Detection of ciguatoxins in shark involved in a ciguatera fish poisoning

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SYNTHESIS

- First identification of ciguatoxins in shark (*Carcharhinus leucas*, bull shark – Madagascar)
- New CTX analogues: I-CTX-5 & I-CTX-6
- First identification of gambieric acid in fish.
POISONING EVENT

- Occurred in November 2013 in Fenerive-Est, Madagascar
- 94 people poisoned, 11 of whom died
- Ciguatera symptoms: neurological and digestive signs.
- After eating the flesh, the liver, the head, and part of the viscera of a bull shark (*Carcharinus leucas*).

PREVIOUS POISONING EVENTS - Sharks


Numerous human poisoning after the consumption of several species of shark have been reported since the 1940s, proposed to be ciguatera events according to the toxicity in animal assays or due to the symptoms in patients.
PREVIOUS POISONING EVENTS - Sharks


Carchatoxins A and B from shark liver. Mice administered with the shark toxins died within 4 hr, or otherwise survived. In contrast, mice may die even after 24 hr in the case of ciguatoxin.
• Gather epidemiologic information of the poisoning event
• Confirm species of shark and individual

• Processing shark tissues: toxin extraction

• Analysis of shark tissues by MBA
• List symptoms in mice

• CTX screening by CBA

• Stomach chromatographic fractionation

• Confirmation and quantification of CTXs by LC-HRMS
SPECIES and INDIVIDUAL CONFIRMATION

Example of a chromatogram for samples A565_4 and A565_5 for the microsatellite locus Cl11. Black peaks represent alleles with allele sizes indicated by the labels.

Assignment test (STRUCTURE software) for shark species identification. Each bar on the x-axis represents one individual and the y-axis represents the probability to belong to one or another cluster. The multilocus genotypes of the unidentified shark samples (presumed to be bull shark) were used in assignment with other bull shark individuals (from Pirog et al. 2015) and other carcharinid species that successfully amplified the loci used (Carcharhinus obscurus or Carcharhinus plumbeus). We found two clusters: the red one corresponds to other shark species and the green one corresponds to bull shark individuals. The unidentified samples clustered with the bull shark cluster.
EXTRACTION

10g tissue

T↑(70ºC)
15 min

20mL acetone
x2

Centrifugation
15 min

Filtration

Evaporation (50ºC)

4mL aq. Residue and water

16mL DEE

Collect upper Phase DEE

Add Diethylether

Evaporate DEE

4mL hexane

2mL MeOH:H2O (4:1)

x2

Collect methanolic phase

Collect upper Phase DEE

Add hexane

Add n-hexane

Collect methanolic phase

Evaporate

Redissolve in MeOH

Filtration + Storage-20ºC
MBA - Mouse bioassay

- Injection of flesh & stomach extrachs → high toxicity
- Symptoms in mice: characteristics of ciguatera (digestive and neurological)

CBA - Cell-based assay (Neuro-2a)

Cytotoxicity of CTX1B on Neuro2A cells with (white dots) and without (black dots) ouabaine/veratridine treatment.
CBA – Crude extracts
LC-HRMS
- LC: Hypersil Gold, 1.9um, 50x2.1 mm
- 200 ul/min
- Orbitrap Exactive-HCD
- Resolució 50000 (2Hz)
- Full scan: 400-1500Da
- ESI +

Linearity range P-CTX-1
12.5-25-50-100 ppb

Quantification:
P-CTX-1+NH4+ + Na+
LC-HRMS
I-CTX-3&4
(Stomach crude)

[\text{I-CTX-3&4+Na}^+]^+
(C_{62}H_{92}O_{20}+Na^+)

 Orbitrap-Exactive

\[ \text{Theorical} \ [C_{62}H_{92}O_{20}+NH_4]^+ \]

\[ \text{Theorical} \ [\text{I-CTX-3&4+Na}^+]^+ \]

\[ \text{NL:} \ 1.53E4 \]
15062917#499 RT: 5.53
AV: 1 T: FTMS \{1,1\} + p
ESI Full ms
[400.00-1500.00]

\[ \text{NL:} \ 4.82E5 \]
C_{62}H_{92}O_{20}NH_4^+
C_{62}H_{92}O_{20}N_4^+
paChrg1

\[ \text{NL:} \ 4.84E5 \]
C_{62}H_{92}O_{20}Na_1
C_{62}H_{92}O_{20}Na_{10}
paChrg1
### CTXs quantifications

Concentration of P-CTX-1 equiv./kg tissue in crude stomach, flesh and fin extracts as determined by mouse bio-assay (MBA), Neuro-2a cell-based assay (CBA) and liquid chromatography coupled to high resolution mass spectrometry (LC-ESI-HRMS).

