Microcystin Produces Disparate Effects on Liver Cells in a Dose Dependent Manner

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Abstract: In this review we present recent studies on the effects of the protein phosphatase inhibitor microcystin on mammalian cells. Whereas high concentrations of microcystin promote liver cell death induced by ROS signalling without the involvement of typical apoptotic proteins, intermediate doses activate classic apoptotic pathways. Low concentrations however, increase liver cell survival and proliferation, and can cause primary liver cancer.

Keywords: Microcystin, protein phosphatases, PP1, PP2A, reactive oxygen species, cancer, apoptosis, mitochondria.

INTRODUCTION

Green waters have been associated with toxicity and disease since pre-historic time. The first scientific reports of death associated to toxic cyanobacteria concerned the enigmatic mortality of domestic animals [1]. There are numerous toxins produced by cyanobacteria, but the most studied by far is the hepatotoxin microcystin (for an introduction to toxin-producing cyanobacteria, see [2]). Almost a hundred years after the first report, a peptide was purified and identified to be a cyclic peptide (Fig. **1**) [3-5]. Since then, more than 20 microcystin variants and several related structures have been identified [2]. Even though the mechanism of action is well documented, namely inhibition of protein serine/threonine phosphatases, the multitude of secondary effects on mammalian cells have been only partly elucidated.

THE TOXICITY OF MICROCYSTINS IN MULTI-CELLULAR ORGANISMS

There have been numerous reports of the toxic effects of microcystin on multi-cellular organisms ranging from planktonic crustaceans to vertebrates such as fish and mammals. Small crustacean (e.g. Daphnids) grazing on cyanobacteria, are postulated to be the main target for the toxin production, though it is claimed in a recent investigation that microcystin itself is "phylogenetically older" than the animals susceptible to its effects [6]. The toxic effects on invertebrates are widespread, but can largely be linked to the inhibition of protein serine-threonine phosphatases (PP) in the affected organism [7, 8]. Microcystin-intoxication in vertebrates mainly affects the

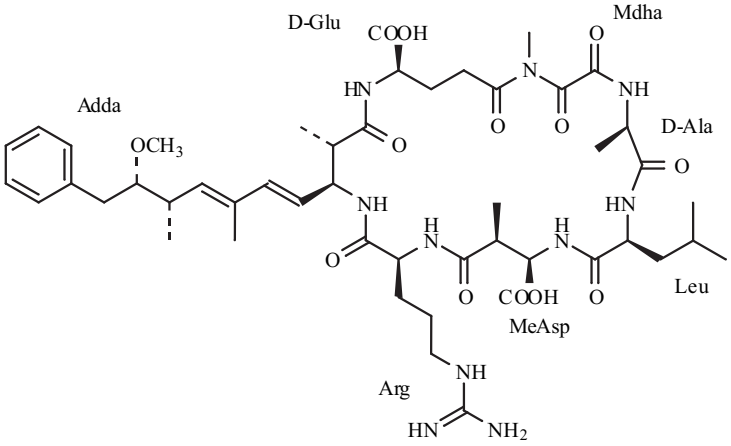

Fig. (1). The molecular structure of microcystin-LR. The cyclic heptapeptide has the structure cyclo-(D-Ala[1]-Leu[2]-D-MeAsp[3]-Arg[4]-Adda[5]-D-Glu[6]-Mdha[7]). Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, D-MeAsp is D-erythro-β-methylaspartic acid and Mdha is N-methyldehydroalanine. The residues Leu[2] and Arg[4] are variable. Adapted from [2] and [22].

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intestine and liver. This tissue specificity is partially a result of the oral administration of toxin, but also that microcystin, which is rather hydrophilic, is unable to penetrate the plasma membrane, and requires active uptake mechanisms to enter cells. Such carriers are found only in the liver, demonstrated to be specific bile acid transport mechanisms [9, 10].

During a particularly severe incident in a haemodialysis unit in Caruaru, Brazil, 126 patients developed toxic hepatitis, and at least 43 died after microcystin intoxication. Typical symptoms were malaise, myalgia and weakness, nausea and hepatomelagy, as well as a range of neurological symptoms. The liver was the hardest affected organ, with abnormal function, and rapid and fatal liver failure [11]. The haemodialysis unit took water from a reservoir contaminated with microcystin-producing cyanobacteria, and microcystin was found in high concentrations in all elements of the water treatment system, as well as in the liver and serum of the dialysis patients [12].

