CYTOTOXICITY STUDIES FOR THE DETECTION AND QUANTIFICATION OF MAITOTOXIN

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Résumé : *la concentration inhibitrice à 50% (IC_{50}) de la maitotoxine (MTX) a été déterminée sur des cellules de fibroblastes (L929, 3T3, FR 3T3, BHK-21 C 13) et de neuroblastes (N18) de mammifère. Le comptage des cellules, le test colorimétrique au tétrazolium (MTT) et la coloration au rouge neutre (NR) ont été réalisés après 3 et 24 heures d'incubation des cellules en présence de solutions de MTX et d'extraits de toxicité inconnue. Le test au rouge neutre est le plus sensible. La IC_{50} des cellules varie suivant le type de cellules de 0,001 à 0,008MU/ml. La détection de la toxicité est observable sous un microscope à phase inverse 1 heure après exposition et la quantification 3 heures après. Le test au rouge neutre est actuellement lu au laboratoire à l'aide d'un spectrophotomètre pour plaques à multi puits, qui permet ainsi un criblage rapide d'un grand nombre de fractions obtenues au cours des différentes étapes de la purification de MTX.*

Abstract : *the 50% inhibitory concentration (IC_{50}) of maitotoxin (MTX) was evaluated on fibroblastic (L929, 3T3, FR 3T3, BHK-21 C 13) and neuroblastic (N18) mammalian cells. Cell counting, the colorimetric tetrazolium test (MTT) and the neutral red uptake assay (NR) were conducted 3 and 24 h after exposure of the cells to MTX solutions and to extracts of unknown toxicity. The sensitivity of the neutral red assay was the highest. The IC_{50} of the cells varied according to the cell type from 0.001 to 0.08 MU/ml. Detection was possible through observation under an inverted microscope 1 hour after exposure and quantification 3 hours after. The neutral red assay is currently conducted in our laboratory using a multi-well plate absorbance detector that allows a rapid screening of a large number of fractions obtained throughout the different steps of MTX purification.*

Maitotoxin (MTX), a ciguateric toxin

Three groups of toxins have been isolated from toxic fish involved in ciguatera: ciguatoxins, scaritoxin and maitotoxin. The latter has been found in the herbivorous fish *Ctenochaetus striatus*. Maitotoxin and, to a less extent, ciguatoxin have also been obtained from the benthic dinoflagellate *Gambierdiscus toxicus*.

The chemical structure of maitotoxin is still not completely known. However, some of its characteristics have been described by Yokoyama *et al.* (1988) : a molecular weight of 3424 ± 0.5, high polarity, two ester sulfate groups, no repeating units, no side chains other than methyls and no carbocycles; the structure of two parts of the molecule has been published. The detection of MTX is conducted using biological tests. Today, this toxin seems to be the most potent marine toxin, presenting a mouse lethality of 0.13 µg/kg intraperitoneally (IP).

The mode of action of MTX has been extensively studied these last years. On many cell types it induces an increase of Ca_i which can result either from the activation of Ca channels or phospholipase C. Such a Ca content increase is likely related with the positive action of the toxin on contraction of muscle cells or secretion of glandular cells. Other MTX effects have been described such as a slow phospholipase A2 activation. In addition, calcium ions are probably not the only ones to be involved in MTX action. MTX is used as a tool to dissect the relation between Ca_i elevation and cell death and also to analyze the topography of

membrane receptors involved in IP formation (Gusovsky *et al.* 1989; Gusovsky *et al.* 1990).

Our laboratory is currently involved in the purification of MTX. In order to detect and quantify maitotoxin through the different steps of purification, we have conducted cytotoxicity studies and adapted colorimetric tests (Durand-Clément *et al.*, 1991).

Maitotoxin purification

Cultures of the dinoflagellate *Gambierdiscus toxicus* isolated from Saint Barthélémy by Dr. Durand-Clément (Strain SB04) were used as source of MTX. Cells were grown on Provasoli's ES natural sea water growth medium at 27±1°C under cool white fluorescent light (700-1000 lux; LD12-12). Cells were harvested at the end of the exponential phase, and stored at -20°C.

