxin concentrations indicated in the top. D. Activation voltage of sodium channels in control conditions and after bath application of 1 µM deltamethrin with increasing CTX3C concentrations. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control currents.

between environmental pollutants that share their mechanism of action has not been considered so far. Since the marine biotoxins ciguatoxins and the pyrethroid deltamethrin have a common molecular target (VGSC), and that the food pyramid recommendations for a healthy diet indicate that humans are exposed to all these pollutants simultaneously (Kumar et al., 2020; Sirohi et al., 2021; Zhang et al., 2021), the present work was undertaken. The main route of human exposure to ciguatoxins is due to the consumption of contaminated fish including tuna fish, salmon, hake, amberjack, bass and grouper fish, and the major contributors of human deltamethrin exposure are maize (49 %), wheat (22 %) and milk (12 %) (EFSA, 2020; EFSA, 2010). Therefore, the aim of this work was to evaluate, for the first time, the existence of interactions between food and water pollutants that share their cellular targets.

Deltamethrin has been reported to prolongate the opening state of VGSC and hyperpolarize the activation voltage of sodium channels (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007; Field et al., 2017) and ciguatoxins have the same effect (Martin et al., 2014; Martin et al., 2015a; Martin et al., 2015b; Raposo-Garcia et al., 2022). Both type of compounds may have a synergistic effect enhancing their combined effect on the peak amplitude of sodium channels currents, and consequently this potential effect was evaluated using a parameter that has historically been used to assess the in vitro potency of compounds (Bothe and Lampert, 2021; Brown and Narahashi, 1992; de la Cerda et al., 2002; OECD, 2018; Raposo-Garcia et al., 2022). The results presented here indicate an increase in the potency of CTX3C by 1000 times after its combination with deltamethrin, while the potency of deltamethrin alone was increased 10 times in the presence of CTX3C. Therefore, a synergistic effect of both compounds on sodium channels, following the Chou & Talalay method (Chou, 2010) either in the sodium current amplitude or in the activation voltage of sodium channels was observed.

Hyperpolarization shifts in the activation voltage caused by type II pyrethroids insecticides, specifically deltamethrin, have been previously reported (Bothe and Lampert, 2021; Chinn and Narahashi, 1986; Davies et al., 2007) and in this study were confirmed since deltamethrin at concentrations of 5 µM deltamethrin caused a 10 mV negative shift in sodium channel activation. Similarly different CTX3C concentrations shifted the activation voltage of human sodium channels to more negative action potentials at concentrations from 1 nM and higher. Remarkably, when cells were exposed to a combination of 1 µM deltamethrin and 0.001 nM CTX3C, that separately did not cause any effect, the activation voltage of sodium channels was significantly shifted by about 19 mV to the negative side. These results are in accordance with previous reports which described that the activation voltage is more sensitive to detect the effect of CTXs on VGSC (Raposo-Garcia et al., 2022). This fact is very important regarding food safety since the consequence of the hyperpolarizing change in the activation voltage of sodium channels, derive in a hyperpolarization at resting cell membrane potentials, which in excitable cells could lead to hyperexcitability and the generation of spontaneous action potentials at very low concentrations (Nicholson and Lewis, 2006).

Although the primary site of action of deltamethrin are voltage-dependent sodium channels (Chinn and Narahashi, 1986), the compound can also decrease the opening probability of voltage-dependent chloride channels (Forshaw et al., 1993; Forshaw and Ray, 1990; Hodgdon, 2008). Therefore, an intracellular chloride load can be expected with is in accordance with the increase in the output chloride currents reported in this work. An effect that supports the idea that the action of pyrethroid compounds in chloride channels is also relevant to pyrethroid poisoning as previously suggested (Forshaw et al., 1993; Forshaw and Ray, 1990).

We conclude that there is a synergistic effect of ciguatoxins and pyrethroids in VGSC. A fact demonstrated after the simultaneous exposure of the cells to a mixture of low concentrations of both compounds which enhanced their separate decrease of the maximum peak inward sodium current and increased the negative shift in the activation voltage of sodium channels caused independently by each environmental pollutant.

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Therefore, this work confirms the need to set regulations concerning levels of compounds that share the same mechanism of action, even though they reach humans through different consumable products because daily diet involves many compounds that enter the body through food and water and share their mechanism of action. As demonstrated in this work, deltamethrin and CTX3C, have a synergistic effect that could be harmful to human health, even when they are ingested in lower amounts than those recommended. The findings reported here are relevant since exposure to CTXs has been reported to cause neurological disorders, such as paraesthesia, reversal temperature perception or ataxia among others (L'Herondelle et al., 2020) and the chronic exposure to pyrethroids may also be associated to neurological deficits such as Parkinson's disease, Alzheimer dementia (Hansen et al., 2017; Van Maele-Fabry et al., 2012; Zaganas et al., 2013) or even, in children, pyrethroid exposure was related to neurodevelopment disorders in early-life exposure (Oulhote and Bouchard, 2013; Richardson et al., 2015; Viel et al., 2017). Thus, as most mammals and humans are exposed continuously to a mixture of pyrethroids and marine biotoxins it remains to be seen how the combined effects of these compounds affect long-term neuronal function in a chronic exposure (Wakeling et al., 2012). Finally, it is important to remark that an acceptable daily intake (ADI) of 0.01 mg/kg bw per day of deltamethrin and an acute reference dose (ARfD) of 0.05 mg/kg bw has been set by the Joint Meeting on Pesticide Residues (JMPR) (https://apps.who.int/food-additives-contaminants-jecfa-database/chemical.aspx?chemID=70). In this regard, EFSA on its latest report on pesticide residues in food reported that, despite the regulations in the legislation and the controls, deltamethrin is one of the substances that appear in levels exceeding their corresponding MRL. Moreover, deltamethrin is one of the pesticides most often found in samples of the EFSA monitoring program (EFSA, 2020). Thus, human exposition to this pollutant should not be despicable. All this, together with the global increase of the incidence of CFP (Botana, 2016; Mattei et al., 2014; Varela Martínez et al., 2021) and the fact that there are still no regulatory limits in the European legislation for ciguatoxins in food, emphasizes the need to further study the combined effect of the exposure to these pollutants especially since synergy has been demonstrated in the present work.

5. Conclusions

There is a synergistic effect of the marine biotoxin CTX3C and the pesticide deltamethrin regarding their effect on VGSC both decreasing the maximum peak inward sodium currents and hyperpolarizing their activation voltage Thus, it is important to remark that the establishment of LMR in the legislation separately for compounds that share the same cellular target could risk the health of consumers and highlights the need of a different approach to study their in vivo and in vitro toxicity in order to establish limits that guarantee the safety of consumers.

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CRediT authorship contribution statement

Sandra Raposo, Celia Costas, writing original draft, analyzing data, Carmen Vale, M. Carmen Louzao formal analysis. Luis M Botana: funding. All authors have read and agreed to the published the version of the manuscript.

Data availability

No data was used for the research described in the article.

Declaration of competing interest

The authors declare that they have not known competing financial interest or personal relationships that could have appeared to influence the study.

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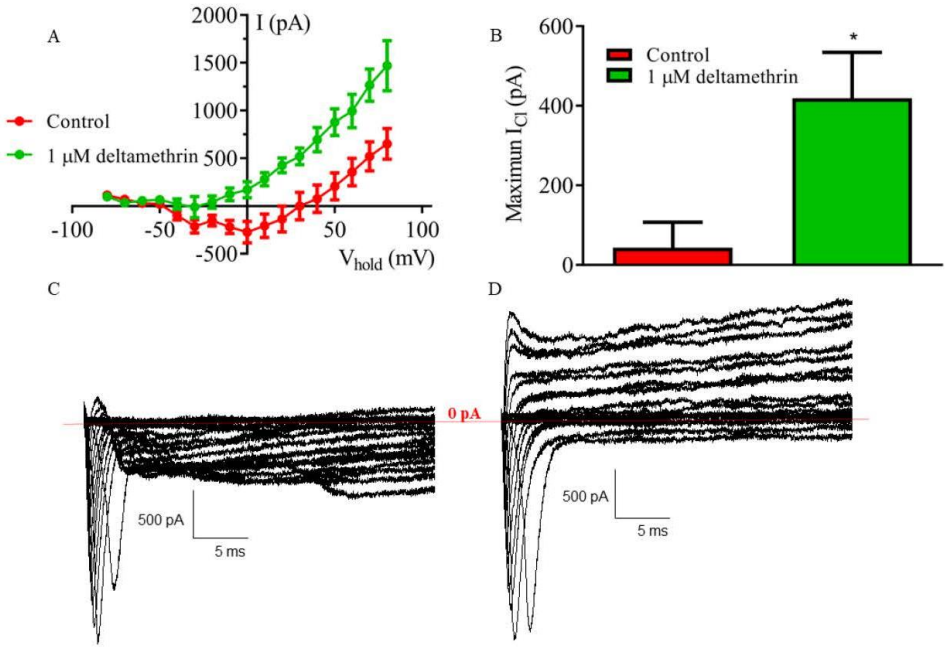
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Synergistic effect of environmental food pollutants: pesticides and marine biotoxins

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Supplementary Figure 1: Effect of deltamethrin on the amplitude of chloride currents. A. I–V curve for the activation of chloride currents in the absence and presence of 1 µM deltamethrin showing a significantly increase in I_{Cl}. Currents were obtained 5 min after bath application of solvent or 1 µM deltamethrin. B. Bar graphs showing the mean I_{Cl} amplitude in absence or in presence of deltamethrin 1 µM. C. Recording traces of chloride currents in control cells. D. Corresponding recording traces of chloride currents in toxin-treated cells and after bath addition of 1 µM deltamethrin. Representative current traces were obtained at voltage steps ranging from −80 to +80 mV with 10 mV step increases. Data are mean ± SEM of four different cells. *p < 0.05 vs control currents.

Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins

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Publicación

Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C in human voltage-gated $Na_v1.6$ sodium channels.

Resumen

Las biotoxinas marinas emergentes, como las ciguatoxinas y las brevetoxinas, se han estudiado amplia e independientemente como contaminantes alimentarios. Sus niveles máximos en los alimentos se fijaron sin considerar sus posibles efectos sinérgicos como consecuencia de su coexistencia en productos del mar y su acción sobre la misma diana celular. La absoluta falta de datos y normativa de los posibles efectos combinados que puedan tener ambas biotoxinas marinas planteó la necesidad de analizar sus efectos directos *in vitro* mediante técnicas de electrofisiología. Los resultados presentados en este estudio indican que las ciguatoxinas y brevetoxinas tuvieron un efecto sinérgico en los canales de sodio dependientes de voltaje $Na_v1.6$ humanos al causar un cambio negativo en sus voltajes de activación e inactivación. Los resultados presentados aquí indican que la brevetoxina 3 actúa como agonista parcial de los canales de sodio humanos, mientras que la ciguatoxina 3C es un agonista completo, lo que explica las diferencias en el efecto de cada toxina en el canal. Por tanto, este trabajo sienta las bases celulares para seguir aplicando este tipo de estudios de toxicidad combinada a otros contaminantes alimentarios que puedan actuar de forma sinérgica y así implementar los límites regulatorios correspondientes considerando su coexistencia y los riesgos para la salud humana y animal derivados de la misma.

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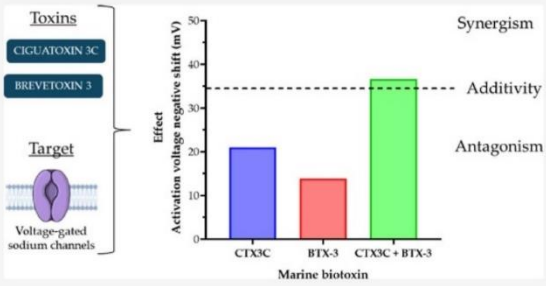
Synergistic Effect of Brevetoxin BTX-3 and Ciguatoxin CTX3C in Human Voltage-Gated $Na_v1.6$ Sodium Channels

Sandra Raposo-Garcia, Celia Costas, M. Carmen Louzao, Mercedes R. Vieytes, Carmen Vale,* and Luis M. Botana*

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ABSTRACT: Emerging marine biotoxins such as ciguatoxins and brevetoxins have been widely and independently studied as food pollutants. Their maximum levels in food components were set without considering their possible synergistic effects as consequence of their coexistence in seafood and their action at the same cellular target. The absolute lack of data and regulations of the possible combined effects that both marine biotoxins may have raised the need to analyze their direct *in vitro* effects using electrophysiology techniques. The results presented in this study indicate that ciguatoxins and brevetoxins had a synergistic effect on human $Na_v1.6$ voltage-gated sodium channels by hyperpolarizing their activation and inactivation states. The results presented here indicate that brevetoxin 3 (BTX-3) acts as partial agonist of human sodium channels, while ciguatoxin 3C (CTX3C) was a full agonist, explaining the differences in the effect of each toxin in the channel. Therefore, this work sets the cellular basis to further apply this type of studies to other food toxicants that may act synergistically and thus implement the corresponding regulatory limits considering their coexistence and the risks to human and animal health derived from it.



