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Minireview

Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response

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Abstract **Microcystins, potent heptapeptide hepatotoxins produced by certain bloom-forming cyanobacteria, are strong protein phosphatase inhibitors. They covalently bind the serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A), thereby influencing regulation of cellular protein phosphorylation. The paralytic shellfish poison, okadaic acid, is also a potent inhibitor of these PPs. Inhibition of PP1 and PP2A has a dualistic effect on cells exposed to okadaic acid or microcystin-LR, with both apoptosis and increased cellular proliferation being reported. This review summarises the existing data on the molecular effects of microcystin-LR inhibition of PP1 and PP2A both in vivo and in vitro, and where possible, compares this to the action of okadaic acid.**

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Key words: Microcystin; Okadaic acid; Protein phosphatase; Apoptosis; Tumorigenesis

1. Introduction

Microcystin-LR (MCLR) is a cyclic heptapeptide toxin produced by several genera of cyanobacteria. The increased occurrence of cyanobacterial blooms emphasises the need to investigate both the effects of the toxin on humans and animals as well as mechanisms of bloom control and toxin removal from human and animal water sources [1,2].

The development of primary liver cancer in China has been linked to long-term chronic exposure to microcystin (MC) [3,4]. People drinking pond or ditch water containing low levels of MC had a higher mortality rate from hepatocellular carcinoma than people drinking well water that did not contain any MC [3–5]. In contrast to these observations is evidence of MCLR-induced programmed cell death or apoptosis both in vivo [6,7] and in vitro [8–10]. This apparent dualistic response is characteristic of another well studied protein phosphatase (PP) inhibitor, okadaic acid (OA). This review attempts to compare the processes of tumour promotion and induction of apoptosis for MCLR and OA.

2. Biochemical cellular effects of MCLR exposure

2.1. PP inhibition

MC is a potent inhibitor of the eukaryote serine/threonine (Ser/Thr) protein phosphatases 1 and 2A (PP1 and PP2A) in vitro [11,12] and in vivo [13] which suggests that the toxic effects of MC observed both in vivo and in vitro result from the resultant so-called activation of protein kinases [14]. MCLR inhibits PP1 and PP2A with an IC_{50} of about 0.1–1.0 nM [11,12]. Pre-incubation of PP2A with OA prevents the subsequent binding of MCLR to the catalytic subunit of the enzyme [12]. OA can be used to differentiate between the activity of these two PPs, with PP1 having an IC_{50} of 10 nM and PP2A of 0.1 nM OA [12]. MCLR does not inhibit PP2C [11,12] and has no effect on the in vitro activity of two of the cellular Ser/Thr protein kinases, protein kinase C (PKC) and cAMP-dependent kinase (PKA) [11].

In vivo inhibition of PP by MC was shown in liver homogenates of mice exposed to toxin and was dose-dependent for both MCYM (dose range 20–200 μg/kg) and MCLR (dose range 15–120 μg/kg) [13]. Both PP1 and PP2A covalently bind MCLR [6,15] at cysteine-273 for PP1 and cysteine-266 for PP2A [15,16].

2.2. Effects of MC on the cytoskeleton

The cellular cytoskeleton consists of three protein components, namely microtubules, intermediate filaments and microfilaments. Hyperphosphorylation of the intermediate filaments is a consequence of exposure to OA [17]. MCLR at 1 μM induced hyperphosphorylation of the major intermediate filament proteins keratin 8 and 18 [18] in a dose- and time-dependent manner [19]. Similar results were found for the desmoplakin proteins DPI and DPII, proving that the Ser/Thr PP1 and PP2A are important regulators of the intermediate filament proteins' phosphorylation status [19]. The in vivo protein kinases responsible for the hyperphosphorylation regulating K8 and K18 have not been identified but candidates are PKC, PKA or the calcium/calmodulin-dependent kinase (CaMKII) [20].

Increased phosphorylation of cytoplasmic dynein resulted in impaired motor function accompanied by a reduction in the recovery of endosomal/lysosomal membranes by microtubules in hepatocytes exposed to both MCLR and OA [21]. The MCLR-induced changes in microfilaments and microtubules in primary rat hepatocytes occurred before lactate dehydrogenase (LDH) leakage with the authors suggesting that

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binding of MCLR directly to the cysteine residues found on the tubulin subunits could have resulted in more free tubulin in the cytoplasm, thereby reducing the stability of the tubulin mRNA and a decrease in cellular tubulin [22].

