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Toxicity of cylindrospermopsin, and other apparent metabolites from *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*, to the zebrafish (*Danio rerio*) embryo

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ABSTRACT

Cyanobacteria produce a diverse array of toxic or otherwise bioactive compounds that pose growing threats to human and environmental health. We utilized the zebrafish (*Danio rerio*) embryo, as a model of vertebrate development, to investigate the inhibition of development pathways (i.e. developmental toxicity) by the cyanobacterial toxin, cylindrospermopsin (CYN), as well as extracts from various isolates of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*. CYN was toxic only when injected directly into embryos, but not by direct immersion at doses up to 50 μg/ml. Despite the dose dependency of toxicity observed following injection of CYN, no consistent patterns of developmental defects were observed, suggesting that toxic effects of CYN may not target specific developmental pathways. In contrast, direct immersion of embryos in all of the extracts resulted in both increased mortality and reproducible, consistent, developmental dysfunctions. Interestingly, there was no correlation of developmental toxicity observed for these extracts with the presence of CYN or with previously reported toxicity for these strains. These results suggest that CYN is lethal to zebrafish embryos, but apparently inhibits no specific developmental pathways, whereas other apparent metabolites from *C. raciborskii* and *A. ovalisporum* seem to reproducibly inhibit development in the zebrafish model. Continued investigation of these apparent, unknown metabolites is needed.

1. Introduction

Cyanobacteria ("blue-green algae") produce a diverse array of toxic or otherwise bioactive metabolites. In freshwater environments, in particular, these compounds can pose serious threats to human and environmental health via contamination of drinking water, recreational exposure to waterborne toxins and possible accumulation of toxins in the food-web (e.g. Chorus et al., 2000; Paerl et al., 2001; Rao et al., 2002; Codd et al., 2005; Falconer and Humpage, 2006). Though toxicoses associated with exposure to cyanobacterial toxins are typically recognized from cases of acute poisoning, emerging studies support a likely role of these compounds in sub-acute health effects (e.g. Milutinovic et al., 2002; Dos et al., 2005; Falconer and Humpage, 2006; Sukenik et al., 2006). One such example is the growing evidence to indicate cyanobacterial (and other marine and freshwater algal) toxins may act as developmental toxins, inhibiting or impairing various pathways of vertebrate development (Oberemm et al., 1997; Papendorf et al., 1997; Pilotto et al., 1999; Jacquet et al., 2004; Wang et al., 2005; Bu et al., 2006; Sukenik et al., 2006; Berry et al., 2007; Palikova et al., 2007; Rogers et al., 2007; Wright et al., 2006; Lecoz et al., 2008).

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The cyanobacterial toxin, cylindrospermposin (CYN; Fig. 1), is a hepatotoxic alkaloid first isolated from *Cylindrospermopsis raciborskii*, and subsequently characterized chemically, by Ohtani et al. (1992). Toxicity of CYN was first recognized when more than 100 children of Aboriginal families on Palm Island in Queensland, Australia were admitted to hospitals for various symptoms of gastroenteritis (Griffiths and Saker, 2003). The illness was eventually linked to contamination of the local water supply with a dense algal bloom, and specifically a strain of *C. raciborskii*. From this isolate, CYN was purified and characterized chemically as a highly water-soluble cyclic guanidinium alkaloid, containing a unique tricyclic hydroxymethyl uracil (Fig. 1; Ohtani et al., 1992). Subsequently, the toxin has been identified in at least five additional genera of cyanobacteria, including *Anabaena*, *Aphanizomenon*, *Raphidiopsis*, *Lyngbya* and *Umezakia* (Harada et al., 1994; Banker et al., 1997; Li et al., 2001a; Seifert et al., 2007), and CYN-producing strains of *C. raciborskii* and other cyanobacteria are being found to be increasingly prevalent in temperate and tropical freshwaters (Saker and Griffiths, 2001; Neilan et al., 2003; Saker et al., 2003).