Recently, research on microcystin toxicity has focused on enhanced cell proliferation, rather than cell death (for a recent review, see [13]). Low doses of microcystin in drinking water have been linked to high incidences of primary liver cancer [14]. These findings correlate with animal and *in vitro* experiments, showing increased susceptibility to develop liver cancer after exposure to microcystin [15, 16] or the related compound nodularin [17].

A safe concentration threshold for microcystin in drinking water has been set at 1μg/L [18, 19], but it has been suggested that 0.01μg/L would be more appropriate due to the aforementioned risk of developing primary liver cancers after chronic exposure to microcystin [14].

MOLECULAR MECHANISMS OF PP-INHIBITION BY MICROCYSTIN

Microcystin is a cyclic heptapeptide with the amino acid sequence D-Ala^1-X^2-D-MeAsp^3-Y^4-Adda^5-D-Glu^6-Mdha^7 (Fig. **1**). There are several unusual amino acids; Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid, D-MeAsp is D-erythro-β-methyl-aspartic acid and Mdha is N-methyldehydroalanine. The residues X^2 and Y^4 are variable L-amino acids, the most common being Leu^2 and Arg^4 (microcystin LR). Other variants of microcystin are also frequently found, such as RR, YR, LW and several more [20, 21]. The binding studies discussed in the following section were done with microcystin LR. The unique structure of microcystin confers the ability to bind with very high selectivity to specific serine-threonine protein phosphatases (PP) (Table **1**).

Table 1. Sensitivity of the Various Serine/Threonine Protein Phosphatases (PP) to Microcystin LR

PP-sub type	IC-50	References
PP1	0.2 nM	[26, 74, 84]
PP2A	0.2 nM	[74, 84]
PP2B	0.2 μM	[84]
PP2C	n. i.	[84]
PP3	0.2 nM	[85]
PP4	0.8 nM*	[86]
PP7	n. i.	[87]

*This value is adjusted to match that of PP2A since this study showed values of 2 and 8 pM for PP2A and PP4 respectively. n.i. is not inhibited.

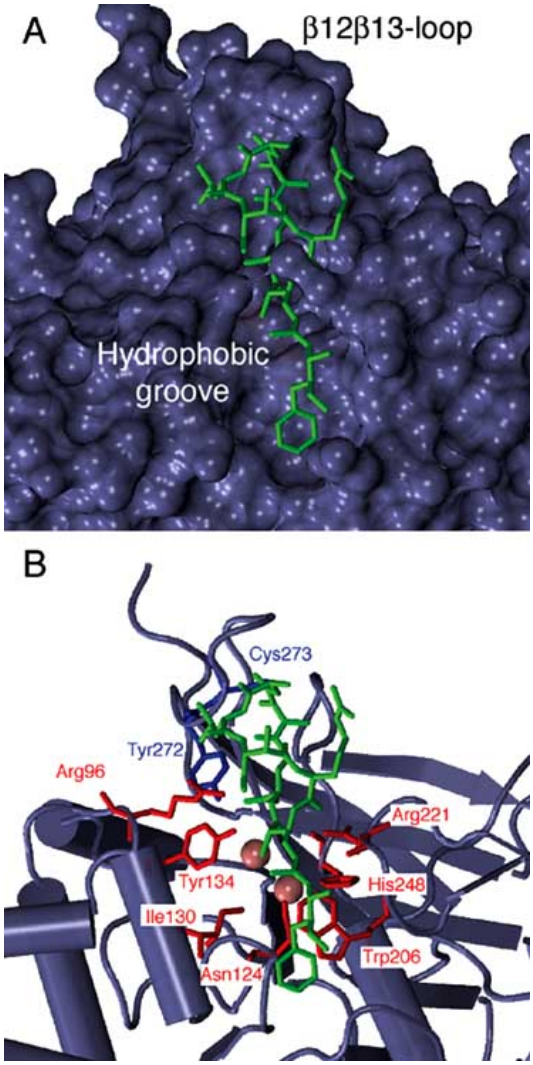

Fig. (2). The protein phosphatase (PP) 1-microcystin-LR complex. A: Surface model of PP1 with microcystin LR (green) bound over the catalytic core. Note that ADDA lies along the hydrophobic groove, and the close interaction between the ring structure of microcystin and the β12-β13-loop. B: Cartoon of the PP1-microcystin-LR complex projected from the same angle as A. Microcystin is shown in green, and the amino acids in the hydrophobic groove that interacts closely with ADDA are shown in red. The blue residues are microcystin-LR-interacting amino acids in the β12-β13-loop. The residue Cys273 makes a covalent bond with the dehydroalanine of microcystin. See text for details concerning the importance of structures on microcystin and PP1-residues on binding and inhibition of phosphatase activity. The images were created in the VMD software system [88] from PDB-files submitted to the RCSD protein data bank (http://www.rcsb.org/pdb/index.html) [25, 89].