1. INTRODUCTION

Brevetoxins (BTXs), produced naturally by dinoflagellates of the genus *Karenia brevis*, and ciguatoxins (CTXs), produced by *Gambierdiscus* and *Fukuyoa* genera, are cyclic polyether toxins with analogous chemical structures, shown in Figure 1. Both BTXs and CTXs can accumulate along the marine food chain, leading to seafood poisoning in humans and animals. The ingestion of BTX- and CTX-contaminated fish and shellfish results in quite similar symptomatology including gastrointestinal, neurological, and cardiovascular symptoms commonly known as neurotoxic shellfish poisoning (NSP)[1] and ciguatera poisoning (CP), respectively. However these symptoms are more severe and longer-lasting for CP.[2] These physiological alterations caused by CTXs and BTXs are a consequence of their effect altering the voltage-gated sodium channels (VGSCs), which are essential transmembrane proteins involved in cellular excitability.[3] However, despite being similar in their mechanism of action, there are differences in the reported effects and potency between the two groups of toxins that indicate that BTXs act at micromolar concentrations, while CTXs present physiological effects at nanomolar concentrations.[4,5]

CTXs and BTXs bind to a common site of the sodium channel located at the cleft created by segment S5 of domain IV, segment S6 of domain I, and the P-loop (P1) that connects segments S5 and S6 of domain IV of the VGSC α-subunit[6,7] but with different affinities. Previous studies indicated that CTXs interact specifically and with higher affinity with sodium channels;[8,9] however, the differences in the chemical structures between the CTX analogues generate modifications in their binding and activity over VGSC.[10,11] BTXs are also considered to have a high binding affinity to sodium channels;[12,13] however, the differences between both groups of toxins are significant since studies of their binding affinity to rat brain sodium channels allowed to calculate an inhibitory constant (ki) of 0.041 nM for ciguatoxin-1B which was more than 50-fold higher than that of BTX-1 (2.24 nM).[10] The difference in binding to VGSC among BTXs and ciguatoxin 3C (CTX3C) is less noticeable since CTX3C has only 20-fold higher affinity (ki = 0.47 nM) than that of BTX-3s, at concentrations of 1 nM of the compounds.[10] Because of the higher binding affinity of CTXs to VGSC, CTX3C can displace brevetoxin 3 (BTX-3) from their binding at site 5[14] of the protein.

The functional consequence of the interaction of CTXs and BTXs with VGSC is a hyperpolarizing shift in the sodium

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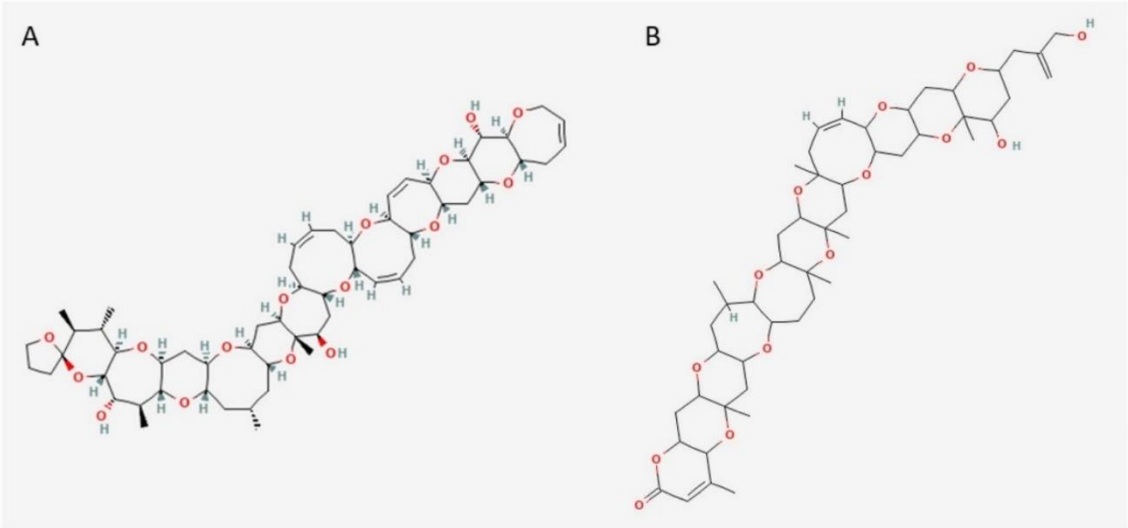


Figure 1. Chemical structure of the studied marine toxins, CTX3C (A) and BTX-3 (B).

channel activation curve[5,7,15] as well as a negative shift of their half inactivation voltage.[16] CTXs are known to have a significant effect on the activation and inactivation state of VGSC at concentrations of 1 nM and higher.[7,11,17] As a consequence, the channels will remain permanently open at resting cell membrane potentials, resulting in depolarization of the membrane and spontaneous and/or repetitive action potential discharges in excitable cells.[6,9] Although both groups of toxins interact with the same site of VGSC, there are important differences between the observed cellular effect of each group since CTXs cause a remarkable decrease in the maximum peak inward sodium current amplitude (I_{Na}),[7,11,17] while no significant effects were reported for BTXs.[4,5] It is important to note that the effects and the affinity of CTXs or BTXs for VGSC depend on the analogue and the channel subtype.[18] Moreover, the binding of BTXs to sodium channels is highly state-dependent, thus, channel opening facilitates BTX binding, feeding back their binding and sodium influx.[15]

Due to the danger that marine biotoxins represent for human and animal health, their limits for international trade in fishery products were set more than a decade ago by the Codex Committee on Fish and Fishery Products (CCFFP) (CODEX STAN 292-2008). The levels of biotoxins in live and raw bivalve mollusk flesh were established for different groups of marine toxins taking into account the limits for each biotoxin group.[19] The maximum levels for BTXs were established as 20 MU (mouse units)/100 g shellfish flesh (800 μg/kg) for BTXs;[19] however, recent studies have reported that this maximum level does not appear protective enough[20] based on the previous lowest observed adverse effect level established at 0.3−0.4 MU/kg body weight.[21] Currently, the American FDA applies a threshold of 800 μg BTX-2 equivalents/kg shellfish flesh and a guidance level for Caribbean CTXs of 0.1 μg/kg C-CTX1 equivalents and 0.01 μg/kg for P-CTX1B.[22] Although these marine biotoxins constitute an emerging risk in Europe, there are still no regulatory official limits, and only a guidance level of 180 μg BTX-3 eq/kg shellfish meat was proposed by the French Agency for Food, Environmental, and Occupational Health and Safety.[20]

The worldwide spread of emergent marine biotoxins, already affecting European coasts, and the scarce reliable information about their biological effects led to several international organizations such as FAO, WHO, and EFSA highlighting the need for a full re-evaluation of the toxicity and relative potencies of these food contaminants.[23,24] Moreover, the coexistence of CTXs and BTXs in fishery products has been previously reported.[25−28] In addition, the possibility of misdiagnosis of NSP with CP has been considered due to their similar symptomatology, and methods to discriminate CTXs from BTXs in fish tissue had been developed.[26] Both, BTXs and CTXs, can also accumulate in water and air,[29] increasing the risk of coexposure to both groups of marine biotoxins. So far, the broad reports on the activity of marine toxins evaluated only their separate effects even when they share the same molecular target. The co-occurrence of BTXs and CTXs may represent a potential risk for human health.[30,31] In this work, the potential risks of the simultaneous presence of both groups of toxins in food based on their effects in the functional activity of human sodium channels have been evaluated for the first time.

2. MATERIALS AND METHODS

2.1. Chemicals and Toxins. Pacific CTX CTX3C was purchased from Wako (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany, purity 99%) and dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 10 μM. For experiments, 1 μM solutions were made in Lockés buffer containing: 154 mM NaCl, 5.6 mM KCl, 1.3 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM *N*-(2-hydroxyethyl)piperazine-N′-ethanesulfonic acid (HEPES), and 5.6 mM glucose. The pH was adjusted to 7.4 with Trizma base. BTX-3 with a 95% purity was purchased from Latoxan (France) and dissolved in ethanol at a final concentration of 50 μM. For experiments, consecutive dilutions were performed in physiological Lockés buffer. The maximum solvent concentration used had no effect on VGSCs. All other chemicals were of reagent grade and purchased from Sigma.

2.2. Human Cell Cultures. The human embryonic kidney cell line (HEK293) transfected with the α subunit of the $Na_v1.6$ sodium channel isoform was kindly provided, under a material transfer agreement, by GlaxoSmithKline R&D (Stevenage, U.K). The cells

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Table 1. Activation Voltage of Human $Na_v1.6$ VGSC after Cell Exposure to CTX3C, BTX-3, and a Combination of Increasing Concentrations of Both Marine Biotoxins

[CTX3C], nM	activation voltage for CTX3C (mV)	activation voltage for CTX3C + BTX-3 (mV)	activation voltage for BTX-3 (mV)	[BTX-3], nM
control	−30.7 ± 2.4	−30.0 ± 2.9	−36.2 ± 1.2	control
0.0001	−30.0 ± 0	−37.8 ± 3.2	−38.3 ± 1.1	0.1
0.001	−30.0 ± 0	−41.3 ± 2.3	−42.6 ± 1.0	1
0.01	−33.3 ± 3.3	−41.4 ± 1.4	−44.0 ± 1.6	5
0.1	−39.0 ± 1.8	−48.3 ± 4.8	−45.0 ± 2.2	10
1	−46.7 ± 2.9	−66 ± 4	−50.0 ± 0	50
5	−51.67 ± 4.8	−66.7 ± 3.3	−50.0 ± 0	100

were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium enriched with glutamax, nonessential amino acid solution (MEM, Gibco, 1% w/v), 10% fetal bovine serum, and 0.4 mg/mL Geneticin (G418, Gibco) and maintained at 37 °C in a humidified 95% O_2/5% CO_2 atmosphere until they reached 80% of confluence, replacing the medium every 2 days. The cells were subcultured in 12-well plates in glass coverslips at a density of 60,000 cells/mL. Twenty-four or 48 h before electrophysiological recordings, the cells were placed at 30 °C to maximize sodium channel expression.[32]

2.3. Electrophysiological Recordings. For electrophysiological recordings, glass coverslips with cells were placed in a recording chamber with 0.5 mL Lockés buffer as the extracellular solution. Recording electrodes were fabricated with borosilicate glass micro-capillaries (1.5 outer diameter) and had resistances ranging from 4 to 10 MΩ. Pipettes were filled with an intracellular solution containing 120 mM CsF, 10 mM EGTA, 10 mM HEPES, and 15 mM NaCl and pH adjusted to 7.25 with CsOH. Voltage-gated sodium currents were recorded at room temperature 5 min after reaching the whole-cell configuration with a Multiclamp 700B amplifier and digitalized with the Digidata 1440A (both from Axon Instruments, California, U.S.A.) maintaining the cells at a holding potential (V_{hold}) of −55 mV. Signals were sampled at 50 kHz after low-pass Bessel filtering at 10 kHz. Compensation circuitry was used to reduce the series resistance by at least 70%. To record the activation of VGSCs, voltage steps from −80 to +80 mV in 10 mV step increments were applied prior to a test pulse of −90 mV. Fast inactivation was measured at 0 mV after prior test pulses of 300 ms duration ranging from −100 to 0 mV in 10 mV voltage step increments after maintaining the holding potential at −90 mV during 10 ms.

2.4. Statistical Analysis. All data are expressed as means ± SEMs of n determinations. Data analysis was performed using GraphPad Prism 8. Statistical comparisons were performed using one-way analysis of variance (ANOVA), followed by post hoc Dunnetts tests. p values ≤ 0.05 were considered statistically significant, and IC_{50} values were determined by fitting the data with a log (inhibitor) vs normalized response model.

The combination index (CI) was calculated with the Chou−Talalay equation[33] following the formula

$$\text{combination index} = \frac{D1}{(DX)1} + \frac{D2}{(DX)2} \quad (1)$$

where $(DX)1$ is the IC_{50} value of CTX3C alone, $(DX)2$ the IC_{50} value of BTX-3 alone, and $D1$ and $D2$ are the IC_{50} values of CTX3C and BTX-3, respectively, in combination. According to the Chou−Talalay method, additivity is established if CI = 1, synergism if CI < 1, and antagonism if CI > 1.

