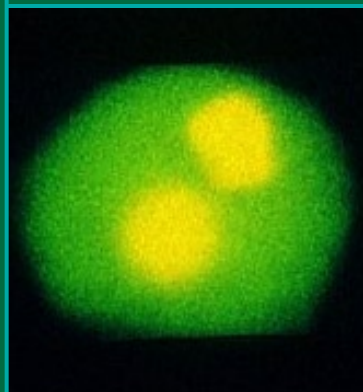
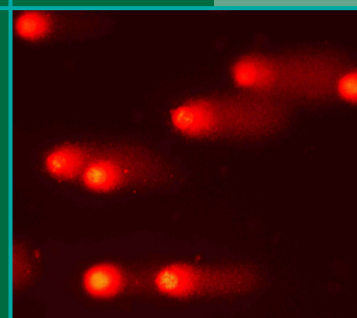




# Cylindrospermopsin Mechanisms of Toxicity and Genotoxicity



Research Report

61

# **Cylindrospermopsin Mechanisms of Toxicity and Genotoxicity**

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## FOREWORD

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## EXECUTIVE SUMMARY

While sufficient data has been produced on the acute and sub-chronic toxicity of cylindrospermopsin (CYN), the lack of data on carcinogenicity, genotoxicity and mechanisms of toxicity has prevented the establishment of guidelines for the toxin.

Preliminary evidence by CRC researchers Prof Ian Falconer and Dr Andrew Humpage has suggested that there is likelihood that CYN is carcinogenic. This project aimed to further investigate the mechanisms underlying toxicity and genotoxicity of CYN and how these may relate to potential carcinogenicity. The data obtained should aid in the risk assessment process. Another issue to be determined was the potential toxicity of the CYN analogue deoxycylindrospermopsin (deoxyCYN) that occurs in source waters with CYN.

Specifically the aims of the project were:

- To gain an insight into the mechanisms of CYN toxicity and genotoxicity
- To assess the toxicity of the CYN analogue, deoxycylindrospermopsin
- To evaluate toxicological or genotoxicological endpoints for use as biomarkers of human exposure to CYN
- To use the toxicology data to undertake human health risk assessment and to provide this information for use in guidelines for CYN in drinking water.

The report is based on a number of journal publications arising from key findings of the project.

### ***Mechanisms of toxicity and genotoxicity***

A range of different endpoints were investigated in order to gain an insight into the mechanisms of toxicity and genotoxicity of CYN. The role of cytochrome P-450 (CYP450) metabolites and oxidative stress were explored using primary mouse hepatocytes (liver cells) as the cell model (Chapter 1), while analysis of CYN effects on tumour suppressor protein p53 regulated genes was carried out on human cell lines (Chapter 2).

Research outcomes in primary hepatocytes showed that genotoxic events measured by the COMET assay were induced at sub-cytotoxic concentrations of CYN, and that pre-treatment of the cells with CYP450 inhibitors reduced the genotoxic events to control levels. These results suggest that genotoxicity is a primary effect of the toxin and that CYP450 derived metabolites are involved in production of the DNA damage.

The reduction in cellular glutathione (GSH) levels (cellular antioxidants) induced by CYN in hepatocytes did not appear to interfere with the ability of the cell to quench reactive oxygen species (ROS). The oxidative stress marker malondialdehyde (MDA) was not raised in the CYN treated cells, and a further block in GSH regeneration did not potentiate cytotoxicity of CYN. It therefore seems unlikely that oxidative stress is involved in CYN toxicity and therefore also of CYN genotoxicity.

Results of the gene expression analysis revealed that CYN induces the tumour suppressor protein p53 in human cell lines (Chapter 2). This p53 transcription factor is known to control the expression of a large number of genes associated with growth arrest, cell death and DNA repair processes. It can be induced by DNA damage as well as other cellular stressors. CYN induced concentration dependent increases in mRNA levels of p53 target genes known as CDKN1A, GADD45 $\alpha$ , BAX and MDM2 following 6 hr exposure, indicating early activation of p53. The mRNA levels for these genes also remained elevated at later time points. It was not possible to confirm whether the p53 response was related to DNA damage or other cellular stressors, however given the importance of p53 in co-ordinating the DNA repair processes and relation to carcinogenicity process, further research work in this area is of importance.

***Toxicity of deoxy-cylindrospermopsin***

Toxicity data for the CYN analogue, deoxy-cylindrospermopsin (deoxyCYN) is limited, although *in vivo* data has previously suggested it is of low potency. In Chapter 3, the *in vitro* toxicity of deoxyCYN was evaluated using cell lines, including the human derived HepG2 (liver) and Caco2 (colon) cells. Results showed that deoxyCYN was cytotoxic with  $IC_{50}$  determinations similar to CYN following 24 hr and 48 hr exposure to the toxin. Morphological changes characteristic to CYN (rounding of cells) were also noted after exposure to the analogue. Furthermore, deoxyCYN inhibited protein synthesis ( $IC_{50}$  = 220 nM) with similar potency to CYN ( $IC_{50}$  = 320 nM) in reticulocyte lysates. The results indicate that deoxyCYN acts by the same mechanism of toxicity as CYN and is equitoxic. In light of the toxicity results obtained in this study, deoxyCYN may also need to be considered in the CYN risk assessment process as is the practice for other cyanobacterial toxin analogues.

***Biomarkers of human exposure to cylindrospermopsin***

In Chapter 4, CYN's interaction with the eukaryotic protein synthesis system was further characterised. This toxicological endpoint was of interest as little is known about how CYN, a potent protein synthesis inhibitor, interacts with the protein synthesis machinery. It was also considered CYN's binding site may be able to be used as a biomarker for human exposure to the toxin, particularly if the interaction was irreversible or reasonably strong, facilitating an extended half life *in vivo*. Previous work has shown that CYN effects on protein synthesis were irreversible in mammalian cell culture, although it could not be determined whether this was due to irreversible binding of the toxin at the site of interaction or the toxin being trapped within the cell.

Using radiolabelled toxin, binding studies were carried out. CYN-ribosome interactions were assessed in reticulocyte lysate following separation of ribosomes from free toxin. Results suggested that the CYN may not target the ribosome itself, but rather one of the soluble proteins associated with the eukaryotic translation system. Displacement studies indicated that CYN binding was non-covalent, although incomplete removal in the presence of 100-fold excess of unlabelled toxin suggests there is a strong association of CYN at the binding site. Given that the results indicate a strong interaction at the binding site, identification of the protein that CYN targets may allow a suitable biomarker to be developed.

***Human health risk assessment / guidelines for cylindrospermopsin in drinking water***

A couple of informative review articles were also published during the course of the project relating to human health risk assessment of cyanotoxins in drinking water (Chapters 5 and 6). CYN toxicology data was reviewed, with respect to human health risk assessment and the information required for guidelines for CYN in drinking water to be set.

The process by which the World Health Organisation (WHO) assesses safe levels of cyanobacterial toxins in water is described in Chapter 5. Experimental data is used to calculate a Tolerable Daily Intake (TDI) for safe human consumption. From this value the Guideline Value (also called the reference dose and the maximum acceptable concentration) is calculated. The toxicology of cylindrospermopsin with respect to development of guideline levels is also discussed in Chapter 6 and highlights the need for further mechanistic studies to be carried out to support current CYN toxicity results, particularly with respect to genotoxicity and mechanism of potential carcinogenicity.

***Other research and related works***

Further work carried out in this project investigating the potential human reproductive effects of CYN will be reported separately as part of a PhD thesis written by Dina Zebian. Related work on cylindrospermopsin toxicology includes CRC Research Report 13: Oral Toxicity of Cylindrospermopsin: No Observed Adverse Effect Level Determination in Male Swiss Albino Mice.

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**ABBREVIATIONS**

AWQC	Australian Water Quality Centre
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BCNU	1,3-Bis(chloroethyl)-1-nitrosourea
BHT	Butylated hydroxytoluene
BSA	Bovine serum albumin
CYN	cylindrospermopsin
CYP450	cytochrome P450
deoxyCYN	deoxy-cylindrospermopsin
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulfoxide
EC50	50% Effective Concentration
EDTA	ethylene-diamine tetraacetic acid
eEF	eukaryotic elongation factor
eRF	eukaryotic release factor
GSH	glutathione
GSSG-Rd	Glutathione disulfide reductase
GV	Guideline Value
HDF	Human Dermal Fibroblasts
IC50	50% Inhibitory Concentration
LDH	lactate dehydrogenase
MDA	malondialdehyde
MS	mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
NMR	nuclear magnetic resonance spectrometry
NOAEL	No Observed Adverse Effect Level
PBS	phosphate buffered saline
PES	polyethersulphone
PSI	protein synthesis inhibition
qRT-PCR	Quantitative reverse-transcription polymerase chain reaction
ROS	Reactive oxygen species
t-BH	tert-butyl hydroperoxide
TDI	Tolerable daily intake

# 1 CYLINDROSPERMOPSIN GENOTOXICITY AND CYTOTOXICITY: ROLE OF CYTOCHROME P-450 AND OXIDATIVE STRESS

This Chapter is based on the following manuscript:

Humpage, A.R., Fontaine, F., Froschio, S., Burcham, P., and Falconer, I. R. (2005).  
Cylindrospermopsin genotoxicity and cytotoxicity: Role of cytochrome P-450 and oxidative stress.  
*Journal of Toxicology and Environmental Health. Part A* **68**, 739-753.

## 1.1 Introduction

The cyanobacterial toxin cylindrospermopsin (CYN) occurs in a number of important drinking-water sources in the world and is being increasingly recognised as a potential threat to drinking water safety (Kuiper-Goodman *et al.*, 1999). It has been strongly implicated in human poisoning events in Australia and Brazil (Hawkins *et al.*, 1985; Carmichael *et al.*, 2001). In the Australian case, 138 children and 10 adults were affected, with the majority requiring hospitalisation. General effects included gastroenteritis, hepatomegaly, and renal insufficiency. A proportion also developed bleeding from mucous membranes or more commonly the gut. All patients recovered within 26 days (Byth, 1980). In the Brazilian case, the co-occurrence of CYN and microcystins confounds the analysis (Carmichael *et al.*, 2001). Various *in vitro* models were previously used to show that the toxin produces multiple cytopathic effects such as protein synthesis inhibition, cytochrome p-450 (CYP450)-metabolism-dependent cytotoxicity, and genotoxicity (Runnegar *et al.*, 1994; Humpage *et al.*, 2000; Froschio *et al.*, 2001, 2003). Protein synthesis inhibition (PSI) occurs (during the elongation step) in the cell-free reticulocyte lysate model of protein synthesis (Terao *et al.*, 1994; Froschio *et al.*, 2001, 2003). Furthermore, in primary hepatocytes, PSI is irreversible and independent of the presence of the broad-spectrum CYP450 inhibitors proadifen and ketoconazole (Froschio *et al.*, 2003). PSI is therefore probably mediated by the parent compound. However, although 100% PSI occurs within 4 h in primary mouse hepatocytes at concentrations above 0.5 M CYN, higher concentrations (1-5  $\mu$ M) are required to produce cell death within 18 h (Froschio *et al.*, 2003). This latter process is inhibitable by SKF525A (proadifen) and ketoconazole *in vitro*. This suggests that the toxicity at low concentrations is primarily mediated via PSI, but at higher concentrations a more rapidly toxic process dominates that is metabolism dependent. This pattern is similar to that seen with acute *in vivo* exposure, where the intraperitoneal (ip) LD<sub>50</sub> for death by 24 h is 2 mg/kg but that for death by 5 d is 1/10th that value (Ohtani *et al.*, 1992). *In vivo*, however, the broad-spectrum CYP inhibitor piperonyl butoxide (PBO) was protective for 7 d against CYN toxicity ip in mice at doses of 0.2-0.8 mg CYN/kg (Norris *et al.*, 2002). In this situation, it might be expected that PSI would still have caused death. However, PBO is known to reduce uptake of other compounds via inhibition of a required CYP450 activity (Sriram *et al.*, 1995; Kuo *et al.*, 1983). Putative CYN metabolites have been observed *in vivo* (Norris *et al.*, 2001). Preliminary evidence suggests that CYN may be carcinogenic in mice (Falconer & Humpage, 2001). DNA adduction has been reported *in vivo* (Shaw *et al.*, 2000), and DNA fragmentation was found to be increased in the livers of animals treated with an LD<sub>50</sub> dose of CYN (Shen *et al.*, 2002), although some effect on DNA integrity due to direct toxicity might be expected at this level of exposure. Humpage *et al.* (2000) also demonstrated genotoxicity using the cytokinesis-blocked micronucleus assay in the lymphoblastoid cell line WIL2-NS. In this system, whole chromosome loss was observed at CYN concentrations of 1  $\mu$ M and above, and strand breakage at 6  $\mu$ M and above. It is possible to speculate that, analogous to acute cytotoxicity, the loss of whole chromosomes might be due to disruption of kinetochore formation secondary to PSI, whereas strand breakage might be due to direct effects on DNA following metabolic activation of the toxin. However, WIL2-NS metabolism of CYN is yet to be confirmed. In contrast, no change in genotoxicity, as measured by the COMET assay, was seen in CYN-treated metabolism-deficient CHO K1 cells (Fessard & Bernard, 2003). Levels of glutathione (GSH) are reduced in affected cells secondary to inhibition of glutathione synthesis (Runnegar *et al.*, 1994, 1995), although *in vivo* evidence suggests that this reduction does not contribute significantly to CYN's acute toxicity (Norris *et al.*, 2002). However, this does not exclude the possibility that increased levels of reactive oxygen species (ROS) in the absence of GSH to quench them may contribute to genotoxicity. In the present study the involvement of metabolism in genotoxicity was investigated by determining whether CYP450 inhibitors protect primary hepatocytes from this outcome as measured by the COMET assay. Levels of the lipid peroxidation marker malondialdehyde (MDA) were also measured to determine whether increased levels of ROS are induced by CYN.

## 1.2 Methods

### 1.2.1 Animals

Adult male albino Swiss mice (4-6 wk old weighing 25-30 g) were obtained from the Adelaide University Animal Breeding Facility (Waite Institute, Adelaide, SA). All animals were supplied with standard rodent food and tap water *ad libitum*, and were housed at 22°C and 40-60% humidity with a 12-h light/dark cycle.

### 1.2.2 Chemicals

Cylindrospermopsin was kindly supplied by the Australian Water Quality Center (AWQC). The toxin was purified from cyanobacterial cultures of *Cylindrospermopsis raciborskii* Seenayya Subba Raju and characterised using nuclear magnetic resonance spectrometry (NMR), mass spectrometry (MS), and liquid chromatography coupled to a photodiode array. The toxin, present as the sodium salt, was free from organic impurities with an estimated purity above 98% according to NMR and full-scan MS. Glutathione (GSH) and tert-butyl hydroperoxide (t-BH) were obtained from Sigma-Aldrich (St. Louis, MO). 1,3-Bis(chloroethyl)-1-nitrosourea (BCNU) was obtained from Bristol Laboratories (Princeton, NJ). Butylated hydroxytoluene (BHT) was obtained from ICN Biochemicals (Cleveland, OH). Lactic acid, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), sodium dithionite, SKF525A, and collagenase (*Clostridium histolyticum*, type IV) were obtained from Sigma Chemical Co. (St. Louis, MO). NAD (free acid, grade III) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). RPMI-1640 media and gentamicin (10 mg/mL) were purchased from Gibco BRL Life Technologies (Cergy Pontoise, France). Omeprazole was purchased from Ultrafine Chemicals Ltd (Manchester, UK). SYBRGreen I was purchased from Molecular Probes, Inc. (Eugene, OR) as a 10,000 x dimethyl sulfoxide (DMSO) concentrate. All other reagents were of analytical grade. Nunclon 96-well plates were purchased from Nunc NS (Roskilde, Denmark), and Iwaki 35-mm tissue culture dishes were from Asahi Techno Glass (Japan).

### 1.2.3 Hepatocyte Isolation

Hepatocytes were prepared from the livers of adult male albino Swiss mice (4-6 wk old) by collagenase digestion (Harman *et al.*, 1987). Cell viability was assessed via trypan blue exclusion, with 75-85% of cells excluding the dye. Hepatocytes were washed 3 times in CaCl<sub>2</sub>-supplemented Krebs Henseleit solution, resuspended in RPMI-1640 media supplemented with 0.2% bovine serum albumin (BSA), 0.03% L-glutamine, and gentamicin (50 µg/mL), and layered onto collagen-coated (Type I rat tail collagen, 50 µg/well) 96-well microtiter plates, or 35 or 60-mm petri dishes. One hundred microliters of RPMI-1640 culture media at a final density of  $3 \times 10^4$  cells/dish, 2 mL at a final density of  $1 \times 10^6$  cells/dish, or 3 mL at a final density of  $3 \times 10^6$  cells/dish was used, respectively. Cells were then placed in a humidified 5% CO<sub>2</sub> incubator at 37°C for 3 h to allow cell attachment.

### 1.2.4 Cytotoxicity Assays

The cytotoxicity of CYN alone or co-incubated with various CYP inhibitors was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide viability assay (MTT assay) (Park *et al.*, 1987) or measuring the lactate dehydrogenase (LDH) activity in culture media (Richards *et al.*, 1975), modified as described below. For both, experiments were carried out in 96-well microtiter plates and measurements for MTT were performed on a microplate reader (Polarstar Galaxy, BMG Labtechnologies GmbH, Germany), while LDH activities were quantified using a spectrophotometer (see details later). CYN, a highly soluble zwitterionic molecule, was used as a 2-mM stock solution in MilliQ water. CYN cytotoxic concentration response was determined using the LDH assay for final concentrations of 0.05, 0.1, 0.5, 1, 2, 4, 6, 8, 10, 15, 20, and 25 µM in fresh media (without BSA). Cells were exposed for 18, 21, or 24 h depending on the experiment. Cell morphology was also closely monitored.

*MTT assay*

This colorimetric assay measures the ability of mitochondrial succinate dehydrogenase to reduce MTT into a DMSO-soluble blue salt, absorbing at 570 nm. At the end of the exposure period, 100 µl of a 0.5-mg/mL MTT solution in RPMI-1640 media was added to each well. After a 2 h incubation at 37°C, 5% CO<sub>2</sub>, the medium was removed and cells were lysed by adding 100 µl DMSO to each well. The optical density of the cell contents was then measured at 570 nm using a microplate reader (Polarstar Galaxy, BMG Labtechnologies GmbH, Germany). This relatively high-throughput assay was used for CYP inhibition experiments so that a range of inhibitors and inhibitor concentrations could be tested on cells from the same mouse. Data were expressed as percent of the optical density measured in the negative vehicle controls, that is, 100% viable cells.

*LDH assay*

LDH activity in culture media was measured using a spectrofluorimetric method modified from Richards and collaborators (1975). NAD reduction into NADH from lactate oxidation was monitored by measuring NADH fluorescence at  $\lambda_{exc}$  = 320 nm and  $\lambda_{em}$  = 460 nm. LDH activities at each time point were expressed as percent of the total activity in individual wells, which was determined at the end of an experiment after lysing cells with Triton X-100.

*CYP inhibitor studies*

Cells were pre-incubated for approximately 30 to 45 min with CYP inhibitors at various concentrations prior to the addition of CYN. The CYP inhibitors omeprazole, ketoconazole, and troleandomycin were prepared as 480 µM stock solutions in BSA-free culture medium and contained approximately 0.5 to 2.4% methanol. SKF525A was prepared in culture medium as a 240 µM stock solution. CYP inhibitor stock solutions were diluted in BSA-free culture medium for use; 125 µL was added to each well and preincubated for 30 to 45 min. Afterward, 25 µL CYN was added to attain a final concentration of 1 µM. For omeprazole, ketoconazole, and troleandomycin, final concentrations of CYP inhibitors were 100 and 10 µM. SKF525A was used at only one concentration 50 µM. Cells were then returned to the incubator for 21 h. All solutions were prepared freshly on the day of use. Final concentrations of methanol in the culture medium did not exceed 0.6%. Negative controls were prepared by adding this concentration of solvent to untreated cells and cells treated with CYN alone.

**1.2.5 Glutathione Determination**

Glutathione (GSH) levels were determined based on the fluorometric method of Hissin and Hilf (1976). This method utilises the reaction of GSH with the fluorescent reagent *o*-phthalaldehyde. The original buffer was modified to allow quantification by assay in a 96-well microplate. The cell culture medium was removed from the hepatocyte monolayer and the cells were washed with Hanks buffered salt solution (HBSS, 2 mL); 500 µL of 3% perchloric acid (PCA) was added to each 36-mm dish. The monolayer was scraped into the PCA and centrifuged at 5000 x g for 5 min to pellet the cells. The supernatant was removed for GSH determination, while the pellet was retained for DNA quantification (Labarca & Paigen, 1980).

The assay buffer consisted of 0.2 M Tris, 0.2 M NaCl, and 1 mM ethylene-diamine tetraacetic acid (EDTA) (pH 8). To maintain pH 8, PCA samples were neutralised with NaOH (16 µL of 2.5 M NaOH to every 100 µL of 3% PCA sample); 200 µL buffer, 50 µL NaOH-treated PCA sample, and 10 µL of 1 mg/mL *o*-phthalaldehyde stock prepared in methanol were loaded sequentially into microplate wells. After incubation at room temperature for 20 min the fluorescence was determined at excitation  $\lambda$  = 340 nm, emission  $\lambda$  = 420 nm, on a Biolumin 960 (Molecular Dynamics) microplate reader. The GSH content of the cells was expressed as nanograms GSH per microgram DNA. On each day of analysis, a GSH standard curve was constructed using reduced GSH. Standards were analysed in triplicate. Samples were analysed in duplicate.

### 1.2.6 Malondialdehyde Assay

The lipid peroxidation product malondialdehyde (MDA) was assayed using the BIOXYTECH LPO-586 kit obtained from OXIS International, Inc. (Portland, OR). The procedure was carried out with minor modification of the manufacturer's instructions. The assay is based on the reaction of N-methyl-2-phenylindole with MDA to yield a chromophore that absorbs strongly at 586 nm (Gérard-Monnier *et al.*, 1998). The cell culture medium was removed and the hepatocyte monolayer was washed with 20 mM Tris buffer (pH 7.4). Cells were scraped from the bottom of the dish using a cell scraper (Costar, MA) into 100  $\mu$ L of 20 mM Tris buffer containing 5 mM butylated hydroxytoluene (BHT) to prevent further sample oxidation. To provide adequate sensitivity in the MDA assay, the contents of three 36-mm hepatocyte dishes were pooled for each sample. Samples were sonicated on ice for 3 x 20 s at 50 W using a Braun Labsonic 1510 sonicator before centrifugation at 3000 x g for 5 min. The supernatant was used for MDA determination, while the pellet was precipitated in 2 washes of 10% TCA and digested in 0.3 M NaOH for DNA quantification. Results were expressed as nanograms MDA per microgram DNA for each sample.