Most of the serine-threonine phosphatase binding studies have been done on PP1 and PP2A, these being the most abundant of serine threonine phosphatases.

The inhibition of PP1 by natural inhibitors such as DARPP 32 (dopamine and cAMP regulated phosphoprotein) and Inhibitor-1 (hI-1) is dependent on several regions of PP1, including parts of the active site. The c-terminal β12-β13 loop, consisting of residues 269 – 281 (Fig. **2A** and **2B**) is an important structure for recognition in the active site, both for substrates and natural inhibitors [22-24]. Microcystin and other PP-inhibitors like okadaic acid and nodularin interact closely to the β12-β13 loop when binding to the active site of PP1 [22, 23] (Fig. **2**). Microcystin has a dehydroalanine (MDHA) residue that covalently alkylates the SH group of Cys273, which is located adjacent to the catalytic centre of PP1 [25]. However, this covalent bond is not the primary cause of PP1-inhibition by microcystin since PP1-mutants with substitution of Cys273 to Ala, Ser or Leu increases the IC-50 only moderately [26]. Interestingly, the covalent binding of microcystin to PP1 has been suggested to be the main reason for the failure to detect microcystin post-mortem in suspected cases of poisoning [26].

Non-covalent interactions between different amino acid of PP and microcystin play a central role in the inhibitory mechanisms of PP by microcystin. The L-Leu of microcystin interacts hydrophobically with the aromatic Tyr272 on the β12-β13 loop (Fig. **2B**). Mutagenesis of Tyr-272 to charged residues does not alter the catalytic activity of PP1, but greatly reduces the affinity of microcystin for PP1, proving the importance of hydrophobic interaction in microcystin recognition and binding to the enzyme [27].

The hydrophobic groove of PP 1 and 2A seems to be especially important for the interactions between microcystin and the protein (Fig. **2**). Hydrophobic interaction between the residues Trp206 and Ile130 in the hydrophobic groove of PP1 and the unusual amino acid ADDA side-chain of microcystin is crucial for the microcystin toxicity [24, 28]. Studies with analogues of microcystin that only consist of Adda and a single additional amino-acid underlines the importance of Adda for inhibition of PP. Gulledge and co-workers found substantial inhibition of PP1 and PP2A using Adda and one additional amino acid [29]. However, similar analogues with modifications to the Adda structure itself were inactive.

MITOCHONDRIA-MEDIATED APOPTOSIS VIA THE CLASSIC BID-BAX-BCL-2 PATHWAY

The mitochondria are involved in several of the apoptotic pathways, but until recently, little was known of the importance of mitochondria in microcystin-mediated cell death. It has been shown that rapid apoptosis induced by high doses of microcystin is independent of Bcl-2 [30]. However, there is some evidence that microcystin in some cases acts via pathways dependent on Bcl-2 proteins.

An early mitochondrial response to apoptotic signalling is permeabilisation of the mitochondrial membrane, the so-called MPT (mitochondrial permeability transition). This initial phase involves opening of the mitochondrial permeability transition pore (PTP), mitochondrial matrix swelling, rupture of the outer membrane and release of pro-apoptotic proteins from the inner mitochondrial membrane into the cytosol (see [31-33] for reviews on the release of death proteins from the mitochondria).

A drop in the electric membrane potential, high [Ca^{2+}] in the mitochondrial matrix, high pH and ROS also induce opening of the mitochondrial permeability transition pore [32]. There is evidence that Ca^{2+} influx into the mitochondria stimulates a cascade beginning with mitochondrial ROS production [34, 35], leading to ROS-induced peroxidation of the phospholipid cardiolipin and subsequent dissociation of cytochrome *c* from cardiolipin [36-38]. Formation of ROS and subsequent cytochrome *c* release from submitochondrial particles are blocked in the presence of the antioxidant enzymes superoxide dismutase (SOD) and catalase [38].

