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Morphological cell transformation of Syrian hamster embryo (SHE) cells by the cyanotoxin, cylindrospermopsin

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ABSTRACT

Cylindrospermopsin (CYN) is a cyanotoxin which has been implicated in human intoxication and animal mortality. Genotoxic activity of this hepatotoxin is known but its carcinogenic activity remains to be elucidated. In this work, CYN was assessed for its cell-transforming activity using the Syrian hamster embryo (SHE) cell transformation assay. This *in vitro* assay is used to evaluate the carcinogenic potential of chemical, physical and biological agents in SHE cells, which are primary, normal, diploid, genetically stable and capable of metabolic activation. We demonstrated that CYN induced a significant increase in morphological cell transformation in SHE cells following a 7-day continuous treatment in the range of non-cytotoxic concentrations 1×10^{-7}–1×10^{-2} ng/mL.

1. Introduction

This study aimed to assess the carcinogenic potential of cylindrospermopsin (CYN) (Fig. 1), a cyanobacterial hepatotoxin mainly produced by *Cylindrospermopsis raciborskii* (Van Apeldoorn et al., 2007). Due to widespread eutrophication, CYN may occur worldwide in drinking-water sources such as lakes and reservoirs and has been implicated in human intoxications and animal mortality (Carmichael et al., 2001; Griffiths and Saker, 2003; Hawkins et al., 1985). CYN is a hepatotoxin when administered orally in rodents but it also caused damage in the kidneys, stomach, intestine, thymus, spleen and white blood cells in mice (Falconer et al., 1999; Hawkins et al., 1985). Foetal toxicity in the mouse after CYN exposure late in gestation has already been found (Rogers et al., 2007). *In vitro* toxicity studies have shown that CYN is a potent inhibitor of protein synthesis in a cell-free system (Froscio et al., 2001) and in cell culture (Froscio et al., 2003), as well as of glutathione synthesis (Runnegar et al., 1995, 1994; Humpage et al., 2005). *In vitro* endocrine disruptor properties were recently reported as inhibition of progesterone production was observed (Young et al., 2008). Preliminary evidence of carcinogenic effects have been reported *in vivo* mainly in liver of mice (Falconer and Humpage, 2001) and an epidemiological study of an exposed population with CYN in Australia indicated an increased rate of gastrointestinal cancers compared to unexposed population, although involvement of CYN was not proved (Falconer and Humpage, 2006). Nevertheless, mechanisms leading to carcinogenesis need to be elucidated. Recently, Gácsi et al. (2009) have shown that CYN affects the chromatin structure by inhibiting chromatin condensation and by preventing the formation of metaphase chromosomes. These authors suggested that the modification of the chromatin

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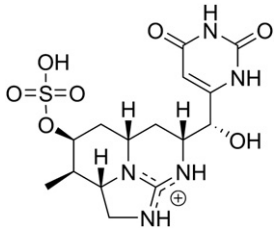

Fig. 1. Chemical structure of CYN.

structure could be related to a possible carcinogenic effect of CYN as it was demonstrated that chemically-induced carcinogenesis affects chromatin structure by causing a high degree of supercoiling (Trencsényi et al., 2007). CYN has been found to be genotoxic *in vivo* after intraperitoneal injection in Balb/c mice of a single dose of 0.2 mg/Kg CYN (Shen et al., 2002) and *in vitro* in primary mouse hepatocytes (0.05–5 μM CYN equivalent to 20.8–208 ng/mL) (Humpage et al., 2005). Investigations with the cytokinesis block micronucleus assay have revealed chromosome alteration properties of CYN by induction of significant increases in the frequency of micronuclei in both differentiated and undifferentiated human intestinal Caco-2 cells (0.5–2 μg/mL CYN) (Bazin et al., in press), in differentiated human hepatoma HepaRG cells (0.04–0.3 μg/mL CYN) (Bazin et al., in press) and in human lymphoblastoid cells (WIL2-NS) (6–10 μg/mL CYN) (Humpage et al., 2000), as well as loss of whole chromosomes in WIL2-NS cells (1–10 μg/mL CYN) (Humpage et al., 2000). CYN seems to act as a progenotoxin involving genotoxic cytochrome P450-generated metabolites since genotoxic effects induced *in vitro* with CYN were inhibited in primary mouse hepatocytes by the cytochrome P450 inhibitors, omeprazole and SKF525A (Humpage et al., 2005), and in Caco-2 and HepaRG cells by ketoconazole (Bazin et al., in press). This conclusion is supported by the absence of DNA damage observed following a CYN treatment (0.5 and 1 μg/mL) in CHO-K1 cells which have low metabolic activities (Fessard and Bernard, 2003). CYN was not found to be clastogenic in CHO-K1 in the range of 0.05–2 μg/mL with or without metabolic activation with S9 fraction (Lankoff et al., 2007).