The different steps of maitotoxin extraction were as follows: methanol extraction, diisopropylether-water partition and butanol-water partition. Further purification of the butanol extract was conducted through chromatography: open silica column ($CHCl_3$:CH_3OH gradient) and reverse phase HPLC: C18 (CH_3OH:H_2O gradient) and C8 (CH_3CN:H_2O gradient). Extracts were stored in methanol at -20°C.

Detection and quantification

Mouse bioassay : detection and quantification of MTX was conducted on 20-22 g female Swiss mice. Extracts obtained at different steps of MTX purification were evaporated, dissolved in a physiological solution (0,9% NaCl, 1% TWEEN 20) and injected intraperitoneally (IP). A mouse unit (MU) was defined as the amount of toxic extract necessary to kill a twenty gram mouse in 24 h. The specific activity of the toxic fraction obtained after C8 HPLC was of 7µg/kg mouse.

Cytotoxicity studies : toxicity was also assessed on mammalian cells: 3T3, L929 fibroblastic and N18 neuroblastoma cells from mouse [MEM medium (GIBCO), 5% foetal calf serum, antibiotics], FR 3T3 fibroblastic cells from rat embryos [DMEM medium (GIBCO), 5% calf serum, antibiotics] and BHK21 C13, hamster fibroblastic cells [BHK21 Glasgow MEM (GIBCO), 5% calf serum, antibiotics]. Cells were cultured on 12 and 96-well plates. Morphological changes were observed under an inverted transmission microscope (Nikon). The 50% inhibitory concentration (IC_{50}) of MTX was estimated by cell counts (Malassez counting chamber), the neutral red uptake assay (Borenfreund *et al.*, 1985) and the tetrazolium test (MTT) (Borenfreund *et al.*, 1988) 3 and 24 h after exposure of the cells to the extracts. Optical density (O.D.) was measured with a Multiscan MCC-340 (Labsystems) absorbance detector. A reference solution of MTX [50 µg/kg mouse (IP)] was used to estimate MTX content of unknown samples.

The stability of MTX in the cell culture media was tested upon mice and cells in culture. There was a decrease of MTX activity after 24 h in the culture medium. Detection of MTX was possible 1 h after addition to the cells. Cells exposed to MTX presented an atypical round shape or were detached from the support at high concentrations. Cell response to MTX did not apparently depend on the degree of purity of the extract (crude, butanol and HPLC extracts). The neutral red assay was more sensitive than the MTT and was selected to conduct MTX detection and quantification. Values of IC_{50} showed that sensitivity to MTX varied according to the cell type tested, N18 cells presenting a high sensitivity (table 1).

Cellular type	IC_{50} (Cell counts) (MU/ml)	IC_{50} (Neutral red O.D.) (MU/ml)
N18	.001-.002	Not tested
L929	.001-.005	.002-.01
3T3	.005-.01	.005-.015
BHK 21 C13	Not tested	.02-.08
FR 3T3	Not tested	.08

Table 1: Cytotoxicity of maitotoxin. (MU: mouse unit; O.D.: optical density)

The IC_{50} estimated 3 h after exposure of the cells to the toxin was lower than that obtained after 24 h ; the unstability of the toxin in the culture medium could account for it.

Total toxicity of MTX extracts estimated by the mouse bioassay did not show significant difference with that estimated by the neutral red assay performed on BHK 21 C13 cells.

The utilisation of a 96-well absorbance detector enabled us to test a large amount of dilutions and fractions obtained through the different steps of MTX purification. Accurate dose-response curves were obtained with this technique. BHK21 C13 cells showed a linear dose-response curve while FR 3T3 presented a slight response at low MTX concentrations (Figure 1). Further studies on the mechanism of action of MTX may clarify this point.