For assay, a 300  $\mu$ L sample or standard was added to 650  $\mu$ L of 2.5 mM N-methyl-2-phenylindole in a mixture of acetonitrile/methanol (3:1). The reaction was started by the addition of 150  $\mu$ L of 37% HCl (12 N). Samples were vortexed briefly and then incubated for 60 min at 45°C before being separated by centrifugation (15,000 x g, 10 min), transferred to a 1 mL cuvette for determination of absorbance at 586 nm (blank = 20 mM Tris buffer). The contribution of the hepatocyte sample to  $A_{586}$  was negligible (sample blank).

Due to the instability of MDA itself, the diethylacetal 1,1,3,3-tetramethoxypropane (TMOP) was used as the source of MDA to create a standard curve. It is hydrolysed to MDA during the 60-min incubation at 45°C in the presence of HCl. TMOP standards were prepared in 20 mM Tris buffer to generate a standard curve of 0.6-9  $\mu$ M TMOP final concentration in the reaction mix. The slope of the observed standard curve was within 5% of that expected based on the molar extinction coefficient ( $\epsilon$ ) of MDA (110,000) and was used to calculate the concentration of MDA in each sample. A buffer blank was included with each assay set.

### 1.2.7 Inhibition of GSSG-Rd Activity

Glutathione disulfide reductase (GSSG-Rd) activity was inhibited by treatment of hepatocytes with 1,3-bis(chloranthyl)-1-nitrosourea (BCNU) as described by Adamson and Harman (1989). BCNU was dissolved in ethanol. Hepatocytes were exposed to 100  $\mu$ M BCNU (0.5% ethanol) for 30 min followed by a 2-h recovery incubation in BCNU-free media. Cells were then exposed to CYN or t-BH for the required incubation period. Vehicle (0.5% ethanol)-treated hepatocytes were used as the control.

### 1.2.8 COMET Assay

For the COMET genotoxicity assay, experiments were carried out in 35-mm tissue culture dishes. CYN was added to attain final concentrations of 0.05 to 0.5  $\mu$ M in fresh media (without BSA) and the cells were returned to the incubator for 18 h (37°C, 5% CO<sub>2</sub>). In some instances, cells were preincubated for approximately 30 to 45 min with SKF525A or omeprazole at final concentrations of 50 and 100  $\mu$ M, respectively, prior to the addition of CYN. Hepatocytes treated with 1 mM clofibric acid for 18 h were used as a positive control in the COMET procedure (Sallustio *et al.*, 1997; Ghaoui *et al.*, 2003). Clofibrate has been shown to be converted in rodent hepatocytes to a genotoxic metabolite via a glucuronide dependent phase II pathway (Ghaoui *et al.*, 2003). CYP inhibitor solutions were prepared freshly on the day of use. Final concentrations of methanol in the culture medium did not exceed 0.5%. Negative controls were prepared by adding this concentration of solvent to untreated cells and cells treated with CYN alone.

On the morning following cell treatment, medium was discarded and cells were washed once with PBS, scraped off the culture dish, and finally resuspended in 100  $\mu$ L PBS (approximately  $0.5 \times 10^6$  cells/mL) as previously described (Fontaine *et al.*, 2004). The COMET assay was performed using the procedure from Singh *et al.* (1988) with a number of modifications for primary cultured hepatocytes (Singh *et al.*, 1988; Ghaoui *et al.*, 2003; Fontaine *et al.*, 2004). Briefly, aliquots of approximately 50,000 hepatocytes resuspended in PBS and mixed with 1% low-melting agarose were layered onto

regular microscope slides precoated with 1% normal agarose. After solidification, slides were coated with a third layer of 0.5% low-melting agarose. The basic steps of the assay modified for primary cultured mouse hepatocytes then included, briefly, lysis of cells to liberate DNA, exposure to alkali (pH>13) to obtain unwound DNA and to express alkaline-labile sites as double-strand breaks, electrophoresis under alkaline conditions, alkali neutralisation, DNA staining with SYBRgreen I, comet visualisation, and scoring (Fontaine *et al.*, 2004).

## 1.2.9 Data Analysis

EC50 values were determined by nonlinear regression. Effects of CYP inhibitors were compared using paired t-tests between the results for the CYP inhibitors matched with the effect of CYN in the same experiment. One-way analysis of variance (ANOVA) was used for analysis of Comet areas and lengths, and for the log-transformed tail moment data. The untransformed tail moment data was also analysed using non parametric tests. For clarity when graphing the tail moment data, the suggestion of Plewa *et al.* (2004) was adapted. That is, each field was assumed to be a random sample of the treated cell population. Therefore, by the central limit theorem, the medians of these fields were assumed to be normally distributed about their mean. This assumption was shown to be valid by examining the distribution of the data about the mean in dot plots. All analyses were performed using GraphPad Prism software. The criterion for significance was set at  $p < 0.05$ .

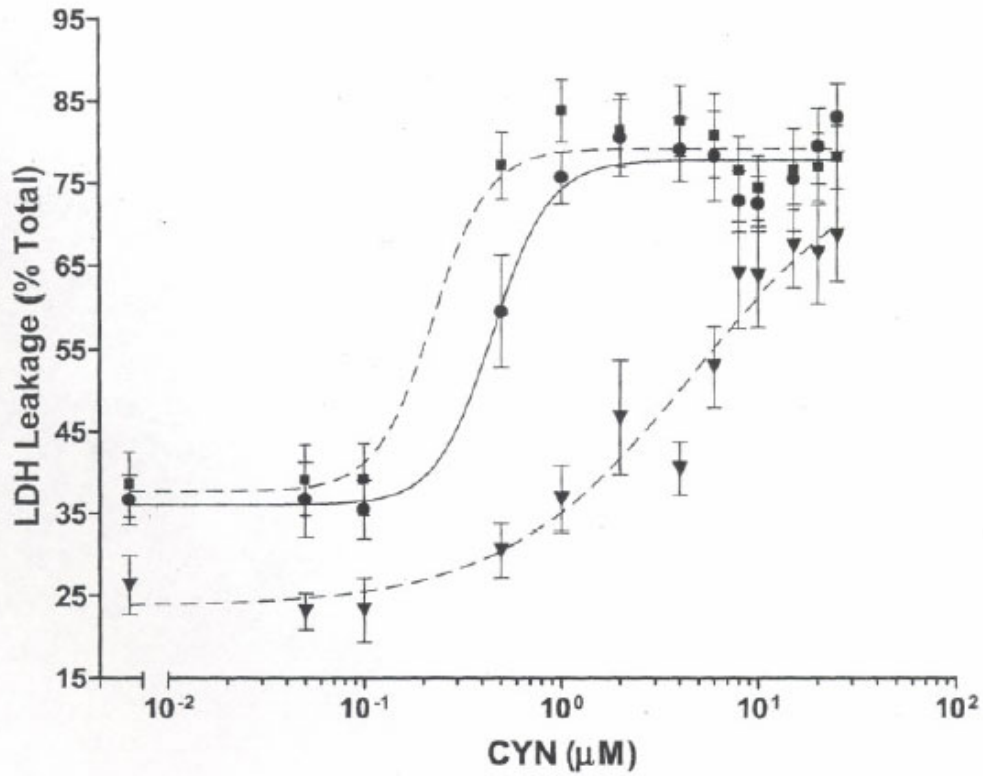
## 1.3 Results

The concentration response for the leakage of lactate dehydrogenase into culture media was determined with 0.05-25  $\mu\text{M}$  concentrations of CYN for up to 24 h. LDH leakage is a sensitive indicator of cytotoxicity in rodent hepatocytes. CYN was toxic to the cells in the low micromolar range, producing marked time- and concentration- dependent increases in LDH leakage. The EC50 at 18 h was 0.47  $\mu\text{M}$  (95% CI = 0.38-0.58  $\mu\text{M}$ ) (see Figure 1.1). The concentration response was very steep, with concentrations of 1  $\mu\text{M}$  and above producing greater than 75% LDH leakage within 18 h whereas concentrations below 0.1  $\mu\text{M}$  had no effect. These data are consistent with previous findings (Froscio *et al.*, 2003; Runnegar *et al.*, 1994). The comparison of 24-h CYN exposures in fresh and day-old cells strongly suggests loss of CYN-metabolising ability in the older cells.

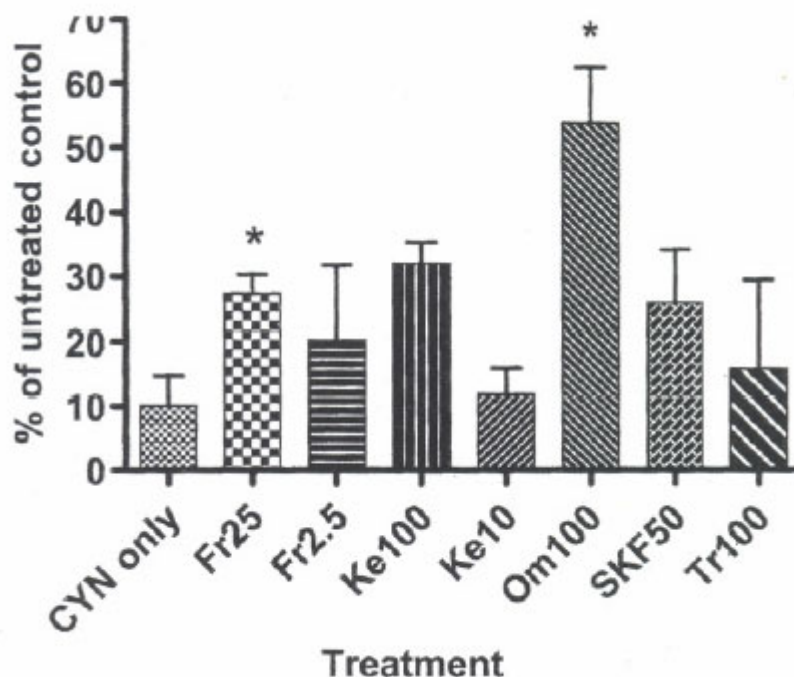
Ketoconazole and SKF525A were not significantly protective (Froscio *et al.*, 2003; Runnegar *et al.*, 1994) (see Figure 1.2). Omeprazole was found to be effective when used at 100  $\mu\text{M}$ , being approximately twice as effective as these compounds. Furfurylline at 25  $\mu\text{M}$  was significantly protective. Inhibitors of CYPs 2A6, 2D6, and 2E1 were not found to be cytoprotective in this system (not shown).

GSH levels were depleted by CYN concentrations of 1  $\mu\text{M}$  and above after an 18-h exposure, and 5  $\mu\text{M}$  produced a significant reduction after 10 h with almost complete depletion after 18 h (data not shown). However, 5  $\mu\text{M}$  CYN did not elevate levels of lipid peroxidation, as measured by malondialdehyde production, and furthermore, inhibition of glutathione reductase by BCNU did not increase MDA production nor the LDH leakage produced by this concentration of CYN (Figure 1.3). In contrast, the positive control tert-butyl hydroperoxide (t-BH, 0.5 mM) produced a threefold increase in MDA. t-BH induced 50% LDH leakage after a 1-h exposure, and this was increased to more than 80% by prior treatment with BCNU (data not shown).

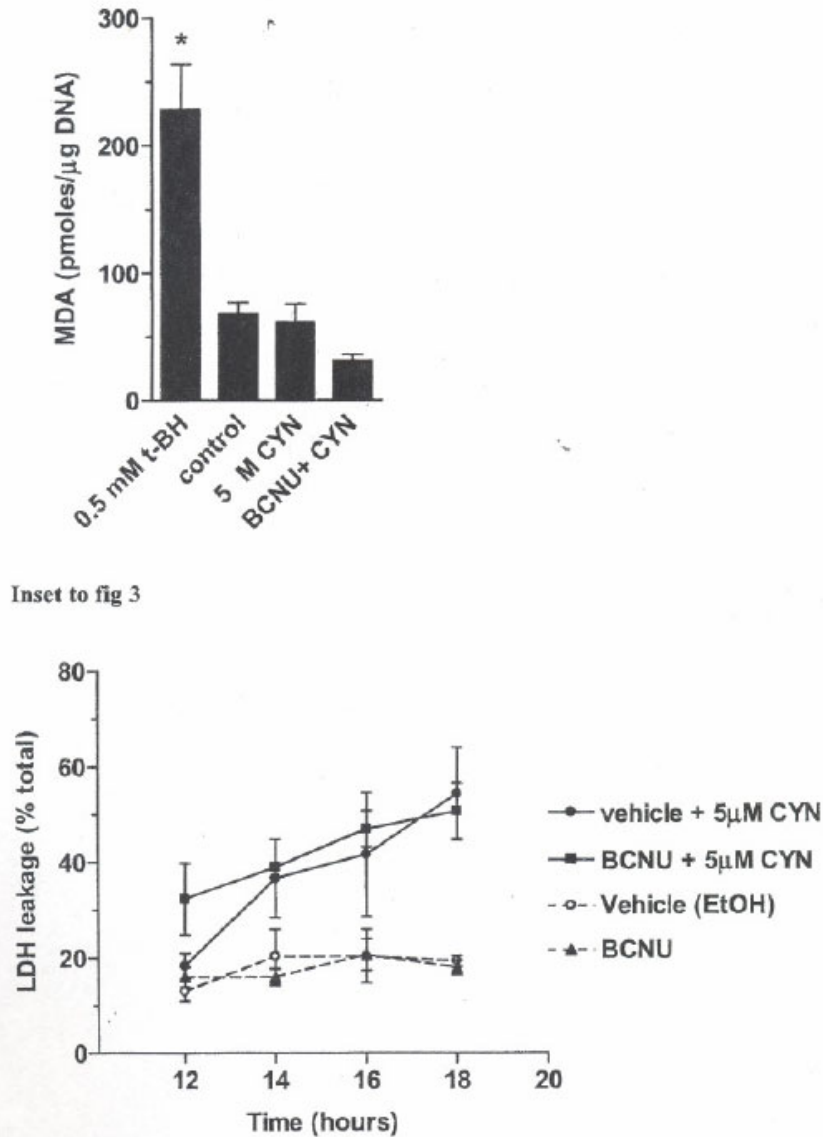




**Figure 1.1** Concentration response for lactate dehydrogenase (LDH) leakage from isolated mouse hepatocyte monolayers maintained in collagen-coated 96-well plates upon treatment with 0, 0.05, 0.1, 0.5, 1, 10, 20 and 25  $\mu\text{M}$  cylindrospermopsin (CYN). The left-most datapoints are the 0- $\mu\text{M}$  controls. Curves are shown for CYN exposures of 18 h (circles), 24 h (squares), and 24 h in 1-d-old cells (triangles). Each data point represents the mean  $\pm$  SD of three independent observations (i.e.,  $n = 3$  mice with 12 replicates for each CYN concentration).

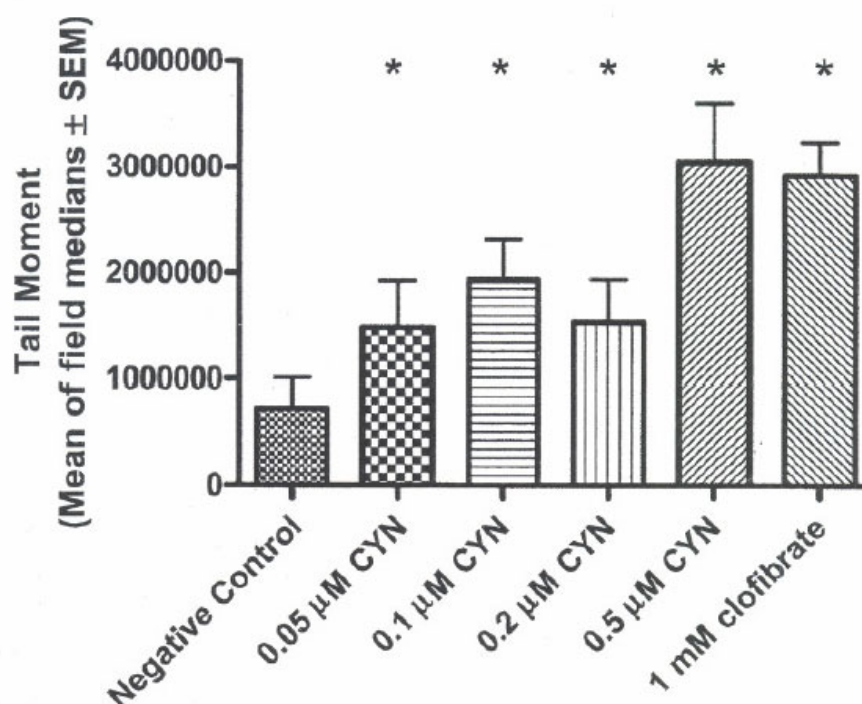


**Figure 1.2** Protection of CYN-exposed cells (1  $\mu$ M) by various CYP450 inhibitors. Fr, furafylline (25 and 2.5  $\mu$ M); ke, ketoconazole (100 and 10  $\mu$ M); Om, omeprazole (100  $\mu$ M); SKF, SKF525A (proadifen, 50  $\mu$ M); Tr, troleandomycin (100  $\mu$ M). Primary hepatocytes were treated with the CYP inhibitors 30-45 min before exposure to CYN, and then MTT was used to quantify the cellular response 21 h later. Results were expressed as percent of untreated controls. Data points represent means  $\pm$  SE of two or three independent experiments; asterisk indicates significant at  $p < 0.05$  when compared to CYN only.



**Figure 1.3** Inset: Malondialdehyde (MDA) levels in control and toxin-treated hepatocytes after exposure to 5  $\mu$ M cylindrospermopsin (CYN) for 12 h with or without BCNU pre-treatment (100  $\mu$ M, 30 min). Positive control cells were exposed to test butyl hydroperoxide (t-BH, 0.5 mM). Values are mean  $\pm$  SE for three independent experiments; asterisk indicates significant at  $p < 0.05$  when compared to control). Main figure: Effect of GSSG-Rd inhibition on LDH leakage from hepatocytes treated with 5  $\mu$ M CYN). Hepatocytes were pre-treated with BCNU to inhibit GSSG-Rd activity or vehicle for 30 min followed by a 2-h recovery period prior to exposure to CYN. The development of CYN toxicity was followed from 12 to 18 h postexposure. Values are mean  $\pm$  SE of three independent experiments.

The potential genotoxicity of CYN was investigated using the well-established COMET assay. A statistically significant concentration-dependent increase in comet tail length, area, and moment occurred in cells treated for 18 h with CYN concentrations ranging from 0.05 to 0.5  $\mu\text{M}$  (Figure 1.4), data not shown for comet tail length and area. To determine whether one or more phase I-derived metabolite(s) contributed to the DNA lesions seen in CYN-treated hepatocytes, the effect of cytochrome P-450 inhibitors on CYN DNA toxicity was explored. SKF525A (50  $\mu\text{M}$ ) and omeprazole (100  $\mu\text{M}$ ) both prevented the increase in comet tail lengths, areas, and moments associated with exposing cells to the noncytotoxic 0.2  $\mu\text{M}$  CYN concentration for 18 h (Figure 1.4, data not shown for length and area).



**Figure 1.4** Concentration-dependent increase in comet tail moment of nuclei from mouse hepatocytes treated with 0.05-0.5  $\mu\text{M}$  concentrations of CYN for 18 h. Between 50 and 65 comets were scored per slide. For each concentration, two different slides were scored (i.e., 100 to 125 comets). The positive control consisted of nuclei from cells treated with 1 mM clofibric acid for 18 h. The experiment was repeated with three different mice and for clarity pooled data are displayed as means of field medians  $\pm$  SEM (see Materials and Methods, Statistical Analyses). Statistical significance was assessed using the original data with a nonparametric Kruskal-Wallis test followed by Dunn's post hoc test to detect differences at specific concentrations; asterisk indicates significant at  $p < 0.05$  when compared with culture medium control.

## 1.4 Discussion

Certain CYP450 inhibitors can attenuate the cytotoxicity of CYN (Froscio *et al.*, 2003; Runnegar *et al.*, 1994, 1995). This is corroborated by the finding here that CYN cytotoxicity after a 24 h exposure is greatly reduced in 1 day old cells compared with freshly isolated cells (Figure 1.1), as primary hepatocytes are known to progressively lose metabolic capabilities in culture (Berry *et al.*, 1991). Most of the compounds that have been used previously were relatively nonspecific inhibitors of CYP450s, so experiments attempted, in the present study, to include more specific inhibitors to identify the key CYP450 isoforms involved in CYN cytotoxicity. The MTT assay was used in 96-well plates in order to increase the number of potential CYP inhibitors that could be studied. Surprisingly, it was found that the effectiveness of ketoconazole and SKF525A, both shown previously to prevent CYN-induced LDH leakage in larger culture dishes, was reduced in this assay format, inhibiting the toxic effect of CYN only by about 25% (Figure 1.2). Furfurylline (CYP1A2) and omeprazole (CYP3A4, but with some CYP2C19 activity in mice; Bogaards *et al.*, 2000) were moderately protective. It may be that in this context the MTT assay was too sensitive a marker of the cytopathic effects of CYN since it may have detected sublethal effects on mitochondrial reductase activity, perhaps secondary to protein synthesis inhibition, which would not be subject to CYP450 inhibition because it is the parent compound that mediates this effect (Froscio *et al.*, 2003).

Runnegar *et al.* (1994, 1995) showed that GSH levels were reduced in hepatocytes incubated with CYN, and that this was due to the inhibition of GSH synthesis. This was further examined *in vivo* by Norris *et al.* (2002), who showed that this effect did not contribute significantly to the acute toxicity of CYN. However, there remained the possibility that this reduction in GSH levels could lead to an increase in oxidative stress in the cell, and that this could contribute to genotoxicity. To test this, the lipid peroxidation product MDA was quantified in CYN-exposed mouse hepatocytes to see whether the toxin produced an increase in this marker of oxidative stress. Exposure of hepatocytes to 5  $\mu\text{M}$  CYN for 12 h was chosen as an appropriate concentration and time point for MDA measurement. At this time, GSH levels in CYN-treated hepatocytes were expected to be <50% of the control and declining. No LDH leakage was expected. The effect of BCNU pretreatment on MDA production and LDH leakage was also examined at this time point. No change in MDA levels was found (Figure 1.3). BCNU inhibition of GSSG reductase to block regeneration of GSH did not potentiate MDA production or CYN cytotoxicity. It therefore seems unlikely that ROS are the mediators of CYN cytotoxicity and therefore also of CYN genotoxicity.