The Bcl-2 family members play a pivotal role in regulation of mitochondrial permeability transition. The Bcl-2 protein family includes both inducers (Bid, Bax, Bak, Bad) and suppressors (Bcl-2, Bcl-X_L, Bcl-w) of mitochondria-mediated apoptosis [39]. The pro-apoptotic Bid protein becomes activated when it is cleaved, and the truncated Bid (t-Bid) translocates and integrates into the mitochondrial outer membrane where it cooperates with Bax, Bak and lipids to form pores in the membrane [40-42]. In contrast, the anti-apoptotic Bcl-2 and Bcl-X_L proteins inhibit translocation and insertion of t-Bid and Bax into the mitochondrial outer membrane [43, 44]. Even though previous findings have shown that microcystin shortcuts the apoptotic machinery by acting independently of caspase- and Bcl-2-regulated pathways [30], a recent study provides new insight on microcystin-induced apoptosis.

It has been shown that arsenic induces MPT and formation of ROS by blocking complex I in the mitochondrial electron chain [45]. Recent evidence suggests that massive accumulation of ROS only occurs at high concentration of arsenic, whereas, lower doses of arsenic induce apoptosis through a classic Bax/Bak-dependent mechanism [46, 47]. Interestingly, a concentration-dependent mechanistic switch between classic Bid-Bax-Bcl-2- and ROS-signalling has also recently been reported for microcystin-induced apoptosis in mouse liver. By using a combined transcriptomic, proteomic and a computer simulation approach it has been found that mice treated with moderate doses of microcystin had liver failure primarily due to apoptosis via a Bid-Bax-Bcl-2 pathway [48]. However, high doses of microcystin led to apoptosis through a ROS-dependent pathway, not dependent on the classic apoptotic machinery [48].

THE EFFECTS OF HIGH DOSES OF MICROCYSTIN ON CELLS *IN VITRO*

The cellular effects of microcystin were first reported by Eriksson and co-workers in 1989, who described reorganisation of microfilaments as well morphological changes in primary rat hepatocytes after treatment with microcystin [49]. Døskeland and co-workers proved evidence for programmed cell death induced by protein serine/threonine phosphatase inhibition by natural toxins [50], and this has been supported by several later studies

[30, 51, 52]. These authors also found that high doses of microcystin or nodularin induce rapid hyperphosphorylation of several proteins that preceded the initial morphological signs of apoptosis in hepatocytes [30]. By microinjecting microcystin, apoptosis was observed within few minutes in many other cell types as well. Interestingly, Bcl-2 or Bcl-X_L overexpression did not prevent the rapid apoptosis induced by phosphatase inhibitors [30]. Thus, emerging evidence indicate that cell death induced by high doses of phosphatase inhibitors is independent of the Bcl-2 family members. Microcystin-induced cell death has been linked to the formation of reactive oxygen species by Ding and co-workers who showed that hepatocytes treated with microcystin produced large amounts of ROS only minutes after addition of microcystin [53]. Furthermore, microcystin-induced ROS-formation was reduced by pre-treatment of cells with an iron-chelator, or an MPT-inhibitor [53], suggesting that the ROS were of mitochondrial origin. However, caspase inhibitors only delayed, while calpain inhibitors significantly inhibited microcystin-induced apoptosis [54]. The authors therefore suggested that the Ca^{2+}-dependent protease calpain, not caspases, plays an essential role in microcystin-induced apoptosis. Moreover, the rapid apoptosis induced by phosphatase inhibitors in hepatocytes and other cell types involves the Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) [55]. It is well known that active autophosphorylated CaMKII becomes dephosphorylated and inactivated by PP1 and PP2A [56]. Thus, inhibition of these phosphatases by microcystin induces activation of CaMKII and subsequent initiation of apoptosis. The fact that both calpain and CaMKII is essential for microcystin-induced apoptosis suggests a co-operative effect between the kinase and the protease.