3. RESULTS

Due to the co-occurrence in seafood products of BTXs and CTXs and the cellular target being the same, the sodium channels, in the present work, the single and combined effects of pacific ciguatoxin CTX3C and BTX-3 in human sodium channels were studied.

3.1. Effect of CTX3C or BTX-3 on Human VGSC. The effects of increasing CTX3C concentrations, between 0.000001 and 10 nM, on the maximum peak inward sodium currents (I_{Na}) and the activation voltage of human VGSC after single cell exposure were first evaluated and reported by our group.[7] As previously reported,[7,34,35] CTX3C elicited a concentration-dependent decrease in the maximum peak amplitude of sodium currents being detected even at the lowest concentrations. In control conditions, the peak sodium current at −10 mV was −1156 ± 189 pA (n = 13) decreasing in a concentration-dependent manner up to −692 ± 372 pA (n = 3) after bath application of 1 nM CTX3C. The percent inhibition of the peak inward sodium currents by different CTX3C concentrations was used to obtain a concentration−response curve with an estimated IC_{50} of 0.173 nM [95% confidence interval (CI) from 0.000647 to 2.1 nM, R^2 = 0.92]. CTX3C also affected the activation voltage of sodium channels and remarkably shifted in the negative direction after bath addition of 1 nM CTX3C as indicated by one-way ANOVA followed by Dunnett's test. At this concentration, the negative shift in the activation voltage of sodium channels reached −15.9 ± 3.8 mV (p = 0.0004; df = 21; t = 4.1). Higher toxin concentrations also hyperpolarized the activation voltage in a concentration-dependent manner, as summarized in Table 1.

The activity of BTX-3 in VGSC was also evaluated but, in this case, bath application of increasing BTX-3 concentrations, from 0.1 to 100 nM, did not cause any remarkable decrease in the maximum peak inward sodium current. The percent inhibition of VGSC amplitude (I_{Na}) by the different BTX-3 concentrations was used to obtain a concentration−response curve with an estimated IC_{50} of 202 nM (95% CI: 55.3 to 6000 nM). A low variance value, very close to zero (0.23) was obtained, indicating that all the values were in a narrow range obtaining a low dispersion of the data since none of the concentrations elicited an important decrease in sodium current amplitude, with 100 nM BTX-3 decreasing peak sodium current amplitude only by 31.2 ± 15%. Noteworthily, at −20 mV, cell exposure to 1 nM BTX-3 did not elicit any significant decrease in the maximum I_{Na}, which was −3696.2 ± 530.8 pA (n = 25) in control conditions and −2887.70 ± 402.0 pA in the presence of 1 nM BTX-3 (n = 23) which supposes a decrease of 21.9 ± 10.9%. However, cell exposure to the same toxin concentration significantly hyperpolarized the activation voltage of human VGSC by −7.3 ± 1.8 mV (p = 0.0003; t = 4.016, df = 32). These results are in agreement with previous reports, where electrophysiological recordings showed that BTX-3 did not affect the amplitude of I_{Na} neither in protist sodium channels[5] nor in other different cell lines,[36] showing only a 33% decrease in peak inward sodium current in presence of 1000 nM BTXs in diatom sodium channels.[37] Thus, the results obtained show that although CTXs and BTXs share their main site of action in VGSC, they trigger different functional effects, with CTX3C decreasing the maximum I_{Na}

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Table 2. Shift in the Activation Voltage of Human $Na_v1.6$ Sodium Channels after Exposure to CTX3C, BTX-3, and a Combination of Increasing Concentrations of Both Marine Biotoxins

[CTX3C], nM	shift in the activation voltage for CTX3C (mV)	shift in the activation voltage for CTX3C + BTX-3 (mV)	shift in the activation voltage for BTX-3 (mV)	[BTX-3], nM
control	0	0	0	control
0.0001	0.71 ± 5.4	−7.78 ± 4.3	−2.07 ± 1.6	0.1
0.001	0.71 ± 4.7	−11.25 ± 3.7	−6.39 ± 1.5	1
0.01	−2.62 ± 5.6	−11.43 ± 3.5	−7.79 ± 1.9	5
0.1	−8.29 ± 3.3	−18.33 ± 5.2	−8.79 ± 2.7	10
1	−15.95 ± 3.8	−36.00 ± 4.9	−13.79 ± 2.6	50
5	−20.95 ± 4.8	−36.67 ± 5.4	−13.79 ± 3.6	100

and hyperpolarizing the activation voltage of VGSC while BTX-3 only affected their activation voltage, shifting it toward more negative potentials, as summarized in Tables 1−3. Representative sodium channel activation traces at −20 mV in presence of 0.001 nM CTX3C, 1 nM BTX-3, and a combination of both are represented in Figure S1.

Table 3. Normalized Maximum Peak of Sodium Currents after Cell Exposure to CTX3C Alone and CTX3C in the Presence of 10 nM BTX-3

[CTX3C], nM	normalized I_{Na} (max)	+10 nM BTX-3 normalized I_{Na} (max)
control	−1 ± 0.02	−1 ± 0.07
0.0001	−0.79 ± 0.05	−0.89 ± 0.08
0.001	−0.62 ± 0.08	−0.89 ± 0.13
0.01	−0.66 ± 0.09	−0.88 ± 0.13
0.1	−0.63 ± 0.09	−0.90 ± 0.11
1	−0.27 ± 0.07	−0.68 ± 0.09
5	−0.32 ± 0.04	−0.46 ± 0.09

3.2. Functional Consequences of the Combined Effects of CTX3C and BTX-3 on Human VGSC. In view of the results obtained separately for CTX3C and BTX-3 and the possibility of the simultaneous presence of both types of marine biotoxins in food, it is important to study the effects elicited by the presence of both marine compounds. The toxin concentrations evaluated for each compound were selected to cover a wide range of concentrations based on their individual effects in VGSC and the IC_{50} value obtained for each single compound. Therefore, cells were exposed to combined increasing concentrations of CTX3C from 0.0001 to 5 nM and BTX-3 from 0.1 to 100 nM.

The addition of combined increasing concentrations of CTX3C (from 0.0001 to 5 nM) and BTX-3 (from 0.1 to 100 nM) elicited a concentration-dependent decrease in sodium current amplitude and a remarkable hyperpolarization of the activation voltage, as shown in Figure 2A. The percent inhibition of the peak I_{Na} by the simultaneous addition of BTX-3 and CTX3C to the bath solution allowed to obtain a concentration−response curve as a function of the concen-

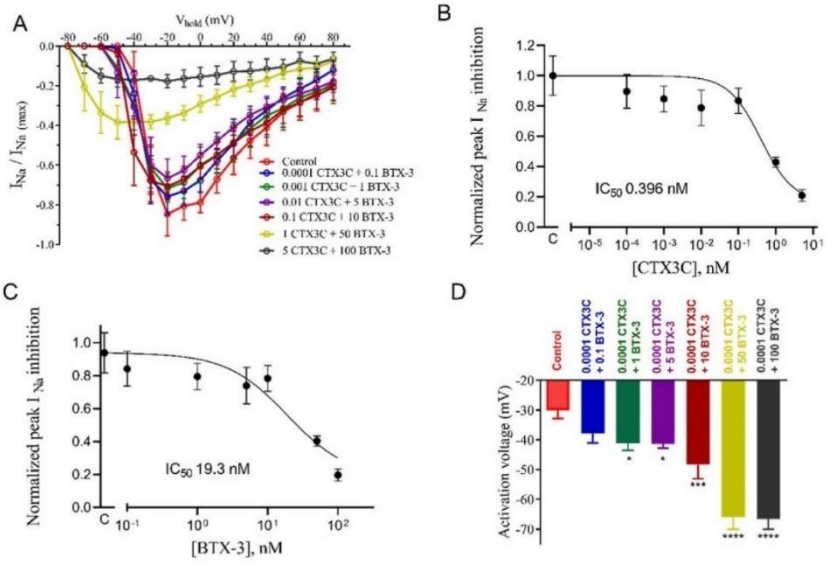


Figure 2. (A) Current−voltage relationship for the effect of different concentrations of combinations of CTX3C and BTX-3 on peak inward sodium currents. Toxin concentrations are expressed in nM. (B) Concentration−response graph for the peak inhibition of sodium currents by different CTX3C and BTX-3 concentrations relative to the CTX3C presence in the recording chamber. (C) Concentration−response graph for the peak inhibition of sodium currents by the simultaneous presence of CTX3C and BTX-3 expressed as a function of the BTX-3 concentration in the recording chamber. (D) Activation voltage of sodium channels in control conditions and after bath application of simultaneous increasing of BTX-3 and CTX3C concentrations, expressed in nM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs* control currents.

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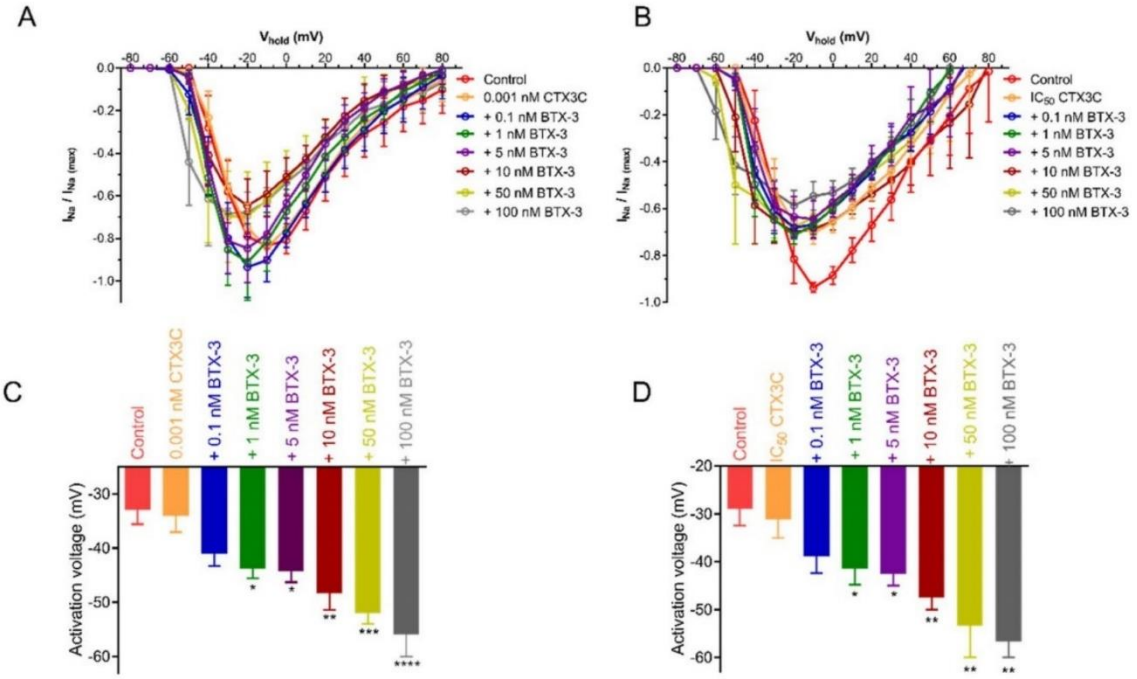


Figure 3. (A) Current−voltage relationship for the effect of CTX3C at 0.001 nM and different BTX-3 concentrations in I_{Na}. (B) Current−voltage relationship for the effect of 0.17 nM CTX3C (concentration that corresponds to its IC_{50} in VGSC) and different BTX-3 concentrations in I_{Na}. (C) Activation voltage of sodium channels in control conditions and after bath application of 0.001 nM CTX3C with increasing BTX-3 concentrations. (D) Bar graph showing the activation voltage of sodium channels in control conditions and after bath application of 0.17 nM CTX3C with increasing BTX-3 concentrations. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs* control currents.

trations of each compound. Nonlinear fit of these data yielded an estimated IC_{50} based on CTX3C concentrations when combined with BTX-3 of 0.396 nM (95% CI: 0.0243 to 3.97 nM, R^2 = 0.85), represented in Figure 2B, while the IC_{50} calculated taking into account the different BTX-3 concentrations in the presence of CTX3C was 19.3 nM (95% CI: 9.37 to 39.2 nM, R^2 = 0.90), as illustrated in Figure 2C.

