PROTEIN PHOSPHATASE INHIBITION AND TOXICITY OF MICROCYSTIN IN THE LITTLE SKATE *RAJA ERINACEA*

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Microcystins (mcyst) are potent hepatotoxic protein phosphatase (PP) inhibitors that are produced by a variety of cyanobacteria. These microorganisms form dense blooms in waters rich in nutrients releasing toxins in the environment. Mcyst (and related toxins) are a family of small cyclic peptides (7 or 5 amino acids) all hepatotoxic but differing in relative toxicity with LD_{50} ranging between 50-800 µg/kg i.p. in mice (Stotts, R.R., et al., *Toxicon* 31: 783-789 1993). Ingestion of water containing these toxins results in significant numbers of deaths of terrestrial and aquatic animals (Carmichael, W., *Sci. Am.* 270: 64-72, 1994). Exposure to mcyst through contaminated water used in a kidney dialysis unit has resulted in the death by liver failure of 55 patients in Brazil (Barreto, V., et al., *Hepatology* 24: 187A, 1996).

The primary toxic effect of mcyst is damage to the liver, although some kidney damage has been reported in fish (Raberg, C.M.I., et al., *Aquatic Toxicol.* 20: 131-146, 1991). Mcyst is cell impermeant. Hepatic targeting of the toxin is the result of carrier mediated uptake of the peptide exclusively by hepatocytes (Eriksson, J., et al., *Biochem. Biophys. Acta* 1025: 60-66, 1990; Runnegar, M., et al., *Toxicon* 29: 43-51, 1991). The uptake of mcyst by rat hepatocytes is inhibited in a concentration dependent manner by a variety of dyes and also by bile acids (Runnegar, M., et al., *Tox. Appl. Pharmacol.* 134: 264-272, 1995). Prevention of mcyst uptake by the liver by pretreatment of mice with rifamycin protected mice from a lethal dose of mcyst (Runnegar M., et al., *Am. J. Physiol.* 265: G224-G230, 1993). In mammals exposed to lethal doses of mcyst the liver increases in size because of massive intrahepatic centrilobular hemorrhaging. This intrahepatic hemorrhage is a consequence of the extensive dissociation of parenchymal cells with loss of sinusoidal integrity and loss of hepatic architecture (Falconer, I., et al., *Aust. J. Biol. Sci.* 34: 179-187, 1981; Hooser, S., et al., *Vet. Pathol.* 26: 246-252, 1989).

Mcyst added to isolated rat hepatocytes causes loss of microvilli and rapid membrane blebbing (Runnegar, M., et al., *Naunyn-Schmiedeberg's Arch. Pharmacol. Exp. Pathol.* 317: 268-272, 1981; Eriksson, J., et al., *Exp. Cell Res.* 185: 86-100, 1989). These morphological changes have been shown to result from the extensive cytoskeletal rearrangement that involves all three cytoskeletal components: microfilaments (Runnegar, M. and Falconer, I. *Toxicon* 24: 109-115, 1986), intermediate filaments (Falconer, I. and Yeung, S. *Chem.-Biol. Interactions* 81: 181-196, 1992) and microtubules (Wickstrom, M., et al., *Toxicol. Pathol.* 23:326-337, 1995; Hamm-Alvarez, S., et al., *Am. J. Physiol.* 271: C929-C943, 1996).

The mode of action of mcyst has been shown by a number of groups to be the specific, potent inhibition of ser/thr protein phosphatases 1 and 2A (PP 1 and PP2A) (MacIntosh, C., et al., *FEBS Lett.* 264: 187-192, 1990). These enzymes are essential components in the control of cellular function by reversible protein phosphorylation (Cohen, P. *Ann. Rev. Biochem.* 85: 453-508, 1989.). Most studies characterizing the toxicity and mechanism of action of mcyst have been done

in laboratory strains of rats and mice.Only limited studies have looked at the effect mcyst on aquatic animals. In this study we aim to characterize the effect of mcyst in the little skate (*Raja erinacea*), using this species as an example of a freeliving aquatic species.

