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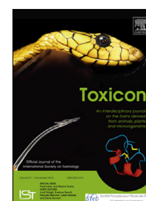

Evaluation of the intestinal permeability and cytotoxic effects of cylindrospermopsin

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A B S T R A C T

Cylindrospermopsin is a freshwater and widespread cyanotoxin considered hazardous for human health. Climate change and eutrophication are the main factors influencing the increasing presence of cylindrospermopsin producers that can contaminate human and animal drinking waters, leading to a rise in ecological and human risk. In order to reach the bloodstream and thus the target receptor, an orally administered drug must first cross the intestinal barrier. The goal of this study was to examine the cylindrospermopsin intestinal permeability and its cellular effects on intestinal and hepatic cells. We explored the human intestinal permeability of cylindrospermopsin by performing *in vitro* permeation studies across the Caco-2 cell monolayer. Cell permeability data indicated a limited passage of the toxin through the intact intestinal epithelium in a time and concentration dependent way. Cylindrospermopsin induced neither damage on the integrity of the monolayer nor cytotoxicity in tests performed with Caco-2 even at micromolar concentration. Opposite, when hepatic Clone 9 cells were exposed to cylindrospermopsin, a noticeable cytotoxicity was observed being more marked at the higher concentrations used. In addition, this cell line showed alterations in reduced glutathione content due to cylindrospermopsin over time. Meanwhile glutamate cysteine ligase levels, the first rate-limiting enzyme of the glutathione route, showed a significant increase. Therefore our results indicate that cylindrospermopsin cytotoxicity is unrelated to protein inhibition or a decrease of reduced glutathione levels in Clone 9 cells.

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1. Introduction

Cylindrospermopsin (CYN) is a widespread trycyclic alkaloid hepatotoxin first isolated in 1992 from the cyanobacterium *Cylindrospermopsis raciborskii* (Ohtani et al., 1992). Its principal analogs include 7-epi-CYN and the deoxygenated form, deoxy-CYN (Banker et al., 2000; Norris et al., 2001). The chemical structure of CYN, is a hydroxyuracil moiety linked to a tricyclic guanidine. The molecule is a sulfated guanidinium zwitterion with relative low molecular weight of 415 Da and highly water-soluble (Ohtani et al., 1992). It is also very stable in conditions of varying light, pH and heat (Chiswell et al., 1999).

C. raciborskii was the first cyanobacterial species reported as CYN producer. However, CYN is also produced by

Abbreviations: CYN, cylindrospermopsin; GSH, reduced glutathione; mBCL, monochlorobimane.

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a range of other cyanobacterial species such as *Umezakia natans* (Harada et al., 1994), *Aphanizomenon flos-aquae*, *Aphanizomenon ovalisporum*, *Anabaena lapponica*, *Raphidiopsis curvata*, *Lyngbia wollei* and more recently, *Aphanizomenon gracile* (Rucker et al., 2007). Eutrophication of waters and the ability of cyanobacteria to adapt to new habitats has allowed these organisms to inhabit new places worldwide such as lakes, reservoirs, rivers, ponds, dams and even swimming pools (Kinnear, 2010). Moreover, climate change has also been proposed as a key factor for the expansion of these species (Bonilla et al., 2012; Sinha et al., 2014). The increasing presence of these CYN producers is a potential health risk to humans and animals, in fact, several cases of intoxication after drinking contaminated water, have already been reported (Bourke et al., 1983; Hawkins et al., 1985).

CYN toxicity studies performed in mice have shown intraperitoneal (ip) LD_{50} ranging from 2 mg/kg after 24 h to 0.2 mg/kg after 5 days (Harada et al., 1994; Ohtani et al., 1992). Orally dosed mice had reduced sensitivity to the toxin showing higher LD_{50} ranging from 4.4 to 6.9 mg/kg with death after 2–6 days (Falconer et al., 1999; Seawright et al., 1999), but similar effects on the animals.

Several studies have revealed that the main target of this cyanotoxin is the liver and its hepatotoxic effects have been demonstrated *in vivo* (Shaw et al., 2000). In addition, some other organs such as the kidneys, lungs, adrenal glands, thymus, heart, bone marrow and even intestinal tract are also affected by CYN regardless of the administration route (Bazin et al., 2012; Falconer et al., 1999; Falconer and Humpage, 2001; Hawkins et al., 1985).

This cyanotoxin also showed to be potentially carcinogenic as it induces cellular transformation and genotoxic effects, including DNA fragmentation, even at non-cytotoxic concentrations (Bazin et al., 2010; Humpage et al., 2005; Shaw et al., 2000). Furthermore, some studies revealed that the uracil group interferes with DNA synthesis and may act as a carcinogen in several mouse tissues (Falconer and Humpage, 2001).