2.3. Molecular mechanisms underlying the response to PP inhibitors

The effects of the PP inhibitors MC and OA on cellular metabolism are confusing. Both are involved in tumour promotion and yet are also involved in the conflicting response of cell death by means of apoptosis. The cell cycle is regulated by a delicate balance between phosphorylation and dephosphorylation of key control proteins [20]. The specific dephosphorylation of Ser/Thr residues plays an essential role in control of the cell, with more than 97% of protein phosphates occurring at Ser/Thr residues [23]. Therefore, by inhibiting both PP1 and PP2A, MCLR and OA are able to interact with a variety of molecular targets, resulting in an overall increase in phosphorylation of regulatory proteins. These effects have been chiefly investigated using OA, as this toxin is effective at very low concentrations on a wide variety of cells [24]. MC, however, requires an active bile transport system to be taken into hepatocytes [25], which is not found in immortalised hepatocyte or non-hepatocyte cell lines [26].

2.3.1. Effects of PP inhibition on protein kinases. The tumour-promoting activity of MC is likely to arise from its ability to potently inhibit PP2A (Fig. 1) which regulates several mitogen-activated protein kinases (MAPK) [27]. OA activates a variety of protein kinases (see Rossini [24] for a comprehensive review) possibly by activating the MAPK cascade via tyrosine kinase-linked G protein receptors which would in turn activate phospholipase C (PLC) as illustrated in Fig. 1. Generation of diacylglycerol (DAG) by cleavage of phosphatidylinositol 4,5-bisphosphate (PIP) would activate PKC which in turn would trigger activation of the MAPK cascade involving Raf-1, MEK, and ERK [20,24]. Two of the major forms of MAPK, ERK1 and ERK2, are regulated by the action of PP2A. Treatment of normal human and mouse fibroblasts with OA resulted in increased phosphorylation of the p42 MAPK, ERK2, within 60 min [28]. Another MAPK, p38, is also phosphorylated in a cascade resembling that of the ERKs and is thought to include some additional kinase regulatory steps [20,24,27]. It is important to note that the effect of OA on MAPKs is dependent on cell type, with no increase in ERK2 phosphorylation being observed in HeLa or HTC cells exposed to OA [28].

The MAPKs in turn regulate transcription of genes required for cellular proliferation, such as the transcription activator protein, c-Jun [20]. OA treatment of Jurkat cells induced an increase in synthesis of nuclear factor κB enhancing binding protein and transcription activator protein complex [17]. An increase in transcription of the proto-oncogenes c-*jun* and c-*fos* was also observed [17]. The activity of Jun-activating kinase (JNK), a PP2A-regulated enzyme [27], was induced in human monocytic leukaemia cells upon exposure to OA [29].

