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Human and rat hepatocyte toxicity and protein phosphatase 1 and 2A inhibitory activity of naturally occurring desmethyl-microcystins and nodularins

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ABSTRACT

Contamination of water, foods and food supplements by various genera of cyanobacteria is a serious health problem worldwide for humans and animals, largely due to the toxic effects of microcystins (MCs) and nodularin (NOD), a group of hepatotoxic cyclic peptides. The toxins occur in variable structures resulting in more than 90 different MCs and 8 different NODs, many of them not having been investigated for their toxic potency. Potent MCs such as MC-LR have been shown to elicit their hepatotoxic potency via inhibition of hepatic protein phosphatases (PP) 1 and 2A leading to over-phosphorylation of vital cellular proteins. This mechanism of action is also thought to be responsible for the long term tumor promoting action of certain MCs and NOD in the liver.

Here, we report on the isolation of certain MCs and NOD as well as a number of their desmethylated derivatives from algae bloom. Subsequently, we determined the cytotoxicity of these compounds in isolated primary human and rat hepatocytes in culture. In parallel experiments, we analyzed the inhibitory potency of these congeners on PP1 and 2A using commercially available enzymes. We found in primary rat hepatocytes that MC-LR, -YR and NOD were cytotoxic, namely in the 10 to >50 nM range, while MC-RR was not. The desmethylated congeners of MC-LR, -YR, and NOD were equally or more-toxic as/than their fully methylated counterparts. In primary human hepatocytes we could show that MC-LR, NOD and the desmethylated variants [^{3}Asp]MC-LR, [^{7}Dha]MC-LR and [^{1}Asp]NOD were cytotoxic in the 20 to >600 nM range. Inhibition data with human, bovine and rabbit protein phosphatases 1 and 2A were roughly in accordance with the cytotoxicity findings in human and rat hepatocytes, i.e. desmethylation had no pronounced effects on the inhibitory potencies. Thus, a variety of naturally occurring desmethylated MC and NOD congeners have to be considered as being at least as toxic as the corresponding fully methylated derivatives.

1. Introduction

Cyanobacteria are abundant in a large number of eutrophic and hypertrophic fresh and brackish water lakes, ponds and rivers throughout the world. In particular during warm seasons cyanobacteria bloom formations occur under these conditions. About 40 genera, e.g. *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngba*, *Microcystis*, *Nostoc* and *Oscillatoria*, can form toxins (Pearson et al., 2010; Dawson, 1998). Among the most frequently found cyanobacterial toxins are members of the cyclic peptide families of microcystins (MCs) and nodularins (NODs). Their presence in water has led to fatalities in wild and domestic animals worldwide and has also been associated with human illness (Sivonen and Jones, 1999; Falconer and Humpage, 2005). The most serious incident of human intoxication occurred 1996 at a hemodialysis clinic in Brazil, where over 50 deaths could be attributed to MCs which were later identified in the water supply (Azevedo et al., 2002). Further exposure routes may be through the consumption of contaminated foods such as snails, mussels, bivalves, crustacean and fish, as well as food supplements such as *Aphanizomenon flos-aquae* products (Ibelings and Chorus, 2007; Schaeffer et al., 1999).

The hepatotoxic MCs contain seven amino acids linked by peptide bonds to form a cycle (Fig. 1). Most of the more than 90 MC congeners found so far in cyanobacteria have in common five peculiar amino acids which do not play a physiological role in vertebrate metabolism. Among these are D-alanine, D-*erythro*-β-methylaspartic acid (D-MeAsp), 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), D-glutamate and *N*-methyldehydroalanine (Mdha), while the amino acids at positions 2 and 4 are variable (Fig. 1). D-MeAsp,

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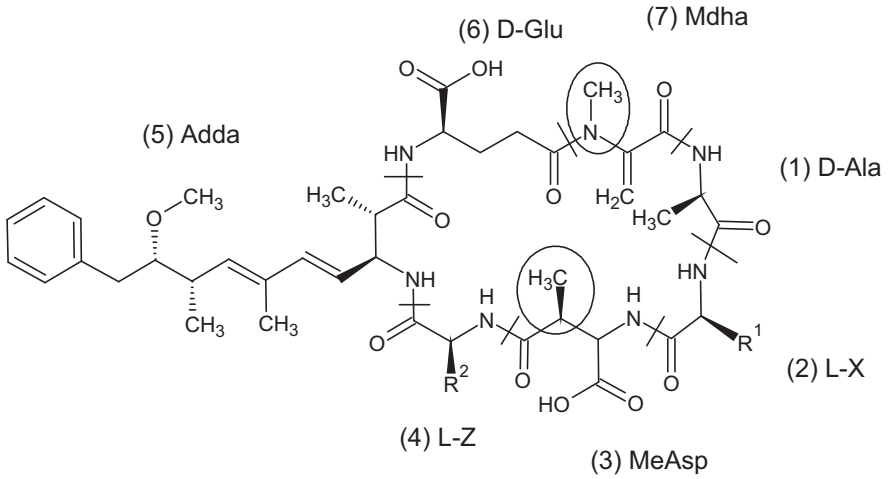

Fig. 1. Chemical structure of microcystins. The methylation sites varying among the test compounds are circled. Adda, [2S, 3S, 8S, 9S]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; D-Glu, D-glutamic acid; Mdha, N-methyldehydroalanine; D-Ala, D-alanine; MeAsp, D-*erythro*-β-methylaspartic acid.