Mcyst-YM (the tyrosine-methionine variant of mcyst) was radiolabelled with ^{125}I (5-10 Ci/mmol) in order to quantitate uptake of mcyst in hepatocytes. Tracer amounts (2-3 nM) of ^{125}I-mcyst together with varying amounts of unlabelled mcyst were added to suspensions of skate hepatocytes (isolated by collagenase perfusion of the liver). Incubations were carried out at 15°C and 5°C. Cell associated mcyst-YM was determined by gamma counting of the cell pellets separated by centrifugation. Ser/thr PP activity of cells lysates or tissue extracts was determined by measuring the release of inorganic ^{32}P-phosphate from ^{32}P-phosphorylase a at 30°C. One unit of activity equals the release of 1 nmol phosphate/min. Results are shown as mean ± SEM of n, the number of animals/cell preparations. Raw values were analyzed across preparations by one-way ANOVA followed by Fisher's test. A p value of ≤0.05 was considered statistically significant (*). To determine the *in vivo* toxicity of mcyst to the skate the toxin was injected in the tail vein of skates and, after sacrifice or death, the liver, kidneys and rectal glands were taken for histology and determination of PP activity.

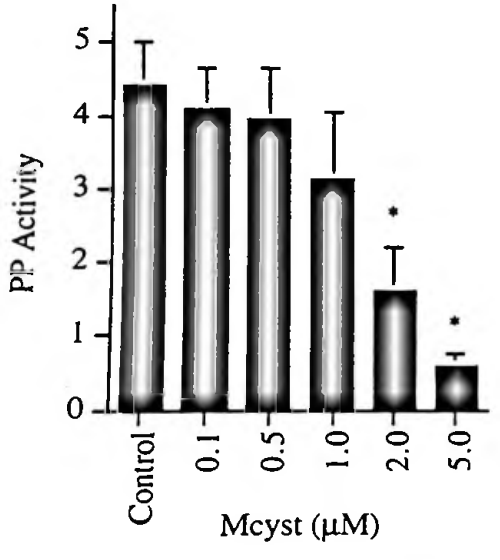

Figure 1a. PP activity of skate hepatocytes 30 min after mcyst addition: dose response (n=3).

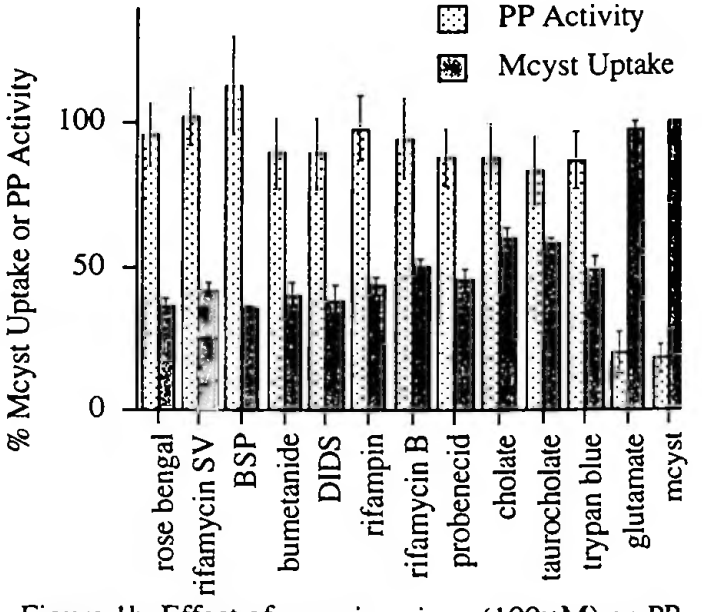

Figure 1b. Effect of organic anions (100μM) on PP activity (% control), and mcyst accumulation (% of 10 μM) in skate hepatocytes (n=4-5, p<0.05 for all treatments except glutamate.

The inhibition by mcyst of the PP activity of hepatocytes is dose dependent (Figure 1a). The concentration of mcyst required to obtain full inhibition of PP activity is considerably larger than that reported for rat hepatocytes (100 nM; Runnegar, M., et al., *J Pharmacol. Exp.Ther.* 273:545-553, 1995). This could reflect a decreased rate of uptake at 15°C the temperature at which these incubations were done when compared to the 37°C used for incubations of rat cells. We have not excluded the possibility that the high content of lipid vesicles in the skate cells might lead to a

sequestration of intracellular mcyst and thereby significantly decrease the amount available to bind to PP. We have previously shown that a number of dyes and bile acids decrease the uptake and accumulation of mcyst by skate hepatocytes (Runnegar, M., et al., *Bull.MDIBL* 36: 77-80, 1997). Here we show that decrease of uptake parallels decreased inhibition of PP by mcyst (Figure 1b). This pattern of protection is similar to that seen in the rat indicating common features of the hepatic carrier(s) responsible for hepatic mcyst uptake in these two groups of animals.

