Genotoxicity Investigation of Chlorinated Degradation Products of a Cyanobacterial Toxin, Cylindrospermopsin

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Abstract

Cylindrospermopsin (CYN), a potent cyanobacterial hepatotoxin produced by *Cylindrospermopsis raciborskii* and other cyanobacteria, is regularly found in water supplies in many parts of the world and has been associated with the intoxication of humans and livestock. Water treatment via chlorination can degrade the toxin effectively but result in the production of several byproducts. In this study, male and female Balb/c mice were injected via the intraperitoneal (IP) route with a single dose of 10 mg/kg 5-chlorouracil and 10 mg/kg 5-chloro-6-hydroxymethyluracil; these two compounds are the predicted chlorinated degradation products of CYN. DNA was isolated from the mouse livers and examined for strand breakage by alkaline gel electrophoresis (pH 12). The median molecular length (MML) of the DNA distributed in the gel was determined by estimating the midpoint of the DNA size distribution by densitometry. The toxicity of 5-chlorouracil (as measured by DNA strand breakage) was significantly influenced by time from dosing. There was no significant difference in MML between mice dosed with 5-chloro-6-hydroxymethyluracil and the controls. In another experiment, mice were dosed with 0, 0.1, 1, 10 and 100 mg/kg body weight 5-chlorouracil and 0, 0.1, 1, 10 and 20 mg/kg 5-chloro-6-hydroxymethyluracil via IP injection. The heart, liver, kidney, lung and spleen were removed, fixed and examined under electron microscopy. Liver was the main target organ. The EM results revealed marked distortion on the nuclear membrane of liver cells in mice dosed with 1.0 mg/kg 5-chlorouracil or 10 mg/kg 5-chloro-6-hydroxymethyluracil, or higher.

Introduction

Cyanobacteria may produce toxins that may present a hazard for drinking water safety. Cylindrospermopsin (CYN) is a potent cyanobacterial hepatotoxin produced by *Cylindrospermopsis raciborskii* and other cyanobacteria such as *Umezakia natans* and *Aphanizomenon ovalisporum* (Duy *et al.,* 2000; Shaw *et al.,* 2000). CYN is a tetracyclic alkaloid, possessing "a tricyclic guanidine moiety combined with hydroxymethyluracil" (Ohtani *et al.,* 1992). It has a molecular weight of 415 Daltons and is zwitterionic. It is therefore highly water-soluble, indicating that difficulties exist for removal of this toxin using conventional treatment techniques such as flocculation and filtration. Of the water treatment procedures, chlorination, possibly micro/ultrafiltration, and especially ozonation are the most effective in breaking down this cyanobacterial toxin (Hitzfeld *et al.,* 2000). Under experimental conditions using samples with a solution pH of 6–9, a residual chlorine concentration of 0.56 mg/L was sufficient to degrade >99% of CYN (Senogles *et al.,* 2000). This type of water treatment, however, may result in the formation of a number of chlorinated byproducts: one is 5-chlorocylindrospermopsin; the other predicted byproducts are 5-chlorouracil (5ClUra) and 5-chloro-6-hydroxymethyluracil (5Cl6HMUra) (Wickramasinghe *et al.,* 2001). In this study, the genotoxic potential of two predicted degradation byproducts of CYN, namely 5ClUra and 5Cl6HMUra, were examined.

Materials and Methods

Balb/c mice (2 months old) of 25–30g body weight were used for the dosing experiments. All mice were acclimatized for at least one week before experimentation. 5ClUra was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5Cl6HMUra was synthesized by Dr. W Wickramasinghe of NRCET. 5ClUra was dissolved in corn oil to produce 6 mg/mL, 600 µg/mL, 60 µg/mL and 6 µg/mL solutions. Via IP injection, mice were administered the above solutions to achieve doses of 0.1 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg, respectively. Mice dosed with pure corn oil were treated as the vehicle control for 5ClUra. 5Cl6HMUra was dissolved in saline and administered to mice via IP injection to achieve doses of 0 mg/kg, 0.1 mg/kg, 1 mg/kg, 10 mg/kg and 20 mg/kg. Mice dosed with saline were treated as the solvent control for 5Cl6HMUra. Mice were autopsied after 96 hr exposure and the liver, heart, spleen, kidneys and lungs of each mouse were put into 10 mL fixative solution (2.5% glutaradehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate trihydate, pH 7.8) and processed for electron microscopy examination. Mortality of the experimental animals was also recorded over the 96-hour period.