Genotoxic and even carcinogenic effects have been reported *in vivo* in mice in major organs, mainly the liver (Falconer & Humpage, 2001; Shen *et al.*, 2002). However, only two *in vitro* studies have been carried out thus far, with only one reporting cytogenetic damage in a human lymphoblastoid cell line (Humpage *et al.*, 2000). The second study (Fessard & Bernard, 2003) did not detect any DNA damage in Chinese hamster ovary K1 cells exposed to approximately 2  $\mu\text{M}$  CYN (1  $\mu\text{g/mL}$ ) for 24 h, a treatment found effective in the lymphoblastoid cell line. The authors recognised, however, that "the hypothesis of CYN metabolism into one or more genotoxic products must be further explored." In the present study using metabolically capable hepatocytes, CYN clearly produced significant DNA fragmentation at concentrations as low as 0.05  $\mu\text{M}$ . To find out if CYP450 metabolites mediated this CYN-induced genotoxicity, the effects of omeprazole and SKF525A on the COMET assay outcome were examined. Both compounds proved effective in protecting the cells from CYN-induced genotoxicity (Figure 1.4). SKF525A was as effective in this experiment as it had previously been in preventing CYN-induced cytotoxicity (Froscio *et al.*, 2003), again suggesting that the MTT assay may have underestimated the protective effect of this compound. This does not exclude the possibility that different metabolites may be responsible for cytotoxicity and genotoxicity. The fact that two phase I inhibitors prevented DNA damage associated with CYN treatment *in vitro* minimises the chance that some property of both SKF525A and omeprazole unrelated to phase I inhibition might explain their inhibitory effect on CYN-induced DNA toxicity.

As has been shown previously and again here, the cytotoxicity concentration response for CYN is extremely steep. The EC<sub>50</sub> for LDH leakage from mouse hepatocytes at 18 h was approximately 0.5  $\mu\text{M}$  but 0.1  $\mu\text{M}$  was nontoxic (Figure 1.1). In contrast, the concentration response for genotoxicity as measured by the COMET assay after an 18 h exposure was much broader and, more importantly extended to concentrations at least 10-fold less than the cytotoxicity EC<sub>50</sub> (Figure 1.4). In particular, CYN genotoxicity occurred at concentrations below the EC<sub>30</sub> where cell death-related DNA digestion should not be detectable (Henderson *et al.*, 1998). The greater effect observed at 0.5  $\mu\text{M}$  could be due

to the additional effect of cell death-related DNA damage, and studies are currently investigating this possibility. In our previous study in WIL2-NS lymphoblasts, micronucleus induction was also seen without an increase in necrotic or apoptotic cells being observed. These findings suggest that the genotoxicity at sub cytotoxic concentrations is a specific and primary effect of CYN. This has important implications for the assessment of the risk to public health posed by CYN.

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## 2 INDUCTION OF P53-REGULATED GENE EXPRESSION IN HUMAN CELL LINES EXPOSED TO THE CYANOBACTERIAL TOXIN CYLINDROSPERMOPSIN

This Chapter is based on the following manuscript

Bain, P., Shaw, G., and Patel, B. (2007). Induction of p53-Regulated Gene Expression in Human Cell Lines exposed to the Cyanobacterial Toxin Cylindrospermopsin. *Journal of Toxicology and Environmental Health, Part A* **70**, 1687-1693.

### 2.1 Introduction

Toxic secondary metabolites are commonly produced by bloom-forming cyanobacteria in surface water bodies. One toxin that has received much attention in recent years is the hepatotoxic alkaloid cylindrospermopsin (CYN). CYN was identified after a human poisoning incident in North Queensland, Australia, resulted in the hospitalisation of a number of individuals who developed symptoms of hepatic and renal injury (Byth, 1980). A cyanobacterial species, *Cylindrospermopsis raciborskii*, was found to be the probable causative agent and CYN was later isolated from this organism (Ohtani *et al.*, 1992). CYN is now known to be produced by a number of freshwater cyanobacterial species worldwide (Harada *et al.*, 1994; Shaw *et al.*, 1999; Banker *et al.*, 2001; Li *et al.*, 2001). The chemical structure of CYN consists of the pyrimidine uracil linked to a reactive cyclic guanidinium moiety (Ohtani *et al.*, 1992). The presence of uracil in the structure led researchers to suggest a possible interaction with nucleic acids. Following moderate doses, DNA fragmentation (Shen *et al.*, 2002) and modification (Shaw *et al.*, 2000) were observed in the livers of treated mice, while in human lymphoblastoid cells, CYN was shown to induce the formation of centromere-negative micronuclei, indicating double-stranded DNA breakage (Humpage *et al.*, 2000). CYN also reduced protein synthesis in cultured cells and inhibited messenger RNA translation with *in vitro* systems (Terao *et al.*, 1994; Froscio *et al.*, 2001, 2003).

DNA damage and various other cellular stresses induce the activation of the tumor suppressor protein p53, a transcription factor controlling the expression of a large number of genes associated with growth arrest, apoptotic cell death, and DNA repair processes (Meek, 1998; Sengupta & Harris, 2005). Under normal conditions, p53 turnover is continual, with degradation being mediated by the ubiquitin ligase Mdm2 (Honda *et al.*, 1997). The MDM2 gene is itself a target of p53 transactivation, providing a negative feedback loop that restores low levels following activation of the pathway. Stabilisation of the p53 protein occurs in response to a range of cellular stresses, the most well-described being DNA damage. Following damage sensing by various kinases, signals are transduced to effectors that operate to inhibit the interaction between Mdm2 and p53. This is achieved through various modifications to either protein. Phosphorylation of p53 at particular residues inhibits Mdm2 binding (Shieh *et al.*, 1997), and auto-ubiquitylation of Mdm2 may occur after DNA damage induced by gamma radiation (Stommel & Wahl, 2004). The ARF tumor suppressor protein inhibits p53–Mdm2 interaction by binding to Mdm2, resulting in the stabilisation of p53 levels (Honda & Yasuda, 1999).

Phosphorylation of p53 at specific residues also modulates its transcription factor activity, altering the expression of genes with various functions in growth arrest, apoptosis, and DNA repair. A major target of p53 transactivation is CDKN1A, encoding p21WAF1/CIP1, a protein that interacts with cyclin dependent kinases, resulting in cell cycle arrest (Yin *et al.*, 1997). p53 partially controls the expression of a gene encoding the growth arrest and DNA damage-related protein GADD45 $\alpha$  (Hollander *et al.*, 1993) that inhibits the transition from G2 phase to mitosis (Wang *et al.*, 1999) and is also required for some types of DNA repair following exposure to genotoxic agents (Smith *et al.*, 2000). Apoptosis may be triggered by p53 through the increased expression of genes encoding the Bcl2- family proteins Bax and PUMA (Green, 2000). These proteins induce apoptosis by increasing mitochondrial outer membrane permeability, resulting in cytochrome c release and initiation of the caspase cascade. There is some recent evidence suggesting that activated p53 may directly induce apoptosis by interacting with Bcl2-family proteins (Erster *et al.*, 2004).



The molecular interactions underlying CYN toxicity remain elusive, particularly with respect to genotoxicity and mutagenicity. In this report, data show that exposing p53-proficient, human-derived cell types to CYN results in elevated mRNA levels for genes known to be regulated by the p53 transcription factor.

## 2.2 Materials and Methods

### 2.2.1 Cylindrospermopsin

Cylindrospermopsin (CYN), from cultures of *Cylindrospermopsis raciborskii* AWT205, was purified and analysed using methods described previously (Norris *et al.*, 2001). Purified CYN dissolved in Milli-Q water was filtered through presterilised 0.22- $\mu$ m filter units (Pall Life Sciences) prior to use.

### 2.2.2 Cell Culture

Human dermal fibroblasts (HDFs), Caco-2 (HTB-37, ATCC), HepG2 (HB-8065, ATCC), and C3A cells (CRL- 10741, ATCC) were grown at 37°C under a 5% CO<sub>2</sub> humidified atmosphere in Dulbecco's modification of Eagle's medium (DMEM, Gibco-Invitrogen), high-glucose, supplemented with 42  $\mu$ g/mL L-glutamine, 110  $\mu$ g/mL sodium pyruvate, and 4  $\mu$ g/mL pyridoxine-HCl. For routine maintenance, media were further supplemented with 10% foetal bovine serum (Gibco-Invitrogen), 100 U/mL penicillin (Gibco-Invitrogen), and 100  $\mu$ g/mL streptomycin (Gibco-Invitrogen). For toxin exposures, the antibiotics were omitted.

### 2.2.3 Cellular Proliferation and Cytotoxicity Assays

Cells were seeded into 96-well plates (5000 cells per well) and grown for 24 h. CYN was diluted in sterile water prior to addition to the growth medium such that each well contained an equal amount of vehicle to allow for any effects resulting from dilution of the growth medium. Vehicle controls contained water and medium only. After exchanging growth media for media containing toxin or vehicle the plates were incubated for 24, 48, or 72 h. Inhibition of cellular proliferation was measured using a commercially available tetrazolium reduction assay according to the manufacturer's instructions (Promega). The assay uses a soluble combination of the tetrazolium compound, 3-(4,5-dimethylthiazol- 2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS), and the electron-coupling reagent phenazine ethosulphate (PES). The solution was added directly to the culture media as recommended by the manufacturer and incubated for 1–3 h prior to transferring the culture medium to fresh 96-well plates and measuring specific absorbance. Cell membrane integrity was measured by quantifying the release of lactate dehydrogenase activity into the culture medium using a commercially available fluorescence-based assay (Promega). For this assay, 96-well plates were set up as already described; however, an extra control was included to enable the measurement of a detergent-lysed vehicle control (i.e., one lysed vehicle control and one unlysed vehicle control for each dilution series). Each treatment was performed in triplicate. Data analysis, graphing, and nonlinear regressions were carried out using GraphPad Prism.

### 2.2.4 Relative Gene Expression Analysis

Cells were seeded into six-well plates and grown for 24–48 h until monolayers reached 60–70% confluence. Culture media were exchanged with fresh medium containing CYN or an equal volume of vehicle (sterile Milli-Q water). Treatments were performed in triplicate for each condition. Following incubation for the indicated times, total RNA was isolated using silica spin-column purification (RNeasy kit, Qiagen) according to the manufacturer's instructions. Briefly, cells were washed 3 times with sterile phosphate-buffered saline (PBS), scraped into lysis buffer directly in cell culture dishes, and transferred to 1.5 mL microcentrifuge tubes. The optional oncolumn deoxyribonuclease treatment was included to prevent contamination by genomic DNA. RNA was eluted with 50  $\mu$ L of RNase-free water (Qiagen), and eluates were passed through the columns a second time to improve yields. Total RNA was quantified by measuring absorbance at 260 nm. The presence of contaminating protein was checked by measuring the A260/A280 ratio, and RNA integrity was confirmed by agarose gel electrophoresis using a denaturing loading buffer. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was undertaken using a two-step procedure. Firstly, complementary DNA (cDNA) was synthesised from 1  $\mu$ g of total RNA using reverse transcriptase (Fermentas) according to the

manufacturer's instructions, primed with a mixed oligo-dT with the sequence dT29-VN. Relative quantification of cDNA complementary to transcripts of interest was achieved using SYBR-green I quantitative PCR. Primers (Table 2.1) were designed against reference mRNA sequences obtained from GenBank, with the aid of Primer3 software (Rozen and Skaletsky, 2000). PCR reactions (25  $\mu$ L) contained 0.4  $\mu$ M forward and reverse primers, 0.5 U HotMaster Taq polymerase (Eppendorf), 1 $\times$  HotMaster Taq buffer (Eppendorf), 0.25 mM each dNTP (Fermentas), 0.5 mg/mL bovine serum albumin (Sigma- Aldrich), 5% glycerol (BDH), 10 nM fluorescein (Sigma-Aldrich), 1/40,000 SYBR green I (Invitrogen Molecular Probes), 2.5% molecular-biology-grade dimethyl sulfoxide (DMSO, Sigma-Aldrich), and 5  $\mu$ L of a 1/50 dilution of cDNA. Reactions were performed in triplicate in a Bio-Rad iCycler fitted with the iQ real-time detection unit. A two-step protocol was used with a combined annealing and extension step of 60 s at 60°C and a denaturation step for 15 s at 95°C. Fluorescence was measured at the end of the annealing/extension step. Dissociation curves confirmed the presence of single PCR products for each primer pair. Relative mRNA quantification was determined with the aid of the Relative Expression Software Tool 2005 (Pfaffl *et al.*, 2002). Quantifications of mRNA levels in treated cultures at a given time were determined relative to vehicle controls at that time and normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Statistical analyses were performed in REST, and graphing was carried out in Microsoft Excel.

**Table 2.1** PCR Primers Used in this study

Gene	Gene product	GenBank accession (mRNA)	Primer sequences (5' $\rightarrow$ 3')
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046	CCAGGTGGTCTCCTCTGACTTGCTGTAGCCAAATTCGTTG
<i>CDKN1A</i>	Cyclin-dependent kinase interacting, p21 <sup>WAF1/CIP1</sup>	NM_000389	GAAGACCATGTGGACCTGTCCGGATTAGGGCTTCCTCTTG
<i>GADD45<math>\alpha</math></i>	45-kD Growth arrest and DNA damage protein	NM_001924	GGCCCGGAGATAGATGACTTTTTCCTTCCTGCATGGTTC
<i>BAX</i>	Bcl-2 associated x-protein	NM_138761	GGGGACGAACTGGACAGTAACAGTTGAAGTTGCCGTCAGA
<i>MDM2</i>	Human homologue of mouse double minute 2	NM_002392	GTATCAGGCAGGGGAGAGTGGAAGCCAATTCTACGAAGG

## 2.2.5 Immunoblotting

Cells were seeded into wells of a 24-well plate ( $10^4$  per well) and grown for 24 h. Media were exchanged for fresh media containing toxins or vehicle controls and incubated for the indicated times. Monolayers were washed 3 times with PBS and harvested by scraping into 0.5 mL PBS. Cells were pelleted and lysed on ice. Proteins were separated on 12% polyacrylamide gels using standard procedures and blotted onto nitrocellulose membranes (Millipore). Membranes were blocked for 1 h with blocking buffer (2% bovine serum albumin and 0.1% Tween-20 in PBS). Immunodetection of total p53 protein was achieved using monoclonal anti-p53 antibody (Sigma-Aldrich). Anti-GAPDH monoclonal antibody (Ambion) was used as a loading control, and bound antibodies were detected with a mixture of goat Fab- and Fc-specific antimouse immunoglobulin (Ig) G antibodies conjugated to alkaline phosphatase (Sigma-Aldrich). Bands were detected with a chemiluminescent alkaline phosphatase substrate (Pierce) and visualised using a cooled CCD system (Fujifilm LAS3000).

## 2.3 Results

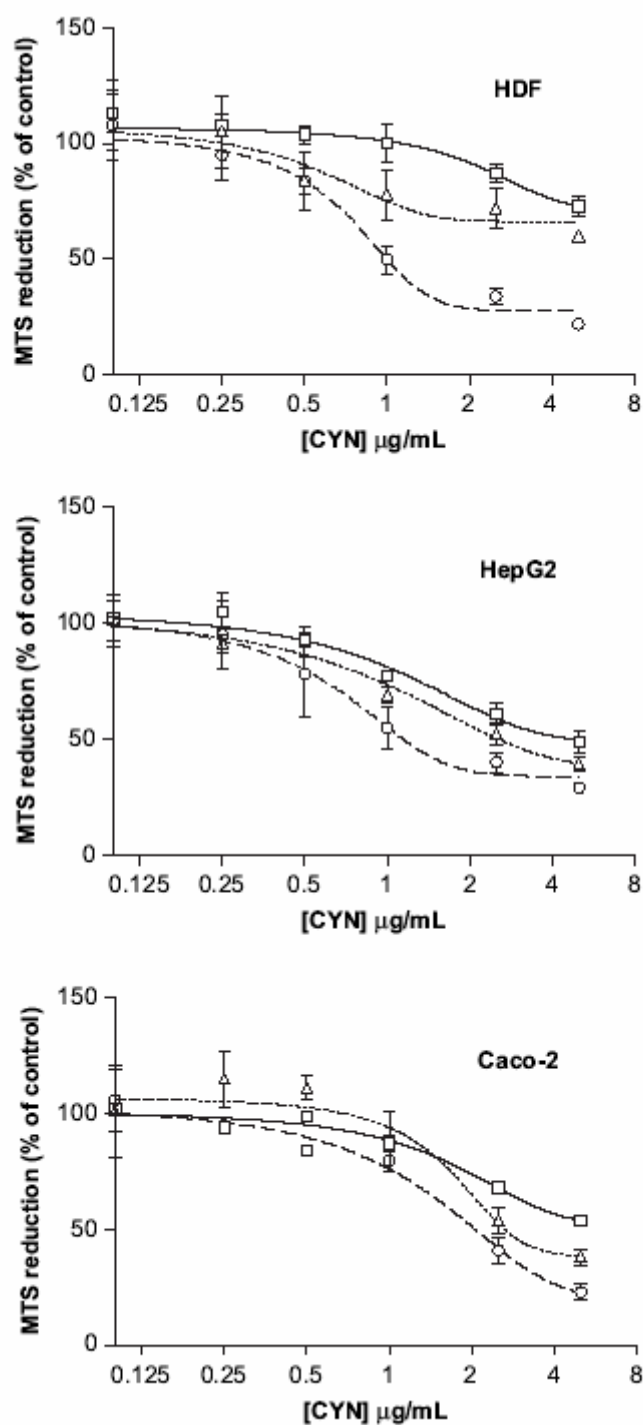
Before undertaking gene expression analysis, it was necessary to optimise experimental conditions to avoid large-scale changes in transcription or mRNA stability resulting from cell death processes. This was achieved by characterising CYN cytotoxicity in the cells using standard assays, and ensuring that mRNA levels for housekeeping genes were stable under the chosen experimental conditions. Concentration-response relationships for the inhibitory effect of CYN on cellular proliferation, as estimated by the MTS assay, were determined in HDF, HepG2, and Caco-2 cells up to 5  $\mu$ g/mL CYN (Figure 2.1). Due to the lack of data for higher concentrations it was not possible to calculate IC50

values accurately; however, a time-dependent effect was seen in each of the three cell types. This was most obvious in the fibroblasts. Disruption of cellular membranes, indicated by lactate dehydrogenase (LDH) release, did not occur over the tested concentration ranges in HepG2 or Caco-2 cells (data not shown), and reached only 30% of the lysed controls at concentrations above 1 µg/mL CYN (2.4 µM) after 72 h in HDFs (Figure 2.2).

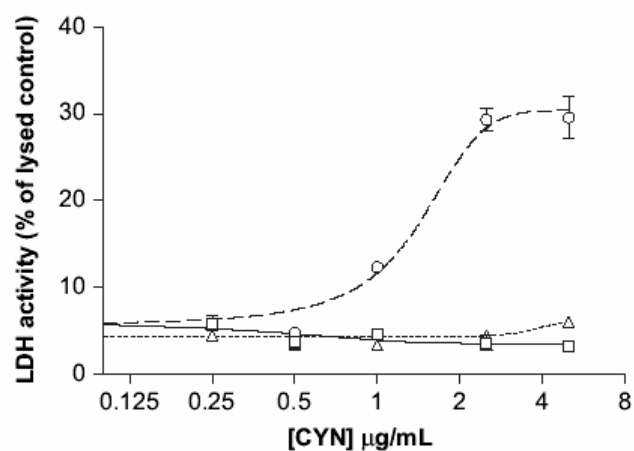
Changes in gene expression were investigated after 6 and 24 h. A CYN concentration of 1 µg/mL (2.4 µM) was selected for the 24 h experiments, which corresponded to MTS reduction measurements of between 80 and 100% of control cultures. At this concentration, GAPDH mRNA levels remained constant (relative to control cultures) in all cell lines tested. GAPDH mRNA levels were stable in HepG2 cells and HDFs after 6 h of exposure to CYN concentrations of up to 5 µg/mL (data not shown). GAPDH was therefore used as the reference gene for all gene expression analyses.

CYN treatment resulted in significant increases in relative mRNA levels for p53 target genes in HDFs and HepG2 cells. After 6 h of treatment, relative mRNA levels for CDKN1A, GADD45α, BAX, and MDM2 generally increased with increasing CYN concentration (Figure 2.3). After 24 h at a concentration of 1 µg/mL CYN, relative mRNA levels for these genes were elevated compared with control cultures (Figure 2.4). with relative expression levels in HDFs markedly higher than in HepG2 cells. CDKN1A expression increased in both HDFs and HepG2 cells after 24 h of exposure to 1 µg/mL CYN. In contrast, CDKN1A expression did not increase under these conditions in Caco-2 cells (Figure 2.4 inset), which express a mutant, inactive form of p53 (Gartel *et al.*, 1996).

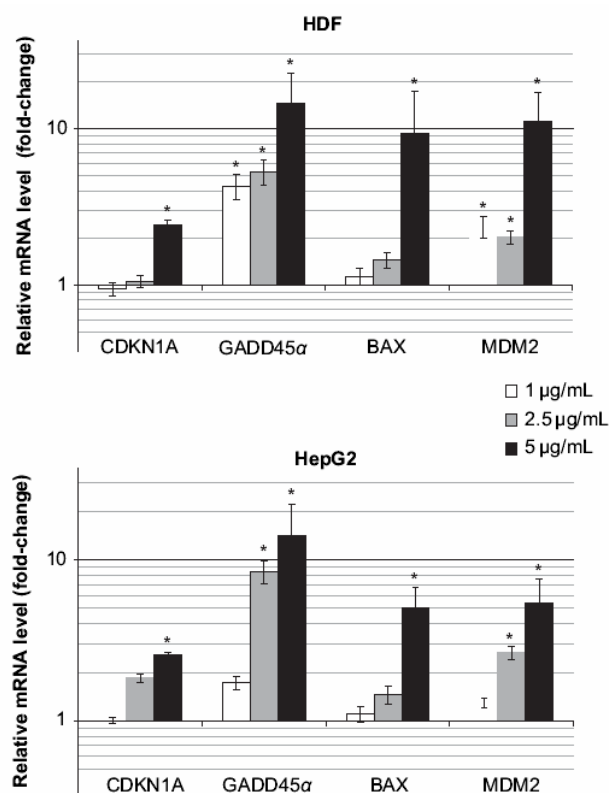
The accumulation of p53 protein rather than phosphorylation of specific residues was investigated, as stabilisation is not wholly dependent on phosphorylation events but also on numerous other modifications such as ubiquitylation, acetylation, and neddylation (Brooks & Wei, 2003; Xu, 2003). p53 is expressed at low levels in most cell types, and the protein was not detected in HDFs or HepG2 cells using our methods. A cell line derived from HepG2, C3A, appeared to express quantitatively higher p53 levels, and an increase in band intensity was noted after 48 h of exposure to increasing CYN concentrations (Figure 2.5).