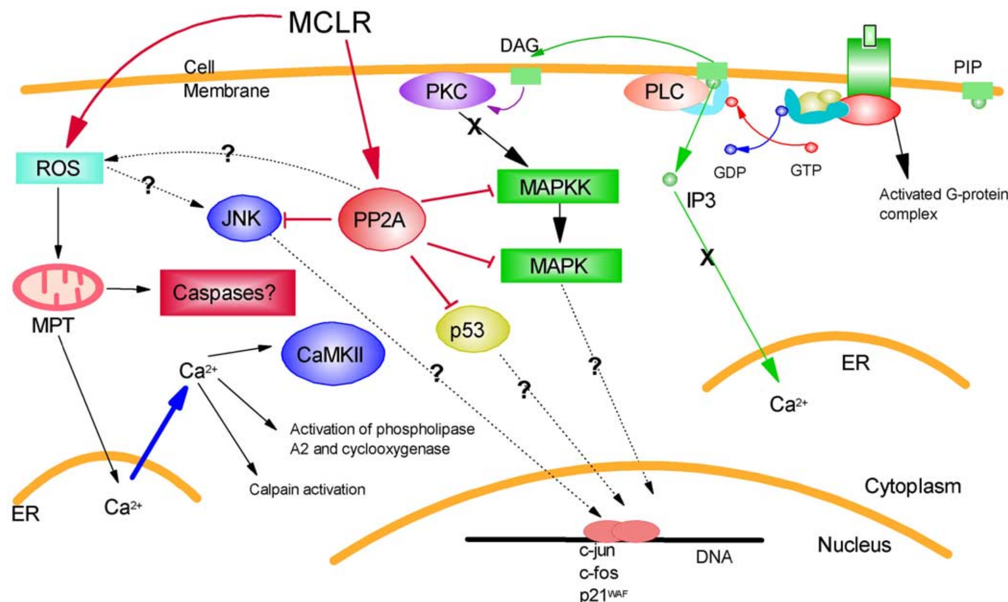

Fig. 1. The proposed scheme illustrating cellular events upon MCLR exposure. The MAPK pathway is usually activated by means of receptor tyrosine kinases activated by G-linked receptors which activate the cleavage of PIP to DAG and IP3 by PLC. IP3 triggers the release of Ca^{2+} from the endoplasmic reticulum (ER), but this does not occur for MCLR as MCLR inhibited vasopressin-induced Ca^{2+} release. DAG release and increased Ca^{2+} are known to activate PKC activation of MAPKK which in turn activates MAPK. PKC was not found to play a role in MCLR-induced kinase activation in vivo. PP2A regulates the phosphorylation of both MAPKK and MAPK so inhibition of this PP by MCLR would allow for the continual activation of both these kinases. Once activated MAPK is translocated to the nucleus where it activates transcription factors such as c-*jun* and c-*fos*, which initiate transcription of genes necessary for growth and differentiation. The MAPK, ERK2, is known to be activated by OA and is responsible for activating nuclear transcription factors, but this is unknown for MCLR (?). Increased phosphorylation of p53 occurs in MCLR-exposed hepatocytes, however the effect on $p21^{WAF}$ remains to be elucidated (?). MCLR induces the formation of reactive oxygen species (ROS) which triggers mitochondrial permeability transition (MPT) prior to the release of Ca^{2+} from the endoplasmic reticulum which activates CaMKII, phospholipase A_2, cyclooxygenase and the calpains. ROS are known to activate the PP2A-regulated kinase, JNK, which leads to increased c-*jun* activity, but the role of JNK in MCLR toxicology is not known (?). Cytochrome *c* release from the mitochondria after MPT usually results in activation of the caspase cascade, however the role of caspases in MCLR-induced apoptosis remains to be clarified.

PP2A negatively regulates several of the kinases involved in the MAPK cascade, and its inhibition by chronic, low level doses of MC could have severe regulatory effects on cell cycling [20]. It has been suggested that activation of MAPK may inhibit apoptosis, thereby providing an underlying mechanism for the tumour-promoting activity of the Ser/Thr PP inhibitors resulting in increased cellular proliferation (Fig. 1) [20].

2.3.2. The effect of PP inhibition on cellular proliferation. The eukaryotic cell cycle has checkpoints between the four phases G_0/G_1, S, G_2 and M, to regulate accurate cell division, the major control sites being found between G_1/S and G_2/M phases. Cyclin-dependent kinases (CDKs) play an important role in regulating the transition from one stage to the next, and their activity is controlled by their state of phosphorylation/dephosphorylation and presence/absence of their regulatory cyclin protein. A CDK1 target late in the G_1 and S phases is retinoblastoma (Rb) protein. In its phosphorylated state, Rb releases e2f transcription factor, which immediately leads to increased mRNA synthesis of the genes required for DNA replication [24]. Phosphorylation of Rb has also been found to be decreased in OA-exposed cells which have been serum-starved and then exposed to growth factors [17,24]. Contradicting results were obtained whereby increased phosphorylation of Rb was observed in ML-1 cells exposed to OA [30]. OA treatment of quiescent hamster and human fibroblasts pushed 30–40% of cells from G_0/G_1 to S phase [31]. This increased proliferation was accompanied by an increase in phosphorylation of Rb and MAPKs.

One can therefore conclude that OA can act as a stimulator of proliferation either by increasing the phosphorylation of proteins, such as Rb, or by preventing critical dephosphorylation of these proteins by PP2A. The effect of MC exposure on Rb phosphorylation has not been investigated.

OA exposure has resulted in increased expression of several genes, the most critical being that of $p21^{WAF1}$. This 21 kDa protein acts as an inhibitor of cyclin D-, E- and A-dependent kinases, thereby blocking the cell cycle in G_1. This deregulation of $p21^{WAF1}$ has been observed in fibroblasts and tumour cells exposed to OA [24].

The protein, p53, is an important regulator of the cell cycle and apoptosis. Increased phosphorylation of p53 halts cell cycling by increasing transcription of $p21^{WAF1}$. Its activity is controlled by conformational changes resulting from phosphorylation/dephosphorylation, which is regulated by PP2A and PP1, or inhibition of its degradation [23,24,32]. OA exposure induced increased phosphorylation of p53 in fibroblasts [33]. The increase in phosphorylation was not accompanied by an increase of transcription of *p53* and was found to precede apoptosis in BALB/3T3 cells exposed to 500 nM OA [34]. In its phosphorylated form, p53 has been found to both increase and decrease transcription rates in OA-exposed cells [35]. The OA-induced increase in phosphorylation of p53 was accompanied by an increase in its association with $p21^{WAF}$ and increased levels of mRNA for $p21^{WAF}$ [35].

There was an overall increase in transcription of repair and detoxification genes in the livers of rats exposed to a sublethal dose of MCLR and killed after 3 or 6 h [36]. Increased phosphorylation of p53 was observed in the livers of rats exposed to either a lethal (100 μg/kg) or sublethal (45 μg/kg) dose of MCLR [23]. This is the first evidence of MCLR-induced effect on cell cycle control and supports the data obtained for OA modulation of cell cycling. However, it is prudent to remember that MC and OA differ at least 10-fold in their inhibition of PP1 which may result in potentially different effects on cellular biochemistry.