It is proposed that microcystin short-cuts the apoptotic decision machinery of the cells, acting directly on the execution elements of the apoptotic process [30]. Mitochondria are acknowledged as the major apoptosis executioner (see previous chapter), and are thus a likely target for the search of the mechanisms behind microcystin-induced apoptosis. The studies of Ding and co-workers strongly suggest that the rapid apoptosis (<10 minutes)

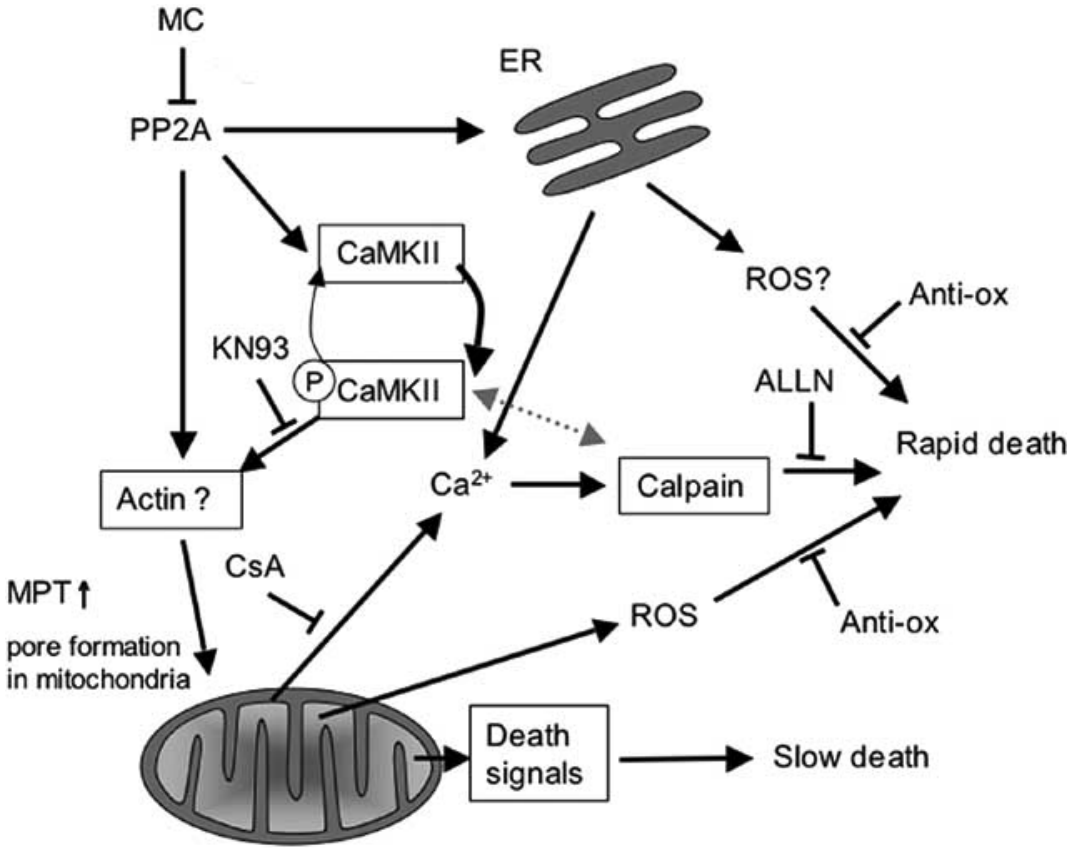

Fig. (3). Schematic representation of some of the signalling pathways involved in microcystin-mediated cell death. Only the main pathways are included: The rapid ROS-dependent cell death commences by microcystin inhibiting the protein serine/threonine phosphatases PP1 and PP2A. This causes hyperphosphorylation of Ca^{2+}/calmodulin dependent kinase II (CaMKII). CaMKII could be the source of ROS-production, possibly *via* alteration of actin and other cytoskeleton elements, and a subsequent influence on the mitochondrial permeability transition (MPT). A gross leakage of ROS from the mitochondria will itself be pro-apoptotic, whereas Ca^{2+} mediates apoptosis via calpain. Microcystin can also recruit ROS and Ca^{2+} from the endoplasmatic reticulum (ER), *via* unknown mechanisms. The various pathways can be blocked by inhibiting CamKII (KN93), calpain (ALNN) or mitochondrial leakage (CsA). Inhibition of microcystin-mediated apoptosis downstream of these check-points may be inhibited by anti-oxidants (Anti-ox). The grey dotted line between calpain and CamKII indicates a possible co-operative effect between these enzymes.

The classic apoptosis pathways are mediated by death signals leaking from the mitochondria due to Bid/Bad/Bax-activity. This produces a slower death that is dependent on various classical pro-apoptotic members.

induced by microcystin are dependent on functional mitochondria [53, 57]. What appears to be one of the main events of rapid microcystin-induced apoptosis is gross formation of ROS.