For the genotoxicity assays, male and female Balb/c mice were injected via the IP route with a single dose of 10 mg/kg 5ClUra or 10 mg/kg 5Cl6HMUra. These doses were chosen based on data from acute toxicity studies (Banker *et al.,* 2001) Animals were selected randomly and sacrificed at 6 hr, 12 hr, 24 hr, 48 hr and 72 hr. Livers were removed from the abdominal cavities of individual animals, frozen in liquid nitrogen and stored at –80°C until further analysis. DNA was extracted and purified with Proteinase K, phenol and chloroform treatment. Each DNA sample was subjected to electrophoresis under alkaline conditions (pH 12) following the procedures described in Shen *et al.* (2002). The median molecular length (MML) of the DNA distributed in the gel was determined by estimating the

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midpoint of the DNA size distribution by densitometry (Black *et al.,* 1996).

Eight DNA samples were examined for each treatment. A two-way analysis of variance (ANOVA) was used to test the null hypothesis that time from dosing and treatment of the degradation products does not cause significant change in DNA integrity (as measured by MML). In the event that ANOVA indicated a significant effect, the dataset was further analyzed using a pair-wise Tukey test. Statistical significance was accepted at $P < 0.05$.

Results and Discussion

No death occurred within the 96-hr exposure period with respect to either chlorinated degradation product. No apparent change in the appearance and behavior was observed in the exposed as compared to the control animals, with the exception that one mouse dosed with 20 mg/kg 5ClUra trembled and moved slowly inside the cage. On this basis, the second lowest dose (*i.e.,* 10 mg/kg) was used in the subsequent genotoxicity test for both degradation products.

Mouse bioassay indicates that the acute IP LD_{50} is 2 mg/kg after 24 hrs (Ohtani *et al.,* 1992) and 0.2 mg/kg after 5 days for CYN (Banker *et al.,* 2001; Ohtani *et al.,* 1992). In this study, the LD_{50} of 5ClUra and 5Cl6HMUra were at least 500 and 100 times higher than the LD_{50} of CYN, respectively. Based on these results, it is postulated that the intact pyrimidine ring is an essential molecular component for the toxicity of CYN and its degradation products. The hydroxyl group at position 7 in cylindrospermopsin is also essential for its acute toxicity as it has been shown that deoxycylindospermopsin (Norris *et al.,* 1999) is much less toxic than cylindrospermopsin. We therefore concluded that after water treatment with chlorine under appropriate condition, the acute toxicity of the water contaminated with CYN was reduced considerately to levels that should not produce unacceptable risks to humans in most situations.

No toxicological effects on heart, lung, kidney and spleen were observable based on electron microscopy. Histological alterations were, however, observed in the liver. Therefore, similar to CYN, the liver appeared to be the target organ for this compound. The most marked alteration was in the cell nuclei. Based on this observation, liver tissues from animals exposed to 0.1, 1 and 10 mg/kg 5ClUra were processed for further examination. In the control mice, the hepatocyte contained a round or oval nucleus with variable amounts of dispersed and peripheral heterochromatin and a single prominent nucleolus. The nuclear membranes were smooth and had numerous nuclear pores. There were no abnormal morphological changes in hepatocytes of the control mice. When mice were administered 0.1 mg/kg of the compound, appearance of all the nuclei still remained normal. When the mice were administered 1 mg/kg of the 5ClUra, fewer normal nuclei could be found. Nucleolus and condensed chromatins were attached to the nuclear membrane. Almost all nucleus membrane changed into irregular shapes. Based on the above morphological standpoint, it could be concluded that the threshold dose was 1 mg/kg. When the mice were administered 10 and 100 mg/kg, all the nuclei were damaged.

There were no abnormal changes in the group treated with 0.1 mg/kg 5Cl6HMUra compared with the control. In the 1 mg/kg group, polynuclei and dispersed heterochromatins could be observed though the shape of the nucleus remained normal. In the 10 mg/kg group, almost all the nuclei showed an irregular shape. In the groups dosed with 10 and 20 mg/kg 5Cl6HMUra, accumulation or aggregation of cell organelles could be found. From the standpoint of histological alterations, it could be concluded that the threshold dose was 1 mg/kg.