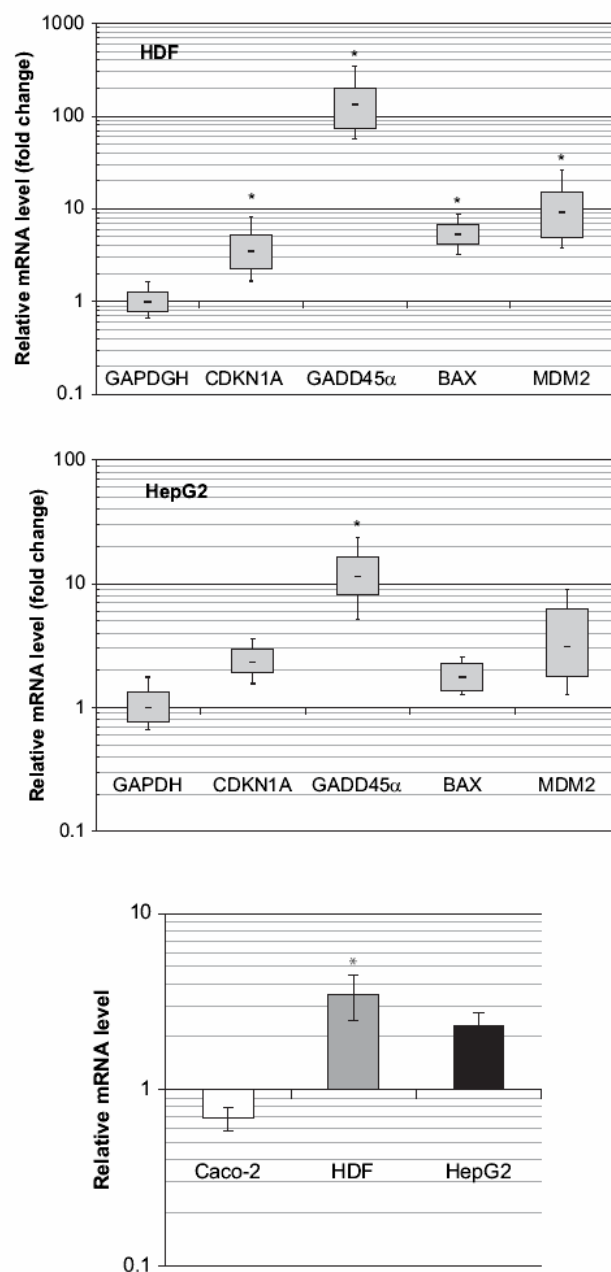
**Figure 2.1** The effects of cylindrospermopsin on cellular proliferation as measured by the MTS assay. Cells were treated for 24 h (—□—), 48 h (---△---), or 72 h (---○---), with 0, 0.1, 0.25, 0.5, 1, and 5 µg/mL cylindrospermopsin. Values are expressed as a percentage of control cultures. Data points represent means  $\pm$  SEM for three replicates.



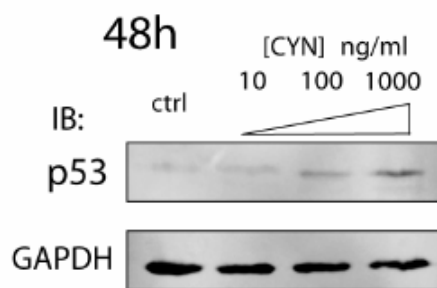
**Figure 2.2** Lactate dehydrogenase release from human dermal fibroblasts treated with varying concentrations of cylindrospermopsin for 24 h ( $-\square-$ ), 48 h ( $\cdots\triangle\cdots$ ), or 72 h ( $---\circ---$ ). Values are expressed as a percentage of activity in vehicle controls lysed with detergent. Data points represent means  $\pm$  SEM for three replicates.



**Figure 2.3** Relative quantification of mRNA levels for selected p53-regulated genes in cells treated for 6 h with 1, 2.5, or 5 µg/mL cylindrospermopsin using SYBR-green I qRT-PCR. Relative mRNA levels are expressed as a factor of those in control cultures ( $\pm$  SEM) and normalised to relative mRNA levels for GAPDH. SEM and significance were calculated using the Relative Expression Software Tool (Pfaffl *et al.*, 2002); asterisk indicates significant at  $p < 0.01$ .



**Figure 2.4** Relative quantification of mRNA levels for p53-regulated genes in cells treated for 24 h with 1  $\mu$ g/mL cylindrospermopsin. Central dashes represent mean relative mRNA levels, boxes represent SEM ranges, and whiskers represent the 95% confidence interval. SEM and significance were calculated using the Relative Expression Software Tool (Pfaffl *et al.*, 2002); asterisk indicates significant at  $p < 0.01$ . Inset: Comparison of relative CDKN1A mRNA levels in Caco-2, HDF, and HepG2 cells after 24 h of exposure to 1  $\mu$ g/mL cylindrospermopsin.



**Figure 2.5** Detection of p53 accumulation in C3A cells treated with increasing concentrations of cylindrospermopsin for 48 h.

## 2.4 Discussion

The time-dependent nature of CYN toxicity observed here is in general agreement with studies using *in vivo* models that found a similar phenomenon using the purified toxin (Hawkins *et al.*, 1997; Metcalf *et al.*, 2002). In contrast, freshly isolated rat hepatocytes are extremely sensitive to CYN cytotoxicity, with relatively short exposures of 18 h inducing LDH activity release of up to 60–80% of lysed controls (Runnegar *et al.*, 1994, 1995; Frosio *et al.*, 2003; Humpage *et al.*, 2005). It is now generally accepted that a CYN derivative resulting from cytochrome P-450 family (CYP450) metabolism is responsible for toxicity in primary hepatocytes, as indicated by the protective effect of CYP450 inhibitors (Runnegar *et al.*, 1994, 1995; Frosio *et al.*, 2003; Humpage *et al.*, 2005). From the lack of LDH activity release after 24 and 48 h in all 3 cell types used in the current study it seems that these cells are not capable of metabolic activation of CYN, so the effects on MTS reduction and p53-dependent gene expression are presumably due to the native toxin.

Despite having transactivation-independent functions associated with apoptosis (Chipuk *et al.*, 2004) and DNA repair (Sengupta & Harris, 2005), p53 predominantly operates by altering the transcription of target genes. Consequently, it is widely accepted that the increased expression of genes known to be regulated by p53 provides an indication of p53 activation. HepG2 are capable of expressing wild-type p53 protein (Bressac *et al.*, 1990), and the HDFs used in this study were originally isolated from a normal patient in a clinical setting, implying normal p53 function (personal communication, D. Watters). In HDFs and HepG2 cells, elevated mRNA levels for p53-regulated genes were apparent after only 6 h of exposure, suggesting an early activation of the pathway. The upstream stress signals contributing to this phenomenon are presumably the result of damage to cellular components, but from the data presented here it is not possible to speculate on the nature of the damage. The finding that broad-spectrum CYP450 inhibitors inhibit genotoxicity in primary mouse hepatocytes (Humpage *et al.*, 2005) suggests that it is unlikely that cells used in this study might undergo DNA damage due to a CYN metabolite. However, a DNA damage-related p53 response cannot be ruled out since native CYN was seen to induce cytogenetic effects in CYP450-deficient human lymphoblastoid cells at relatively low concentrations (Humpage *et al.*, 2000). An alternative possibility is that native CYN may induce p53 activation through processes unrelated to DNA damage. This may be associated with the molecular interactions underlying translational inhibition, since toxins known to inhibit protein synthesis by damaging ribosomal RNA rapidly induce the activation of p53 via the mitogen-activated protein kinase pathway (Zhou *et al.*, 2005). Although the induction of a cohort of p53 target genes suggests that CYN treatment switches on the transactivation functions of the p53 protein, accumulation of the protein at time points earlier than 48 h, that is, 3, 6, and 24 h, was not detected with CYN concentrations up to 5 µg/mL (data not shown). This may be due to a reduction in *de novo* p53 expression arising from CYN-mediated translational inhibition, or increased expression of Mdm2 and subsequent degradation of p53. In these scenarios, the pool of p53 protein existing at the time of treatment would be responsible for the observed induction of target gene expression, although this does not explain the apparent prolonged activation over 24 h. While the gene expression data suggest a possible role for p53 in

CYN-induced growth arrest, the antiproliferative effects of CYN were comparable in treated Caco-2 cells (mutant p53) and HepG2 cells (wild-type p53), indicating that alternative inhibitory pathways may also be induced by the toxin. Diminished cell growth in all three cell types used in this study might simply result from a reduction in overall protein synthesis, with little impact from putative p53-dependent pathways occurring in HepG2 and HDF cells. Furthermore, translational inhibition by CYN may conceivably reduce the magnitude of p53-dependent responses by preventing, at least in part, protein synthesis from mRNA transcribed after exposure to the toxin. The involvement of p53 in CYN toxicity needs to be a priority for future research, particularly because of the important role the protein has in coordinating DNA repair processes. It would be interesting to examine the phosphorylation status of various key residues in the p53 protein using specific antibodies following CYN treatment, to help identify upstream signaling events occurring after CYN exposure.

## 2.5 References

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### 3 STUDIES OF THE COMPARATIVE IN VITRO TOXICOLOGY OF THE CYANOBACTERIAL METABOLITE DEOXYCYLINDROSPERMOPSIN

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#### 3.1 Introduction

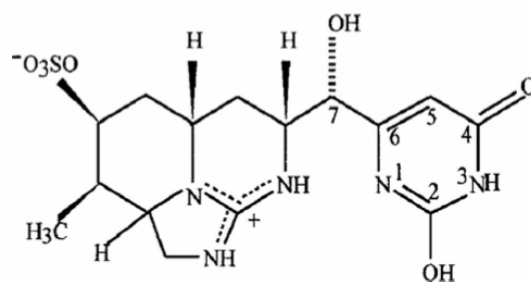
Cyanobacteria produce a variety of toxins, which are classified functionally into hepato-, neuro-, and cytotoxins (Falconer, 1999). One cytotoxic alkaloid from cyanobacteria that is highly toxic is cylindrospermopsin (CYN). This toxin is currently under active investigation because the CYN-producing organisms are becoming increasingly common. CYN is produced by different cyanobacterial species, namely, *Cylindrospermopsis raciborskii*, *Anabaena bergii*, *Aphanizomenon ovalisporum* (Banker *et al.*, 1997; Shaw *et al.*, 1999), *Rhaphidiopsis curvata* (Li *et al.*, 2001a), and *Umezakia natans* (Harada *et al.*, 1994). These organisms occur widely in Australia, North and South America, Africa, Europe, and Central and Southeast Asia (Duy *et al.*, 2000). CYN has been detected in Australia, New Zealand, the Americas, Europe, and Asia (Li *et al.*, 2001b; Stirling & Quilliam, 2001). It has been shown that different isolates can vary widely in their toxicity levels (Griffiths & Saker, 2003).

CYN is highly water-soluble and zwitterionic (Moore *et al.*, 1998) and stable to extremes of temperature and pH (Chiswell *et al.*, 1999). The chemical formula is  $C_{15}H_{21}N_5O_7S$  with a molecular weight of 415. The structure of CYN is given in Figure 3.1. Deoxycylindrospermopsin (DeoxyCYN) is an analog of CYN and features the replacement of the hydroxyl group at position 7 with hydrogen. DeoxyCYN is slightly less polar than CYN as evidenced by a slightly longer retention time on C18 reverse-phase high-performance liquid chromatography (HPLC) columns (Norris *et al.*, 1999). DeoxyCYN exists in tautomeric forms and its structure is given in Figure 3.2.

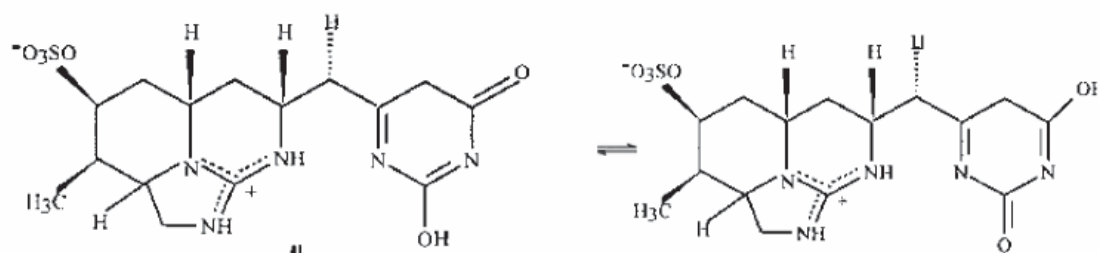
In animals dosed with CYN *in vivo*, organ damage is seen widely, with the liver being the major target. When given orally or intraperitoneally to mice, CYN can cause injury to the gastric mucosa (Seawright *et al.*, 1999) and damage to the liver, kidney, and other organs (Hawkins *et al.*, 1985; Terao *et al.*, 1994; Hawkins *et al.*, 1997; Falconer *et al.*, 1999). The subchronic toxicity of CYN has been investigated by dosing mice in drinking water (Shaw *et al.*, 2001) and by gavage (Humpage & Falconer, 2003). Tentative guideline values have been determined from these data, and range from 1 to 10  $\mu\text{g/L}$ . Mechanisms for liver toxicity were proposed as inhibition of protein synthesis and disruption of metabolic activity, producing lipid accumulation and subsequent hepatocyte death (Terao *et al.*, 1994).

Studies on the *in vitro* toxicology of CYN demonstrate the cytotoxicity and genotoxicity of this toxin. The release of lactate dehydrogenase (LDH) into the medium after cell lysis has been used as an indicator of *in vitro* toxicity. With fresh isolated rat hepatocytes, the increasing LDH level demonstrated the cytotoxicity of CYN (Runnegar *et al.*, 1994). An investigation using cultured rat hepatocytes also showed significant increase of LDH in the medium (Runnegar *et al.*, 1995). The 72-h LC<sub>50</sub> for CYN in primary rat hepatocytes was shown to be 40 ng/mL (Chong *et al.*, 2002).

The toxicity of CYN may depend on metabolic transformation in the liver by cytochrome P450 (Norris *et al.*, 2002). Glutathione, which may be required to inactivate CYN, is inhibited in the hepatocytes themselves (Runnegar *et al.*, 1995). The genotoxicity of CYN has been demonstrated by production of significant DNA strand breakage in the livers of mice exposed to this toxin (Shen *et al.*, 2002) and the induction of micronuclei in human white cells in culture (Humpage *et al.*, 2000).



**Figure 3.1** Chemical structure of cylindrospermopsin



**Figure 3.2** Structure of the tautomers of deoxy-cylindrospermopsin

Falconer and Humpage (2001) conducted dosing trials using *C. raciborskii* material containing CYN with mice to determine tumor initiation. They concluded that CYN was potentially carcinogenic, with the reservation that the study was not a carcinogenicity study.

A possible link to human poisoning from CYN is indicated by an incident on Palm Island in Northern Queensland, Australia, in 1979. There was a major outbreak of hepatoenteritis in a community on the island, requiring the hospitalisation of 140 children and 10 adults (Hawkins *et al.*, 1985).

The possibility of the presence of toxins additional to CYN in *C. raciborskii* extracts has been raised. Hawkins *et al.* (1997) compared the toxicity of crude extracts of *C. raciborskii* with purified CYN. They demonstrated increased toxicity for extracts of *C. raciborskii* above that reported by Ohtani *et al.* (1992) for purified CYN. Falconer *et al.* (1999) suspected more than one toxin in *C. raciborskii* when they compared the toxicity of different batches of this cyanobacterium with similar cylindrospermopsin content. The damage to liver and kidneys of mice treated with CYN were different with every batch.

During the analysis of water samples using HPLC–mass spectroscopy (MS)/MS, Norris *et al.* (1999) found an analog of CYN. After purifying sufficient quantities of this analog, nuclear magnetic resonance (NMR) studies were conducted that showed the structure to be deoxycylindrospermopsin (deoxyCYN), which exists in tautomeric forms, shown in Figure 3.2. This toxin has been recently chemically synthesised, and it has been shown to inhibit protein synthesis in hepatocytes (Looper *et al.*, 2006).

DeoxyCYN has been detected in *C. raciborskii* (Norris *et al.*, 1999) and *Raphidiopsis curvata* (Li *et al.*, 2001a). The *R. curvata* strain produced twice as much deoxyCYN as CYN. In a Thailand strain of *C. raciborskii*, the content of deoxyCYN was only 10% of the level of CYN (Li *et al.*, 2001b). More recently, deoxyCYN has been found in the filamentous benthic cyanobacterium *Lyngbya wollei* (Seifert *et al.*, 2006). Interestingly, deoxyCYN was produced at much higher levels than CYN in this organism.

In an attempt to determine if deoxyCYN added to the toxicity of extracts of *C. raciborskii* compared with pure CYN, Norris *et al.* (1999) dosed three mice (due to limited toxin supplies) with purified

deoxyCYN. The mice were dosed intraperitoneally (ip) with 0.8 mg/kg, which was approximately 4 times the median lethal dose of CYN over 5 days. Because the three mice did not show observable toxicity after 5 days, Norris *et al.* (1999) proposed that deoxyCYN does not play a significant role in the toxicity of *C. raciborskii*. They suggested that the hydroxyl group on the uracil bridge or the keto-enol status of the uracil moiety is important for the toxicity of CYN (Norris *et al.*, 1999). Given the structural similarity of deoxyCYN to that of CYN and the fact that deoxyCYN is commonly formed by CYN producing cyanobacteria, it is important to produce scientific information on its potential toxicology. To contribute to that information, this study determined the *in vitro* toxicology of deoxyCYN in comparison with that of CYN and further investigated the relative potency of deoxyCYN in terms of inhibition of protein synthesis in a cell-free system.

## 3.2 Materials and Methods

### 3.2.1 General Reagents

All reagents were purchased from Sigma-Aldrich USA unless otherwise specified.

### 3.2.2 Culturing of *C. raciborskii*

*Cylindrospermopsis raciborskii* (strain AWT205) was kindly supplied by Dr. Peter Hawkins of Sydney Water. This was cultured in Jaworski's medium in 20-L glass bottles. An aquarium heater was placed in the medium to maintain 27°C. The culture was aerated and illumination was by natural light. Media was added to the batch culture at regular intervals to maintain log phase.

### 3.2.3 Isolation and Purification of CYN and deoxyCYN

The *C. raciborskii* cultures were vacuum filtered through glass-fibre filters to separate cellular material from the filtrate. CYN and deoxyCYN in the filtrate were isolated by the use of graphitised carbon cartridges as described in Norris *et al.* (2001), and toxins in the cellular material were extracted using methanol. Isolates containing the toxins were purified using reverse-phase HPLC according to the method of Norris *et al.* (2001). The concentration of CYN and deoxyCYN was determined using HPLC-MS/MS according to the method of Eaglesham *et al.* (1999). In addition, the CYN and deoxyCYN were quantified using nitrogen elemental analysis by the National Research Centre in Halifax, Canada.

### 3.2.4 Cell Culturing

All media, phosphate-buffered saline (PBS), trypsin, and other substances used for cell culturing were warmed in a water bath to 37°C. The cells were split when they were confluent. The medium was aspirated and discarded. The cells were washed twice with 10 mL PBS; 2 mL trypsin/ethylenediamine tetraacetic acid (EDTA) solution was added to the cells and incubated at 37°C for 2 to 5 min, depending on the cell line. The cells were dislodged from the flask by flicking the flask. The efficiency of the trypsinisation process was checked under a microscope. After that, Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum was added to the cells and mixed carefully by pipetting to inactivate the trypsin. An aliquot of 3 mL of the cell suspension was transferred to a new flask containing 7 mL of medium. Flasks were incubated under standard conditions. Cells were passaged 1 in 2 or 1 in 4, depending upon when confluent cells were required.

Cells were routinely cultured in 75 cm<sup>2</sup> vented culture flasks at 37°C in a humidified 5% CO<sub>2</sub>/95% atmosphere. All cell lines were maintained in DMEM medium (Invitrogen USA). All cultures were tested regularly for possible Mycoplasma contamination by the Virology Section of Queensland Health Scientific Services, Coopers Plains, Australia.

The most commonly used human hepatocellular carcinoma cell line is HepG2. This cell line was established from the tumor tissue of a 15-yr-old Argentine boy with hepatocellular carcinoma in 1975. They are adherent cells that grow in monolayers as small yellow aggregates.

BE-2 was established from a human Caucasian bone marrow neuroblastoma. It was isolated from the bone marrow of a 22-mo-old male with disseminated neuroblastoma in 1972. The cells show a retractile morphology with short neurite-like cell processes.

The Caco-2 human colon adenocarcinoma cell line was established from the primary colon tumor of a 72-yr-old Caucasian man in 1974. They are epithelial adherent cells and grow in colonies.

MNA is a mouse neuroblastoma cell line. HDF cells are human diploid fibroblasts that are derived from the dermis of normal adult skin.

### 3.2.5 Trypan Blue Exclusion Assay

The trypan blue dye exclusion assay was used to evaluate cell number and viability. BE-2 and MNA cells were trypsinised and counted from subcultures. Cells were seeded in 500  $\mu$ L dimethyl sulfoxide (DMSO) medium (100,000 per well) in 24-well plates and incubated overnight. The medium was exchanged for 500  $\mu$ L of toxin-containing medium whereby cells were dosed with 0, 1, 2.5, and 5  $\mu$ g/mL of CYN or deoxy-CYN. The microtiter plates were incubated for 48 and 72 h. To evaluate cell viability, a portion of the medium was removed from each well, stained with 0.2% trypan blue and counted on a hemacytometer. Live cells exclude the dye, whereas dead cells are stained. After that, attached cells in the wells were trypsinized, stained, and counted.

### 3.2.6 MTS Cell Proliferation Assay

Cells were collected from subcultures, trypsinized, and counted using a hemocytometer by the trypan blue exclusion method. Viable cells in 100  $\mu$ L DMEM media were seeded into individual wells of a 96-well plate (50,000 cells per well) with the exception of the blank, where only 100  $\mu$ L media was added. Microtiter plates were incubated overnight. Medium was exchanged for 100  $\mu$ L of toxin-containing medium. Cells were exposed to deoxyCYN and CYN at 0, 0.1, 0.25, 0.5, 1.0, 2.5, and 5.0  $\mu$ g/mL for 24 h and 48 h, respectively. The cell viability was assessed using an aqueous solution of 3-(4,5-dimethylthiazol-2-yl)-5-(-3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and the electron-coupling reagent phenazine ethosulfate (PES). The MTS was reduced by metabolically active cells into a colored formazan product, with a maximal absorbance at 490 nm. At the end of incubation, 20  $\mu$ L of aqueous solution was added to each well and the cells were then incubated for a further 4 h at 37°C. The optical density was then measured at 490 nm using a microplate reader. Cell viability was estimated as the percentage absorbance of test sample relative to control.

### 3.2.7 Morphological Changes in Treated Cells

Morphologic changes were monitored by phase-contrast microscopy. A total of 100,000 viable cells per well were seeded into 24-well plates and incubated overnight. The medium was then exchanged for toxin-containing medium. The cells were treated with 0, 0.5, 2.5, and 5  $\mu$ g/mL deoxyCYN and CYN. The incubation periods were 24 and 48 h, respectively. After incubation, images were taken using a digital camera connected to a phase-contrast microscope.

### 3.2.8 Protein Synthesis Inhibition Assay

The inhibition of *in vitro* protein synthesis was determined using a commercially prepared rabbit reticulocyte lysate system (Promega, USA) with some adaptations from the manufacturer's instructions. The kit consisted of a rabbit reticulocyte lysate system, luciferase assay system, ribonuclease inhibitor, and nuclease-free water. In this system, luciferase mRNA is the message and can be detected by a standard luciferase assay.