Relevant to this discussion, is the finding that the use of anti-oxidants can inhibit ROS-induced, as well as microcystin-induced apoptosis [58]. A recent study showed that the CamKII-dependent commitment point for apoptosis was prior to the ROS-dependent commitment point [59]. By adding anti-oxidants to microcystin-treated hepatocytes, the apoptosis could be prevented after the CamKII dependent commitment point. However, the specific link between CamKII and ROS is not yet known. It has been suggested that full complement of β-actin is needed to transduce cell death signals from mitochondria [60]. Aggregates of F-actin have been described as a hallmark of microcystin-induced apoptosis [49], and are postulated to drive the mitochondria-derived release of ROS in yeast [61]. It has been shown that microcystin induce CaMKII-dependent phosphorylation of myosin light chain (MLC), probably through activation of MLC-kinase [30]. This phosphorylation is further enhanced since MLC is a substrate for PP1 [62]. Together with other findings of cytoskeleton rearrangements following microcystin-treatment of hepatocytes [63], it is reasonable to believe that the cytoskeleton is one of the key factors in mitochondria-derived ROS-formation.

Finally, a hormone- or agonist-stimulated increase of cellular [Ca^{2+}] has been suggested to activate a mitochondrial protein phosphatase that dephosphorylates cytochrome *c* oxidase and turns off the allosteric ATP-inhibition. This results in increase of mitochondrial membrane potential and a subsequent ROS formation. ATP-synthase has been shown to bind to microcystin [64]. Whether this binding alters the activity of the enzyme is not yet reported, but an inhibition of this enzyme could very well contribute to the gross production of ROS in the mitochondria following microcystin-treatment.

As described in this chapter, there are several ways that microcystin can induce ROS-production. This increase in ROS also corresponds to findings that microcystin causes a decrease in the level and activity of several cytoplasmic ROS scavenger proteins, such as SOD and catalase [65].

PROLIFERATIVE EFFECTS OF TRACE DOSES OF MICROCYSTIN

Studies from China have shown that long-term exposure to low doses of microcystin can produce primary liver cancers [14] and neoplastic changes in mouse liver [66]. This is in contrast to the cell death promoting mechanisms induced by microcystin. In the previous section, we have discussed the apoptosis-promoting effects of high doses of microcystin via mechanisms involving generation of a gross burst of ROS However, a recent study showed that reduction of active NF-κB led to a small increase in ROS which made mice lacking IKKβ more susceptible to chemical hepatocarcinogenesis [67]. ROS itself was not carcinogenic, but augmented proliferation of cells surviving the chemical treatment used to provoke tumour formation. If anti-oxidants were given to the animals shortly before exposure to the chemical carcinogen, the tumour-formation was abolished [67]. Thus, a low dose of microcystin might give a small ROS-response, not enough to kill the cells, but enough to support enhanced survival.

Apparently the tumorigenic effects of microcystin are a result of a combination of enhanced activation of survival factors, as well as a chronic increase in ROS-formation. ROS can, like ionising radiation cause DNA-damage (for more on ROS and cancer, see [68, 69] and references therein). In line with this, sub-toxic doses of microcystin (0.01 – 1 μM in hepatoma cells) induced ROS-mediated increase in intermediates formed during DNA repair and a rise of the levels of oxidised pyrimidines and purines [70, 71]. Inhibition of nuclear PP may also contribute to the carcinogenic effect of microcystin [72] but this effect has been little investigated. In addition, death after activation of classic apoptotic pathways can be increased if an anti oxidant is present, whereas addition of H_2O_2 reduces apoptosis [73].

By continuously down-regulating the effects of protein phosphatases, microcystin can alter the phosphorylation status and activity of a number of downstream proteins. Even though there are no clear evidence between long-term exposure to low doses of microcystin and up-regulation of survival factors, there are several possible links that should be discussed. Many of the following mechanisms are elucidated by the use of okadaic acid, a dinoflagellate toxin which inhibits PP1 and PP2A similarly to microcystin, but with a higher affinity to PP2A [22, 74].