Despite considerable research, much remains to be clarified with respect to the toxicity of CYN. Initial evaluation of CYN indicated that the most likely mode of action of CYN is inhibition of protein synthesis, including observed detachment of ribosomes from membranes of the rough endoplasmic reticulum or possible interaction with soluble proteins involved in protein translation (Terao et al., 1994; Froscio et al., 2001; Runnegar et al., 2002; Froscio et al., 2008). Froscio et al. (2001), for example, using an *in vitro* assay system based on the rabbit reticulocyte lysate translation system, reported an IC_{50} of 120 nM and detection limit of 50 nM (equivalent to approximately 50 ng/mL and 21 ng/mL, respectively) for inhibition of protein synthesis by CYN. Subsequent studies (e.g. Runnegar et al., 2002; Froscio et al., 2008) using this same *in vitro* assay system have consistently confirmed inhibition at these concentrations. Likewise, evaluation of cellular inhibition of protein synthesis, specifically in mouse hepatocytes, similarly suggests complete inhibition at concentrations above 0.5 μM (Froscio et al., 2008). In addition, dose-dependent cytotoxicity of CYN was also observed in both rat and mouse hepatocytes at concentrations above 1 μM (Runnegar et al., 1994; Froscio et al., 2003). However, attenuation of this cytoxicity, and not protein synthesis, by cytochrome P450 inhibitors suggests that this acute toxicity to liver cells may be related to biotransformation products rather than inhibition of protein synthesis by CYN (Runnegar et al., 1995). Moreover, recent studies suggest CYN, with a potentially reactive guanidine, may also act through covalent binding and breakage of DNA in cells exposed at concentrations in the range of 1–10 μg/mL (Humpage et al., 2000; Shaw et al., 2000; Shen et al., 2002) leading to various cellular abnormalities. More recent studies though have suggested that cellular abnormalities may be unrelated to direct interaction with DNA (e.g. Fessard and Bernard, 2003). Clearly, given the increasing recognition of the apparently widespread distribution of CYN and CYN-producing cyanobacteria in freshwater systems, continued toxicological investigation will be essential to clarifying potential threats to human and environmental health.

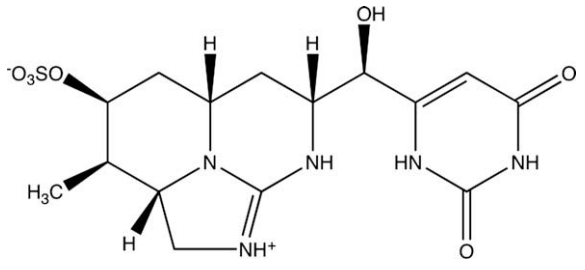

Fig. 1. Chemical structure of cylindrospermopsin. Cylindrospermopsin is a water-soluble cyclic guanidinium alkaloid containing a tricyclic hydroxymethyl uracil.

Identification and characterization of developmental toxins from marine and freshwater algae has been facilitated by the use of several aquatic models, specifically including embryos of the zebrafish (*Danio rerio*) and other teleost fish species (reviewed by Berry et al., 2007). Owing to a number of practical advantages including small size, nearly transparent embryos, ease of husbandry, short embryogenesis and rapid sexual maturation, as well as growing knowledge of the species' genome, the zebrafish has emerged as an especially important model system (Teraoka et al., 2003; Hill et al., 2005). This includes the use of zebrafish embryos to characterize developmental toxicity of a number of well-described toxins from marine algae, including saxitoxin (LeFebvre et al., 2004) and domoic acid (Tiedeken et al., 2005), and from freshwater cyanobacteria, particularly including microcystin-LR (Oberemm et al., 1997; Wang et al., 2005). In addition, the zebrafish embryo has been used to screen algal isolates for developmental toxins (Berry et al., 2007), and guide purification and characterization of novel or less well-known cyanobacterial metabolites with respect to their developmental toxicity, including the previously unknown mueggelone (Papendorf et al., 1997) from blooms of *Aphanizomenon flos-aquae*, and hapalindole alkaloids from *Fischerella* (Berry et al., 2007). Here we employ the zebrafish embryo as a model of vertebrate development to investigate the developmental toxicity of CYN, as well as other possible metabolites from *C. raciborskii* and other CYN-producing taxa of cyanobacteria.

2. Materials and methods

2.1. Chemicals

Solvents, including methanol (MeOH; Omnisolv®, 99.9% purity minimum) and HPLC-grade chloroform ($CHCl_3$; 99.8% purity minimum), from EMD Chemicals, Inc. were purchased from Fisher Scientific. CYN, purified as described below, was obtained from the National Research Centre for Environmental Toxicology at the University of Queensland, Australia. All other chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.).

2.2. Isolation and culture of cyanobacteria

Seven strains of *C. raciborskii* (Table 1), and one strain of *Aphanizomenon ovalisporum* (Table 1), were isolated