The activation voltage has been reported to be more sensitive to detect the effects of VGSC modulators;[11] therefore, this parameter was evaluated to obtain the combination index of both marine biotoxins. In this case, the effect observed was very pronounced, as shown in Figure 2D. A significant negative shift of the activation voltage was obtained after cell exposure to concentrations of CTX3C as low as 0.001 nM; that alone did not elicit any change, with 1 nM BTX-3 hyperpolarizing the activation voltage by −6.39 ± 1.5 mV by itself, while the combination produced a hyperpolarization of the activation voltage of −11.25 ± 3.7 mV. The activation voltages in each condition are shown in Tables 1 and 2.

In summary, the concomitant increase of the concentrations of both marine biotoxins in the bath chambers shifted the VGSC activation voltage to more negative potentials, as shown in Table 2, which summarizes the changes in the activation voltage of human VGSC caused either by CTX or BTX alone or the combination of both compounds.

3.3. Functional Consequences Elicited by a Constant Low CTX3C Concentration and Increasing BTX-3 Concentrations on Human VGSC. In view of the results obtained over VGSC after cell exposure to different CTX3C and BTX3 combinations and to further explore their predominant effects, the cells were exposed to a constant CTX3C or BTX-3 concentration for 5 min in the recording chamber and increasing concentrations of the other toxin were added.

3.3.1. Evaluation of the Effect of a Single Low Concentration of CTX3C with Increasing BTX-3 Concentrations on Human VGSC. Previous studies have reported a higher affinity of CTX3C to sodium channels than for BTXs;[8,9,14] therefore, the next step was to study the effect of a constant low CTX3C concentration and progressively increasing the concentration of BTX-3 in the recording chamber. First, CTX3C at 0.001 nM was tested in combination with increasing BTX-3 concentrations. These data were compared with the effects of a constant CTX3C concentration equal to its IC_{50} concentration in the presence of different BTX-3 concentrations. As shown in Figure 3A, the addition of 0.001 nM CTX3C and increasing BTX-3 did not elicit any change in I_{Na}. However, in cells exposed to 0.17 nM CTX3C, a decrease of the maximum I_{Na} peak was elicited, but it was not enhanced by the presence of further increasing BTX-3 concentrations, as represented in Figure 3B. In addition, the activation voltage of VGSC in these conditions was analyzed. As shown in Figure 3C, cell exposure to CTX3C at 0.001 nM did not modify the activation voltage of VGSC when added alone to the recording chamber, but its effect was enhanced after the addition of BTX-3 at concentrations of 1 nM and higher. Similarly, as shown in Figure 3D, when cells were exposed to 0.17 nM CTX3C, no changes in the activation

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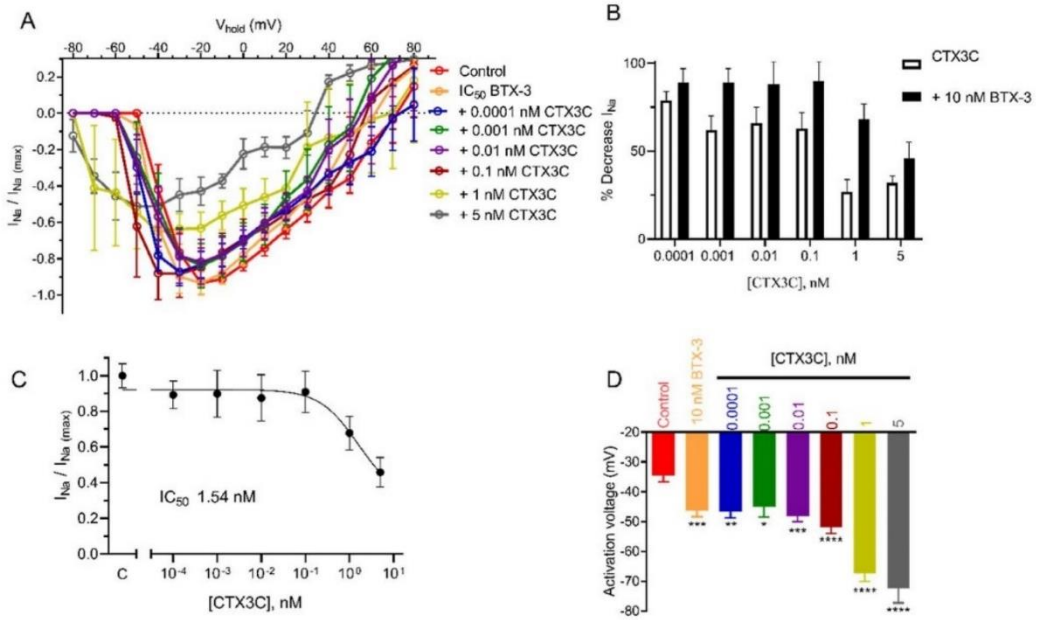


Figure 4. (A) Current−voltage relationship for the effect of 10 nM BTX-3 and increasing CTX3C concentrations on sodium current amplitude. (B) Percent decrease of I_{Na} caused by BTX-3 alone and the simultaneous presence of increasing CTX3C concentrations. (C) Concentration-curve graph for the peak inhibition of sodium currents by different CTX3C concentrations in the presence of 10 nM BTX-3 in the recording chamber. (D) Activation voltage of VGSC in control conditions and after bath application of 10 nM BTX-3 with increasing CTX3C concentrations. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ *vs* control currents.

voltage were observed; however, bath application of BTX-3 at concentrations of 1 nM and higher caused a negative shift in their activation voltage. These effects are summarized in Table 2.

3.3.2. Evaluation of the Effect of a Single BTX-3 Concentration with Increasing CTX3C Concentrations in Human VGSC. Previous studies have reported that CTX3C was able to displace BTX-3 from site 5;[14] therefore, the effect of CTX3C in sodium channels was evaluated in the presence of an active BTX-3 concentration of 10 nM. BTX-3 alone hyperpolarized the activation voltage of human VGSC by −8.79 ± 2.7 mV, but higher CTX3C concentrations were needed to decrease peak sodium currents, as represented in Figure 4A,B. Thus, the presence of BTX-3 in the bath decreased the effect of CTX3C alone in I_{Na}, as summarized in Table 3.

Nonlinear fit of these data yielded an estimated IC_{50} for CTX3C in the presence of 10 nM BTX-3 of 1.54 nM (95% CI: 0.797 to 3.03 nM, R^2 = 0.95), represented in Figure 4C. As expected, the activation voltage of the sodium channels was significantly affected by the presence of 10 nM BTX-3 alone, but this effect was exacerbated after addition of different CTX3C concentrations to the recording chamber, as shown in Figure 4D.

The negative displacement in the activation voltage of VGSC indicated that CTX3C acted as a full agonist of the channels, while BTX-3 was a partial agonist. This fact is demonstrated by the observation that a high concentration of BTX-3 caused a response typical of a competitive antagonist decreasing the effect observed with the full agonist alone. However, increasing the concentration of CTX3C displaced the partial agonist from their binding site and led to the decrease in I_{Na} caused by CTX3C alone. These results are illustrated in Figure 5.

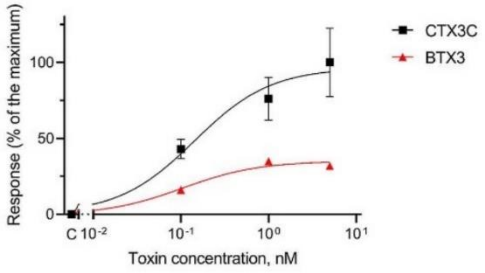


Figure 5. Graphical representation of the profile of the percentage of response on the negative change of the activation voltage of human VGSC produced by the full agonist CTX3C (black) and the partial agonist BTX-3 (red) added in the same concentrations to the bath chamber.

3.4. Combined Effects of BTX-3 and CTX3C on Sodium Channel Activation: Combination Index. The results obtained in this study, as previously described for CTXs alone,[11] led to the conclusion that the change in VGSC activation could be the most sensitive parameter to analyze their functional effects in sodium channels. Figure 6 summarizes the effect of cell exposure to the different BTX-3 and CTX3C concentrations employed. These values of the activation voltage of human VGSC caused by CTX3C, BTX-3, and the combination of both were used to obtain the concentration−response curves. Nonlinear fit of the data

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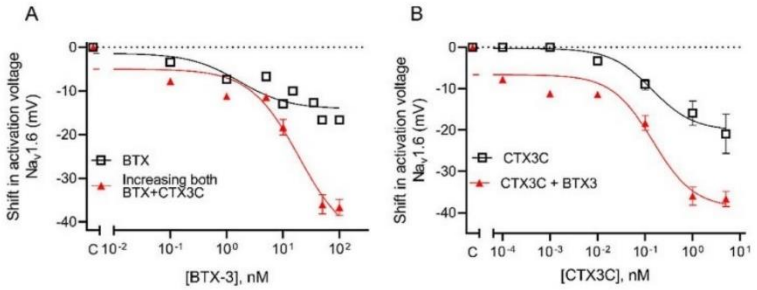


Figure 6. Single and combined effects of different CTX3C and BTX-3 concentrations in the activation voltage of human $Na_v1.6$ VGSC. (A) Concentration−response graph for the negative shift in the activation voltage in different conditions represented against the BTX-3 concentrations in the bath chamber. (B) Concentration−response graph for the negative shift of the activation voltage in different conditions as a function of the CTX3C concentrations added to the cells.

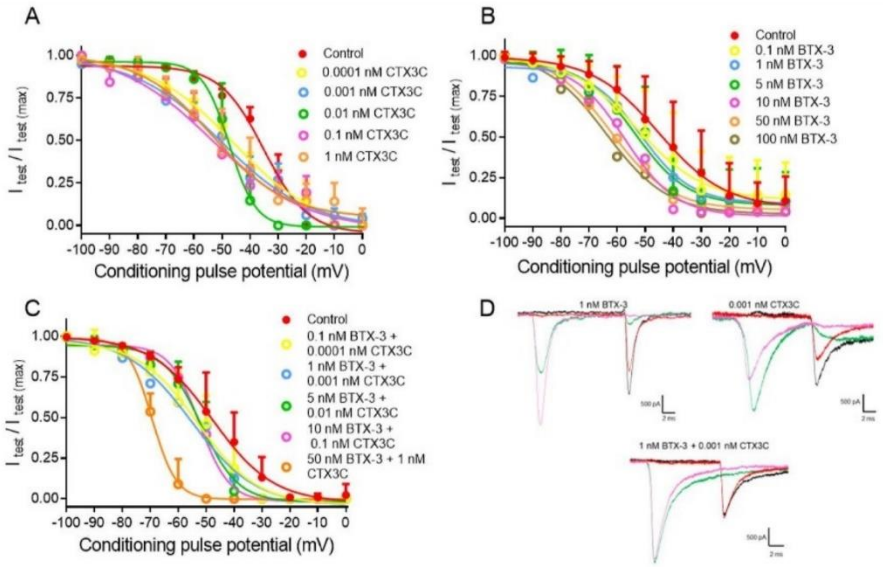


Figure 7. Single and combined effect on the fast inactivation of human VGSC of different CTX3C and BTX-3 concentrations and combinations. (A) Effect of CTX3C on $Na_v1.6$ VGSC inactivation. (B) Effect of BTX-3 over the fast inactivation of $Na_v1.6$ VGSC inactivation. (C) Effect of different CTX3C and BTX-3 combinations on the fast inactivation of human sodium channels. (D) Sodium current inactivation traces under different treatment conditions obtained at a prepulse potential of −60 mV (black for control and red for treated traces) and a prepulse of −30 mV in control (green traces) and treated cells (pink traces).

yielded an estimated IC_{50} of 46.2 nM (95% CI: 35.5 to 60.8 nM) in the basis of the bath concentration of BTX-3 and an IC_{50} of 7.45 nM (95% CI: from 5.39 to 1.03 nM) for the simultaneous presence of CTX3C and BTX-3 based on BTX-3 concentrations, represented in Figure 6A as black and red lines respectively. Similarly, an IC_{50} for CTX3C of 2.58 nM (95% CI: 1.78 to 3.79 nM) and 0.195 nM (95% CI: 0.078 to 0.184 nM) was found for the simultaneous presence of CTX3C and BTX-3 based on CTX3C concentrations (Figure 6B). With these values, the combination index was determined and yielded a value of 0.24. This value is lower than 1, which indicates that the effect of these two marine biotoxins in the activation voltage of human VGSC is synergistic.