3. Apoptosis and oxidative stress

3.1. In vitro investigations

OA-treated hepatocytes clearly undergo apoptosis as evidenced by internucleosomal DNA fragmentation, bleb formation and packaging of cytoplasmic organelles [17,37]. The first ever in vitro investigation into the toxic effects of a MC extract on primary hepatocytes demonstrated the characteristic apoptotic feature of cell membrane blebbing within 5 min of exposure to the toxin [38]. Primary rat hepatocytes have been used extensively to investigate the induction of oxidative stress and apoptosis arising from exposure to MCLR and this is the subject of a comprehensive review [10].

Exposure to MCLR extract resulted in increased lipid peroxidation, indicative of oxidative stress, in primary hepatocytes [39]. Increases in levels of reactive oxygen species (ROS) preceded the onset of mitochondrial permeability transition (MPT) and initiation of apoptosis in hepatocytes exposed to 1 μM MCLR [40]. Pre-exposure of hepatocytes to the iron chelator deferoxamine or cyclosporin A, a specific inhibitor of MPT, prevented MPT and cell death, thereby demonstrating the requirement of MPT in the MCLR-induced apoptotic cascade [39,40]. Generation of ROS (determined by changes in H_2O_2 and O_2^- concentrations) preceded the MCLR-induced collapse of the cytoskeleton and subsequent release of LDH in hepatocytes exposed to 1 μM MCLR in the medium [41]. Addition of a superoxide dismutase mimic prevented the collapse and LDH release, presumably by inactivation of the ROS [41].

OA-induced apoptosis in hepatocytes and the human hepatoma cell line Hep3B was found to be caspase-dependent [37]. Both DNA fragmentation and cleavage of poly(ADP-ribose) polymerase (PARP) were clearly visible after 48 h treatment with 50 nM OA [37]. Although cleavage of caspases 3/7, 2 and 9 was recorded, the exact mechanism of activation of OA-induced caspase-dependent apoptosis is not known [37,42]. Interestingly, no cleavage of caspase 8 was noted in either study.

Ceramide is a cell-signaling molecule implicated in the conflicting processes of either cell differentiation and growth inhibition or cellular proliferation [43]. It is known to interact with at least four cellular targets, including the ceramide-activated PP1 [44]. Serine/arginine-rich proteins (SR) are substrates of PP1 and known modulators of mRNA splicing. Ceramide activation of PP1 resulted in dephosphorylation of SR proteins [45]. More recently, it has been proposed that ceramide-induced apoptosis results from the alternative splicing of pre-mRNA for caspase 9 and $BclX_L$ mediated by PP1 [46]. This stage in the apoptotic pathway could conceivably be inhibited by MC.

Cytochrome *c* release was induced in primary hepatocytes exposed to 1 μM MCLR and addition of inhibitors of MPT markedly reduced the release of cytochrome *c* into the cytosol and significantly reduced the leakage of LDH from the hepatocytes [47]. The release of cytochrome *c* did not surprisingly activate caspase 9. Caspase 3 activities were reduced after exposure to MCLR and no PARP cleavage was observed. MCLR did, however, activate the calcium-activated cysteine

proteinase calpain [47]. The authors suggested that the increase in calpain activity resulted from MCLR-induced increase of Ca^{2+} in the cytoplasm; however, the exact mechanism of how MCLR affects the intracellular Ca^{2+} homeostasis is not yet known [10,47]. Increased activity of both milli- and micro-calpain were observed in MCLR-exposed Caco2 [48] cells suggesting that this mechanism extends to non-hepatic cells.

Apoptosis was induced in rat and salmon hepatocytes exposed to either 1 μM MCLR or OA [49]. Addition of the CaMKII inhibitor KN93 inhibited the development of apoptosis in hepatocytes exposed to MCLR. Both MCLR (16 μM) and nodularin (5 μM), a toxic pentapeptide cyanotoxin with PP inhibitory properties, induced apoptosis in primary hepatocytes [50]. Nodularin-induced apoptosis was inhibited by the addition of the general caspase inhibitor Z-VAD.fmk, but was independent of Bcl-2 levels in rat leukaemia cells and human breast carcinoma cells (MCF-7) treated with 250 or 500 μM toxin respectively [50].