To study the carcinogenic potential of CYN, we used an *in vitro* short-term assay, the cell transformation assay (CTA) on Syrian hamster embryo (SHE) cells (Combes et al., 1999) that has been recommended by OECD Guidelines as a alternative to *in vivo* long term experiment for carcinogenic potential of chemicals (OECD, 2007, http://www.oecd.org/dataoecd/56/5/37863750.pdf). SHE cells are primary, normal diploid cells, genetically stable and capable of metabolic activation. They provide a suitable model for the research of mechanisms of morphological cell transformation (Alexandre et al., 2003; Maire et al., 2005a,b; 2007), a phenotypic feature expressing the first steps of the conversion of normal to neoplastic-like cells with oncogenic properties (DiPaolo et al., 1971). SHE cells used at early passages constitute the basis of the *in vitro* cell transformation assay for the detection of genotoxic and non-genotoxic carcinogens. Cells derived from morphologically transformed colonies produce tumours when injected back into isologous animals, which validated the use of phenotypically transformed SHE cells as an indicator of neoplastic properties of chemicals. The morphologically-transformed phenotype is characterised by the lost of contact inhibition and density-dependent inhibition of cells, resulting in a random orientation pattern of growth in three dimensions (Fig. 2). This short-term assay is commonly used to identify non-genotoxic and genotoxic rodent carcinogens. More than 500 substances and chemicals have been evaluated with this assay since it was first reported by Berwald and Sachs in the 1960s (Berwald and Sachs, 1963, 1965; OECD, 2007).

2. Material and methods

2.1. Chemicals

CYN [CAS #143545-90-8] was dissolved in water. Purified CYN was supplied by the Australian Water Quality Centre (>98% purity, Adelaide, Australia). In all experiments, benzo(a)pyrene (BaP) [CAS #50-32-8] was used as positive control and DMSO [CAS #67-68-5] as vehicle solvent used at final concentration of 0.02%. The chemicals were diluted in culture medium just before use.

2.2. Syrian hamster embryo cell culture

Cryopreserved SHE cells were isolated from a Syrian golden hamster embryo at day 13 of gestation using the procedure described by Pienta et al. (1977) and in accordance with the modifications suggested by Elias et al. (1989). Stock cells were preserved in liquid nitrogen. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gibco 31600 without phenol red). The lyophilised DMEM medium was reconstituted with ultrapure water, then adjusted to pH 7.0 at 37 ± 1 °C in a 10 ± 0.5% CO_2 humidified atmosphere with 1.38 g/L $NaHCO_3$ and sterilised by membrane filtration (0.2 μm porosity). The complete medium was prepared with addition of 12% heat-inactivated foetal calf serum (FCS, SH30070, batch AQL25247, Hyclone).

2.3. Cell transformation assay

The transforming potency of each chemical was studied according to the procedure previously described (Tu et al., 1986; Elias et al., 1989; Bessi et al., 1994). To summarise, 60,000 X-irradiated cells (50 Gy, to prevent cell division and used to feed target SHE cells) were plated in 60 mm Petri dish (Corning) with 2 mL complete medium. The next day, 150–200 target SHE cells were seeded with 2 mL complete medium in each dish onto the feeder layer of irradiated SHE cells. The cells were treated 24 h after target cell seeding with 4 mL complete medium containing CYN or BaP. At least, ten dishes were used per treatment for the cytotoxicity assay and at least forty dishes were used per treatment group in each series of cell transformation assays. Two series of experiments have been carried out. The concentrations for the first experiment were 1×10^{-5}–1 ng/mL and 1×10^{-7}–1×10^{-3} ng/mL for the second one. The results of both experiments were pooled. The cells were exposed to the test substance at 37 ± 1 °C in