A translation master mix was prepared on ice according to the manufacturer's instructions. For the negative control (no mRNA) it was necessary to take 12.5  $\mu$ L of the master mix out, before the mRNA was added. Toxin dilutions from 100 to 100,000 nM were prepared using nuclease-free water; 1.25  $\mu$ L of each toxin dilution was added to an Eppendorf tube and 12.5  $\mu$ L of translation master mix (prepared as per the manufacturer's instructions) was mixed to it carefully. One negative control (no toxin) was

processed with nuclease-free water. The results for test samples were expressed as a percentage of the control. Samples were assayed in triplicate according to the method of Froscio *et al.* (2001, 2003).

### 3.3 Results and Discussion

#### 3.3.1 Cytotoxicity Determined by Trypan Blue Exclusion

Table 3.1 shows the IC<sub>50</sub> values of BE-2 and MNA cells incubated with different concentrations of deoxyCYN and CYN for 48 and 72 h. Incubation with CYN produced fewer viable cells in the culture than with deoxyCYN. By comparing the IC<sub>50</sub> values of deoxyCYN and CYN, it was apparent that the cytotoxicity of deoxyCYN was between 20 to 40% lower than CYN. The MNA cells were more sensitive to both toxins than BE-2 cells. DeoxyCYN was toxic in differing cell types (neuroblastoma and bone marrow derived).

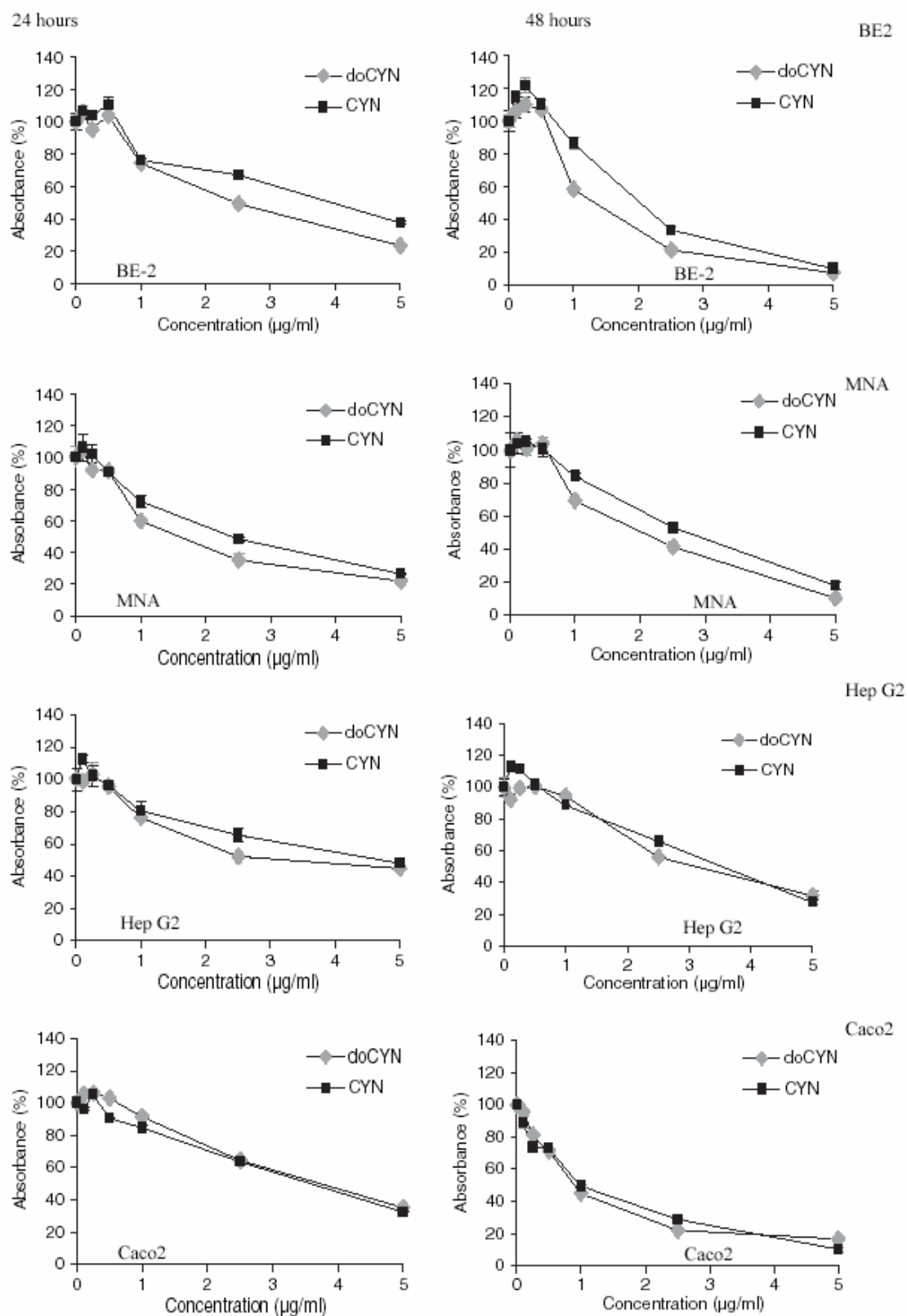
**Table 3.1** IC<sub>50</sub> Values of BE-2 and MNA Cells Incubated With Different Concentrations of DeoxyCYN and CYN for 48 hr and 72 hr.

Cell type	IC <sub>50</sub> (µg/ml), 48 h		IC <sub>50</sub> (µg/ml), 72 h	
	DeoxyCYN	CYN	DeoxyCYN	CYN
BE-2	3.1	2.6	2.1	1.7
MNA	2.2	1.6	1.7	1.2

#### 3.3.2 Antiproliferative Effects Determined by MTS Assay

Cell viability was determined by the MTS dye assay. Figure 3.3 shows the percentage viability of BE-2, MNA, HepG2, and Caco2 cells incubated with different concentrations of deoxy-CYN and CYN for 24 and 48 h. For all cell lines and both toxins, clear dose-response curves were obtained. The toxicity was shown to be time dependent as expected. Incubation with 5 µg/mL deoxyCYN and CYN, respectively, led to a reduction of cell viability of 52% to 92% of the control, depending on the cell line. The IC<sub>50</sub> values for the four different cell lines tested are given in Table 3.2. The results indicate that deoxyCYN was at least as potent as CYN in terms of antiproliferative effects with the cell lines tested.

This assay demonstrated that the Caco2 cells were the most sensitive to deoxyCYN and CYN, eliciting a response at 0.25 µg/mL for the 48-h treatment, while the other cell lines only responded at concentrations of 1 µg/mL or higher. The toxicity of CYN has been tested before by Chong *et al.* (2002) with rat hepatocytes using the MTT assay. Using their data, IC<sub>50</sub> for 48 h for CYN was approximately 0.2 µg/mL. With MNA, BE-2, HepG2, and Caco2 the IC<sub>50</sub> values varied between 0.9 and 3.6 µg/mL. This difference may be explained by the use of transformed cancer cell lines in this study, which appear to be less sensitive than the primary cell lines used by Chong *et al.* (2002). This is potentially due to a number of factors, including lower levels of metabolic activation and possible toxicokinetic differences.



**Figure 3.3.** Percentage viability of BE-E, MNA, HepG2, and Caco2 cells incubated with different concentrations of deoxyCYN and CYN for 24 h and 48 h. Data are means of three replicates.



**Table 3.2** IC<sub>50</sub> Values for Four Cell Lines Dosed with Deoxy-CYN and CYN

Cell type	IC <sub>50</sub> (µg/ml), 24 h		IC <sub>50</sub> (µg/ml), 48 h	
	deoxyCYN	CYN	deoxyCYN	CYN
BE-2	2.5	2.9	1.3	2.0
MNA	1.6	2.4	2.0	2.7
HepG2	3.2	4.6	3.1	3.6
Caco2	2.6	2.7	0.9	1.0

### 3.3.3 Morphological Effects of CYN and DeoxyCYN on Treated Cells

Microscopic examinations indicated that BE2, MNA, and HepG2 cells treated with both toxins had undergone significant morphological changes. The BE2 and MNA cells in particular demonstrated cell shrinkage and cell rounding to various degrees. These morphological features are indicative of apoptosis. A summary of these results for the four cell types is given next.

#### *BE2*

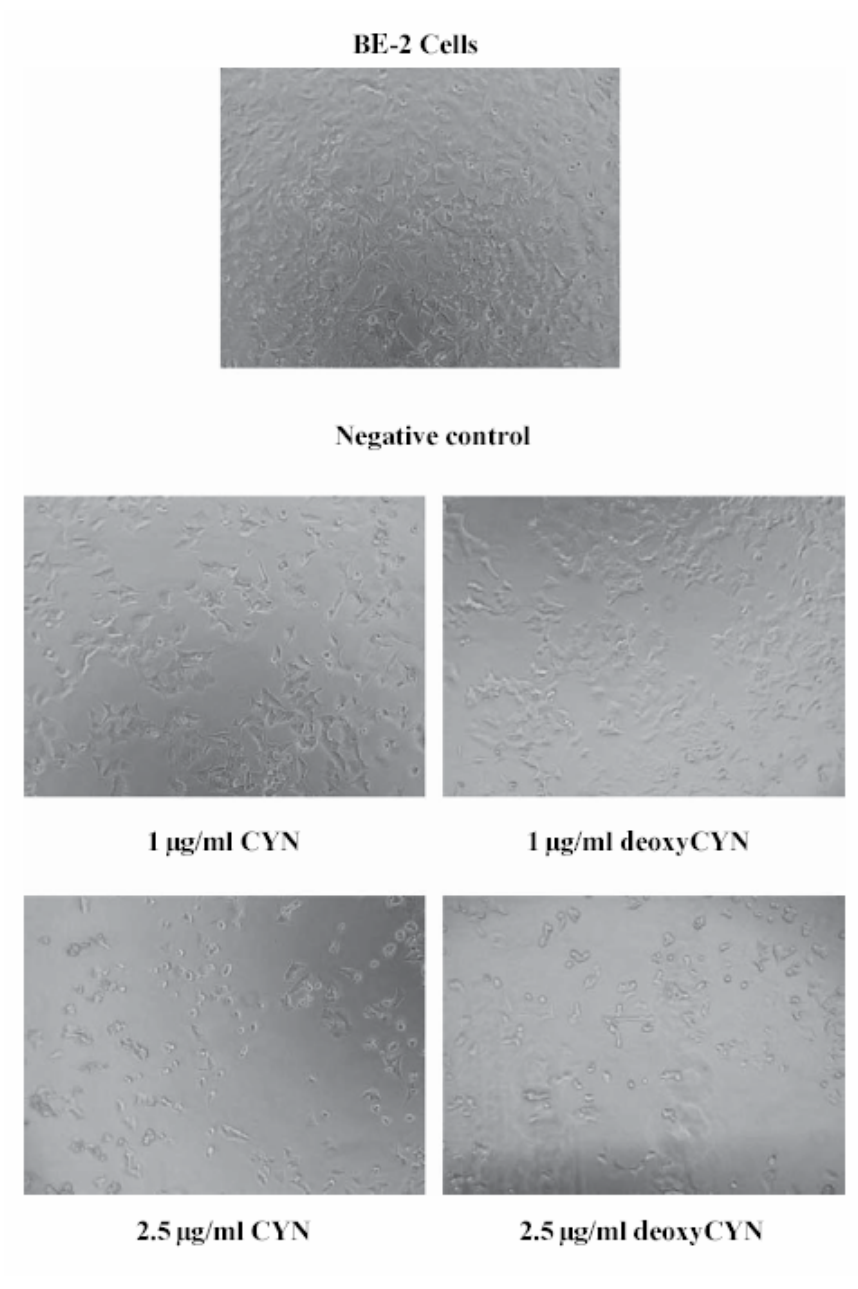
This cell line grew as a monolayer of irregular shaped cells. At 1 µg/mL of both toxins for 24 h, the cells did not change shape, but the number of cells decreased by more than 50%. At 2.5 µg/mL for both toxins the cells appeared shrunken and rounded up which are signs of cytotoxicity. These changes are demonstrated in Figure 3.4.

#### *MNA*

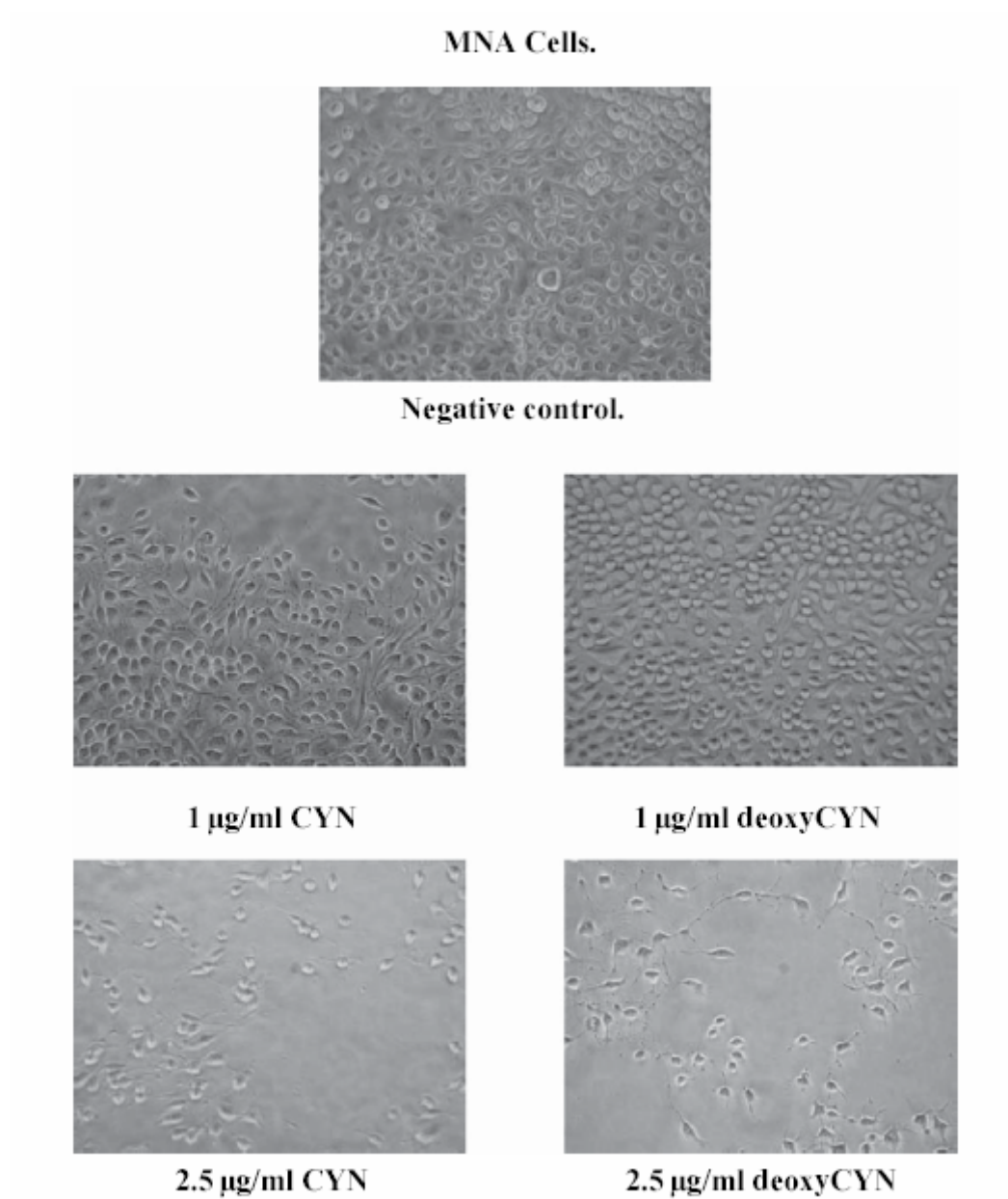
The MNA cells grew close together and had a rectangular shape. Exposure to 1 µg/mL of deoxyCYN produced no observable effect, whereas the CYN-treated cells changed their morphology to resemble interconnected neuronal-like cells with extended axonal processes. At 2.5 µg/mL of both toxins, the cell numbers decreased considerably and the deoxyCYN treatment produced cells with long axonal processes.

#### *HepG2*

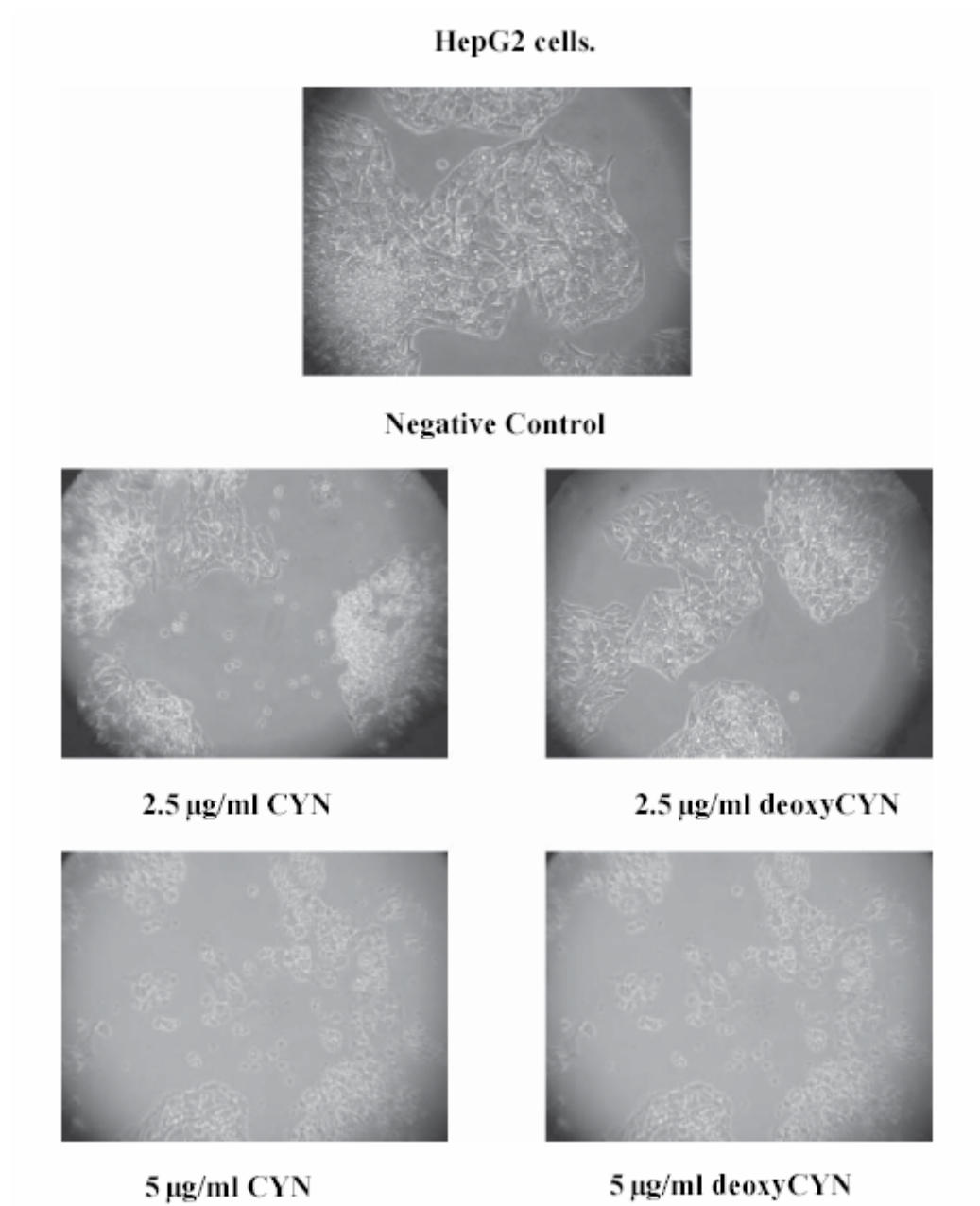
The HepG2 cells grew with irregular size and shape. At 2.5 µg/mL, CYN produced significant numbers of floating cells, whereas 5 µg/mL of deoxyCYN was required to produce a similar effect. These effects are demonstrated in Figure 3.6.



**Figure 3.4** Morphological Changes in BE-2 cells treated with CYN and deoxyCYN



**Figure 3.5** Morphological Changes in MNA cells treated with CYN and deoxyCYN

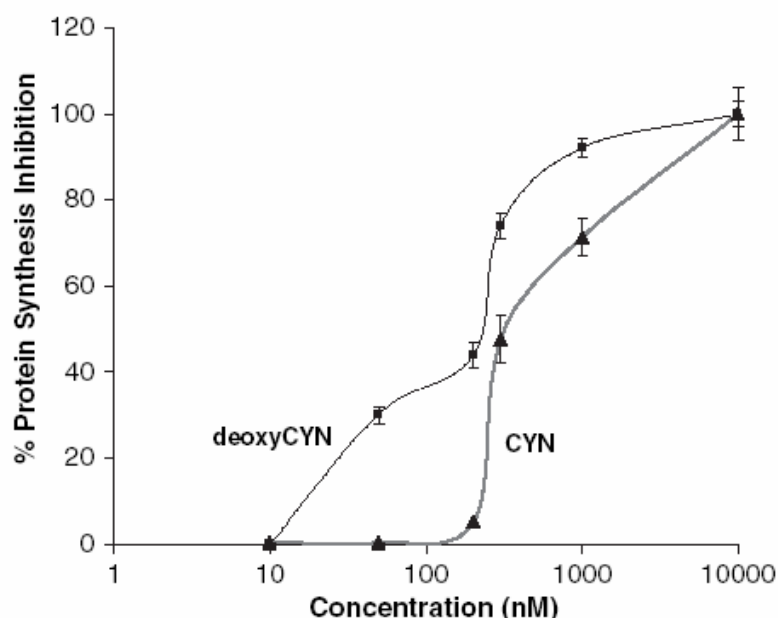


**Figure 3.6** Morphological Changes in HepG2 cells treated with CYN and deoxyCYN

### 3.3.4 Comparative Inhibition of Protein Synthesis by DeoxyCYN

The inhibition of protein synthesis by varying concentrations of deoxyCYN and CYN is shown in Figure 3.7. At 1000 nM, the inhibition was 90% for deoxyCYN and 70% for CYN. The IC<sub>50</sub> for deoxyCYN was 220 nM (0.088 µg/mL), and 340 nM (0.14 µg/mL) for CYN. The inhibition of protein synthesis was dose dependent and a sigmoidal relationship was produced on the semi-log plot. CYN and deoxyCYN inhibited protein synthesis to a similar degree. In addition, the potency of protein synthesis inhibition with CYN in this study was similar to that found by Frosio *et al.* (2001). Looper *et al.* (2005) also found that deoxyCYN inhibited protein synthesis in hepatocytes.