MCLR-exposed hepatocytes exhibited an increase in overall cellular protein phosphorylation, which correlated with an increase in apoptogenic events within the cell [51]. The authors assumed that there could only be increased protein phosphorylation in cells exposed to a PP inhibitor if there was increased kinase activity. The multifunctional CaMKII is activated by intermolecular autophosphorylation at Thr^{286} in the presence of calcium and calmodulin. PP1 and PP2A are able to reverse this autophosphorylation at Thr^{286}, however inhibition of these PPs by MCLR would lead to activated CaMKII. Addition of the CaMKII-specific inhibitors KN93 and KN5926 to MCLR-exposed hepatocytes completely inhibited the increase in protein phosphorylation and prevented the apoptotic cascade seen in cells not treated with the inhibitor [51]. Inhibitors targeting casein kinase, cyclin-dependent kinases and tyrosine kinases did not prevent apoptosis when added to the medium with 0.4 μM MCLR. Microinjection of inhibitors specific for myosin light chain kinase and PKC together with various MCLR concentrations into rat hepatocytes did not protect the cells from death [51].

Cells not able to actively take up MC have been shown to undergo morphological changes indicative of apoptosis in response to exposure to MC, albeit at concentrations considerably higher than observed for primary rat hepatocytes [8,9,52]. The protection offered by CaMKII inhibitors was shown to be effective in non-hepatocyte cell lines: mouse embryonic fibroblasts (C3H C18 and Swiss 3T3), normal rat kidney cells (NRK), human embryonic kidney cells (HEK293), monkey kidney cells (COS-1) and rat promyelocytic leukaemia cells (IPC-81) [51].

As previously mentioned, CaMKII kinase is activated by an increase in Ca^{2+} in the cytosol. Inhibition of vasopressin-induced receptor-mediated and G protein phosphoinositide turnover occurred in rat hepatocytes exposed to 1 μM MC [53]. MC also inhibited the inositol 1,4,5-triphosphate (IP3) induction of angiotensin II and epinephrine. A consequence of IP3 release is the increase of intracellular Ca^{2+} release from the endoplasmic reticulum. An increase in cytosolic Ca^{2+} alone is not sufficient to induce apoptosis, as activation of CaMKII by the Ca^{2+}-elevating agent vasopressin did not result in apoptosis [51]. CaMK was also implicated in the increased phosphorylation of the intermediate filament K8 in hepatocytes exposed to 10 μM MCLR, but did not affect the phosphorylation state of K18 [19].

Nobre et al. [54] found that MCLR impaired the renal function of rats and these impairments could be antagonised by dexamethasone and indometacin. A previous study found that dexamethasone inhibited the release of arachidonic acid induced by MCLR in rat hepatocytes [55]. It is also known that MCLR induces cyclooxygenase via the release of arachidonic acid and that these events are related to the release of calcium in the cell [54]. The PP inhibitor activity of MCLR may lead to the activation of PKC which could in turn also activate phospholipase A_2 [54]; however, as PKC does not play a role in MCLR-induced cell death [51], phospholipase A_2 activation presumably arises from increased release of Ca^{2+} in the cytoplasm. Phospholipase A_2 activation is also observed in OA-exposed hepatocytes [24]. Recent work suggests a role for tumour necrosis factor α in MCLR-induced activation of phospholipase A_2 [56].

3.2. *In vivo investigations*

Mice given a lethal dose of MCLR (57.6–101.3 μg/kg), showed extensive bleeding in the centrilobular regions at death [57]. Both the cytoplasm and nuclei of the centrilobular hepatocytes surrounded by haemorrhaging stained strongly for MCLR. Mice given a lower dose (57.6 and 69.1 μg/kg) were killed at 24 h. Sections of liver exhibited focal coagulative necrosis, mainly in the midzonal areas extending towards the central veins which did not stain for MCLR. Mice dosed with sublethal toxin doses (20, 30 and 45 μg/kg) and killed after 7 days exhibited high levels of apoptotic cells in the centrilobular and perinecrotic regions, some of which correlated with positive immunostaining for MCLR. The authors suggested a role for MCLR-induced apoptosis; however, the fact that no MCLR was found in the necrotic regions suggested a role for damage due to ischaemia/hypoxia [57].

The role of oxidative stress in MC-induced toxicosis was investigated in rats continually dosed with purified MCLR at three different dose levels: low (16 μg/kg), medium (32 μg/kg) and high (48 μg/kg) [7]. There were no significant changes in liver weights, a clear indication of haemorrhage induced by exposure to MCLR, between the control and experimental animals. High dose rats showed apoptosis in the centrilobular areas, with fewer apoptotic cells visible in the medium dose rats. Apoptotic cells were a rare occurrence in the low dose rats. Levels of malondialdehyde, a by-product of lipid peroxidation, were significantly raised in the medium and high dose rats, with levels of malondialdehyde in low dose level rats approaching those of the controls [7]. As lipid peroxidation is a good indicator of oxidative stress in cells, the authors concluded that chronic exposure to sublethal doses of MCLR resulted in oxidative stress and suggested that it may play an important role in the chronic liver damage induced by MCLR toxicosis at sublethal levels. Lipid-derived free radicals were formed in the livers of rats treated with 50 μg/kg MCLR [58]. Repeated exposure of mice to a 75% LD_{50} dose of MCLR resulted in an increase in malondialdehyde levels in the liver [59]. An increase in lipid peroxidation levels was also recorded in the livers of mice receiving a single sublethal dose of MCLR [60]. The MCLR-induced increase in lipid peroxidation preceded an increase in transcription and activity of glutathione peroxidase in the liver.