3.5. BTX, CTX, and Their Combinatory Effects on the Inactivation State of Human VGSC. BTX-3 inhibits the fast inactivation of VGSC by its interaction with multiple active centers of the protein, specifically the A-ring lactone and the C-42 of the R side chain interacting with site 5 of the channel.[15,16] Similarly, CTX3C shifts the half inactivation voltage ($V_{1/2}$) in the negative direction.[38,39] Thus, the effect of both compounds in the fast inactivation voltage of the channels was analyzed. As shown in Figure 7, BTX-3 elicited a significant change in the fast inactivation of human VGSC, $V_{1/2}$ was −46.8 ± 1.8 mV (n = 20) in control conditions and −62.1 ± 3.9 mV (n = 18; p = 0.04) after bath application of 1 nM BTX-3. Cell exposure to higher toxin concentrations altered the inactivation state of VGSC, as shown in Figure 7A. In the same context, CTX3C shifted $V_{1/2}$ of inactivation toward more negative potentials (Figure 7B) even at the lowest concentration studied. In control conditions, $V_{1/2}$ of inactivation was −35.1 ± 1.2 mV (n = 7) and −45.8 ± 3.3 mV (n = 8) after bath application of CTX3C at 0.0001 nM (p = 0.0076). Thus, the combination of both marine biotoxins showed an enhanced effect on the fast inactivation state of

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Table 4. Inactivation Voltage of Human VGSC After Cell Exposure to CTX3C, BTX-3, and a Combination of Increasing Concentrations of Both Marine Biotoxins

[CTX3C], nM	inactivation voltage for CTX3C (mV)	inactivation voltage for CTX3C + BTX-3 (mV)	inactivation voltage for BTX-3 (mV)	[BTX-3], nM
control	−36.1 ± 1.2	−41.7 ± 1.0	−45.1 ± 1.8	control
0.0001	−45.8 ± 3.3	−53.1 ± 1.7	−52.9 ± 2.1	0.1
0.001	−50.1 ± 4.1	−55.3 ± 2.2	−51.6 ± 2.1	1
0.01	−47.4 ± 1.4	−51.9 ± 1.2	−53.6 ± 2.8	5
0.1	−53.4 ± 6.8	−52.4 ± 0.9	−57.6 ± 2.4	10
1	−54.9 ± 5	−69.1 ± 0.4	−62.1 ± 3.9	50

human VGSC, as represented in Figure 7C and summarized in Table 4. A bar graph representation of the changes in $V_{1/2}$ of human VGSC after cell exposure to all studied conditions are shown in Figure S2.

The combination index for the effect of BTX-3 and CTX3C in the fast inactivation voltage of human VGSC was also calculated using the values of the hyperpolarizing shift in the inactivation voltage elicited by CTX3C, BTX-3, and the combination of both. Nonlinear fit of the data yielded an estimated IC_{50} for BTX-3 of 4700 nM (95% CI: 3300− 7800 nM) and 2500 nM (95% CI:11,580− 6260 nM) for the simultaneous presence of CTX3C and BTX-3 in function of the BTX-3 concentrations. An IC_{50} for CTX3C of 306 nM (95% CI:180− 900 nM) and 59.9 nM (95% CI: 33.8−247 nM) was obtained for the simultaneous presence of CTX3C taking into account the concentration of CTX3C. The combination index yielded a value of 0.73. This value is lower than 1 confirming a synergistic effect of CTX3C and BTX-3 on the inactivation state of human VGSC.

4. DISCUSSION

The increasing expansion of marine biotoxins leads to a worldwide health concern that prompted public organizations to effectively evaluate the risks they pose to human health.[19,22,40] CTXs and BTXs are two groups of compounds which share this characteristic, acting on the same cellular target, leading to similar neurological symptomatology in humans,[1,2] and they cause similar *in vitro* effects. The combined effects of both food contaminants on sodium channel functionality have not been previously studied. CTXs decreased the maximum peak inward sodium currents at very low concentrations and cause a negative change in the activation voltage of up to 20 mV of VGSC, which is in accordance to previous reports[7,11,17] and also their fast inactivation state.[35,38] BTXs also caused an important negative displacement in the activation voltage of sodium channels of up to 13 mV not modifying the maximum I_{Na}[4,5] and hyperpolarizing their fast inactivation voltage.[16]

The study of the functional effect elicited by the simultaneous presence of both marine toxins demonstrated that there is an enhancing effect on the negative shift of the activation voltage of human sodium channels when both BTXs and CTXs were combined. Therefore, a synergistic effect of both compounds in the activation voltage of human VGSC was demonstrated. The results presented here constitute the first report displaying an increased potency of CTX3C and BTX-3 on the activation voltage of human VGSC by 10 times after its combination. The consequence of the negative change in the activation voltage of VGSC triggered by both marine biotoxins in excitable cells is hyperexcitability.[41] The results obtained for the fast inactivation state of sodium channels supported their synergistic effects and were in accordance with previous studies that found a shift of the $V_{1/2}$ of inactivation by −15 or −18 mV[15,16,38,39] in the negative direction. Since VGSC inactivation is a critical determinant of action potential frequency, defective inactivation is a hallmark of channel alterations, underscoring the critical importance of the inactivation function of VGSC in excitable cells.[42,43] All these results explain the effects previously reported indicating spontaneous depolarization and oscillations of the membrane potential[17,35,44,45] as well as the *in vivo* neurological symptomatology caused by these toxins.[1,2] In summary, the results presented here indicate that CTXs and BTXs have a synergistic effect on human VGSC since the simultaneous exposure of the cells to a mixture of low concentrations of both compounds enhanced the effect elicited independently by each toxin on the activation as well as on the inactivation voltage of human VGSC. Therefore, the potentiation effect between BTXs and CTXs could pose a risk for human health, even at very low amounts since both compounds lead to neurological symptomatology that would be exacerbated by the concomitant presence of both seafood toxins.

The description of BTXs as partial agonists of the site 5 of VGSC has been previously reported indirectly in cerebellar granule cell neurons using calcium influx measurements.[46] However, these experiments were performed in cells containing a wide array of receptors[47] and in the presence of 0.04% pluronic acid that could alter cell membrane integrity,[48] but no direct effect using electrophysiological methods has been reported so far. Therefore, the results presented in this paper constitute the first evidence of the synergic action of BTXs, acting as partial agonists of human VGSC, and CTX CTX3C as a full agonist of human VGSC. Despite acting on the same cellular target, the differences in the activity of both compounds on VGSC can be explained by the full agonist and partial agonist model. Remarkably, VGSCs undergo conformational changes reflecting their transition from resting to activated/open and to inactivated/closed states.[49] In this paper, the functional interaction of BTXs and CTXs with VGSC was directly evaluated, and it was confirmed that the activation voltage of VGSC was more sensitive than the amplitude of sodium currents to detect the CTX and BTX groups of marine toxins.[11]

So far, the commonly used cell-based method for BTX and CTX detection was based on the evaluation of cell viability in the presence of ouabain and veratridine in mice neuroblastoma cells.[50] However, N2a cells express a low number of VGSC with peak inward sodium currents between −200 and −400 pA.[51] Therefore, the standardized protocol for CTX and BTX detection makes it difficult to establish a cell bioassay as a potential reference method for CTXs and BTXs.[52] In this regard, the data presented here present the first approach to set up electrophysiology methods to evaluate the presence of marine toxins that can be automatized.[53]

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There is one last subject that requires being clarified in further work. The synergistic interaction between CTXs and BTXs might suggest an allosteric effect that could be explained by two different binding sites at the same receptor that modify the binding energy of each toxin if the other is bound. We cannot provide a model with the evidence shown in this paper, but this would need further exploration.

5. CONCLUSIONS

There is a synergistic effect of the marine biotoxins CTX3C and BTX-3, potentiating their separate effect on VGSC, with both hyperpolarizing the activation voltage. It is also important to remark that the behavior of both compounds on VGSC is different since CTX3C acts as a full agonist, and BTX-3 act as partial agonist of human VGSC, being a determinant for the effect that they exert jointly and separately on the receptors. Noteworthily, for compounds that act on the same cellular target, it is important to determine the toxicity of each compound and their combinations at a wider level, both *in vivo* and *in vitro*, to establish safety limits for consumers.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.3c00267.

Representative traces of sodium current activation recordings in human sodium channels and effect of different CTX3C and BTX-3 concentrations alone or the combinations of both toxins on their inactivation state (PDF)

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Sandra Raposo, Celia Costas, and Carmen Vale: writing original draft, analyzing data, and formal analysis. Luis M Botana, M. Carmen Louzao, and Mercedes Rodriguez Vieytes: conceptualization and funding. All authors have read and agreed to the published the version of the manuscript. CRediT: **Sandra Raposo-Garcia** data curation, formal analysis, writing-original draft, writing-review & editing; **Celia Costas** formal analysis, writing-review & editing; **M Carmen Louzao** conceptualization, funding acquisition; **Mercedes R. Vieytes** conceptualization, funding acquisition; **Carmen Vale** formal analysis, supervision, writing-review & editing; **Luis M. Botana** conceptualization, funding acquisition, project administration.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

BTXs, brevetoxins; CCFFP, Codex Committee on Fish and Fishery Products; CI, confidence interval; CP, ciguatera poisoning; CTXs, ciguatoxins; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; EFSA, European Food Safety Authority; FDA, Food and Drug Administration; HEK, human embryonic kidney; I_{Na}, VGSC current amplitude; mU, mouse units; nM, nanomolar; NSP, neurotoxic shellfish poisoning; V_{hold}, holding potential; VGSC, voltage-gated sodium channels

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Supporting Information for:

Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C in human voltage gated Nav1.6 sodium channels

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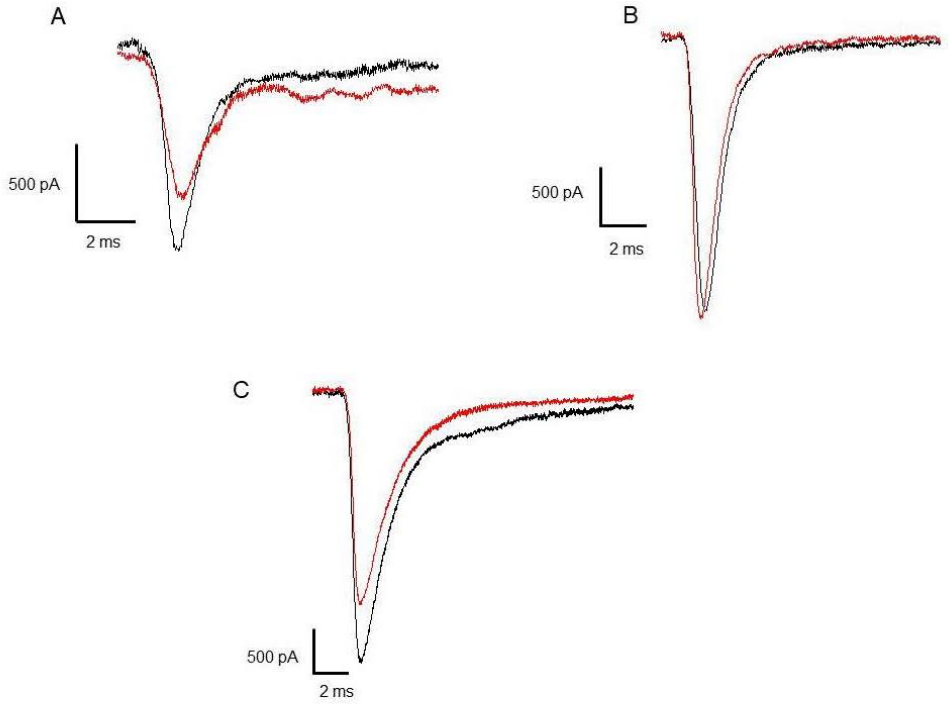


Figure S1: Representative traces of sodium current activation recordings at -20 mV in control conditions (black trace) and cells treated with A: 0.001 nM CTX3C. B: 1 nM BTX-3. C: 0.001 nM CTX3C and 1 nM BTX3.