Reversible protein phosphorylation and dephosphorylation has been proposed to be the main mechanism that regulates the activity of Bcl-2 family proteins [75]. Site-specific phosphorylation of Bcl-2 at Ser 70 has been found to be essential for full anti-apoptotic signalling activity [76]. Both PP1 and in particular PP2A have been associated with apoptosis promotion [75]. PP1 binds to the survival factors Bcl-2 and Bcl-X_L leaving the phosphatase in favourable position to dephosphorylate phospho-Bad when the latter is associated to Bcl-2 [77]. In addition, PP1 is involved in the dephosphorylation and inactivation of the survival factor Bcl-2 [78, 79]. Bcl-2 is believed to maintain membrane integrity in the mitochondria and thereby prevent leakage of death proteins. In contrast to Bcl-2, the mode of action of the pro-apoptotic Bad protein is inactivated by phosphorylation [75]. The Ser155 phosphorylation of Bad prevents heterodimerisation of Bad with Bcl-X_L, leading to Bcl-X_L association with Bax in mitochondria. The

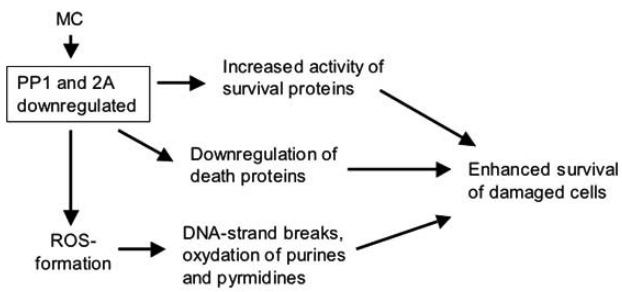

Fig. (4). Some of the ways of which low doses of microcystin can enhance cell survival and promote liver cancer. See text for details on the specific mechanisms behind regulation of the survival and death proteins, and DNA-damage.

interaction between Bcl-X_L and Bax antagonises Bax-induced pore-formation in the mitochondria and subsequent release of pro-apoptotic proteins [80]. Likewise, phosphorylation of Bad at Ser 112 and Ser 136 promotes cytosolic interaction between Bad and 14-3-3 proteins [75, 81]. Thus, it is reasonable to suggest that inhibition of PP1 or PP2A enhance cell survival via signalling leading to increased phosphorylation of Bcl-2 and Bad. In fact, microcystin-mediated inhibition of PP1 and PP2A also enhance survival signals upstream of Bad-phosphorylation. The PI3K/Akt survival pathway is negatively regulated by PP1 and PP2A [82]. Akt phosphorylates Bad at Ser 136 [81], and an up-regulation of the Akt activity (initiated by inhibition of PP1 and PP2A) will enhance the pro-survival effects of phosphorylated Bad.

Several other pro- or anti-apoptotic Bcl-2-family members are reported to have PP1 consensus docking motifs, and are putatively regulated by PP1, PP2A or other microcystin-inhibitable phosphatases. In addition, there are numerous PP1 interacting proteins that can be regulated either by phosphorylation, or by binding in complexes with the phosphatases [83]. Clearly, a shift in the activity of these may alter the cells ability to respond to death or survival signals, but the exact mechanisms behind microcystin-induced cell survival is not yet understood.

SUMMARY

When reading reports on microcystin-induced cell death or proliferation, one must bear in mind that different cell types respond differently to specific doses of microcystin, primary hepatocytes being the most sensitive by far. In addition, during *in vivo* studies it is difficult to determine precisely the effective concentration of microcystin in the liver, making such experiments rather inaccurate in comparison to *in vitro* studies. However, results from both *in vitro* (specifically addressed in this review) and animal studies (see [13] for a recent review) consistently support the mechanisms detailed above.

In this review, we have focused on recent advances in our understanding of the opposing response of liver cells to low and high concentrations of microcystin. In a dose-dependent manner microcystin tune PP activity and by this means disturb the delicate balance of downstream transduction-pathways that determine the biological outcome. Exposure to constant low doses of microcystin causes slight down-regulation of the PP-activity, leading to alterations in the activity of some proteins, which provide enhanced survival. In addition, it is known that microcystin can induce alterations of the DNA during long-term-exposure. In contrast to this, several studies provide compelling evidence that high doses of protein phosphatase inhibitors induce apoptosis through a ROS-dependent pathway, and recent evidences suggest that intermediate doses of microcystin predominantly initiate apoptosis via classic mitochondrial death pathways.

There is still much to be known about the biological effects of microcystin on mammalian cells, and the diseases associated with microcystin intoxication. We would like to emphasise the ability to promote primary liver cancer, since this is caused by lower concentration of microcystin than the present lower threshold of 1μg/L. A better understanding of the proliferative effects of microcystin may eventually lead to a revision of the safety guidelines.

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