Apoptosis was only detectable in the livers of mice 24 h after a single sublethal dose of 45 μg/kg MCLR [6]. No apoptotic cells were visible in the livers of mice killed at 2, 4 and

12 h. Mice killed after 2 h exhibited enlarged hepatocytes accompanied by a loss in vacuolation, an indication of glycogen depletion, which stained intensely in the cytoplasm for the presence of MCLR thereby indicating a strong correlation between the presence of the toxin in the cell and the altered morphology. Mice exposed to four or seven doses of MCLR showed extensive hepatocytomegaly, but again, only small numbers of apoptotic cells were observed in both treatment groups [6].

4. Tumorigenesis

Regular monitoring of formal water sources aims to reduce exposure of individuals to the WHO level of MC of 1 μg/l. Studies in China, however, suggest that long-term chronic exposure to much lower concentrations of toxin may pose a far greater risk to people exposed on a regular basis to toxin in their drinking water [3,5]. The elucidation of the process of MCLR-induced tumorigenesis has been approached from several directions.

4.1. In vitro tumorigenesis

The ability of MC to act as a tumour initiator would require it to induce damage at the DNA level in addition to inhibiting the PPs to promote cellular proliferation. Baby hamster kidney cells (BHK-21) and mouse embryo fibroblast (MEF) cells exposed to MC containing extract or pure MC exhibited severe fragmentation of DNA at the higher dose levels of 100 μg/ml extract, and 1 and 2 μg/ml MCLR [61]. The fragmentation was time-dependent with the most fragmentation observed after 3 h exposure to the toxins. OuaR mutations, a measure of genotoxicity, were induced in a dose-dependent manner in human Rsa cells exposed to 15 μg/ml MCLR with 170 OuaR mutants per 10^4 survivors [62]. Control cells had only one OauR cell per 10^4 survivors. MC-exposed cells showed mutations at K-ras codon 12. A MC-containing extract induced DNA damage in hepatocytes and showed strong mutagenicity in the Ames test [63]. MCLR-induced DNA strand breaks in the human hepatoma cell line HepG2 occurred in a time- and dose-dependent manner [64].

By far the most comprehensive in vitro investigation on MCLR-induced increased cellular proliferation was done in hepatocytes derived from C3H/HeJ mice [65]. Cells numbers increased significantly after exposure to 1 nM MCLR for 18 and 42 h. This increase was not, however, accompanied by an increase in total DNA levels or DNA synthesis, suggesting that octaploid and tetraploid hepatocytes had undergone cell division without a round of DNA replication. Hepatocytes exposed to 3 nM MCLR increased slightly in cell number after 18 and 42 h, whereas exposure at 10 nM reduced cell numbers by 50% within 18 h. Repeated exposure of hepatocytes to MCLR at concentrations ranging from 10 to 100 pM resulted in an increase in hepatocyte cell numbers as seen for a single dose of 1 nM MCLR. OA was approximately 10-fold more toxic than MCLR at 10 nM with DNA synthesis being inhibited at all concentrations greater than 0.3 nM OA [65].

4.2. In vivo tumorigenesis

The obvious approach to investigate MCLR tumorigenesis is to conduct long-term, oral dose studies in susceptible animals. Four of 71 mice receiving the highest concentration of an extract of MC developed liver tumours, whereas only two of the 223 mice receiving the lower dose developed any tumours after a year [66].

The tumour promoter activity of OA was demonstrated using a two-stage carcinogenesis model whereby an initiator is first administered to the test animal prior to the potential tumour promoter under investigation [17]. A topical epithelial tumour initiator, dimethylbenzanthracene, was applied to the skin of the backs of mice prior to repeated administration of MCLR [67]. There were significantly bigger tumours on MC-exposed mice than on the control mice after 52 days supporting the theory that MCLR was indeed a tumour promoter.

This was confirmed in rats dosed with levels of MCLR below acute toxicity levels, after initially being primed with the liver-specific tumour initiator diethylnitrosamine (DEN) [68]. Rats treated repeatedly with 10 μg/kg, twice a week for 5 weeks, demonstrated increased numbers of placental glutathione *S*-transferase (GST-P)-positive foci per square centimetre of liver tissue when compared to the lower dose group and the control animals. The development of MCLR-induced GST-P-positive liver tumour foci was found to be dose-dependent [68].