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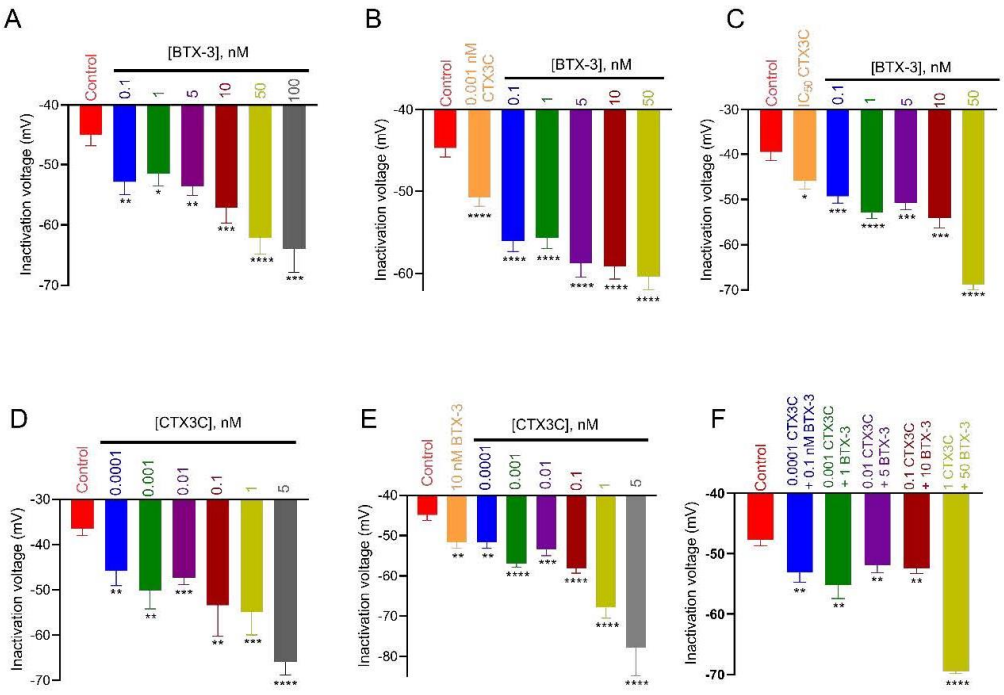


Figure S2: Single and combined effect on the inactivation state of human sodium channels of different CTX3C, BTX-3 concentrations and their combinations. **A**: Inactivation state of sodium channels in control conditions and after bath application of increasing BTX-3 concentrations. B: BTX-3 effect after cell exposure to 0.001 nM CTX3C. C: or an IC_{50} CTX3C concentration of 0.17 nM. **D**: Inactivation state of sodium channels in control conditions and after bath application of increasing CTX3C concentrations. E: CTX3C effect after cell exposure to 10 nM BTX-3. Inactivation state of human sodium channels in control conditions and after bath application of combined increasing BTX-3 and CTX3C concentrations, expressed in nM (F). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs control currents.

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Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C in human voltage-gated $Na_v1.6$ sodium channels.

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5 . DISCUSIÓN

Los canales iónicos dependientes de voltaje son proteínas muy abundantes y relevantes en el organismo y cualquier compuesto que actúe sobre los mismos, sus propiedades o su funcionamiento puede originar alteraciones fisiológicas o tener efecto terapéutico (Berend et al. 2012; Catterall et al. 2005; Catterall et al. 2020; Strazzullo and Leclercq 2014). Los canales de sodio dependientes de voltaje pueden verse alterados por contaminantes alimentarios entre ellos las ficotoxinas marinas y los plaguicidas. La presente tesis doctoral se centró en el estudio de compuestos neuroactivos que actúan sobre canales iónicos dependientes de voltaje. Los compuestos evaluados incluyeron biotoxinas marinas producidas de forma natural por microorganismos fitoplanctónicos y compuestos sintéticos como son los pesticidas piretroides, usados ampliamente en agricultura y cuyo empleo está actualmente en entredicho ya que la legislación europea ha planteado recientemente reactualizar su uso para el control de plagas, además de obligar a realizar muestreos periódicos de los mismos durante los años 2024, 2025 y 2026 (European Commission 2023). Además de su efecto sobre los canales de sodio, muchos de estos compuestos también alteran el normal funcionamiento de los canales de cloro dependientes de voltaje, como es el caso de los piretroides (Forshaw et al. 2000). Estos compuestos tóxicos tanto naturales como sintéticos, cuyo estudio se abordó en la presente tesis doctoral, afectan a los animales y al ser humano después de la ingestión de alimentos contaminados. En el caso de las biotoxinas marinas, entran en la cadena trófica principalmente a través de moluscos, ya que se alimentan por filtración, pudiendo llegar a concentrar grandes cantidades de toxinas en su vianda (Botana 2014). Asimismo, también pueden incorporarse a la cadena trófica a través de peces como ocurre en el caso de la ciguatoxina (Banach et al. 2020). Dado el peligro que entrañan para la seguridad alimentaria, las autoridades sanitarias han regulado los niveles máximos permitidos para algunos grupos de toxinas en productos pesqueros, así como también han establecido métodos oficiales de detección. Sin embargo, la creciente expansión y frecuencia de las floraciones algales en las costas europeas, constituye cada vez un mayor riesgo para la salud pública, y requiere más investigación para obtener datos toxicológicos y nuevos métodos eficaces para su detección. Por otro lado, la legislación sobre pesticidas establece sus LMR tanto en productos vegetales como animales (European Commission 2023).

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En el ámbito de las biotoxinas marinas, esta tesis doctoral se centró en el estudio de las ciguatoxinas, ya que se trata de biotoxinas marinas emergentes en las costas europeas para las cuales aún no se han establecido límites en la legislación. La rápida expansión de estas toxinas hacia aguas europeas junto con la falta de material certificado de referencia y la escasez e incongruencia de los datos toxicológicos existentes hace cada vez más necesario el desarrollo de nuevos estudios para establecer su toxicidad y garantizar la seguridad de los consumidores (Solino and Costa 2020). Con el fin de aportar nuevos datos toxicológicos y contribuir a la caracterización del riesgo que supone la presencia en alimentos de las toxinas involucradas en la intoxicación por ciguatera, se han llevado a cabo estudios *in vivo* de toxicidad aguda y subcrónica. Los resultados obtenidos se han publicado en el artículo titulado "***In vivo* subchronic effects of ciguatoxin-related compounds, reevaluation of their toxicity**" donde se estudiaron en primer lugar los efectos agudos de la administración intraperitoneal de MTX1 a distintas dosis, así como los efectos de diferentes dosis de CTX3C y MTX1 tras la exposición oral aguda de ratones a estos compuestos. En el mismo trabajo se evaluó la toxicidad subcrónica de CTX3C, gambierona, MTX1 y MTX3 administradas por vía oral durante 28 días mediante sonda esofágica. Los resultados obtenidos en este trabajo mostraron en primer lugar que la MTX1, históricamente considerada como uno de los compuestos naturales más tóxicos, producía la muerte de los animales a dosis mucho más elevadas que las previamente descritas, con una dosis letal 50 (LD_{50}, por sus siglas en inglés lethal dose 50) por vía intraperitoneal de 1107 ng/kg, mucho mayor que la LD_{50} históricamente referenciada de 50 ng/kg (Murata et al. 1993). Los resultados de este primer estudio de Murata et al. generan controversia ya que en el artículo no se describía la pureza de los compuestos ni la metodología empleada ni fueron realizados siguiendo procedimientos estandarizados y aprobados para los ensayos *in vivo* (OECD 2008; OECD 2022b). Este hecho, añadido a que los resultados obtenidos a lo largo de las siguientes décadas por diferentes autores eran muy variados y ambiguos en cuanto a metodología, origen de la toxina y resultados (Legrand and Bagnis 1984; Munday et al. 2017; Murata et al. 1993; Reyes et al. 2014; Yokoyama et al. 1988) llevó a recalcar en este trabajo la necesidad de nuevos estudios toxicológicos que reevalúen la toxicidad de la MTX1. En cuanto a la exposición aguda de ratones a MTX1 por vía oral no se observaron síntomas ni alteraciones bioquímicas a dosis de 800 ng/kg confirmando el menor efecto de la MTX1 por esta ruta de acuerdo a lo previamente descrito (Munday et al. 2017). Y en el caso del tratamiento oral repetido durante 28 días, la MTX1 a dosis de 800 ng/kg tampoco causó alteraciones,

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observándose una muerte dosis-dependiente de los animales, pero en este caso, a dosis de 2560 ng/kg y mayores, mucho más altas que para el tratamiento intraperitoneal (1107 ng/kg). Es importante tener en cuenta que este trabajo se realizó siguiendo las directrices establecidas por la OECD para la evaluación de la toxicidad de compuestos químicos, no obstante, la gran discrepancia entre la toxicidad oral y la parenteral es probable que se deba a la baja absorción oral de la MTX1 debido a su alto peso molecular y su carácter hidrofílico (Pisapia et al. 2017), si bien hasta el momento no existen datos sobre la farmacocinética de esta molécula después de su administración oral.

A pesar del creciente impacto en la salud pública de las toxinas del grupo de las ciguatoxinas, existe una falta de datos sobre la toxicidad oral crónica de todos los compuestos involucrados en la intoxicación por ciguatera. En el estudio realizado en esta tesis doctoral, tras un periodo de 28 días de administración oral de CTX3C, gambierona y MTX3 no hubo muertes a ninguna de las dosis administradas. Sin embargo, tras la administración oral aguda de CTX3C se observó una disminución en la producción de orina de hasta un 85% a la dosis de 1050 ng/kg CTX3C. Estos efectos observados pueden estar relacionados con el efecto de las ciguatoxinas sobre los canales de sodio dependientes de voltaje, ya que un efecto similar ha sido documentado para la tetrodotoxina, un conocido bloqueante de los canales de sodio dependientes de voltaje (Boente-Juncal et al. 2019). Durante el tratamiento oral subcrónico, en cambio, la producción de orina no se vio afectada, ya que las dosis empleadas fueron mucho más bajas, sin embargo, si que se detectó un incremento en los valores de potasio sanguíneo tras la administración de la dosis más alta de CTX3C. Este dato es relevante ya que la hiperpotasemia es considerada un indicador de fallo renal (Palmer and Clegg 2018). Un aumento de los valores de potasio en sangre se detectó también tras el tratamiento oral subcrónico con gambierona a las dosis más altas. El resto de las toxinas estudiadas no alteraron los valores bioquímicos ni electrolíticos de los animales en ninguno de los tratamientos.

Debido a la falta de estudios toxicológicos, en la legislación europea actual no existen límites máximos para la presencia de ciguatoxinas en productos pesqueros. El Reglamento 853/2004 (y posteriores modificaciones) recoge textualmente que “No se pondrán en el mercado los productos de la pesca que contengan biotoxinas tales como la ciguatoxina”. A este respecto, la FDA Americana estableció unas concentraciones de referencia de 0,01 μg de equivalentes de P-CTX1B/kg de tejido y 0,1 equivalentes de C-CTX1/kg de tejido (FDA 2020), las cuales fueron determinadas basándose en los TEF obtenidos a partir de valores de LD_{50} intraperitoneal

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aguda en ratones (Lewis et al. 1991). Después de analizar los estudios existentes la EFSA (EFSA 2010) estableció los siguientes TEF para los distintos análogos de las ciguatoxinas: P-CTX1 = 1, P-CTX3C = 0,2, P-CTX-4A = 0,1, P-CTX3 = 0,3, C-CTX-1 = 0,1. Sin embargo, el principal problema de establecer factores de equivalencia tóxica para las toxinas que causan la intoxicación por ciguatera basándose en estudios *in vivo* es que se toma como referencia de toxicidad la muerte de los animales, cuando en esta intoxicación rara vez se llega a producir la muerte, siendo los síntomas causados su principal peligro para la salud de los consumidores afectados por ciguatera (WHO 2020). En concreto los síntomas crónicos, especialmente los trastornos neurológicos que pueden durar hasta años (Johnson-Arbor 2023; Mullins et al. 2023; Traylor and Singhal 2023) . Para tratar de aportar datos ante este problema, se publicó el trabajo titulado "**Determination of the toxicity equivalency factors for ciguatoxins using human sodium channels**" donde se establecieron factores de equivalencia tóxica para tres análogos pacíficos de la ciguatoxina (CTX1B, CTX3B y CTX4A) y para otros compuestos implicados en la intoxicación por ciguatera (gambierol, gambierona y MTX3) en función de su efecto sobre su principal diana, los canales de sodio dependientes de voltaje. Para ello, se estudió el efecto de estas toxinas sobre la amplitud de las corrientes de sodio y sobre el voltaje de activación de los mismos. Los resultados obtenidos demostraron que el voltaje de activación de los canales de sodio dependientes de voltaje era el parámetro más sensible para la detección de ciguatoxinas y los compuestos relacionados ya que el efecto que causan sobre él es más representativo que el efecto provocado en la amplitud máxima de la corriente de entrada de sodio, permitiendo la detección de los compuestos a concentraciones mucho más bajas. Los análogos de las ciguatoxinas estudiados (CTX1B, CTX3B y CTX4A) mostraron un marcado efecto disminuyendo la intensidad máxima de las corrientes de entrada de sodio en la célula, así como un marcado desplazamiento negativo del voltaje de activación de los canales. Mientras que compuestos como el gambierol, la gambierona y la MTX3 no tuvieron efecto significativo en la disminución de la corriente máxima de entrada de sodio, pero sí que causaron un marcado efecto hiperpolarizando su voltaje de activación. Dado que un desplazamiento negativo del voltaje de activación en los canales de sodio en una célula excitable desencadena potenciales de acción repetitivos, este hecho podría estar asociado a la sintomatología nerviosa desarrollada en la intoxicación por ciguatera. Por ello se sugirió que todos los compuestos relacionados con la ciguatera, incluyendo el gambierol, la gambierona y la MTX3 podrían ser relevantes en esta intoxicación contribuyendo a la hiperexcitabilidad celular. Los resultados obtenidos en este

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trabajo permitieron establecer factores de equivalencia tóxica en base a los cambios producidos en el voltaje de activación para cada toxina tomando como referencia la CTX1B por su mayor efecto, siendo los siguientes: CTX1B (1) > CTX3B (0,28) > CTX4A (0,1) >> gambierol (0,01) > gambierona (0,005) > MTX3 (0,002). Además, en este estudio demostró un efecto de la gambierona sobre canales de cloro dependientes de voltaje, en los cuales una concentración de 200 nM de gambierona causó una disminución en la intensidad máxima de la corriente de cloro, resaltando la necesidad de más estudios toxicológicos que aporten datos para determinar la implicación de la homeostasis del cloruro en la intoxicación por estas biotoxinas marinas ya que el mantenimiento del equilibrio iónico es imprescindible en el organismo.