The findings of Frosio *et al.* (2003) using a protein synthesis inhibition assay with primary hepatocytes, suggested that the protein inhibition produced by CYN could not be reversed following removal of the toxin; therefore, the inhibition is either irreversible or the toxin is trapped within the intracellular environment. Since inhibition of protein synthesis is related to the mechanism of toxicity of CYN, it is suggested that deoxyCYN has the potential to exert *in vivo* toxicity to a similar extent to CYN.



**Figure 3.7** Sigmoidal dose-response curve for the inhibition of protein synthesis by deoxyCYN and CYN. Values are means  $\pm$  SEM of three replicates.

### 3.4 Conclusions

This work has demonstrated that deoxyCYN produces cytotoxicity and reduces cell proliferation in a range of cell types using *in vitro* systems. Additionally, the toxicity of deoxyCYN *in vitro* is of a similar magnitude to that produced by CYN. Changes in the morphology of a number of different cell types upon exposure to deoxyCYN were similar to the changes produced by CYN. The results have shown that the main acknowledged mechanism of toxicity for CYN, inhibition of protein synthesis, also operates with deoxyCYN, with the potency of the two toxins being similar. Given that the *in vitro* toxicology of deoxyCYN has been demonstrated, and that this compound co-occurs with CYN in water supplies, it is strongly suggested that further *in vivo* animal toxicology research be conducted to provide information for the human health risk assessment of deoxyCYN.

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## 4 INTERACTION OF THE CYANOBACTERIAL TOXIN CYLINDROSPERMOPSIN WITH THE EUKARYOTIC PROTEIN SYNTHESIS SYSTEM

This Chapter is based on the following manuscript

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### 4.1 Introduction

Cylindrospermopsin (CYN) is an alkaloid toxin produced by a number of species of cyanobacteria, most commonly *Cylindrospermopsis raciborskii*. The occurrence of CYN producing species in freshwater used for drinking water poses a public health risk. In fact, the toxin was first identified following its implication as the causative agent in an outbreak of severe hepatoenteritis on Palm Island in 1979 (Byth, 1980; Hawkins *et al.*, 1985).

The toxin structure consists of a sulphated and methylated tricyclic guanidine linked to hydroxymethyluracil group (Ohtani *et al.*, 1992). Exposure to CYN produces hepatonecrosis in experimental animals, with extrahepatic lesions of variable location and severity including in the kidneys, lungs and thymus (Terao *et al.*, 1994; Hawkins *et al.*, 1997; Falconer *et al.*, 1999).

Early toxicological investigation demonstrated that CYN was a potent inhibitor of protein synthesis, inhibiting globin synthesis in rabbit reticulocyte lysates with complete cessation at 120 nM (Terao *et al.*, 1994). In this study, the contribution of protein synthesis impairment in the pathogenesis of CYN toxicity *in vivo* was also demonstrated, with the authors showing dissociation of ribosomes from the endoplasmic reticulum in the livers of toxin treated mice.

In primary hepatocytes the effects on protein synthesis are an early indicator of exposure to CYN (0.5 – 5 µM), and occur at subtoxic concentrations and well before the onset of toxicity at higher concentrations (Froschio *et al.*, 2003). These inhibitory effects were thought to be irreversible as no recovery from inhibition was observed after removal of the toxin from the culture medium. The irreversible effects may arise from covalent binding or strong non-covalent interaction of the toxin with the ribosomes and/or their components, or intracellular modification of the toxin preventing its exit from the cell. CYN has also been observed to cause long lasting effects *in vivo* with unexpected mortalities following repeated administration of toxin below expected LD<sub>50</sub> (7 day) doses. Daily intraperitoneal dosing of mice with 64-128 µg kg<sup>-1</sup> CYN (2-3 fold below the LD<sub>50</sub>) produced mortality following 3 doses (Rogers *et al.*, 2007). In another study, oral administration of a cyanobacterial extract containing CYN (1500 mg extract /kg) resulted in mortality when the second dose was given after a two-week recovery period (Falconer and Humpage, 2001). In this case, the minimum oral lethal dose (7 days) had been determined to be 2500 mg/kg.

Mechanistically, little is known about how CYN interferes with the complex protein synthesis machinery. The addition of CYN to reticulocyte lysates causes an immediate cessation of protein synthesis (Froschio, 2002) indicating that the toxin interferes with the elongation step. While the uracil moiety in the toxin's structure has been shown to be important in maintaining biological activity of the toxin *in vivo* (Banker *et al.*, 2001), it is not known whether this moiety facilitates the toxin's effects *in vitro*. It is suggested that the uracil may be involved in mediating the response on the ribosome by interacting with nucleotides in rRNA. Synthetic CYN analogues have also been used to show that by disrupting the functionality of the tricyclic guanidine rings, the potency of protein synthesis inhibition is decreased more than 100 fold (Runnegar *et al.*, 2002).

In this study we further characterise the interaction of CYN with eukaryotic ribosomes. Given previous indications that CYN produces irreversible effects on protein synthesis in mammalian cell culture we wanted to determine whether the toxin binding is also irreversible. Using radiolabelled toxin, binding studies were carried out and provide evidence that the primary target for the toxin may not be the ribosome itself.

## 4.2 Methods

### 4.2.1 Preparation of CYN and [ $^{14}\text{C}$ ] CYN

Purified cylindrospermopsin (CYN) was obtained from the Australian Water Quality Centre (AWQC), prepared as detailed in (Humpage *et al.*, 2005). [ $^{14}\text{C}$ ] CYN was prepared from *C. raciborskii* strain AWT 205/1 that was cultured in Jaworski's medium (Thompson *et al.*, 1988) with 25% of the  $\text{NaHCO}_3$  replaced by [ $^{14}\text{C}$ ] labelled  $\text{NaHCO}_3$ . The culture (5 L) was grown at  $25 \pm 2^\circ\text{C}$  with 14:10 (light/dark) illumination. After 4 months growth, the dissolved CYN concentration was  $620 \mu\text{g L}^{-1}$ . This solution was filtered through a GF/A glass fibre filter (Whatman, UK) and then gravity fed through two Carbograp SPE cartridges (Alltech, IL, USA). The cartridges had been pre-washed with 5% formic acid in methanol ( $2 \times 12 \text{ mL}$  each) and Milli-Q water ( $2 \times 12 \text{ mL}$  each). CYN was eluted from the cartridges with 5% formic acid in methanol ( $2 \times 12 \text{ mL}$ ). Organic solvents were removed under reduced pressure (Roto-Vac) at  $40^\circ\text{C}$  and resuspended in 5 mL Milli-Q water. CYN was purified from the aqueous extract. The extract was filtered ( $0.4 \mu\text{m}$ ) and a portion (1 mL) loaded onto a Prep Nova HR C18 Radially Compressed Column ( $25 \times 100 \text{ mm}$ ;  $6 \mu\text{m}$ , 60A); Shimadzu Class LC-10 HPLC System with SPDM 10A diode array detector. The chromatography consisted of a linear gradient of 7-20% methanol over 15 mins, followed by a linear gradient of 20-7% methanol over a further 10 mins. The flow rate was  $5 \text{ mL min}^{-1}$ . Fractions were monitored at 262 nm and CYN eluted at 11.9 min. Toxin fractions were pooled, diluted with Milli-Q water (1:1) and freeze dried overnight to yield a white, amorphous powder. Further purification of CYN was achieved by a second round of HPLC. This was carried out as described above, but now using an Alltech Apollo C18 column ( $150 \times 22 \text{ mm}$ ;  $5 \mu\text{m}$ ), linear gradient of 7-20% methanol over 15 mins was followed by linear gradient of 20-7.5% methanol over 13 mins. The toxin was quantified by HPLC/MS/MS (Eaglesham *et al.*, 1999). The specific activity of [ $^{14}\text{C}$ ] CYN was measured using a Packard Tricarb 1600TR liquid scintillation counter and determined to be  $9.88 \text{ mCi mmol}^{-1}$ . The solid toxin was stored in sealed vials under argon at  $4^\circ\text{C}$ .

### 4.2.2 *In vitro* Translation Reactions

Translation reactions were carried out in rabbit reticulocyte lysate obtained from Promega (WI, USA). Reaction mixes (25  $\mu\text{L}$ ) were treated with CYN (60-600 nM) for 5 mins and then protein synthesis was initiated by the addition of 20  $\mu\text{g/mL}$  luciferase mRNA template. Samples were incubated for 90 mins at  $30^\circ\text{C}$ . The amount of luciferase formed was quantified in a white 96-well microplate (PerkinElmer, MA, USA). Samples were diluted 100 fold in lysis buffer (25 mM tris-phosphate, 2mM DTT, 2 mM 1,2-diaminocyclohexane N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) containing 1mg/mL BSA and then 2.5  $\mu\text{L}$  added to 60  $\mu\text{L}$  of BrightGlo reagent (Promega, WI, USA). Luminescence of samples was read (10 sec read time) on a Victor<sup>3</sup> 1420 luminescence microplate reader (Perkin Elmer, MA, USA).

### 4.2.3 CYN Binding in Reticulocyte Lysate Fractions

Binding of [ $^{14}\text{C}$ ] CYN to 80S ribosomes and/or translation components was assessed in reticulocyte lysates. Translation reaction mixes (90  $\mu\text{L}$ ) were prepared and incubated with 60-600 nM [ $^{14}\text{C}$ ] CYN for 30 min at  $30^\circ\text{C}$ . Sub-samples (5  $\mu\text{L}$ ) were then taken for  $A_{260}$  determination and luciferase quantification, while unbound toxin was removed by centrifugation through Sephadex G-50 mini Quick Spin size exclusion columns (Roche Applied Science IN, USA). A load volume of 65  $\mu\text{L}$  was used and samples were centrifuged at  $1000 \times g$ , 4 mins. By this method, greater than 90% of the ribosomes were recovered in the flow through solution and in the absence of ribosomes  $\leq 3\%$  of the free toxin was eluted (data not shown). To account for the total amount of [ $^{14}\text{C}$ ] CYN loaded, the solution retained in the spin-column was then eluted in a series of washes using 60 mM Tris-HCl buffer, pH 7.4. For the first wash 100  $\mu\text{L}$  of buffer was added, and the columns were centrifuged at  $1000 \times g$ , 4 mins. This was followed by 2 washes of 300  $\mu\text{L}$  buffer. The radioactivity in 60  $\mu\text{L}$  of the ribosome and wash fractions was determined by scintillation counting by addition to 10 mL StarScint scintillation fluid (Perkin Elmer, MA, USA) and counting (Beckman LS3801 Scintillation counter). Ribosome concentrations were calculated from  $A_{260}$  determinations using an extinction coefficient of  $\epsilon_{260} = 5 \times 10^7 \text{ M/cm}$  (Lorsch and Herschlag, 1999). The data were used to calculate the amount of ribosome bound [ $^{14}\text{C}$ ] CYN. Controls were run in parallel to determine background counts in each of the fractions. In this case, [ $^{14}\text{C}$ ] CYN was incubated in sterile MilliQ water (absence of reticulocyte lysate) and the samples were processed on the Sephadex G-50 spin columns as detailed above.

#### 4.2.4 Displacement Studies

In competition experiments, reticulocyte lysate translation mixes (90  $\mu$ L) were pre-incubated with 300 nM [ $^{14}$ C] CYN for 30 mins at 30°C. Then 300 nM, 3  $\mu$ M or 30  $\mu$ M concentrations of unlabelled CYN were added and incubation continued at 30°C for a further 60 mins. Samples were applied to Sephadex G-50 Quick spin columns and the amount of ribosome-bound [ $^{14}$ C] CYN determined as detailed above. Data were corrected for background counts.

#### 4.2.5 Separation of Ribosomes from [ $^{14}$ C] CYN using Molecular Weight Cut-off Filters

Translation mixes (90  $\mu$ L) were incubated with 300 nM [ $^{14}$ C] CYN for 30 mins at 30°C. At the end of the incubation, samples were diluted to 150  $\mu$ L with sterile MilliQ water. Separation of ribosomes from material with a defined molecular weight cut off (MWCO) was carried out using Vivaspine polyethersulphone (PES) membranes (Sartorius, Germany). Samples (100  $\mu$ L) were loaded to 5, 10, 30, 50 or 100 kDa filters and centrifuged at 12,000  $\times g$  for 45 mins. Samples of 300 nM [ $^{14}$ C] CYN prepared in MilliQ water were applied to each MWCO filter in parallel. The eluate was collected and 60  $\mu$ L used for scintillation counting as described previously.

#### 4.2.6 Statistical Analysis

GraphPad Prism Version 4.03 for Windows (GraphPad Software, SanDiego, CA, USA) was used for all statistical analyses and graphing.

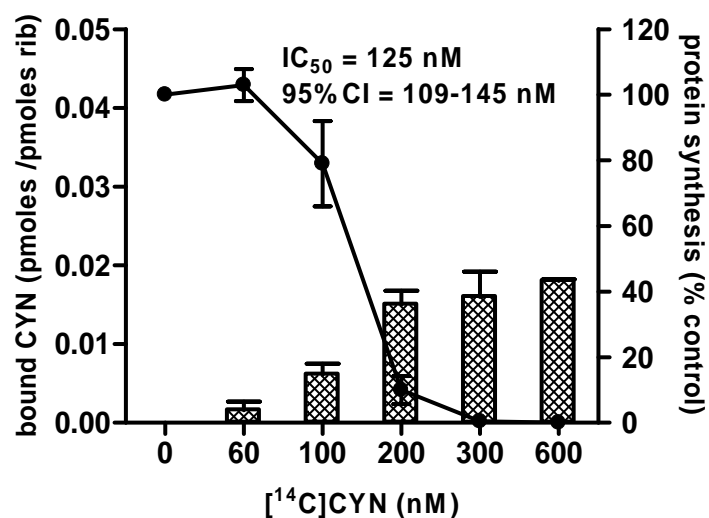
### 4.3 Results

#### 4.3.1 Binding of [ $^{14}$ C] CYN in Reticulocyte Lysate Fractions.

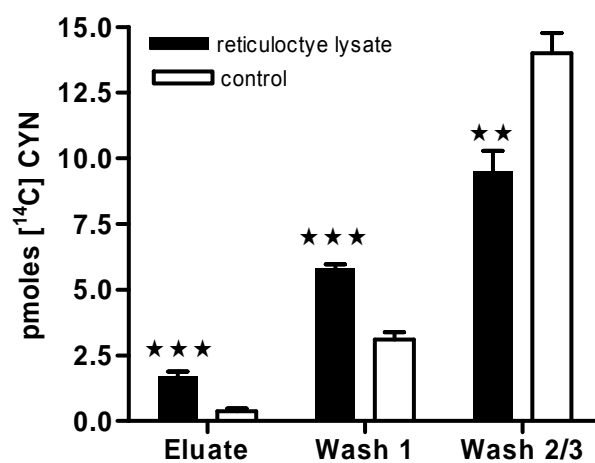
Binding of [ $^{14}$ C] CYN to its target was assessed in reticulocyte lysate fractions following separation on Sephadex G-50 size exclusion columns. By this method,  $\geq 90\%$  of the ribosomes eluted after centrifugation, while the remaining  $\sim 10\%$  of ribosomes eluted in the first of 3 buffer washes. As shown in Figure 4.1, the radiolabelled toxin was demonstrated to inhibit protein synthesis,  $IC_{50}$  of 125nM; (95% confidence interval 109-145 nM), and was associated with concentration-dependent binding of the toxin in the ribosome fraction. Binding reached a plateau at concentrations resulting in complete cessation of protein synthesis (300-600 nM). The binding stoichiometry at plateau was determined to be 0.02:1 CYN to ribosome molecules.

In addition to toxin binding in the ribosome eluate, distribution profiles showed that significant binding of [ $^{14}$ C] CYN occurred in the Wash 1 fraction despite the low ribosome content (10% ribosomes). See Figure 4.2 for example. Following incubation of reticulocyte lysate with 300 nM [ $^{14}$ C] CYN to cause complete cessation of protein synthesis, the amount of toxin in the ribosome eluate was  $1.7 \pm 0.21$  pmoles compared with  $0.42 \pm 0.093$  pmoles in the control ( $p < 0.001$ , t-test); while the first wash fraction contained  $5.8 \pm 0.19$  pmoles toxin compared with  $3.1 \pm 0.34$  pmoles in the control ( $p < 0.001$ , t-test). Wash 2 and 3 (combined) contained  $9.5 \pm 0.78$  pmoles toxin compared to  $14 \pm 0.77$  pmoles in the control ( $p < 0.01$ , t-test), relating to the amount bound in previous fractions. Treatment of reticulocyte lysate with 200 nM [ $^{14}$ C] CYN also showed a similar distribution profile (data not shown).

Toxin binding was also assessed in the absence of mRNA (non initiated samples). Following incubation of the non initiated reaction mixes with 300 nM [ $^{14}$ C] CYN, the amount of toxin in the ribosome eluate was  $2.2 \pm 0.3$  pmoles. Wash 1 contained  $6.6 \pm 0.50$  pmoles of toxin and Wash 2 and 3 (combined) contained  $9.8 \pm 0.63$  pmoles. There was no significant difference from the amount of toxin determined in the non initiated sample fractions to the corresponding fractions obtained from samples incubated in the presence of mRNA.



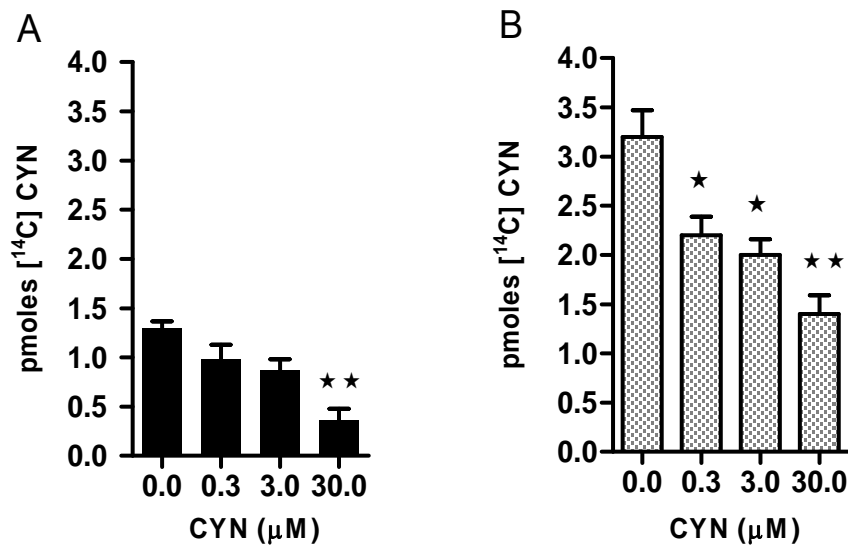
**Figure 4.1** Inhibition of protein synthesis by [<sup>14</sup>C] CYN in reticulocyte lysate (—●—) and associated binding in the ribosome fraction (—■—). Data are mean ± SE of 3-5 independent experiments. The IC<sub>50</sub> was determined by non-linear regression (variable slope).



**Figure 4.2** Binding of 300 nM [<sup>14</sup>C] CYN in reticulocyte lysate fractions separated on Sephadex G50 columns. Ribosomes were distributed as follows: 90% in the eluate fraction, 10% in Wash 1, absent in Wash 2/3. Significant differences from the control ([<sup>14</sup>C] CYN in MilliQ water) were determined by t-test (\*\* p < 0.01, \*\*\* p < 0.001).

### 4.3.2 Displacement of Bound [ $^{14}\text{C}$ ] CYN

To determine whether CYN was bound reversibly or irreversibly to the target, displacement studies were carried out by incubating reticulocyte lysates with 300 nM [ $^{14}\text{C}$ ] CYN for 30 min followed by the addition of unlabelled CYN (0.3-30  $\mu\text{M}$ ) for a further 60 mins. As shown in Figure 4.3 the addition of unlabelled toxin was able to partially displace [ $^{14}\text{C}$ ] CYN from both the ribosome fraction and the Wash 1 fraction. Incubation with 100-fold excess (30  $\mu\text{M}$ ) toxin resulted in significant displacement of [ $^{14}\text{C}$ ] CYN from the ribosome fraction (Figure 4.3A); while incubation with 1-100 fold toxin (0.3-30  $\mu\text{M}$ ) resulted in significant displacement of [ $^{14}\text{C}$ ] CYN in the Wash 1 fraction (Figure 4.3B). All analyses were carried out by one-way ANOVA. Displaced [ $^{14}\text{C}$ ] CYN migrated to the fraction containing washes 2 and 3 (data not shown).



**Figure 4.3** Displacement of [ $^{14}\text{C}$ ] CYN from the (A) ribosome fraction and (B) Wash 1 fraction following incubation with increasing concentrations of unlabelled toxin. Values are the mean  $\pm$  SE of 3 independent experiments and are corrected for background counts. The data were analysed by one-way ANOVA followed by Dunnett's test. A significant difference from the control is indicated by \* ( $p < 0.05$ ) or \*\* ( $p < 0.01$ ).

### 4.3.3 Molecular Weight Cut-Off Filters

Molecular weight cut off filters were used to separate free [ $^{14}\text{C}$ ] CYN from ribosomes and associated high molecular weight material. A range of cut off filters were used (5, 10, 30, 50 or 100 kDa) that would cover the expected size range of a number of the soluble proteins (initiation and elongation factors) known to be associated with protein synthesis. It was first determined that free [ $^{14}\text{C}$ ] CYN (prepared in Milli-Q water), migrated equally well over the range of filters, with 100% recovery of  $13 \pm 0.2$  pmoles toxin. Following incubation of reticulocyte lysate with 300 nM [ $^{14}\text{C}$ ] CYN, the amount of toxin recovered from the lysate following filtration averaged 38% of the amount loaded. This remained unchanged over the range of filters tested;  $4.8 \pm 0.3$  pmoles was recovered from the 5kDa filter and  $5.3 \pm 0.5$  pmoles was recovered from the 100 kDa filter. The remaining 62% was retained on the column with the ribosomes/higher molecular weight material of  $> 100$  kDa. The addition of high salt concentrations (0.5M KCl) to the reticulocyte lysate prior to filtration was observed to increase the amount of [ $^{14}\text{C}$ ] CYN eluting from the molecular weight cut off filters (data not shown).

## 4.4 Discussion

Cylindrospermopsin's (CYN) effects on protein synthesis play an important role in toxicity in mammalian cells, yet little is known about how the toxin interacts with this target.