In order to further investigate the duodenal and/or adenocarcinoma tumour-promoting activity of MC, mice were dosed with *N*-methyl-*N*-nitrosourea, a tumour initiator, followed by oral *Microcystis* toxin in their drinking water for 154 days [69]. No significant difference in tumour formation was recorded between the control and experimental groups suggesting that MC is not a tumour promoter for duodenal and/or adenocarcinomas [69]. Male C57BL/6J mice were dosed with the tumour initiator azoxymethane and provided with MC extract (equivalent to a daily dose of 382 and 693 μg/kg) in their drinking water to investigate the potential of MCLR to promote tumours in the gastrointestinal tract [70]. The size of aberrant crypt foci, indicative of the development of colon cancer, was significantly raised in the colons of MCLR-exposed animals, suggesting an increased risk for the development of bowel cancer upon exposure to MC [70].

Exposure to MCLR coupled with a demonstrated tumour initiator such as aflatoxin B_1 may result in an increased rate of liver cancer [71]. Aflatoxin B_1 did not, however, act synergistically with MCLR in increasing the numbers of GST-P-positive foci in the livers of DEN-initiated rats exposed to both agents. A synergistic effect was observed in rats initiated with aflatoxin B_1 and subsequently exposed to MCLR at 1 or 10 μg/kg, supporting the role of MCLR as a tumour promoter [71].

The ability of MC to act as a tumour initiator was also investigated. Neoplastic nodules were identified in the livers of ICR mice injected with 100 doses of MCLR (20 μg/kg) for 28 weeks [72]. Orally administered toxin (80 μg/kg MC, 100 times for 28 weeks) did not induce the formation of any liver damage or nodule formation.

MCLR induced random DNA fragmentation in the livers of mice injected with MCLR, which correlated to increased hepatocellular damage as measured by release of LDH, alkaline phosphatase and γ-glutamyl transferase [73]. MCLR-induced DNA damage was time- and dose-dependent [61]. MCLR targeting of hepatocyte nuclei was demonstrated after 2 and 4 h in the livers of mice having received a single dose of 45 μg/kg [6]. These studies suggested that MCLR may exert

an effect at the DNA level which could possibly lead to increased cellular proliferation.

5. Discussion

It is apparent after reading the current literature that there is no clear picture of the exact mechanism of toxicity of MC in the liver. That it inhibits PP1 and PP2A is a crucial pivot with far-reaching consequences in cellular biochemistry, most noticeably the 'activation' of cellular kinases. Whether the hepatocyte undergoes apoptosis or increased cellular proliferation appears to be dependent on the effective concentration of toxin to which it is exposed as demonstrated in vitro [65]. Generation of ROS is also an important consequence of MC exposure [41] with far-reaching effects on cellular activities including activation of MPT [74], calpain [47], CaMKII [51] and phospholipase A_2 [54] activities. In addition, oxidative stress is a known stimulus of MAPK and H_2O_2 can induce the activity of JNK [75]. Either or both of these mechanisms could induce hepatic damage leading to necrosis/apoptosis and/or increased cellular proliferation [10,20]. The extent of injury in vivo is in turn dependent on the level of exposure to the toxin [57], efficiency of uptake [25] and the detoxification process within the liver to remove both MC and MCLR-induced ROS [10]. It is difficult to compile a complete overview of the toxic effects of MC on the liver as some investigators make use of purified toxins while others choose to use the more realistic model of a MC extract. An additional complication is comparing results obtained from studies undertaken in vitro which may not necessarily apply to the in vivo scenario.

To summarise, the induction of apoptosis in vitro in MCLR-exposed hepatocytes is closely linked to the generation of ROS, MPT, Ca^{2+} release into the cytoplasm and inhibition of PP1 and PP2A. The role of caspases and proteases in MCLR-induced programmed cell death in vivo is an active area of research. In vitro exposure to MCLR induces increased lipid peroxidation in the liver, indicating increased