En la última década, además de los análogos pacíficos, se ha producido un aumento de los reportes de aparición de ciguatoxina caribeña en costas europeas (Canals et al. 2021; Costa et al. 2018; Estevez et al. 2019; Estevez et al. 2020; Otero et al. 2010). Los estudios y datos disponibles del análogo caribeño son aún más escasos que para análogos pacíficos ya que las ciguatoxinas caribeñas no se encuentran disponibles comercialmente. Los pocos estudios llevados a cabo hace varias décadas reportaron una toxicidad *in vivo* 10 veces menor del análogo caribeño con respecto al pacífico (Lewis et al. 1999; Vernoux and Lewis 1997), sin embargo, la falta de información sobre el procedimiento seguido en aquellos estudios y la pureza de los extractos empleados generó la necesidad de reevaluar la toxicidad de las ciguatoxinas pacíficas y caribeñas. En base a los resultados obtenidos en el artículo anterior, en el trabajo titulado "**In silico simulations and functional cell studies evidence similar potency and distinct binding of Pacific and Caribbean ciguatoxins**" se demostraron por primera vez las diferencias entre los análogos pacíficos (CTX1B y CTX3C) y caribeños (C-CTX1) estudiando sus efectos sobre la intensidad máxima de la corriente de entrada de sodio, así como sobre el voltaje de activación de los canales. Los 3 análogos disminuyeron la entrada de sodio en las células de manera dosis dependiente. La disminución en la intensidad máxima de las corrientes de entrada de sodio, causada por el análogo caribeño C-CTX1 fue similar a la disminución observada para la CTX3C y no muy diferente al obtenido para la CTX1B. Así se estableció una potencia relativa en base a las IC_{50} obtenidas para la disminución de la intensidad máxima de la corriente de entrada de sodio de 1 para CTX1B y de 0,74 para la CTX3C y C-CTX1. Estas potencias fueron similares a las calculadas a partir de las IC_{50} obtenidas en otro laboratorio para los mismos análogos de las ciguatoxinas calculadas utilizando ensayos de citotoxicidad *in vitro* en la línea celular de neuroblastoma de ratón N2a. En cuanto al efecto de

estos compuestos sobre el voltaje de activación, tanto para la CTX1B como para la CTX3C se registró un desplazamiento negativo en el mismo, dependiente de concentración, que fue de 20 mV para 1 nM CTX1B y 16 mV para 1 nM CTX3C. Mientras que, por el contrario, el análogo caribeño C-CTX1 a la concentración más alta empleada (1 nM) no causó ninguna modificación en el voltaje de activación de los canales. En vista de estos resultados la hipótesis propuesta para explicar las diferencias entre los efectos de los 3 análogos sobre el voltaje de activación de los canales, similar para los análogos pacíficos e inexistente para la ciguatoxina caribeña fue que la orientación en la unión al canal de sodio de cada uno de los análogos era diferente. Esta hipótesis se corroboró mediante estudios *in silico* que demostraron que el análogo caribeño de la ciguatoxina se une con el anillo A (cabeza) de la molécula hacia el lado extracelular mientras que los análogos pacíficos lo hacen al revés, con la cabeza hacia la parte intracelular del canal. Esta diferencia en la orientación en la unión de los compuestos podría llevar a una interacción diferente de cada análogo con el sensor de voltaje en el segmento S4 del canal, hecho que fue puesto de manifiesto por primera vez en este trabajo mediante estudios *in silico* de docking y dinámica molecular en colaboración con el Centro Singular de Investigación en Química Biológica y Materiales Moleculares. Estas observaciones concuerdan con la sintomatología causada por las toxinas, ya que los análogos pacíficos se asocian a síntomas neurológicos más intensos, mientras que C-CTX se asocia a efectos gastrointestinales y posteriores síntomas nerviosos periféricos (Boucaud-Maitre et al. 2018).

En Europa, la salud de los consumidores está protegida frente a las ciguatoxinas por la legislación ya que el Reglamento CE 853/2004 establece la prohibición de la puesta en el mercado de pescados que contengan ciguatoxinas. Sin embargo, a la hora de garantizar la ausencia de toxinas en los alimentos entra en juego otro factor muy importante que es la detección de estas toxinas en productos pesqueros. En este sentido, aunque las células de neuroblastoma de ratón se emplean habitualmente con este fin, tanto por autoridades legisladoras como la FDA y en diferentes laboratorios, este método no se encuentra validado, hecho relevante teniendo en cuenta que las células de neuroblastoma son células procedentes del tubo neural que presentan baja expresión de canales iónicos dependientes de voltaje (Badisa et al. 2018; Khan et al. 2018). Por consiguiente, en el trabajo titulado **"Mouse N2a neuroblastoma assay: Uncertainties and comparison with alternative cell-based assays for ciguatoxin detection"** se ha comparado el método *in vitro* de detección de ciguatoxinas actualmente empleado con otros métodos alternativos con la finalidad de aumentar la

sensibilidad de detección y cuantificación de estos compuestos. El método internacionalmente empleado es la determinación de la viabilidad celular mediante el ensayo de MTT en células de neuroblastoma de ratón N2a sensibilizadas con ouabaína y veratridina (EFSA 2010). Sin embargo, este método tiene múltiples variaciones y cada laboratorio usa sus propias condiciones de ensayo ya que por el momento no ha sido estandarizado debido a la falta de estándares certificados de ciguatoxina, la ausencia de efecto citotóxico por sí mismas en algunos cultivos celulares y las pequeñas cantidades en las que a veces se encuentran estos compuestos puros, entre muchas otras razones (Li et al. 2023; Viallon et al. 2020). Debido a la ausencia de citotoxicidad de las ciguatoxinas por sí mismas, este ensayo requiere la adición de ouabaina, que bloquea la bomba sodio/potasio (Na^+/K^+) ATPasa, junto con el activador de los canales de sodio veratridina. Bajo este tratamiento con ouabaína y veratridina (O/V) se potencia el efecto citotóxico de las CTXs permitiendo detectar y cuantificar su presencia en las muestras. Sin embargo, como ha sido previamente documentado, el tratamiento con O/V tiene un efecto tóxico sobre las células (Loeffler et al. 2021; Viallon et al. 2020). Esto ha sido constatado en este trabajo, en el que se constató la citotoxicidad del tratamiento con O/V a las concentraciones más bajas comúnmente empleadas. Sin embargo, esta disminución de la viabilidad celular ocasionada por el pretratamiento con O/V es aceptada y por lo general, se parte de una viabilidad celular de en torno al 80% cuando se va a realizar los ensayos de detección de CTXs (Castro et al. 2022; Loeffler et al. 2021; Viallon et al. 2020). La baja disponibilidad de canales de sodio funcionales en la línea de neuroblastoma de ratón N2a así como el hecho de que la exposición durante 24 h a O/V no aumentaba su funcionalidad ha sido también demostrada en este trabajo. Estos hallazgos demostraron que los efectos de citotoxicidad detectados en la línea N2a tras el tratamiento con CTXs y O/V no pueden deberse al efecto directo de las toxinas sobre los canales de sodio como históricamente se afirma, ya que la línea celular N2a no posee canales de sodio funcionales en cantidad significativa como para poder detectar efecto sobre ellos y cuantificar los compuestos. Durante la realización del método del MTT para la detección de ciguatoxinas se observó que tras el tratamiento de las células durante 24 h con O/V y CTX, las células aunque viables (teñidas de morado por la metabolización del colorante MTT por las mitocondrias) modificaban su apariencia debilitándose y despegándose del fondo de los pocillos, lo cual se detecta como una disminución en la viabilidad celular con el método del MTT ya que estas son eliminadas cuando se realizan los procesos de aspiración y lavado que requiere el ensayo. Los cambios morfológicos que se producían en las células y su separación

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del fondo del pocillo se constataron mediante microscopía confocal, revelando una alteración del citoesqueleto de actina tras los tratamientos. Los daños causados en el citoesqueleto por las ciguatoxinas (Jiang et al. 2012; Sauviat et al. 2006) así como por la ouabaína (Contreras et al. 1999; Lopina et al. 2020; Qiu et al. 2007; Zhao et al. 2013) fueron previamente documentados en otras líneas celulares. Por lo tanto, este resultado indicó que la combinación de ambos tratamientos en las células N2a alteraba de forma importante el citoesqueleto celular. Esta hipótesis también fue respaldada al evaluar la viabilidad celular mediante otros métodos que no requerían la retirada de medio, como son el método de la liberación de LDH y el método del Alamar blue, ambas técnicas indicaron que no existía una disminución de la viabilidad, ya que, en este caso, las células se encuentran viables, esté o no dañado su citoesqueleto.

En vista de los resultados obtenidos, el siguiente objetivo planteado en este trabajo fue aumentar la sensibilidad de los métodos *in vitro* para la detección de las ciguatoxinas empleando su principal diana, los canales de sodio dependientes de voltaje. Con este fin, las células N2a se expusieron durante 24 h a una concentración10 µM de forskolina, una molécula cuya eficacia, promoviendo la diferenciación celular en líneas de neuroblastoma (Ammer and Schulz 1997; Yusta et al. 1988), y aumentando las corrientes de sodio dependientes de voltaje (Bonvallet et al. 1990; England et al. 1996; Fitzgerald et al. 1999), ha sido demostrada previamente. Los registros electrofisiológicos junto con el aumento de la citotoxicidad causada por las ciguatoxinas sobre células N2a detectada con los métodos de LDH y Alamar blue apoyaron la hipótesis previa de que en células N2a no era posible detectar el efecto directo de las ciguatoxinas sobre los canales de sodio en esta línea celular. Debido a esta falta de consenso con el método de detección mediante MTT en N2a, a lo largo del tiempo se han propuesto múltiples alternativas tratando de mejorar la detección. Una de estas alternativas ha sido el uso de neuroblastoma humano SH-SY5Y (Coccini et al. 2017) como línea celular para la detección de ciguatoxinas basándose en que esta línea, al tener origen humano podría simular mejor los efectos causados por las ciguatoxinas en el sistema nervioso. Sin embargo, uno de los principales inconvenientes que es la mayor sensibilidad de esta línea a la ouabaina ya determinada en el primer estudio (Coccini et al. 2017) y confirmada en los experimentos de esta tesis doctoral, así como la baja expresión de canales de sodio dependientes de voltaje mostrados en los registros llevados a cabo en este trabajo no permitió proponer este modelo humano como una alternativa mejor para la detección de ciguatoxinas. Por último, en este estudio se empleó la línea HEK293 que sobrexpresa la subunidad alfa 1.6 humana de los canales

de sodio dependientes de voltaje. En este caso se observó una mejor sensibilidad del modelo para detectar la presencia de ciguatoxinas, ya que las ciguatoxinas produjeron una disminución de la viabilidad celular dependiente de la concentración, empleando el método del MTT sin necesidad de pretratamiento con ouabaína y veratridina. Aun así, en estas condiciones de ensayo, el límite de detección sería más alto. Sin embargo, cuando las mismas células se coincubaron con 100/10 µM O/V y ciguatoxinas, el límite de detección disminuye hasta permitir detectar una disminución de la viabilidad celular causada por la presencia de 0,0001 nM CTX3C. Cabe destacar que, para estos ensayos de evaluación de la citotoxicidad, se ha empleado el análogo pacífico CTX3C, siendo este menos potente que la CTX1B, para asegurar la evaluación del método detección con mayor rango.