CYN toxicity in mice is often characterized by an appearance of foamy lipid vacuolation in the liver, and periacinar coagulative necrosis was also consistently observed. CYN also causes extrahepatic lesions involving the kidney, heart and thymus (Falconer *et al.,* 1999; Terao *et al.,* 1994). The main target of CYN was the liver, while thymus, kidneys and heart were also affected (Terao *et al.,* 1994). It has been suggested that the toxicity of CYN is associated with four consecutive phases of pathological changes in the liver. The initial phase was that of inhibition of protein synthesis; the second phase of membrane proliferation was followed by the third phase of fat droplet accumulation and finally, phase of cell death. In addition, ribosomes on the membrane of the rough surfaced endoplasmic reticulum in the hepatocytes were detached from the membrane and accumulated in the cytoplasm. Nucleoli in the nuclei of the hepatocytes became dense, rounded and reduced in size. The symptoms in the hepatocytes of mice dosed with 5ClUra and 5Cl6HMUra appeared to be different from those of CYN-intoxicated animals, suggesting that different toxicity mechanisms are involved for the degradation byproducts as compared to their parent toxin molecule (CYN). One of the established mechanisms of toxicity of CYN is inhibition of protein synthesis. No evidence has been produced that this mechanism is operating with the chlorouracils.

There was no consistent change in MML value over time for mice exposed to 5ClUra. A significant decrease in MML value was observed 6 hours post dosing, suggesting an impact on DNA integrity due to exposure to 5ClUra (Fig. 1). There is, therefore, some evidence that 5ClUra may be genotoxic, as would be expected due to incorporation of this uracil into DNA (Pal *et al.,* 1981). Furthermore, Pal *et al.* (1981) reported that mice exposed to 5ClUra through drinking water showed heavy incorporation of the base in the liver and testes DNA (1 in 250 nucleotides), although no obvious adverse effects were observed. Notwithstanding, possible long-term effects associated with the incorporation of 5ClUra into DNA molecules do deserve further investigation. Based on our results, there was no apparent effect on DNA integrity due to single exposure to 5Cl6HMUra (Fig. 2).

In conclusion, we have shown that potential chlorination byproducts of CYN have considerably reduced acute tox-

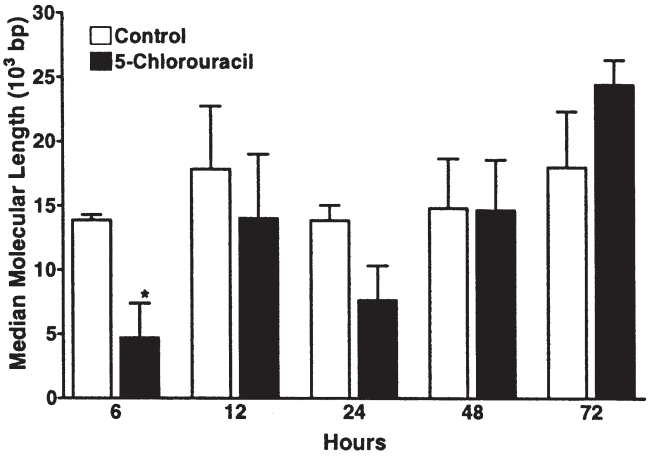

Figure 1 Median molecular lengths of DNA from exposed mice (treated with 10mg/kg 5-chlorouracil) and control mice (treated with 10 mg/kg corn oil) in different time course (n = 8). Significance level: * = $P < 0.05$.

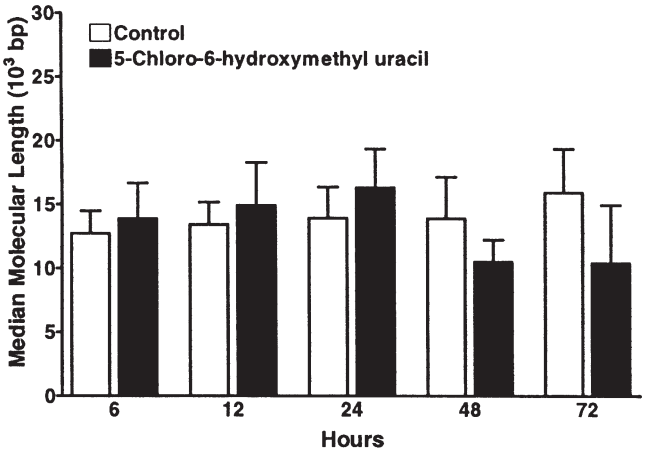

Figure 2 Median molecular lengths of DNA from exposed mice (treated with 10 mg/kg 5-chloro-6-hydroxymethyluracil) and control mice (treated with 10 mg/kg saline) in different time course (n = 8).

icity compared with CYN, but that incorporation into DNA is likely to result in the observed nuclear changes and possible effects on DNA integrity. Overall, our findings cannot eliminate the possibility that some degradation products may have genotoxic potential, and suggest that this aspect should be thoroughly investigated to provide for assessing the risks associated with cyanobacterial toxins and their derivatives following treatment by chlorination.

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