Adda, and Mdha can occur as desmethylated variants, e.g. as dehydroalanine instead of Mdha in [^{7}Dha]MC-RR (Martin et al., 1992, Pearson et al., 2010). The nomenclature of MCs follows the pattern of substitution at the positions 2 and 4, e.g. with [L-leucine2; L-alanine4] in MC-LA or [L-leucine2; L-arginine4] in MC-LR (Carmichael et al., 1988). The NODs are cyclic pentapeptides (Fig. 2) containing D-MeAsp, L-arginine, Adda, D-glutamate and *N*-methyldehydrobutyrine (Mdhb) (Karlsson et al., 2003). Their desmethylated variants contain D-Asp, DmAdda, or Dhb (Lankoff et al., 2006). The L-arginine can be replaced by a few other amino acids, e.g. with L-homoarginine in nodularin-Har and L-valine in Motuporin (deSilva et al., 1992; Saito et al., 2001).

After ingestion of cyanobacteria and/or MCs or NODs with contaminated water or food, the toxins are absorbed from the ileum into the blood circulation and, taken up into the liver via transmembrane multispecific organic anion transporters (bile salt transporters), which are expressed in liver, kidney, gastrointestinal tract and brain (Dietrich et al., 2005; van Apeldoorn et al., 2007). Inhibition of hepatic protein phosphatases 1 and 2A (PP1 and PP2A) is widely recognized as a major toxic mode of action of MCs and NOD (Yoshizawa et al., 1990; Dawson, 1998; Kuiper-Goodman et al., 1999) in the liver. This inhibition involves the disintegration of hepatocyte structure, apoptosis, liver necrosis and internal hepatic hemorrhage that may lead to death by hemorrhagic shock (Figueiredo et al., 2004). In experimental animals, e.g. in rats, repeated dosing of MCs produced liver swelling and hepatic injury (Heinz, 1999). Other organs are affected such as kidneys, lungs and intestine (van Apeldoorn et al., 2007). In mice, NOD treatment caused acute liver hemorrhage and liver failure (Duy et al., 2000; Eriksson et al., 1988). Furthermore, in rat and/or human hepatocytes in primary culture, MC-LR induced oxidative stress, cytotoxic cell damage, and apoptosis (Bouaïcha and Maatouk, 2004; Chong et al., 2002; Mankiewicz et al., 2001). Similar effects were reported for NOD (Pearson et al., 2010).

As a consequence of reports on intoxication episodes, the World Health Organization has recommended a maximum allowable level of 1 μg/l of MC-LR in water (WHO, 1998). Since MC-LR is only one out of a large number of MCs occurring in cyanobacteria, there is a need for comparative determination of the relative toxicity of other MC congeners and NODs. Wolf and Frank (2002) have tried

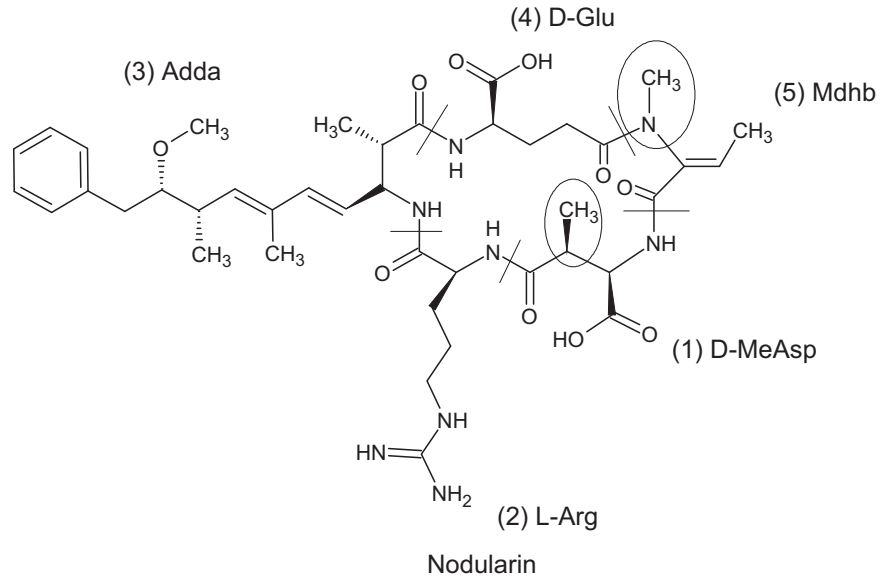

Fig. 2. Chemical structure of nodularin(s). The methylation sites varying among the test compounds are circled. Adda, [2S, 3S, 8S, 9S]-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; D-Glu, D-glutamic acid; Mdha, N-methyldehydrobutyric acid; D-MeAsp, D-*erythro*-β-methylaspartic acid; L-Arg, L-arginine.