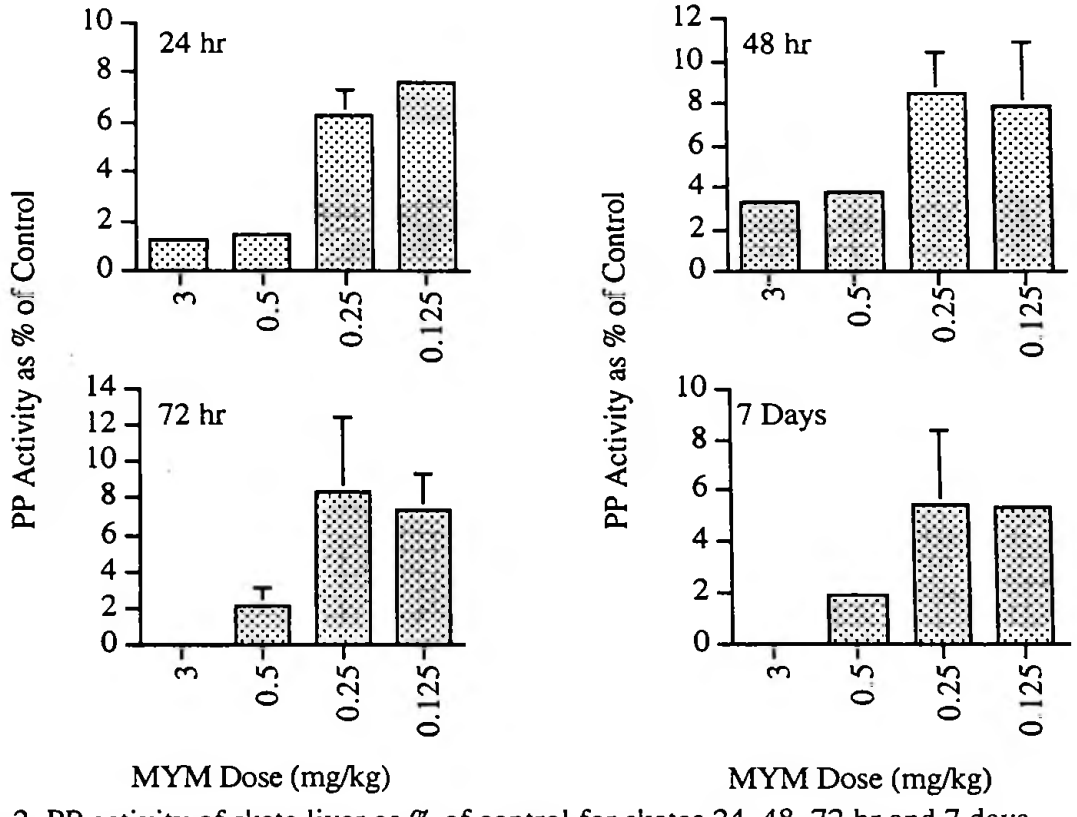

Figure 2. PP activity of skate liver as % of control for skates 24, 48, 72 hr and 7 days after dosing with mcyst. PP activity for controls was 3.20±0.40 units/mg protein, n=5, for treated skates, n=1-3, for n=2 mean given, for n=3 mean ± sem given.

Pilot studies of mcyst *in vivo* toxicity to skates showed severe liver injury and death between two and three days after iv dosing with 2-4 mg/kg. The toxic effects of varying doses of mcyst were investigated over a period of 7 days. On autopsy the liver was grossly affected. Even at doses as low as 0.125 mg/kg there were macroscopic hemorrhages. Detailed histological studies remain to be done, but it is clear that the skate is quite sensitive to low doses of mcyst. Even seven days after dosing the PP activity of liver remained profoundly inhibited (Figure 2), indicating the persistence and lack of clearance of the toxin from liver.

