Bioguided fractionation of *Gambierdiscus* extracts

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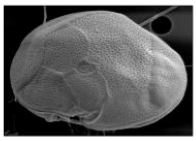


Background

- Species in the benthic dinoflagellate genus *Gambierdiscus* produce ciguatoxins (CTXs) and maitotoxins (MTXs), among the most potent marine toxins isolated to date. Over the last few years an increasing number of *Gambierdiscus* species have been identified, e.g. *G. excentricus* from Canary Islands (Fraga et al., 2011).
- Consumption of fish tainted with sufficient quantities of CTXs causes Ciguatera Fish Poisoning (CFP), globally the largest cause of non-bacterial food poisoning. Originally known as a tropical disease, CFP has recently been reported from areas previously not considered endemic (e.g. Canary Islands).
- MTXs are water soluble and do not readily accumulate in fish tissues, but can reach significant concentrations in the guts of fish and may contribute to CFP if consumed. Three MTX analogs are known to date. The structural elucidation by NMR technique has been obtained only for MTX-1.
- 13 *Gambierdiscus* strains have previously been screened for their toxicity using two *in vitro* assays (N2a and hemolytic assays). The most toxic ones are shown in **Table 1**.

Table 1: The most toxic *Gambierdiscus* strains cultured at Phycotoxins laboratory (Ifremer, Nantes).

Strain	fg CTX3C eq cell^{-1}	pg MTX eq cell^{-1}
***G. excentricus* VGO 791**	**1,426**	**86**
***G.* sp. Vietnam**	**41**	**70**
***G. scabrosus* KW070922_1**	**28**	**1.5**

Materials & Methods

Step 1: Harvesting of *Gambierdiscus* cells

***G. excentricus* VGO 791**
30 flasks (225 cm² surface)
V_{tot} = 9 L

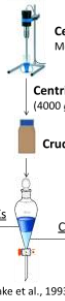
25 µm sieve

Centrifugation
(3000 *g*, 10 min, 20°C)

Cell pellet
2.2 millions cells
3.3 g (wet weight)

Fig. 1: Laboratory cultures of *Gambierdiscus* and cell harvesting.

Step 2: Cell pellet extraction and liquid-liquid partitioning



(Satake et al., 1993)

Fig. 2: Sonication of *Gambierdiscus* (MeOH, 3x) and liquid-liquid partitioning between MeOH:H_2O (3:2, v/v) and CH_2Cl_2.

Step 3: Size-exclusion chromatography

MSF 1.1 million cells eq.
94.9 mg

MTXs

Sephadex® LH-20

1) 10 g in MeOH
height: 52.7 cm
diameter: 1 cm
($V_{wet\ resin}$ = 41.4 mL)

2) eluted with MeOH
50 fractions (2.5 mL)

Fig. 3: Example of fractionation on a Sephadex® LH-20 column.

Step 4: Toxicity screening on neuro-2a (N2a) cells

4.1. Cytotoxicity (MTT) assay

1) Neuro-2a cells (30,000 per well)
2) MTX standard or sample (2.5 h)
3) **MTT** (0.8 g L^{-1}) assay
4) Absorbance at 544 nm

↓

N2a cell survival %
(Caillaud et al., 2010)

4.2. High content screening (HCS) assay

1) Neuro-2a cells (10,000 per well)
2) **Fluo-4-AM** (5 µM), Hoechst 33342 (3 µg mL^{-1})
3) MTX standard or sample (3 min)
4) Fluorescence at 488 nm (HCS)

↓

N2a intracellular calcium
(O'Brien et al., 2006)

Fig. 4: Toxicity screening on neuro-2a cells using (1) MTT to determine cell survival % or (2) Fluo-4-AM to label Ca^{2+}.

Step 5: Acquisition of data using LC-HRMS

LC: Kinetex C_{18} (50 x 2.1 mm, 2.6 µm)
- A: H_2O + HCOOH (50 mM)
- B: aq. CH_3CN (95%) + NH_4^+ $HCOO^-$ (2 mM)

Time [min]	A [%]	B [%]	Flow [mL/min]	Max. Pressure Limit [bar]
0.00	90.00	10.00	0.400	1000.00
10.00	5.00	95.00	0.400	---
12.00	5.00	95.00	0.400	---
12.10	90.00	10.00	0.400	---
16.00	90.00	10.00	0.400	---

ESI ionization technique

HRMS full scan mode
(negative ion acquisition)

Fig. 5: LC method and HRMS Q-Tof 6550 iFunnel (Agilent) for MTX detection.

Preliminary results & Discussion

N2a cytotoxicity (MTT) assay

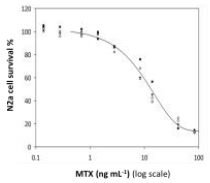


Fig. 6: Sigmoidal dose-response curve (SigmaPlot® 12) of MTX standard on the N2a cytotoxicity (MTT) assay after 2.5 h exposure.

Mitochondrial activity was determined using the quantitative colorimetric MTT assay (Tecan Infinite® M200 plate reader; Tecan, Austria, GmbH) as an indicator of cell viability.

- MTX standard produces mortality in neuro-2a cells in a dose-dependent manner (**Fig. 6**).
- Crude extract and aq. MeOH soluble fraction (MSF) of *G. excentricus* VGO 791 caused more than 90% N2a cells to die (**Fig. 7**).
- Among the 50 LH-20 fractions collected, only those from #06 to #09 (V_{MeOH} = 12.5-22.5 mL) showed to be neurotoxic (**Fig. 7**).
- The early elution of the toxic compound(s) suggests a high molecular weight.