<table>
<thead>
<tr>
<th>Crude extract</th>
<th>MBA (µg P-CTX-1 equiv./kg tissue)</th>
<th>CBA (µg P-CTX-1 equiv./kg tissue)</th>
<th>LC-ESI-HRMS (µg P-CTX-1 equiv./kg tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I-CTX-1&amp;2</td>
</tr>
<tr>
<td>flesh stomach</td>
<td>n.q.</td>
<td>0.06</td>
<td>n.d.</td>
</tr>
<tr>
<td>fin 1</td>
<td>83</td>
<td>92.78</td>
<td>6.54</td>
</tr>
<tr>
<td>fin 2</td>
<td>-</td>
<td>0.12</td>
<td>-</td>
</tr>
<tr>
<td>fin 3</td>
<td>-</td>
<td>0.79</td>
<td>n.d.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.17</td>
<td>-</td>
</tr>
</tbody>
</table>
## Fractionation Conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>Acquity UPLC BEH C18, 1.7 µm (2.1 mm x 50 mm)</td>
</tr>
<tr>
<td><strong>Mobile phase A</strong></td>
<td>Water with 2mM ammonium formate and 50mM formic acid</td>
</tr>
<tr>
<td><strong>Mobile phase B</strong></td>
<td>ACN: Water (95:5) with 2mM ammonium formate and 50mM formic acid</td>
</tr>
<tr>
<td><strong>Gradient</strong></td>
<td>Started 35%B, linearly increasing up to 100%B at 5.0min. Hold up to 10min</td>
</tr>
<tr>
<td><strong>Temperature and flow rate</strong></td>
<td>40°C and 200 µL/min</td>
</tr>
<tr>
<td><strong>Fractions</strong></td>
<td>Collected every 30 sec (n=28). All fractions were evaporated and re-dissolved in 500 µL MeOH</td>
</tr>
<tr>
<td>Fractions</td>
<td>CBA (% P-CTX-1 equiv.)</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>F8</td>
<td>0.23</td>
</tr>
<tr>
<td>F9</td>
<td>5.15</td>
</tr>
<tr>
<td>F10</td>
<td>5.80</td>
</tr>
<tr>
<td>F11</td>
<td>9.37</td>
</tr>
<tr>
<td>F12</td>
<td>8.05</td>
</tr>
<tr>
<td>F13</td>
<td>1.79</td>
</tr>
<tr>
<td>F14</td>
<td>1.44</td>
</tr>
<tr>
<td>F15</td>
<td>2.30</td>
</tr>
<tr>
<td>F16</td>
<td>0.62</td>
</tr>
<tr>
<td>F17</td>
<td>0.29</td>
</tr>
<tr>
<td>F18</td>
<td>0.27</td>
</tr>
<tr>
<td>F19</td>
<td>0.33</td>
</tr>
<tr>
<td>F20</td>
<td>0.23</td>
</tr>
<tr>
<td>F21</td>
<td>0.19</td>
</tr>
<tr>
<td>F22</td>
<td>0.12</td>
</tr>
</tbody>
</table>
LC-HRMS I-CTX-1&2, I-CTX-5 (F12 stomach)

[I-CTX-1&2+Na]+ (C62H92O19Na+)

[I-CTX-5+Na]+ (C62H90O19+Na+)

tR: 6.56 min
LC-HRMS I-CTX-5 and I-CTX-6

Stomach F12 fraction

\[[\text{I-CTX-5}+\text{Na}]^+\]
\[tR: 6.95\text{min}\]

Stomach crude extract

\[[\text{I-CTX-6}+\text{Na}]^+\]
\[tR: 5.82\text{min}\]
Software identification of I-CTX-5
CTXs structures
I-CTX-1&2 \( C_{62}H_{92}O_{19} \)

I-CTX-3&4 \( C_{62}H_{92}O_{20} \)

I-CTX-5 \( C_{62}H_{90}O_{19} \)

I-CTX-6 \( C_{62}H_{90}O_{20} \)
## DISTRIBUTION / FRACTIONS

<table>
<thead>
<tr>
<th>Fractions</th>
<th>LC-ESI-HRMS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I-CTX-1&amp;2</td>
</tr>
<tr>
<td>F9</td>
<td>n.d.</td>
</tr>
<tr>
<td>F10</td>
<td>n.d.</td>
</tr>
<tr>
<td>F11</td>
<td>+</td>
</tr>
<tr>
<td>F12</td>
<td>++</td>
</tr>
<tr>
<td>F13</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not detected; n.q. = not quantifiable
GAMBIERIC ACID

Flesh crude extract

\[ \text{GAMBIERIC ACID D: } \text{C}_{66}\text{H}_{102}\text{O}_{19}^+\text{NH}_4^+ \]

\[ [\text{GA D} + \text{NH}_4]^+ \]

\( (\text{C}_{66}\text{H}_{102}\text{O}_{19}^+\text{NH}_4^+) \)
Conclusions and Discussion

- CTXs were identified for the first time in sharks.
- Sharks in the Indian Ocean have to be considered a ciguatera hazard.
- It is important to consider different tissues within the same organisms, especially the viscera if available.
- According to the CBA, the stomach contained about 9,300 times of P-CTX-1 equivalents in relation to the safety limit established (FDA).
- There was lack of correspondence of CTX quantification between the toxicological assays (MBA and CBA / same order 83 and 93 ppm) and the instrumental analytical approach (16 ppm).
- Two novel CTX congeners, CTX-5 and CTX-6 have been identified. Structural elucidation is pending.
- Gambieric Acid was identified for the first time in fish.
(Heinmann et al., 2011)
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