We were interested to see whether the kidneys are a partial target for mcyst toxicity as has been described for the trout (Raberg, C.M.I., et al., *Aquatic Toxicol.* 20: 131-146, 1991). PP activity of the kidneys of these skates (Figure 3) was decreased but the effect was very mild in comparison to the profound inhibition seen in the liver (compare Figure 2). It is not possible from these *in vivo* studies to conclude that the decrease in PP activity in the kidney really reflects

inhibition by mcyst, indicating that the skate kidney has carrier(s) with some capacity for the uptake mcyst. An alternate possible explanation for the decrease in PP activity could be that changes in feeding patterns in mcyst treated skates result in metabolic alterations that include decreased specific activity for kidney PP.

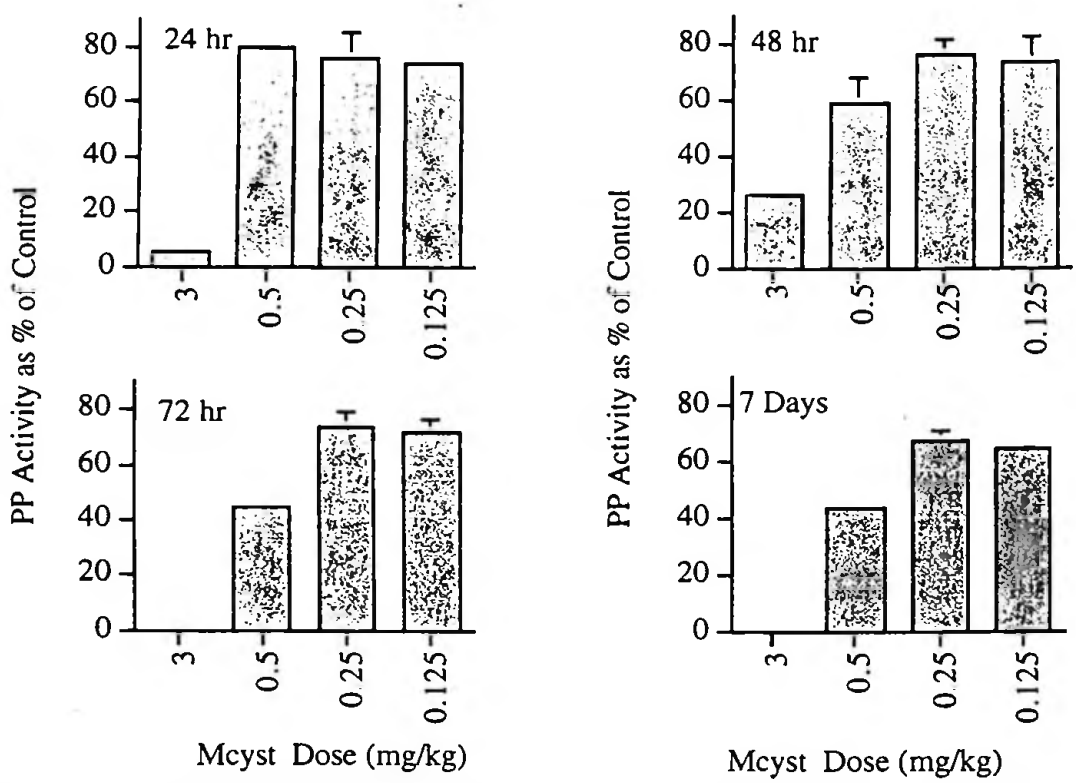

Figure 3. PP activity of skate kidney as % of control for skates 24, 48, 72 hr and 7 days after dosing with mcyst. PP activity for control was 4.32±0.19 units/mg protein, n=5, for treated skates, n=1-3, for n=2 mean given, for n=3 mean ± sem given.

In contrast to the kidney, PP activity of the rectal gland was only decreased at the highest dose (3.0 mg/kg) and in glands taken from animals that had been dead some time. For other animals it did not differ from controls. Detailed histological studies in progress will show how hepatic and possibly kidney lesions correlate with the inhibition of PP activity in these organs. From our findings we can conclude that skates are extremely sensitive to the effect of aquatic toxins like mcyst and related peptides. The persistence of the hepatic inhibition of PP activity would be accompanied by profoundly disturbed metabolism making these animals particularly vulnerable to any other environmental insult.

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