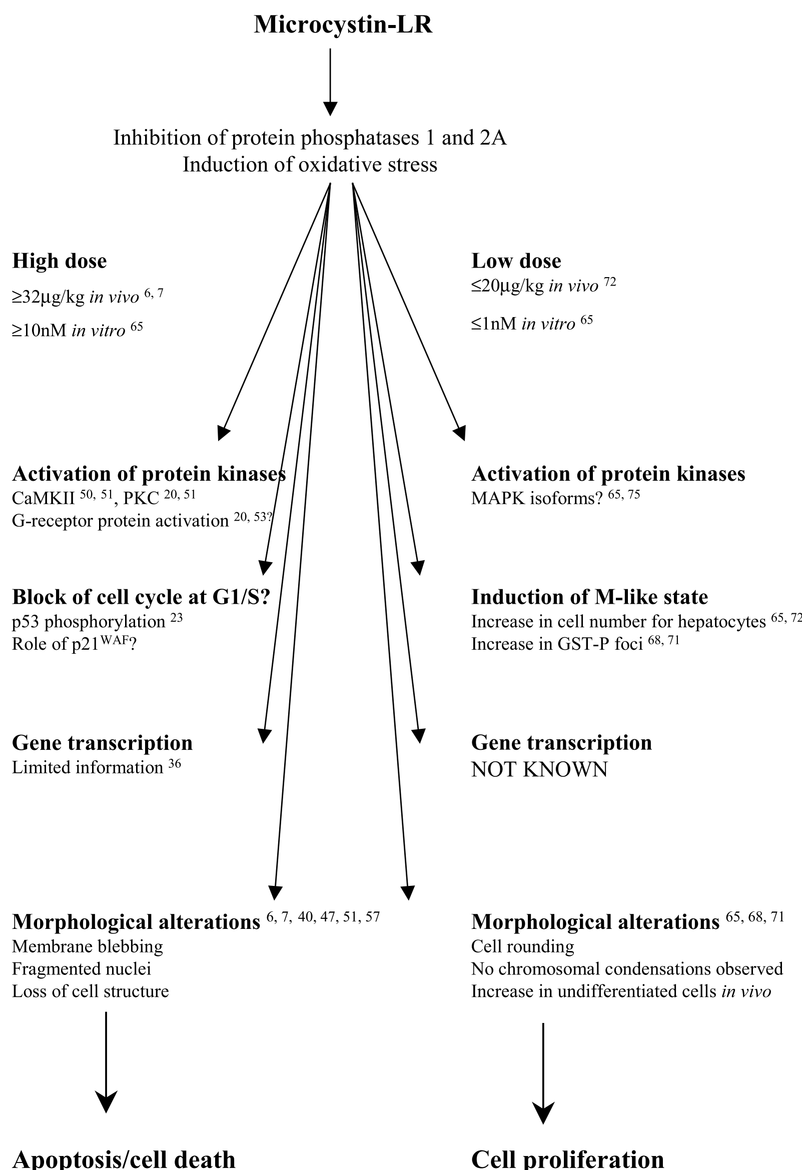

Fig. 2. Simplified view of the molecular basis underlying the dualistic responses triggered in cells exposed to the PP inhibitor MCLR. Modified from Rossini [24] for MCLR.

oxidative stress; however, the causal link to apoptosis is not as clear as in vitro studies suggest. Apoptosis occurs after a delayed period in animals given a sublethal dose of toxin and in patches within the liver tissue. The occurrence of apoptotic cells on the periphery of necrotic areas suggests a role for ischaemia in the development of apoptosis in MCLR-treated animals. The difference in the induction of apoptosis between in vivo and in vitro studies may be attributed to single hepatocytes being exposed to varying levels of toxin, the level dependent on two processes: uptake by the bile transport system and clearance by the glutathione detoxification pathway.

That MC targets the nucleus [76] and appears to damage the DNA [62,73] may influence expression of DNA in a MC-exposed hepatocyte. In addition to this, inhibition of PPs would influence activation of transcription factors such as c-*fos* and c-*jun*, as demonstrated for OA (Fig. 1).

In vitro studies have clearly illustrated the ability of MCLR to induce DNA damage in exposed cells. They have also more clearly differentiated between cell death and proliferation induced in mouse hepatocytes exposed to varying levels of MCLR. Low doses of MCLR (≤ 1 nM) appear to cause an increase in cell numbers while higher doses of toxin (≥ 10 nM) reduce cell viability [65]. In vivo studies indicate a clear role for MCLR to act as a tumour promoter at dose levels in the range of 1–10 μg/kg i.p. or 382–693 μg/kg orally, being associated with the development of liver, skin and possibly colon cancer.

The fact that apoptosis is generally only reported at high doses of MC both in vivo and in vitro and increased cellular proliferation is observed at lower concentrations supports the dualistic response to this toxin as summarised in Fig. 2. It is accepted that apoptosis and proliferation often occur simultaneously within the same cell in the liver [51,75]. It is possible that the observed tumour promotion observed for MCLR could be due to MAPK signaling which stimulates cellular proliferation while inhibiting apoptosis. There is also very little evidence to suggest that one of the direct causes of hepatic injury resulting from chronic low level MC exposure in vivo is the result of apoptosis. It is more likely that the apoptosis observed at higher dose levels is the result of hypoxia resulting from obstruction of blood flow to the hepatic tissues. Apoptosis has not been noted at the low levels of toxin used to induce tumours or cellular proliferation either in vivo or in vitro. It is clear that more information is required to further elucidate the toxic effects of MC on the liver of exposed individuals, be it acute or long-term chronic exposure.

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