En la actualidad, la evaluación de los riesgos que los contaminantes alimentarios suponen para la salud humana se ha centrado en la determinación de los efectos y la toxicidad de los compuestos por separado, con el fin de establecer un nivel seguro independientemente para cada uno de ellos. Sin embargo, cuando se detecta la presencia de toxinas marinas en un producto pesquero, como se ha documentado en múltiples estudios, lo habitual es que no solamente aparezca un tipo de toxinas, sino una mezcla (Aasen et al. 2011; Amzil et al. 2021; Rodriguez-Cabo et al. 2021). Por este motivo, en esta tesis se han estudiado por primera vez los efectos simultáneos que tienen las ciguatoxinas y las brevetoxinas sobre los canales de sodio dependientes de voltaje puesto que comparten la misma diana molecular. Estos estudios dieron lugar al trabajo titulado "**Synergistic effect of brevetoxin BTX-3 and ciguatoxin CTX3C inhuman voltaje-gated Na_v1.6 sodium channels**" en el cual se demostró un efecto sinérgico de ambos grupos de toxinas sobre los canales de sodio dependiente de voltaje. En este caso, es de resaltar que las brevetoxinas y las ciguatoxinas actúan de forma diferente, ya que la BTX-3 no modifica la entrada de sodio y solamente hiperpolariza el voltaje de activación de los canales hacia potenciales más negativos, mientras que la CTX3C altera ambos parámetros, como se ha mencionado anteriormente, disminuyendo la intensidad de la corriente de entrada de sodio y activando el canal a potenciales más negativos. Cuando las células se expusieron durante 5 min a distintas combinaciones de ambas toxinas, se observó una potenciación del efecto causado por cada una de forma individual sobre el voltaje de activación, mientras que no se observó este efecto sobre la intensidad de la corriente de sodio dependiente de voltaje. Tal como se ha descrito para otras toxinas involucradas en la intoxicación por ciguatera (Raposo-Garcia et al. 2022), este caso confirma que el voltaje de activación es mucho más sensible para la detección

de compuestos cuya principal diana son los canales de sodio dependientes de voltaje que el efecto provocado en la amplitud máxima de la corriente de entrada de sodio. Por ello se usó el voltaje de activación para calcular el índice de combinación siguiendo el método de Chou & Talalay (Chou and Talalay 1983), y se obtuvo un valor inferior a 1, lo cual indica sinergia de los compuestos. Los resultados presentados mostraron por primera vez el efecto en la hiperpolarización del voltaje activación de los canales de sodio era 10 veces mayor tras la combinación de CTX3C y BTX-3. Este hecho es importante ya que la exposición a dosis muy bajas de ambos compuestos, que por sí solas no causarían efecto, cuando aparecen en combinación podrían desencadenar una hiperexcitabilidad nerviosa. A este hallazgo se le suma el hecho de que los registros electrofisiológicos realizados directamente sobre los canales de sodio han permitido confirmar que la BTX-3 y la CTX3C modularían los canales de sodio dependientes de voltaje de forma diferente. Un hecho notable es que, en este caso, cuando las células se expusieron en primer lugar a una única concentración alta de BTX-3 y posteriormente se añadió CTX3C, la IC_{50} calculada para la disminución del pico máximo de entrada de sodio obtenido fue de 1,54 nM mientras que la IC_{50} para la CTX3C sola fue de 0,17 nM. Es decir, en presencia de BTX-3 son necesarias concentraciones mucho más altas de CTX3C para lograr el mismo efecto sobre la intensidad máxima de la corriente de entrada de sodio, lo cual puede ser explicado por la activación previa del canal en presencia de BTX-3, que disminuiría la capacidad de activación del mismo en presencia de CTX3C bien sea por competir por el mismo lugar de unión al canal o por modificar la conformación o el estado del mismo, una observación que está de acuerdo con estudios previos en los que se demostró, empleando toxinas marcadas radioactivamente, que la CTX3C era capaz de desplazar a la BTX-3 de su sitio de unión (Inoue et al. 2003). Este hecho también puede explicar las diferencias en el efecto que cada toxina tiene sobre los canales.

Estudios previos habían demostrado que tanto la brevetoxina como la ciguatoxina afectan modifican la inactivación rápida de los canales de sodio dependientes de voltaje (Jeglitsch et al. 1998; Konoki et al. 2019; Yamaoka et al. 2004), si bien no se habían estudiado los efectos conjuntos de combinaciones de ambos compuestos sobre este parámetro. Los resultados obtenidos mostraron un efecto sinérgico de BTX-3 y CTX3C sobre el voltaje de inactivación, lo que sugiere que la combinación de ambas toxinas podría tener efectos importantes en células excitables por ejemplo modificando la frecuencia del potencial de acción (Goldin 2003). Por consiguiente, este estudio puso de manifiesto que la potenciación de los efectos de estos

compuestos sobre los canales de sodio puede requerir analizar nuevamente sus límites en productos pesqueros para garantizar la salud de los consumidores, teniendo en cuenta la posible presencia de ambos en productos alimenticios lo que implicaría un riesgo para la salud humana.

Siguiendo con la línea de posibles efectos combinados entre contaminantes alimentarios, es importante destacar que el consumo de una dieta equilibrada según la Organización Mundial de la Salud (OMS) incluye una ingesta de productos pesqueros al menos dos veces por semana y un consumo de frutas y verduras diario. Esto junto con el hecho de que muchos de los contaminantes que contienen los alimentos comparten una misma diana celular y modo de acción, hace lícito estudiar la toxicidad combinada de estos compuestos para lograr una caracterización real del riesgo que representan estos compuestos para los consumidores cuando están expuestos simultáneamente a ellos. Los pesticidas piretroides son compuestos sintéticos diseñados para actuar directamente sobre los canales de sodio. A pesar de que la legislación recoge unos límites máximos de residuos permitidos para cada uno de los pesticidas, el aumento de la frecuencia de uso en la producción agrícola hace que estos límites no se respeten y aparezcan residuos en los alimentos que se comercializan (EFSA 2015). Entre estos compuestos, la deltametrina es uno de los piretroides más ampliamente empleado y que actúa sobre los canales de sodio dependientes de voltaje. Por ello, en esta tesis doctoral se han estudiado los efectos combinados de las ciguatoxinas y la deltametrina, los cuales comparten diana molecular en el trabajo titulado "**Synergistic effect of environmental food pollutants: Pesticides and marine biotoxins**". Este estudio demostró por primera vez la existencia de sinergias entre contaminantes alimentarios de distinto origen, es decir toxinas naturales, como son las ciguatoxinas y pesticidas sintéticos. Empleando células HEK que expresan la subunidad alfa 1.6 humana de los canales de sodio dependientes de voltaje, se demostró que la presencia conjunta de ambos tipos de compuestos tenía un efecto sinérgico sobre la amplitud de las corrientes de sodio, así como sobre la hiperpolarización de su voltaje de activación que se observó a los 5 minutos de la exposición simultánea de las células a una mezcla de bajas concentraciones de ambos compuestos. Los cambios más significativos, fueron los producidos sobre el voltaje de activación de los canales, ya que las concentraciones de ciguatoxinas y deltametrina que por separado no alteraban este parámetro, en combinación produjeron un desplazamiento negativo del voltaje de activación de -19 mV. Estas observaciones podrían explicar o estar asociadas en parte a los trastornos neurológicos descritos después de la exposición crónica a CTX o a piretroides (Hansen et al. 2017; L'Herondelle et al. 2020; Zaganas

et al. 2013). Aunque el principal sitio de acción de la deltametrina son los canales de sodio dependientes del voltaje, el compuesto también actúa disminuyendo la probabilidad de apertura de los canales de cloruro dependientes del voltaje (Forshaw et al., 1993; Forshaw y Ray, 1990; Hodgdon, 2008). Por lo tanto, se podría esperar una carga de cloruro intracelular que esté de acuerdo con el aumento en las corrientes de salida de cloruro reportadas en este trabajo y demostrando la implicación de los canales de cloro dependientes de voltaje en el efecto tóxico causado por la deltametrina, como previamente había sido sugerido (Eshleman and Murray 1991).

Por lo tanto, siendo la deltametrina uno de los pesticidas que se encuentran con mayor frecuencia en las muestras del programa de monitorización de la EFSA a pesar de todas las limitaciones vigentes (EFSA 2020), unido al aumento global de la incidencia de la intoxicación por ciguatera (Botana 2016; Varela Martínez et al. 2021), este trabajo enfatizó la necesidad de llevar a cabo más estudios sobre el efecto combinado de la exposición a estos contaminantes, así como a otros que compartan mecanismo y diana celular para poder establecer regulaciones sobre los niveles de compuestos ya que en la dieta diaria ingresan al organismo una gran cantidad de residuos y tóxicos a través de los alimentos y el agua.

Los resultados obtenidos a lo largo de la presente tesis doctoral han permitido evidenciar la importancia de los contaminantes alimentarios que actúan sobre canales de sodio dependientes de voltaje y la necesidad de llevar a cabo estudios conjuntos para compuestos que compartan diana celular, con el fin de poder establecer de forma efectiva límites seguros tanto para toxinas marinas como para otros contaminantes alimentarios. Así mismo, para garantizar la seguridad de los consumidores frente a compuestos que actúan sobre canales de sodio, es necesario garantizar que los límites establecidos se cumplan y para ello el establecimiento de un método de detección rápido, barato y eficaz es imprescindible.

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6 . CONCLUSIONES

De acuerdo con los resultados obtenidos en este trabajo se han formulado las siguientes conclusiones:

1. La LD_{50} obtenida por vía intraperitoneal para la MTX1 es mucho mayor que la históricamente documentada.
2. La potencia de los análogos pacíficos y caribeños de la ciguatoxina sobre los canales de sodio dependientes de voltaje fueron: CTX1B > CTX3C ≈ C-CTX1, que se atribuyen a las diferencias de la unión de las ciguatoxinas caribeñas y pacificas al canal de sodio dependiente de voltaje.
3. Los factores de equivalencia toxica establecidos para compuestos relacionados con la ciguatera fueron CTX1B > CTX3B > CTX4A >>> gambierol > gambierona > MTX3.
4. La línea celular HEK Na_v 1.6 constituye una buena alternativa para la detección de ciguatoxinas debido a su mayor disponibilidad de canales de sodio.
5. Existen sinergias entre contaminantes alimentarios (pesticidas y ficotoxinas) con la misma diana celular, demostrada para la combinación de deltametrina y la ciguatoxina CTX3C
6. Existen sinergias entre biotoxinas marinas que actúan sobre canales de sodio dependientes de voltaje observadas con la brevetoxina 3 y la ciguatoxina CTX3C.

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7 . CONCLUSIONS

According to the results obtained in this work, the following conclusions have been formulated:

1. The LD_{50} obtained intraperitoneally for MTX1 is much higher than historically documented.
2. The potency of Pacific and Caribbean ciguatoxin on voltage-gated sodium channels were: CTX1B > CTX3C ≈ C-CTX1, which are attributed to the differences in the binding of Caribbean and Pacific ciguatoxins to voltage dependent sodium channels.
3. The toxic equivalency factors established for ciguatera-related compounds were CTX1B > CTX3B > CTX4A >>> gambierol > gambierone > MTX3.
4. The HEK Na_v 1.6 cell line constitutes a good alternative for the detection of ciguatoxins due to its higher availability of sodium channels.
5. There are synergies between food contaminants (pesticides and phycotoxins) with the same cellular target, demonstrated for the combination of deltamethrin and the ciguatoxin CTX3C.
6. There are synergies between marine biotoxins that act on voltage-gated sodium channels observed with brevetoxin 3 and ciguatoxin CTX3C.

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En la presente tesis doctoral se estudian compuestos que actúan sobre canales iónicos modificando su actividad, en concreto se estudian toxinas marinas implicadas en la intoxicación por ciguatera, como son las ciguatoxinas, cuya principal diana son los canales de sodio dependientes de voltaje. En concreto, se reevaluó la potencia de estas toxinas, estableciendo factores de equivalencia tóxica in vitro. Estos datos se ampliaron con estudios toxicológicos in vivo para analizar su efecto agudo y subcrónico. El método de detección de ciguatoxinas también fue reevaluado. Un segundo apartado se centró en el estudio de las posibles sinergias entre las ciguatoxinas y otros compuestos que actúan sobre los mismos canales de sodio dependientes de voltaje como son brevetoxinas y el pesticida deltametrina.