Binding of [ $^{14}\text{C}$ ] CYN to ribosomes was investigated in reticulocyte lysates, with separation of ribosomes from free toxin achieved using Sephadex G-50 size exclusion columns. The results obtained in these experiments suggest that CYN may not bind to the ribosome itself. While toxin binding in the ribosome fraction was concentration-dependent over the range inhibiting protein synthesis, it did not correspond to ribosome content. The molar ratio of toxin to ribosomes was 0.02:1 at plateau in samples that had been incubated with 300 nM [ $^{14}\text{C}$ ] CYN to cause complete cessation of protein synthesis. Even if it was considered that not all ribosomes in the lysate were active, or that one CYN molecule could block a number of ribosomes on a polysome, the results still could not be explained. On average, ribosomes in polysomes occur once every 80-100 nucleotides (Mathews *et al.*, 2000). For a 2.0 kb mRNA such as that coding for luciferase, a maximum of 20 ribosomes per mRNA could be expected. In contrast, toxins that target the ribosome illustrate 1:1 binding stoichiometry. Studies using [ $^{14}\text{C}$ ] erythromycin (Xiong *et al.*, 2005) or [ $^{14}\text{C}$ ] evernimicin (McNicholas *et al.*, 2000), both inhibitors that target the prokaryotic ribosome, show that binding reaches a plateau when the molar concentration of the toxin equals that of the ribosome.

Further supporting the theory that CYN does not target the ribosome itself, a greater amount of [ $^{14}\text{C}$ ] CYN was observed in the first wash fraction obtained from the Sephadex G-50 columns than from the ribosome fraction. This fraction contained a low ribosome content ( $<10\%$  of the total), but soluble high molecular weight material that was not excluded by the column during the first centrifugation. The fact that [ $^{14}\text{C}$ ] CYN distribution profiles were also similar in the absence of mRNA (non-initiated samples) indicates that the toxin binding is not dependent on an active translation process.

Molecular weight cut off filters were used to determine if the [ $^{14}\text{C}$ ] CYN binding was associated with a defined molecular weight fraction. The range of cut off filters used, 5 -100 kDa covers the molecular weights of a number of the soluble proteins (total complex or subunits of) that are associated with protein synthesis. As previous work has indicated that CYN may interfere with the elongation step of protein synthesis (Frosio, 2002) the elongation factors were considered a potential target. The proteins eEF1 and eEF2 modulate the activities of elongation in eukaryotes (refer to (Proud, 2000) for review of their action). The monomeric protein eEF2 (95 kDa) is known to be targeted by a number of toxins including soradarin (Dominguez and Martin, 1998; Justice *et al.*, 1998) and bisamidine (Gajko-Galicka *et al.*, 2002) and is also inhibited by ADP-ribosylation activity catalyzed by *Diphtheria* and *Pseudomonas A* toxins. Given that CYN was found to be associated with material  $>100$  kDa, eEF2 is unlikely to be the target. The eEF1 complex consists of a number of peptides, eEF1A and eEF1B ( $\alpha$ ,  $\beta$  and  $\gamma$  subunits). While each peptide alone is smaller than the molecular weight cut off, interaction of CYN with the 150 kDa complex cannot be excluded as a potential target. Other soluble factors involved in the eukaryotic translation process include the initiation factors - 11 have been identified, comprising more than 25 peptide chains (Hershey and Merrick, 2000), and a 50 kDa eukaryotic release factor (eRF1) that is involved in termination of translation. While initiation factors were considered a less likely target, it is of interest to note that a number of the peptides involved in the initiation process form complexes of a large molecular weight mass, some  $>600$  kDa. Furthermore,

many of the initiation factors are reported to be present at low molar ratios (0.2-0.5) compared with the ribosome content (Browning *et al.*, 1990). It is plausible that results obtained in the current study could be explained if CYN targeted a high molecular weight protein of low abundance. Confirmation of the exact target will require further work isolating the soluble proteins from reticulocyte lysates and detailed fractionation for biochemical analysis. Finally, previous work has shown that the effects of CYN on protein synthesis were irreversible in a cell based model (Froscio *et al.*, 2003). In the current study bound [<sup>14</sup>C] CYN could be partially displaced by the addition of unlabelled toxin. This displacement occurred both in the ribosome and the Wash 1 fraction. While the displacement indicates that CYN binding is non-covalent, the incomplete removal even in the presence of 100-fold excess of unlabelled toxin suggests that there is a strong association of CYN at the binding site, or perhaps a structural modification of the protein following interaction with the toxin that hinders re-release.

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## 5 HEALTH RISK ASSESSMENT OF CYANOBACTERIAL (BLUE-GREEN ALGAL) TOXINS IN DRINKING WATER

This Chapter is based on the following manuscript

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### 5.1 Introduction

Cyanobacterial toxins are well recognised as a cause of livestock poisoning, which has been extensively reported in the Americas, Europe, Asia and Australasia (Falconer, 2005a). Livestock are inevitably vulnerable to poisoning as they are restricted in access to water by topography and by fences, and hence may have no choice but to drink water infested by toxic cyanobacteria. Most stock deaths have resulted from the formation of cyanobacterial waterblooms in ponds and lakes on farms, but several major poisoning events were through water blooms on rivers and drinking water reservoirs (Steyn, 1945, Bowling, 1992).

Human poisoning has also occurred, but the reports are less well documented. The symptoms of poisoning by the main toxic cyanobacteria in drinking water reservoirs overlap with a range of other gastrointestinal illnesses, largely caused by infectious disease organisms. As a consequence during an outbreak of enteric disease the pathogens are investigated first, as the most probable cause, and only after exhaustive exploration are toxins of any type evaluated. Agricultural chemicals and industrial pollutants such as heavy metals are likely to be next suspected, with cyanobacterial toxins ignored until well after the event (Teixera *et al.* 1993).

Epidemiological data for human poisoning by cyanobacterial toxins only exists for a small number of events. The most well characterised case was the poisoning of renal dialysis patients in a clinic in Caruaru, Brazil, in 1996. In this instance the patients treated in a dialysis clinic during one week suffered severe illness following perfusion, with hepatic failure and, in more than 50 cases, death. Investigation of the water treatment unit at the clinic found contamination of the filters by two types of cyanobacterial toxin, microcystins and cylindrospermopsins (Jochimsen *et al.* 1998; Carmichael *et al.* 2001). Microcystins were detected in the blood and liver of poisoned individuals (Azevedo *et al.* 2002). Because of the severity of the poisoning a thorough investigation was carried out, which showed up major defects in the operation of the water treatment unit at the clinic.

Exposure to toxins through renal dialysis is a particularly potent route of poisoning, equivalent to an intravenous injection in the case of water soluble toxins. The volume of water used in perfusion is large, about 120L, so that the total amount of toxin to which a dialysis patient is exposed is much greater than possible through drinking water. Exposure to cyanobacterial toxins through consumption of contaminated drinking water has however also resulted in poisoning. The earliest demonstration of this was in 1983, when the population of a rural town in Australia was supplied with drinking water from a reservoir carrying a dense water bloom of a toxic species of cyanobacterium, *Microcystis aeruginosa*. The toxicity of this water bloom was being monitored in the reservoir. The controlling authority dosed the reservoir with copper sulphate to destroy the cyanobacteria, which caused the cells to lyse and release toxin into the water. Epidemiological data for liver injury in the affected population, a control population and comparison of the time periods before the bloom, during the bloom and lysis, and afterwards, showed clearly that liver damage had occurred only in the exposed population and only at the time of the water bloom (Falconer *et al.* 1983). In another less well characterised event, about 140 children and 10 adults were hospitalised, after the water supply authorities treated a cyanobacterial bloom in a small drinking water supply reservoir with copper sulphate, to resolve taste and odour problems. Within a week severe hepatoenteritis was apparent in the population, with about 20 cases requiring intravenous therapy. No-one died though several children were placed in intensive care (Byth, 1980). Subsequent investigation demonstrated a "new" toxic cyanobacterial species in the reservoir, with a potent general toxin (Hawkins *et al.* 1985). Later work on this strain of cyanobacterium led to the identification of an alkaloid cytotoxin, with considerable liver toxicity (Ohtani *et al.* 1992).

## 5.2 How Abundant are Cyanobacterial Toxins?

Cyanobacteria are a normal component of the worldwide biota, with a wide tolerance of climatic conditions and environment. As a very ancient life-form they occupy every conceivable ecological niche, and their abundance is limited by nutrient and light availability (Edwards *et al.*, 1992). In aquatic systems cyanobacteria are always present, though the population density varies from very small numbers to more than  $10^6$  organisms/mL. There is a strong relationship between phosphorus concentration in the water and cyanobacterial numbers and also a similar though less linked relationship between dissolved nitrate/ammonia and cyanobacteria (Mur *et al.*, 1999). Thus in general toxic cyanobacterial species will be present in all water bodies, with numbers dependent on the available nutrients and light.

As human population density rises the inflow of nutrients into water bodies increases through agricultural fertiliser use, urban run-off and sewage discharge. This increase in aquatic nutrients is termed eutrophication, and it is observed worldwide. Phytoplankton in general becomes more abundant, and among these organisms are the cyanobacteria. Cyanobacteria can utilise nutrients competitively with eukaryotic phytoplankton, and will proliferate more successfully at lower nutrient concentrations than the green algae. As a result many rivers, lakes and reservoirs worldwide develop high cyanobacterial cell concentrations, especially in the summer months, which appear as greenish suspensions in the water. Some species float to the surface under warm, still conditions, forming scums with extreme cell concentrations above  $1 \times 10^6$  cells/mL. Dried scums often appear blue-green or red through liberation of phycocyanin pigment, leading to the common name of these organisms - blue-green algae.

The scum-forming cyanobacterial species are largely toxic, and the majority of domestic animal poisonings have occurred from the animals drinking scum (Falconer *et al.* 2005). Cell populations carry over from year to year, and once a reservoir or lake has an established water bloom of cyanobacteria in summer, it is very difficult to reverse this phenomenon. Cyanobacteria proliferate in warmer weather, and often form extensive blooms in late summer. With an increase in global temperatures, cyanobacterial populations are likely to increase also. With the growth in human populations, demand for drinking water has resulted in water being drawn from water bodies carrying substantial cyanobacterial populations, thus presenting a risk to human populations.

Annual or even permanent blooms of toxic cyanobacteria are becoming increasingly common in drinking water reservoirs. To give an illustration the three main reservoirs supplying Brisbane in Australia carry substantial populations of the toxic *Cylindrospermopsis raciborskii*. This cyanobacterium forms dense layers 5-10 m below the surface, so that the first indication of the proliferation of the organism may be the blocking of filters in the drinking water treatment plant. Other examples are the main drinking water supply reservoirs for the cities of Sao Paulo in Brazil and Lodz in Poland, which contain heavy blooms of the toxic *Microcystis aeruginosa* in summer.

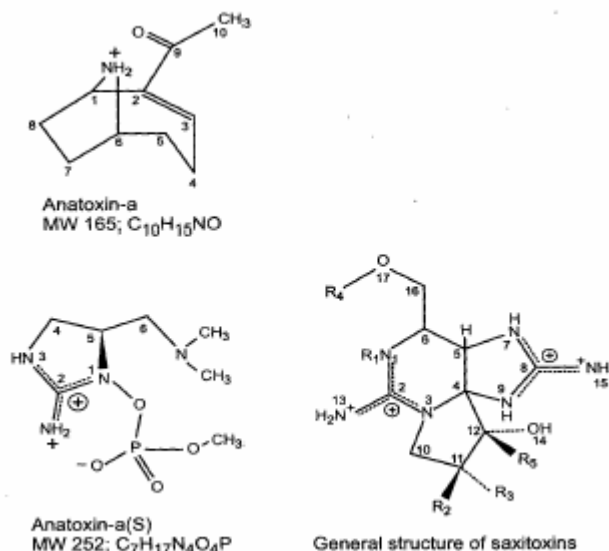
## 5.3 Cyanobacterial Toxins

*Neurotoxins* form one of the major groups of cyanobacterial toxins. They are produced by several genera of cyanobacteria growing in freshwater which have the capacity to form dense waterblooms and floating scums at the edge of lakes and rivers. The neurotoxins are alkaloid compounds, fast acting and have caused many deaths of dogs and livestock (Falconer, 2005). Three types of alkaloid have so far been described (Figure 5.1).

The first to be characterised was anatoxin-a (Figure 5.1), a neuromuscular blocking agent which causes death by respiratory paralysis (Carmichael and Mahmood, 1984). This toxin has been found in three common genera of cyanobacteria, *Anabaena*, *Aphanizomenon* and *Planktothrix*, all filamentous planktonic organisms capable of high cell concentrations and potential scum formation. There has been no clear evidence of human poisoning from these organisms, though a coroner in Wisconsin in 2003 resolved that the death of a male teenager who was diving and playing in a pond containing neurotoxic *Anabaena* had died as a consequence of ingestion of these cyanobacteria (Behm, 2003).

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To verify this cause of death, evidence of toxin in the gastrointestinal tract or tissues was required, however there has been no published report of the presence of toxin. By contrast, dogs poisoned by anatoxin-a have shown the toxin in stomach contents (Edwards *et al.*, 1992). The compound is stable in the environment, as exhibited by the dogs having died after eating decaying lumps of cyanobacteria on the lakeside. Anatoxin-a has been identified in the water of lakes in North America and in Europe, which are largely used for recreation.



**Figure 5.1** Structures of cyanobacterial neurotoxic alkaloids.

There is the possibility of consumption of moderate quantities of water during swimming and especially water skiing, and hence a risk exists for anatoxin-a poisoning of recreational water users (Chorus *et al.*, 2000). Many authorities in developed countries have warning procedures for cyanobacterial blooms at popular recreational areas, to reduce risk to water users (Fromme *et al.*, 2000). There has been little attention paid to the assessment of risk to drinking water consumers from anatoxin-a, largely because of the rapid excretion of the toxin from the body, no evidence of residual effects and low free-water concentrations in lakes.

Anatoxin-a(s) is much less common in cyanobacterial waterblooms, though it was first identified following cattle deaths in the USA (Mahmood and Carmichael, 1987). The alkaloid closely resembles an organophosphorus insecticide (Figure 5.1), and acts as an anticholinesterase. The characteristic feature of this poisoning is excessive salivation, which is the reason for the designation (s). The compound is highly unstable and unlikely to persist in water supplies, and as a result is also unlikely to present any risk.

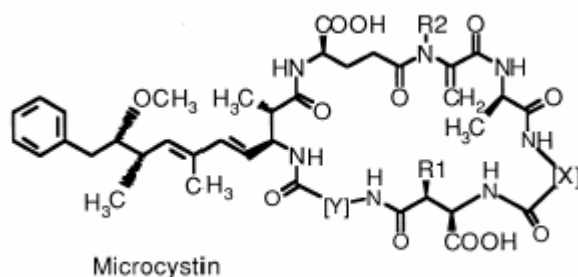
The saxitoxin-type neurotoxins are well known as the cause of paralytic shellfish poisonings, which have resulted in many hundreds of human deaths worldwide (Falconer, 1993). As a result, legislation controls the allowable concentration of saxitoxins in shellfish harvested for human consumption (80 µg/100 g fresh shellfish tissue) and there is a substantial monitoring program in many countries. Saxitoxins are however not limited to marine waters, and also occur in freshwater cyanobacteria. *Anabaena*, *Aphanizomenon* and *Lyngbya* genera of cyanobacteria have species that produce saxitoxins (Carmichael *et al.* 1997; Ferreira *et al.*, 2000; Velzeboer *et al.* 2000).

The massive waterbloom of *Anabaena circinalis* on 1,000 km of the Darling River in Australia in 1991 killed a large number of sheep and cattle, and also resulted in detectable neurotoxicity in town water supplies (Bartram *et al.*, 1999). Saxitoxins are heat-stable molecules, which are not easily removed in conventional water treatments unless pH and chlorine residuals are carefully controlled, but can be effectively removed by ozone or activated carbon (Falconer *et al.* 1989).

The toxicity of saxitoxin is considerable, as the alkaloid blocks sodium conduction in axons preventing nerve impulse transmission, leading to paralysis. The oral LD50 in mice is about 260 µg/kg bodyweight (Kuiper-Goodman *et al.* 1999). Acute poisoning in humans is unlikely to occur from contaminated water supplies, as the human body can tolerate about 100 µg of saxitoxin without ill effect (Falconer, 1993), which, translated to drinking water is 50 µg/L assuming 2L water drunk per day. No cumulative effects have been demonstrated, though there is limited evidence of resistance to toxicity in exposed human populations (Kuiper-Goodman *et al.* 1999). New Zealand is considering a Maximum Acceptable Value in drinking water of 3 µg/L of saxitoxin equivalents in their new drinking water guidelines, which should be ratified shortly (New Zealand Ministry of Health). This value has also been proposed in Australia (Fitzgerald *et al.* 1999). The data on which this value was based were the intraperitoneal toxicity of saxitoxin to mice, and incorporated a safety factor of 1,000. This issue will be discussed further in the section on hepatotoxins (microcystins). There are no data for the concentrations of saxitoxin-type neurotoxins in drinking water, and there were no reports of neurotoxic symptoms in the town population when neurotoxicity was detected in the drinking water supply (Bartram *et al.* 1999).

## 5.4 Hepatotoxins

These toxins have received the greatest attention, as they are the source of the most likely risk to consumers of drinking water. The predominant genera of cyanobacteria forming the peptide toxins called microcystins are *Microcystis*, *Planktothrix* and *Anabaena*. Species from these genera are common in Europe, the Americas, Africa and Asia, and poisoning of domestic animals has been widely reported (Falconer *et al.*, 2005). Only two epidemiological investigations have so far shown human injury from microcystin in drinking water, one in Australia (Falconer *et al.*, 1983) and one in China (unpublished). As a consequence of the frequency of cyanobacterial blooms containing hepatotoxins in drinking water reservoirs, the WHO carefully examined the need for the major toxins, the microcystins, to be included in the drinking water guidelines. An 'expert group' was established to examine the whole issue of cyanobacterial toxins in drinking water, which resulted in a comprehensive assessment of the risks involved (Chorus and Bartram, 1999). The outcome was a recommendation that the microcystins should be included among the chemicals for which Guideline Values be determined. These peptide toxins are cyclic, and contain a majority of D-amino acids (Figure 5.2). The positions shown as [X] and [Y] are L-amino acids, and are variable between species and strains of cyanobacteria. The most abundant variant has L-leucine (L) and L-arginine (R) respectively at [X] and [Y] (microcystin-LR). The amino acid at the left of the molecule is unique, is connected into the ring through an amino group at the β-carbon atom, and has the trivial name of ADDA.



**Figure 5.2** Structure of the peptide hepatotoxin microcystin, first isolated from the cyanobacterium *Microcystis aeruginosa*.

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Microcystins are resistant to digestion in the gastrointestinal tract of eukaryotes, as peptide bonds linking to the D-amino acids are not susceptible to normal hydrolytic enzymes. The toxins are concentrated into the liver by an active transport system, similar to the bile acid transporter (Runnegar *et al.*, 1995). Microcystins specifically inhibit protein phosphatases 1 and 2A, which have a vital role in cell control and in intracellular structure (Falconer *et al.*, 2005). Acute poisoning is through destruction of the liver architecture, leading to blood loss into the liver and hemorrhagic shock (Falconer *et al.*, 1981). Later death is through liver failure with massive destruction of hepatocytes, seen in large animal deaths and human fatalities (Jochimsen *et al.*, 1998, Jackson *et al.*, 1984). Chronic exposure to these toxins in drinking water led to ongoing active liver injury in mice (Falconer *et al.*, 1988).

There is experimental evidence for tumour promotion by microcystins, and limited data for carcinogenesis in rodents (Ito *et al.*, 1997). In rural areas in Southern China some villages showed hyper-endemic rates of hepatocellular carcinoma, which have been shown to be linked to hepatitis, aflatoxin in the food, and drinking surface water. Microcystins in the ponds and ditches used as water sources were suspected of contributing to the cancer rates (Ueno *et al.*, 1996; Yu, 1995).

These toxins are highly stable in water and are resistant to boiling. Hence they present a risk to consumers in less developed regions and countries who are collecting water from surface sources to drink. Many lakes, ponds, ditches and streams in rural and outer urban areas suffer from eutrophication through excessive nutrient leaching from housing, sewage, and intensive agricultural use, leading to cyanobacterial proliferation. In tropical and temperate regions of the world the genus *Microcystis* is the most abundant cyanobacterium forming toxic blooms, with toxin concentrations sufficient to poison domestic animals. If these contaminated water sources are used for human consumption, there is a risk of human poisoning. Conventional Western drinking water treatment may not be effective under bloom conditions in removing microcystins from drinking water and hence there is a risk to consumers. Advanced water treatment using ozone and activated carbon will reliably remove microcystins (Falconer *et al.*, 2005).

WHO have carried out an assessment of the safe level of microcystins in drinking water, based on data from a subchronic toxicity trial in mice, with supporting data from growing pigs (Falconer *et al.*, 1999). The calculation used the No Observed Adverse Effect Level for male mice during a 13 week oral toxicity trial, of 40µg of microcystin-LR/kg/day (Fawell *et al.*, 1994). This was used to calculate a Tolerable Daily Intake (TDI) for safe human consumption, by the incorporation of uncertainty or safety factors. While these are subjective, a factor of 10 for interspecies uncertainty between rodents and humans, a further 10 for variability in sensitivity between people, and an uncertainty of 10 for inadequate data, possible tumour promotion and lack of lifetime exposure are generally accepted.

Thus the:

$$\text{TDI} = \frac{40}{10 \times 10 \times 10} = 0.04\mu\text{g/Kg/day}$$

From this value the Guideline Value (also called the reference dose and the maximum acceptable concentration) was calculated from the standard bodyweight of 60kg, an assumption of the proportion of the dose from drinking water of 0.8 (some may come from food and particularly blue-green algal diet supplements) and a standard water consumption of 2L/day.

$$\text{GV} = \frac{0.04 \times 60 \times 0.8}{2} = 0.96$$

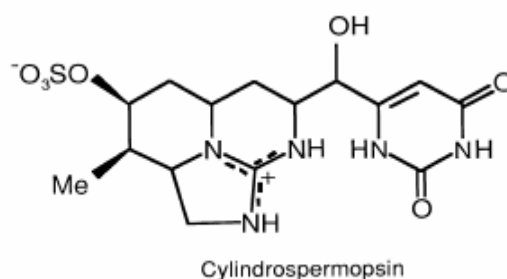
= 1µg/L of microcystin-LR in drinking water.

This value was determined from the toxicity of microcystin-LR, so the WHO Chemical Safety Committee set the Guideline Value for microcystin-LR. Since there are some 60 variants of the molecule, and some highly toxic blooms do not contain any of the -LR variant, it is necessary to

interpret this as toxicity equivalent to microcystin-LR. Where this Guideline Value has been adopted as the basis for national legislation, the need for monitoring of all the toxin variants has been recognised and the equivalent total toxicity calculated (Australian National Health and Medical Research Council, 1994). Individual countries have also adopted a higher standard bodyweight, and a different proportion of the consumption from drinking water. All of the Guideline Values adopted so far lie between 1 and 2 µg/L of microcystin equivalents, which for practical purposes are the same. A larger potential adjustment to this value may result from re-classification of microcystin as a carcinogen, rather than a non-carcinogenic poison. While there is experimental evidence for tumour promotion by microcystin in liver, skin and colon, the only data indicating carcinogenesis have been obtained by continued very high intraperitoneal doses of toxin in mice which cause extensive liver damage (Falconer, 2005). In China there is ongoing investigation into the relationship between surface water consumption and cancer of liver and colon (Zhou *et al.*, 2002). This issue is discussed in detail elsewhere (Falconer *et al.*, 2005), concluding that there is insufficient evidence at present to determine that microcystin is a probable carcinogen but the possibility requires continual evaluation.