Several studies, performed mainly in rat and mice primary hepatocytes, revealed high sensitivity to CYN and an apparent first cellular effect through the inhibition of protein synthesis (Froscio et al., 2003; Runnegar et al., 2002; Terao et al., 1994). Cytotoxicity has also been reported in a range of different mammalian cell lines including hepatic, renal, gastric and intestinal derived cells (Froscio et al., 2009b). It was also reported that the toxin interferes with glutathione metabolic route inhibiting its synthesis (Norris et al., 2002; Runnegar et al., 1995). Both effects are presumed to be long-term toxicity effects, as acute toxicity seems to be exerted by cytochrome P450 (CYP450) generated metabolites when the toxin is metabolically activated at high doses (Bazin et al., 2010; Froscio et al., 2003; Humpage et al., 2005).

How CYN into cells is not yet understood. The molecular size (415 Da) and the hydrophilic nature of the toxin mean that it is unlikely to be readily cell-permeant and may require transport into the cells. Compared to other cyanotoxins, CYN is mainly produced as an extracellular toxin; appearing consequently in a large quantity in environmental samples (van Apeldoorn et al., 2007), being a potential hazard in drinking water worldwide (Carmichael et al., 2001). The actual lack of precise and reliable information about CYN intestinal permeability impairs the establishment of human and animal safe limits in freshwater.

The aim of this study was to elucidate if CYN can cross the intestinal epithelium and enter systemic circulation by using an *in vitro* model with differentiated Caco-2 cells that emulates the human intestine epithelium. In addition we performed a screening of CYN cytotoxic effects on these Caco-2 cells and, due to the hepatotoxic nature of the toxin, also on a rat hepatic cell line, Clone 9.

2. Materials and methods

2.1. Chemicals and biological materials

Cylindrospermopsin (CYN) was purchased from Enzo Life Sciences Inc (Farmingdale, NY, USA). Rabbit Anti-GCLC (Glutamate cysteine ligase catalytic subunit) antibody was supplied by Abcam plc (Cambridge, United Kingdom). Falcon Multiwell 12-well polystyrene plates were purchased from Becton Dickinson (Le Port de Claix, France). BRAND plates 96 well pure Grade non-sterile black F-bottom were from Brand GMBH (Werheim, Germany). Costar 96-well assay plates with clear bottom and tissue culture-treated surface were supplied from Corning Inc. (New York, NY, USA). Sheep ECL Mouse IgG (Inmunoglobulin G), HRP-linked whole antibody was obtained from GE Healthcare (Madrid, Spain). AlamarBlue®, Novex® 10% Tris-Glycine Mini Gels 1.0 mm, 15 Well, Novex® Tris-Glycine SDS Sample Buffer, Novex® SeeBlue® Plus2 Pre-Stained Standard and Phycoerythin (PE) were from Invitrogen (Camarillo, CA, USA). 12-well Milicell hanging culture inserts with 0.4 μm pore size polyethylene terephthalate (PET) membrane, Chicken Anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) antibody, Mouse Anti-Actin antibody clone C4, Goat anti-Rabbit IgG antibody peroxidase conjugated, Rabbit anti-Chicken IgG, HRP conjugate, Immobilon-P Transfer Membrane pore size 0.45 μM, calibration and performance verification kits for Luminex 200™, Luminex sheath fluid, multiscreen 96 well filter plates (Durapore® membrane), 33 mm Milex filter with 0.22 μm and 0.45 μm pore size Ultrafree-MC centrifugal filters (Durapore® membrane) and 0.45 μm pore size Ultrafree-CL centrifugal filters (Low binding Durapore® PVDF membrane) were from Millipore Corporation (Billerica, MA, USA). Fetal Bovine Serum (FBS), HAM's F-12 with stable glutamine medium and Eagle's Minimum Essential Medium (EMEM) were obtained from PAA (Pasching, Austria). Glutathione Assay Kit Fluorimetric, 2-Mercaptoethanol, DL-Buthionine-(S,R)-sulfoximine (BSO), Nutrient Mixture F-12 Ham Kaighn's Modification and Mouse monoclonal Anti-β-Tubulin I antibody, N-hydroxysuccinimide (NHS), sodium tetraborate decahydrate, jeffamine (2,2′-(ethylenedioxy) bis(ethylamine)), ethylenediamine, boric acid, sodium phosphate monobasic, ethanolamine and Tween-20 were purchased from Sigma–Aldrich (St. Louis, MO, USA). Super Signal ELISA Femto Maximum Sensitivity Substrate and Super Signal West Pico Chemiluminescent Substrate were supplied by Thermo Scientific (Rockford, IL, USA). 1-ethyl-

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