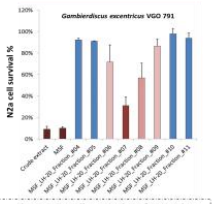


Fig. 7: N2a cell survival % after 2.5 h exposure with *G. excentricus* VGO 791.

N2a high content screening (HCS) assay

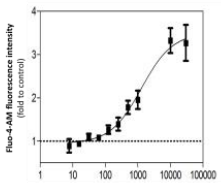


Fig. 8: Sigmoidal dose-response curve (GraphPad Prism 6.0) of MTX standard on the N2a HCS assay after 3 min exposure.

N2a cell nuclei were detected using Hoechst 33342 staining. Fluo-4-AM fluorescence was measured using an ArrayScan VTI HCS Reader (Thermo Scientific, Waltham, MA, USA) and expressed as a fold of intensity compared to vehicle control condition.

- MTX standard increased intracellular calcium in a dose-dependent manner (**Fig. 8**).
- Crude extract and MSF of *G. excentricus* VGO 791 increased Ca^{2+} levels in N2a cells (**Fig. 9**), indicating that the toxic compounds exhibit a similar effect on cells as that of MTX.
- LH-20 fractions of MSF from #06 to #10 (V_{MeOH} = 12.5-25 mL) showed an increase in intracellular calcium levels between 1.5 and 1.8 fold to control (**Fig. 9**).
- Hence, cytotoxicity and changes in intracellular calcium coincided well in early eluting fractions.

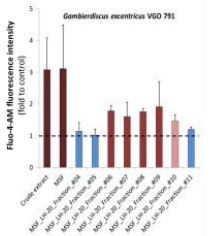


Fig. 9: Fluo-4-AM fluorescence intensity after 3 min exposure with *G. excentricus* VGO 791.

Data-mining of HRMS results

Data-mining via LC-HRMS was carried out to manage data complexity and to correlate MS data to toxicity. Raw data were retreated using MassHunter Qual software (v B.07) using an abundance cut-off of 500 counts. MassHunter algorithms recognize isotope clusters and simple adducts such as water losses. Compound identification was carried out using an in-house developed database. The identification algorithm uses both exact mass and isotope abundance of clusters for assignment to a molecular formula.

- No MTX was found in any of the extracts (crude or purified) suggesting that a hitherto undescribed compound (or more than one) is responsible for the activity found in the cellular assays.
- The crude extract contained 1,312 compounds, while the MSF contained 725 compounds (**Fig. 10**). Thus, the liquid-liquid partitioning step resulted approx. in a purification of a factor 1.8.
- Toxic LH-20 fractions (from #06 to #10) contained between 25 and 51 compounds, with the most toxic fraction (#07) containing only 40 compounds (**Fig. 10**). Comparison of toxic with non-toxic fractions is underway to pinpoint to potential candidates as causative agents for the cytotoxicity observed.
- Size-exclusion chromatography is an efficient clean-up step for high molecular weight compounds as it allows for a purification to a stage where individual compounds may be evaluated as candidates.

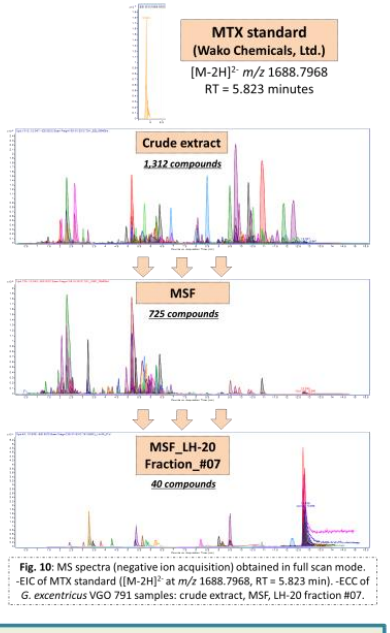


Fig. 10: MS spectra (negative ion acquisition) obtained in full scan mode. -EIC of MTX standard ([M-2H]$^{2-}$ at *m/z* 1688.7968, RT = 5.823 min). -ECC of *G. excentricus* VGO 791 samples: crude extract, MSF, LH-20 fraction #07.

Conclusions & Perspectives

Conclusions

- *G. excentricus* VGO 791 produces hydrophilic (aq. methanol-soluble) compound(s) that are lethal to neuro-2a cells, causing a rapid increase of intracellular calcium such as MTX.
- Fractionation by size-exclusion chromatography suggests that the toxic components elute early (from 12.5 to 22.5-25 mL) consistent with high molecular weight compounds.
- LC-HRMS data (Q-Tof 6550) in full scan mode (negative acquisition) showed the efficacy of liquid-liquid partitioning and size-exclusion chromatography as purification steps.
- LC-HRMS is a useful approach to identify hitherto undescribed toxic compound(s).

Perspectives

- Preparative isolation for identification of the MTX candidate(s) using a bioguided fractionation approach.
- Screening for toxicity and LC-HRMS analysis of the CH_2Cl_2 soluble fraction (DSF) to pinpoint to lipophilic toxins (CTXs).
- What about the other *Gambierdiscus* strains?

References

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