## 5.5 Cylindrospermopsins

These alkaloid cytotoxins were relatively recently discovered, following the widespread human poisoning at Palm Island, Australia due to contamination of the water supply (Byth, 1980). Cyanobacteria from the supply reservoir were collected, cultured and evaluated for toxicity, showing potent toxicity to liver, kidney, adrenals, lymphoid cells and other tissues in mice (Hawkins *et al.*, 1985). Subsequent investigation of the oral toxicity of the cyanobacterium responsible, *Cylindrospermopsis raciborskii*, further demonstrated the tissue damage caused by the toxin (Falconer *et al.*, 1999, Seawright *et al.*, 1999). The toxic alkaloid was isolated and identified as a potent inhibitor of protein synthesis (Ohtani *et al.*, 1992; Froscio, 2002). There are on-going investigations into the mechanism of cylindrospermopsin toxicity, which may involve activated metabolites of the alkaloid (Runnegar *et al.*, 1995; Froscio, 2002.). The alkaloid has several reactive groups, including a hydroxymethyl uracil, which may be vulnerable to biological oxidation (Figure 5.3).



**Figure 5.3** Molecular structure of the cyanobacterial alkaloid toxin cylindrospermopsin. The bridging hydroxyl group may be in either stereochemical position, and may also be replaced by hydrogen.

Cylindrospermopsin has been found in water bodies that have blooms of the cyanobacterial species *Aphanizomenon ovalisporum* (Banker *et al.*, 1997) and *Umezakia natans* (Harada *et al.*, 1994) as well as those with *Cylindrospermopsis* (Li *et al.*, 2001, Stirling and Quilliam, 2001), and recently in Germany, in the absence of any of those species (Fastner *et al.*, 2003). It is apparent from this data that cylindrospermopsin is likely to occur widely in freshwater sources, and that only a beginning has been made in identifying species producing this toxin. Monitoring of water supplies for cylindrospermopsin has found concentrations in natural water bodies, reservoirs and in drinking water that are above 10 µg/L, which is a cause for concern (Griffiths and Saker, 2003, Flewelling *et al.*, 2001).

On the basis of the experimental toxicity of cylindrospermopsin, the reported human poisoning associated with *Cylindrospermopsis* and the toxin concentrations measured in water bodies, it was apparent that risk assessment for this toxin in drinking water was required. A subchronic oral exposure trial of cylindrospermopsin in male mice provided a No Observed Adverse Effect Level of 30 µg of cylindrospermopsin/kg/Day. On the assumption of standard uncertainty factors and the total intake arising from drinking water, a Guideline Value of 1 µg/L resulted (Humpage and Falconer, 2003).

Examination of the molecular structure of cylindrospermopsin indicated that it may be able to interact with DNA or RNA in cells, through the uracil group, assisted by the planar shape of the molecule. If this proves to be the case, then evaluation of the possible carcinogenicity of the molecule is required. Preliminary data indicated that cylindrospermopsin may form DNA adducts (Shaw *et al.*, 2000), and there is evidence for clastogenicity and micronucleus formation in a cultured human white cell line incubated with cylindrospermopsin (Humpage *et al.*, 2000). A preliminary trial of carcinogenicity in mice indicated the presence of excess tumors, providing support for a more definitive carcinogenicity trial (Falconer and Humpage, 2001). It is premature at present to attempt to classify cylindrospermopsin as a possible human carcinogen, because of the very limited current data. Further experimental and epidemiological research on this toxin is required to clarify these issues, and cylindrospermopsin is now on the 'Candidate Contaminant List' of the US Environmental Protection Agency.

### 5.6 Evaluation of the Risks to the Population from Cyanobacterial Toxins.

There are two areas in which more data is necessary to make a clear case for national action on minimising health risks from cyanobacterial toxins. The first is the need for widespread monitoring for the presence of toxic cyanobacterial species and toxins in drinking water sources, to identify the abundance of locations of potential risk. This is in progress in Europe, and an initial survey has been carried out through the American Water Works Association in the USA. From data arising from these surveys, the extent of the problem has become apparent, and the location of a proportion of the populations at most risk.

The second and more difficult aspect is the need for epidemiological studies on at-risk populations to quantify the adverse health effects. Exposure biomarkers will have to be developed, in addition to quantitating the toxin concentrations in tap water. The commonly used clinical measures of liver and kidney function, and clinical records for hepatoenteritis, provide relevant health information. The earlier data on population injury from microcystins indicates the clinical parameters of particular interest (Falconer *et al.*, 1983).

### 5.7 Conclusion

The cyanobacterial toxins provide a risk to human health when the population of toxic cyanobacteria in drinking water sources rises to bloom proportions. The present assessments of Guideline Values for these toxins as chemical, non-carcinogenic contaminants indicate that a safe concentration in drinking water is in the region of 1 µg/L, a concentration that has been exceeded in numerous water storages. Carcinogenicity of these toxins is not yet established, though both microcystins and cylindrospermopsin have caused excess tumors in rodent experiments. With the increased eutrophication of water supplies and global warming, cyanobacterial populations and hence toxic risks are likely to rise in the immediate future. The extent and potential severity of the risks need further evaluation.

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## CYLINDROSPERMOPSIN MECHANISMS OF TOXICITY AND GENOTOXICITY

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## 6 CYANOBACTERIAL (BLUE-GREEN ALGAL) TOXINS IN WATER SUPPLIES: CYLINDROSPERMOPSINS

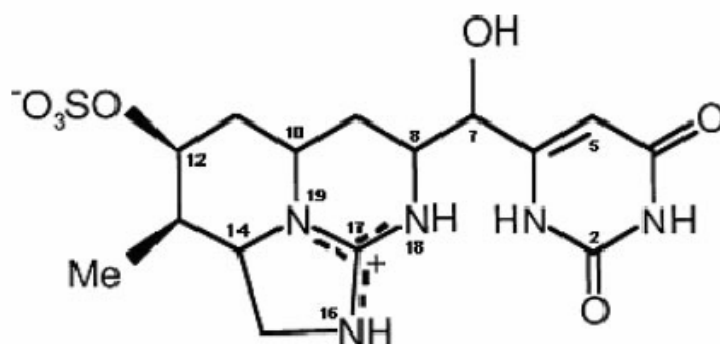
This Chapter is based on the following manuscript

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Cyanobacterial toxins have become recognised as a potential hazard in drinking water throughout the world. This has arisen partly through a long history of livestock poisoning due to drinking of water containing high concentrations of toxic cyanobacteria. More recently, human injury and death have been attributed to cyanobacterial toxins (Falconer, 2005). The two toxin groups from which human injury has been clearly identified are the microcystins and cylindrospermopsins. Several genera of cyanobacteria have species that can produce these toxins, and there are a wide range of ecological habitats in which these organisms can flourish. For example one toxic genus, *Planktothrix*, can grow under ice in frozen water storages and lakes, whereas others such as *Microcystis* and *Cylindrospermopsis* grow well in tropical reservoirs (Chorus and Bartram, 1999). The genetic basis which underlies toxin production by cyanobacteria is increasingly understood. Microcystins are coded by a very large gene sequence for nonribosomal peptide synthesis. These genes can be identified and used as a method of assessing potential toxicity in a natural cyanobacterial population (Tillett *et al.*, 2001; Christiansen *et al.*, 2004; Rouhiainen *et al.*, 2004). Similarly the polyketide synthetase genes coding for the alkaloid toxin cylindrospermopsin have been identified in several genera of cyanobacteria (Schembri *et al.*, 2001; Shalev-Alon *et al.*, 2002). Use of these genetic techniques will complement toxin analysis as the methodology for identifying actual or potentially toxic species. Both approaches need to be applied when toxins are suspected to be present in water, but the source organisms have not been identified. For microcystins, there is a considerable body of information on sources of the toxin and nature of the toxic effects, but this is not the case for cylindrospermopsins (Falconer, 2005). The focus of this review is therefore on the more recently identified drinking water toxin cylindrospermopsin, discussing the present knowledge of this toxin and the areas in which further research is needed to fully establish the nature and extent of the risk to human health.

### 6.1 Cylindrospermopsin Structure

This cyanobacterial toxin was first isolated from a culture of *Cylindrospermopsis raciborskii* grown from trichomes (filaments) obtained from the drinking water supply reservoir on Palm Island, Queensland, Australia (Ohtani *et al.*, 1992). Earlier an extensive human poisoning had occurred among consumers of this water supply, leading to investigation of the cause of the injury (discussed later). The toxin is a stable tricyclic alkaloid containing a guanido group linked at C7 to hydroxymethyl uracil (Fig. 6.1). At the hydroxyl bridge, there are two possible epimers, cylindrospermopsin and 7-epicylindrospermopsin. Both occur naturally and are equally toxic (Banker *et al.*, 2001). Because of the negatively charged sulfate group and the positively charged guanido group, the molecule is a zwitterion and very water soluble. The structural formula has been verified by total synthesis (White and Hansen, 2005). A further naturally occurring variant is 7-deoxycylindrospermopsin, which has been found together with cylindrospermopsin in drinking water reservoirs. The toxicity of this molecule is presently under investigation, and may be similar to that of the hydroxy form (Looper *et al.*, 2005; Humpage *et al.*, unpublished data; Shaw, personal communication). Complete loss of the uracil group from the cylindrospermopsin molecule effectively removes toxicity (Banker *et al.*, 2001).



**Figure 6.1** Chemical structure of cylindrospermopsin.

## 6.2 Cylindrospermopsin Sources Species Distribution

Cylindrospermopsin is biosynthesised by species from at least five genera of freshwater cyanobacteria. The species so far identified are *Cylindrospermopsis raciborskii*, *Anabaena bergii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae*, *Umezakia natans*, and *Raphidiopsis curvata* (for review, see Falconer, 2005). *Aphanizomenon flos-aquae* has recently been identified as the source of cylindrospermopsin in German lakes (Preussel *et al.*, 2006). The original isolations of these toxic organisms were from Australia, Israel, Germany, Japan, and China, respectively. These species are all filamentous organisms, and may be closely related genetically (Gugger *et al.*, 2005). Cylindrospermopsin identified in water in Brazilian drinking water reservoirs has not yet been attributed to any particular species of cyanobacterium.

The organism which has been most studied to the present time is *Cylindrospermopsis raciborskii*, which is abundant in lakes and rivers in many tropical and subtropical areas, and is increasingly recorded in temperate locations. The species has been found in reservoirs, rivers and lakes in Australasia, Europe, Japan, East Asia, South-east Asia, South America, and the USA (Falconer, 2005). In some locations, the organism does not appear to be appreciably toxic, and in other places neurotoxins are present, including saxitoxins (Lagos *et al.*, 1999). There is evidence that *C. raciborskii* is spreading northward in the Northern Hemisphere, into cooler regions where it had not previously been reported (Padisak, 1997; Griffiths and Saker, 2003). Whether this is an effect of global warming or the distribution of more cold-tolerant strains is not yet clarified. The extending distribution does emphasise that the potential for human health risk from this toxic organism is not limited to the tropics, but relevant also to cooler temperate climates.

*Aphanizomenon ovalisporum* was first identified as a cylindrospermopsin-producer in Lake Kinneret (Sea of Galilee), when a water bloom of this organism developed abundantly in the lake, which is Israel's main water supply (Banker *et al.*, 1997). The toxin was purified and found to be of the same composition as the toxin isolated by Ohtani *et al.* (1992), but with the C7 hydroxyl being in the opposite orientation (Ohtani *et al.*, 1992). This epimer was also present in *Umezakia natans* from Japan, showing that the Japanese and Israeli samples of cylindrospermopsin have the same epimeric form, which is of opposite orientation to the toxin form *C. raciborskii* in Australia (Harada *et al.*, 1994). Large toxic blooms of this species have also been reported in shallow artificial lakes in Australia (Shaw *et al.*, 1999). The epimeric form of the toxin was not reported. *Raphidiopsis* is a widely distributed genus, for example in China and Brazil, so that water contaminated with cylindrospermopsin from this origin can be expected to be identified from many locations (Li *et al.*, 2001; Gugger *et al.*, 2005).

### 6.3 *Cylindrospermopsin* in Water Bodies

Monitoring of the actual toxin content of water in drinking water reservoirs with blooms of *C. raciborskii* has been done extensively in Queensland, Australia (McGregor and Fabbro, 2000; Shaw *et al.*, 2000), and in Florida, USA (Burns *et al.*, 2000). The highest concentrations of cylindrospermopsin in both locations were in the region of 100 µg/L of bulk water. More commonly, concentrations of 1–10 µg/L occur. Cylindrospermopsin was recently detected in lakes in Germany (Fastner *et al.*, 2003) and from reservoir water in Brazil (Carmichael *et al.*, 2001). As *C. raciborskii* does not form surface scums, and in clear stratified lakes in summer forms dense bands well below the lake surface (3–10 m down), it is not immediately visible to reservoir managers (Fabbro and Andersen, 2003). In shallow mixed lakes, the filaments distribute throughout the water column, giving a general discoloration. The toxin content of the free water solution is considerable, as cylindrospermopsin readily leaks from cells under normal growth conditions. This leakage was observed in waterblooms of both *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum*. In two instances of *A. ovalisporum* blooms around 80% of the total toxin content of the water was in free solution (Shaw *et al.*, 1999). Hence simple water treatment by flocculation, sedimentation, and filtration, which removes the filaments of the organism, does not satisfactorily remove the toxin.

### 6.4 *Cylindrospermopsin* Toxicity

The toxicity of cylindrospermopsin to the human population was brought to attention by the “Palm Island Mystery Disease” in 1979, during which 148 cases of severe hepatoenteritis with renal tubular damage were recorded on an island off the Queensland, Australia, coast (Byth, 1980; Hawkins *et al.*, 1985). Investigation of the cause eliminated pathogens and was attributed to a water bloom of *C. raciborskii* on the drinking water supply reservoir. Immediately prior to the outbreak, there were complaints of taste and odour in the drinking water, which resulted in the managing authority treating the reservoir with copper sulfate to kill the algae. Within a week, large numbers of children became sick, many of whom were transferred to intensive care for intravenous therapy due to electrolyte loss. Fortunately none died. The Palm Island reservoir was the source of the culture material from which the toxin was later purified and characterised (Ohtani *et al.*, 1992). The Brazilian dialysis clinic tragedy, in which more than 50 patients died from cyanobacterial toxicity in the dialysis water supply, was identified to be due to a combination of microcystins and cylindrospermopsin passing through the unit filters (Carmichael *et al.*, 2001).

Experimental studies in rodents of the toxicity of culture material and of the toxin have provided a picture of general organ damage from intraperitoneal or oral administration of extracts or purified toxin (Hawkins *et al.*, 1985; Ohtani *et al.*, 1992; Falconer *et al.*, 1999; Seawright *et al.*, 1999; Humpage and Falconer, 2003). The toxin causes damage to liver, kidneys, lungs, heart, stomach, adrenal glands, the vascular system, and the lymphatic system (Hawkins *et al.*, 1985). Liver damage is likely to be severe and dose-dependant. Centrilobular necrosis appears within the first 12 h of intraperitoneal dosage and progresses with lipid accumulation in surviving hepatocytes, with animals dying up to 6 days after a single dose. The kidneys exhibit changes in glomerular blood supply and proximal tubular epithelial necrosis. After oral dosing of a culture of *C. raciborskii* to mice, the stomach became ulcerated and the stomach and small intestine contained fresh blood (Seawright *et al.*, 1999). A characteristic feature of damage seen in the thymus and spleen is the selective necrosis of lymphocytes, with atrophy of the thymus and shrinkage of the spleen. Vascular damage has been shown in the heart muscle, eye orbit, and tail. It has been shown that the LD<sub>50</sub> for cylindrospermopsin decreases greatly between 24 h and 5 days, with the 5–6 day intraperitoneal LD<sub>50</sub> of 200 µg/kg bodyweight being one tenth of the 24 h LD<sub>50</sub> (Ohtani *et al.*, 1992). This demonstrates a slow and progressive poisoning, in keeping with the mechanism of toxicity discussed below.

Subchronic oral toxicity studies demonstrated the most sensitive responses to be in increased liver, kidney, and testis weights, together with a decrease in urine protein content. These studies were carried out in order to find the maximum no observed adverse effect level (NOAEL) for oral cell extracts of *C. raciborskii* or purified cylindrospermopsin. Extensive chemico- and histopathological investigation was carried out on male mice dosed by gavage with purified cylindrospermopsin. The NOAEL was concluded to be 30 µg/kg/day, on the basis of urine analysis. The authors proposed a “Guideline Value” for drinking water of 1 µg/L, based on standard safety or uncertainty factors of 10 x 10 x 10 for conversion of rodent toxicity data to human safe intake.

The final 10-fold factor was based on the lack of data for a second species, lack of data for teratogenicity and reproductive effects and the possibility of carcinogenesis (Humpage and Falconer, 2003).

## 6.5 Cylindrospermopsin Carcinogenicity

Cylindrospermopsin has been found to be genotoxic in a number of *in vitro* assay systems. Using the micronucleus assay, cylindrospermopsin caused DNA fragmentation and loss of whole chromosomes in a human white blood cell-line (Humpage *et al.*, 2000). These findings could not be confirmed in CHO K1 cells, suggesting that metabolism of the toxin to an active product was required for genotoxic action (Fessard and Bernard, 2003). This hypothesis was confirmed in primary mouse hepatocytes where cylindrospermopsin-induced DNA fragmentation was detected by the COMET assay, an effect that could be eliminated by application of CYP450 inhibitors (Humpage *et al.*, 2005). DNA fragmentation has since been confirmed as the major genotoxic mechanism using a range of standard *in vitro* assays (Humpage *et al.*, unpublished data).

DNA fragmentation has also been described in the livers of cylindrospermopsin-treated mice (Shen *et al.*, 2002), which provides corroboration of evidence that cylindrospermopsin treatment can induce the formation of overt cancers in mice (Falconer and Humpage, 2001). This report presents the clearest evidence yet that cylindrospermopsin may be a carcinogen, a finding obviously needing urgent attention, as detailed below.

It is of particular importance to ascertain whether cylindrospermopsin is a human carcinogen. As poisoning of children by this toxin occurred in 1979, cancer incidence has been investigated through Cancer Registry data based on residential postcode. The numbers of individuals in the exposed population are insufficient for statistical comparison; however, an indication of an increased rate of gastrointestinal cancer between 1982 and 1999 was seen when cancer rate in the exposed population was compared with a similar but unexposed population (Falconer, unpublished data).

## 6.6 Mechanism of Toxicity

The mechanisms of cylindrospermopsin toxicity are under investigation. Early reports of *in vivo* acute toxicity indicated that the toxic potency was considerably higher if measured 5 days after exposure than if it was measured after 24 h, indicating a relatively slow mechanism of action. Recent data from primary hepatocytes show two routes of toxic action, a rapid route probably through toxicity of a CYP450 oxidation product of the toxin (Runnegar *et al.*, 1995; Froscio *et al.*, 2001) and a slower mechanism through the well-documented inhibition of protein synthesis, which does not require toxin metabolism (Froscio *et al.*, 2001). Uptake of the toxin is relatively rapid since complete and irreversible block of protein synthesis occurs after a 1 h exposure *in vitro*. Inhibition of protein synthesis occurs at the ribosome during the peptide chain elongation step (Froscio *et al.*, 2003). Glutathione synthesis is also reduced via a CYP450-dependant mechanism (Runnegar *et al.*, 1995), but this does not lead to an increase in oxidative stress in the cell (Humpage *et al.*, 2005), suggesting that this is not a primary mechanism of cylindrospermopsin toxicity.

The genotoxicity of cylindrospermopsin may be through an active oxidation product, since CYP450 inhibitors appear to prevent DNA damage by cylindrospermopsin (Humpage *et al.*, 2005). The main genotoxic outcome appears to be DNA fragmentation (Humpage *et al.*, 2000, 2005; Humpage and Falconer, unpublished data), although loss of whole chromosomes has also been shown to occur (Humpage *et al.*, 2000). Determining whether these effects translate into carcinogenicity *in vivo* should be a major focus of future research.

## 6.7 Monitoring and Water Treatment

The basis of reservoir monitoring for cyanobacterial toxins remains species identification and cell counting, supported by chemical analysis or microbiotests for toxins when a risk is perceived (Chorus and Bartram, 1999). Cylindrospermopsin may be measured in water by several techniques, the most definitive being liquid chromatography followed by tandem mass spectrometry (Eaglesham *et al.*, 1999). Other methods include HPLC, which has recently been validated by an interlaboratory

comparison trial (Torokne *et al.*, 2004), and is a practical chemical methodology since most analytical laboratories have access to the equipment.

Microbiotests have a place in toxicity screening for cyanobacterial toxins in reservoirs, and the brine shrimp *Artemia salina* and the anostracan crustacean *Thamnocephalus platyurus* have been shown to be sensitive to cylindrospermopsin at toxicologically relevant concentrations (Torokne, 2000; Metcalf *et al.*, 2002).

## 6.8 Research Needs

A number of areas of cylindrospermopsin toxicology require investigation before the human health risk can be fully assessed.

At the level of mechanisms of action, we do not yet know by which route toxic metabolites are formed to produce rapid toxicity and, more importantly, genotoxicity. There is considerable evidence for involvement of CYP450s but the specific isoforms have yet to be identified. Without this information, it is difficult to make accurate predictions about the applicability of animal data to human risk assessment, because it cannot show whether these mechanisms are likely to occur in humans.

Detailed toxicokinetics are still lacking. Some preliminary work has been done in this regard (Norris *et al.*, 2001), but more work needs to be done to determine rates of uptake, tissue distribution and excretion from an oral dose. Effects of cylindrospermopsin on nonhepatic tissues have not been well investigated. Renal effects seemed to be important after chronic low dose exposure (Humpage and Falconer, 2003), and thrombotic effects have been described by a number of workers. Long-term oral toxicity studies are needed in both sexes of animal, and so far only male mice have been investigated in detail. Reproductive and teratogenic toxicity assessments are also required.

Finally, and most importantly, *in vivo* carcinogenicity trials need to be undertaken to assess the potential carcinogenicity of cylindrospermopsin. Cylindrospermopsin has been placed on the “candidate list” for full toxicological studies by the USEPA and NIEHS. However, a lack of adequate quantities of purified toxin has delayed that process and chemical synthesis of the toxin is very complex. It may be necessary to employ a characterised toxic extract from cultured *Cylindrospermopsis* or *Aphanizomenon* to obtain sufficient toxin for long-term studies. Smaller-scale studies therefore remain important in filling in the research gaps identified earlier.



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