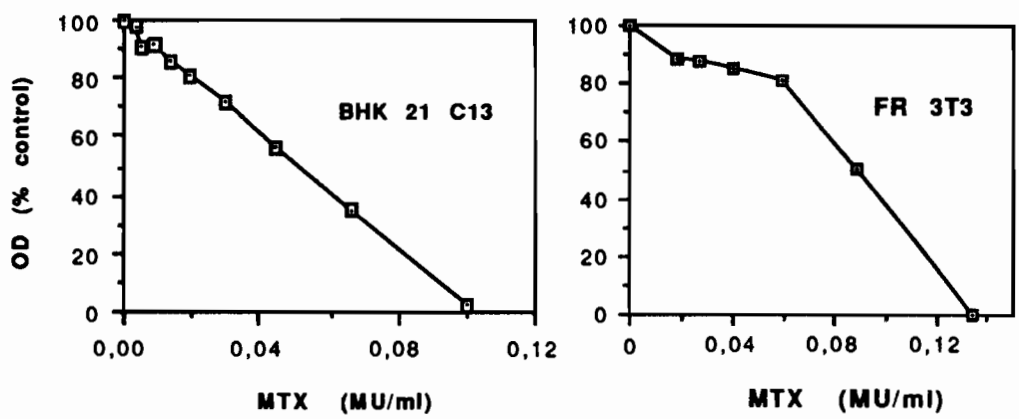

Figure 1 : Toxicity of MTX on BHK 21 C13 and FR 3T3 fibroblasts determined by the neutral red uptake assay. (MU: mouse unit; O.D.: optical density)

Cellular models are thorough, highly sensitive tools to detect and quantify MTX.The high sensitivity of the neutral red uptake assay does not only allow us to precisely establish the finest protocole of MTX purification but it also reduces the amount of toxin used when estimating the content of MTX in unknown extracts. The possibility of semi-automatisation of the test facilitates the screening of a large number of samples and reduces the number of mice used for MTX detection and quantification.

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References

Borenfreund E. and Puerner J.A., *Toxicology Lett.* **24**, 119-124 (1985)
Borenfreund E., Babich H and Martin-Alguacil N., *Toxicology in vitro* **2**, 1-6 (1988)
Durand -Clément M., Diogène G., Legay C. and Lièvremont M., *Océanis* **17**(3), 227-228 (1991)
Gusovsky F., Yasumoto T. and Daly J.W., *FEBS Lett.* **243**(2), 307-312 (1989)
Gusovsky F. and Daly J.W., *Biochem. Pharmacol.* **39**(11), 1633-1639 (1990)
Yokoyama A., Murata M., Oshima Y., Iwashita T. and Yasumoto T., *J. Biochem.* **104**(2), 184-187 (1988)

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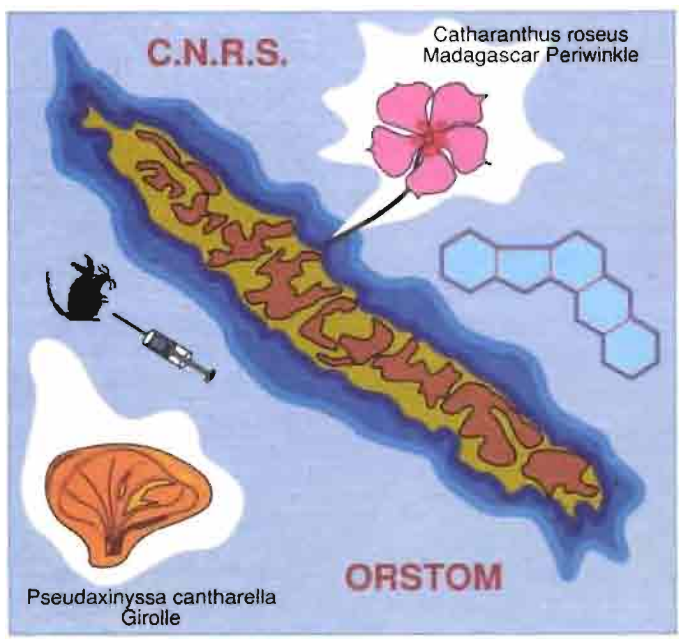

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