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Microcystin-LR Induced Liver Injury in Mice and in Primary Human Hepatocytes is Caused by Oncotic Necrosis

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Abstract

Microcystins are a group of toxins produced by freshwater cyanobacteria. Uptake of microcystin-leucine arginine (MC-LR) by organic anion transporting polypeptide 1B2 in hepatocytes results in inhibition of protein phosphatase 1A and 2A, and subsequent cell death. Studies performed in primary rat hepatocytes demonstrate prototypical apoptosis after MC-LR exposure; however, no study has directly tested whether apoptosis is critically involved *in vivo* in the mouse, or in human hepatocytes. MC-LR (120µg/kg) was administered to C57BL/6J mice and cell death was evaluated by alanine aminotransferase (ALT) release, caspase-3 activity in the liver, and histology. Mice exposed to MC-LR had increases in plasma ALT values, and hemorrhage in the liver, but no increase in caspase-3 activity in the liver. Pre-treatment with the pan-caspase inhibitor z-VAD-fmk failed to protect against cell death measured by ALT, glutathione depletion, or hemorrhage. Administration of MC-LR to primary human hepatocytes resulted in significant toxicity at concentrations between 5nM and 1µM. There were no elevated caspase-3 activities and pretreatment with z-VAD-fmk failed to protect against cell death in human hepatocytes. MC-LR treated human hepatocytes stained positive for propidium iodide, indicating membrane instability, a marker of necrosis. Of note, both increases in PI positive cells, and increases in lactate dehydrogenase release, occurred before the onset of complete actin filament collapse. In conclusion, apoptosis does not contribute to MC-LR-induced cell death in the *in vivo* mouse model or in primary human hepatocytes *in vitro*. Thus, targeting necrotic cell death mechanisms will be critical for preventing microcystin-induced liver injury.

Keywords

Microcystin; apoptosis; hepatocytes; transporters; necrosis; primary human hepatocytes

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1.1 Introduction

Microcystins are a family of structurally similar toxins produced by freshwater cyanobacteria, a blue-green algae species, that includes the species *Microcystis aeruginosa* (Hooser et al., 1989). The most commonly studied microcystin species is microcystin leucine arginine (MC-LR), where leucine and arginine replace the variable amino acid residues present on the microcystin backbone (Bischoff et al., 2001). Intoxication with microcystin toxin results in potent inhibition of protein phosphatases in mammalian cells (Honkanen et al., 1990; MacKintosh et al., 1990). Subsequent to this inhibition, a number of different potential toxic mechanisms have been proposed (Ku et al., 1998; Krakstad et al., 2006; Liu and Sun, 2015). In rat primary hepatocyte culture, exposure to MC-LR results in rapid mitochondrial dysfunction, and prototypical apoptosis (Ding et al., 2000). As such, apoptosis has become the most commonly cited mechanism for MC-LR induced cell death in most models (Kleppe et al., 2015; Chen and Xie, 2016); however, considerable alanine aminotransferase (ALT) release (van der Merwe et al., 2012), and hemorrhagic necrosis (Theiss et al., 1988; Bautista et al., 2015), have been consistently noted in pathological reports of animal exposure to MC-LR. Moreover, MCF-7 breast cancer cells with a defective caspase-3 enzyme, which are highly resistant to apoptosis, are sensitive to MC-LR (Fladmark et al., 1999). The toxicity is sensitive to caspase inhibitors, but only after caspase-3 activity is exogenously restored (Fladmark et al., 1999), indicating that these cells are likely undergoing necrosis, and not apoptosis, in the presence of a defunct caspase-3 enzyme. As such, it is clear that MC-LR is also capable of inducing oncotic necrosis (referred to simply as necrosis hereafter) in lieu of apoptosis under some conditions. Understanding which modality of cell death MC-LR induces in clinically relevant models is imperative, as apoptosis and necrosis are fundamentally different from a therapeutic perspective (Jaeschke and Lemasters, 2003). This is even more striking given that caspase inhibitors that might completely prevent apoptosis after MC-LR poisoning are currently in clinical trials (Baskin-Bey et al., 2007), and offer a potent potential therapeutic if apoptosis is also present in human overdose cases.

The World Health Organization has a suggested threshold value of 1µg/L (1nM) for microcystin concentrations in water designated for human use. Human poisonings with microcystin are relatively rare due to constant water monitoring. Previous instances of microcystin poisoning present with severe toxicity though including acute liver failure (ALF) and death in a majority of patients (Pouria et al., 1998). In the case of an undetected toxic water source, substantial morbidity and mortality is likely without a greater understanding of microcystin toxicity. While patient samples are difficult to attain, understanding microcystin toxicity in human patients is imperative. As such, primary human hepatocytes remain the gold standard for understanding toxins in humans, especially in cases where there are a limited number of patient samples. Reports of microcystin toxicity in primary human hepatocytes are limited (Ikehara et al., 2015), but generally indicate humans undergo many of the same mechanisms present in rodent models, including inhibition of protein phosphatases at nM concentrations of model microcystin toxins such as MC-LR (Batista et al., 2003; Ikehara et al., 2015).

Thus, the purpose of this study was to confirm the presence of apoptosis both *in vivo* in the murine model and *in vitro* in primary human hepatocytes. Surprisingly, we found inhibition of caspases failed to protect against any of the common biochemical or histological abnormalities associated with microcystin toxicity *in vivo*. More importantly, while human hepatocytes are acutely sensitive to even very low doses of MC-LR, there is no evidence for increased apoptosis in these cells.

2.1 Methods

2.1.1 Animals and Experimental Protocol

Eight to ten week old male C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals were maintained in an environmentally controlled room with *ad libitum* access to both food and water. All procedures herein were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee before the onset of experimentation. Microcystin-LR (Cayman Chemical, Ann Arbor, MI) was diluted into PBS and administered at 120µg/kg i.p. to mice in the morning, whereupon mice were sacrificed after 45 minutes or 90 minutes by exsanguination and cervical dislocation. Alternately, some mice were given 700mg/kg galactosamine and 100µg/kg lipopolysaccharide i.p. as a separate model of established apoptosis (Jaeschke et al., 1998). Blood was stored on ice until centrifugation at 12,000 $\times g$ for 3 minutes to acquire plasma. Liver sections were snap frozen in liquid nitrogen for later usage. A portion of the liver was stored in buffered formalin for immunohistochemistry.

2.1.2 Isolation and Culture of Human Hepatocytes

Primary human hepatocytes were freshly isolated from liver resections by the Biospecimen Core in the Department of Pharmacology, Toxicology and Therapeutics at the University of Kansas Medical Center as described previously (Xie et al., 2014; Woolbright et al., 2016). Informed consent was obtained from all patients before liver samples were processed, and the study was approved by the Institutional Review Board at University of Kansas Medical Center. Cells were allowed to attach for 3 hours before the onset of experimentation. Williams' E Media was changed during this period and MC-LR was directly added to the media at the indicated concentration. In some experiments, z-VAD-fmk, a potent pan-caspase inhibitor, or vehicle control was added 30 minutes prior to the addition of MC-LR, or galactosamine and TNF- α as described previously (Woolbright et al., 2015).

2.1.3 Histology

Liver sections were cut into 5µM sections after fixation and stained with hematoxylin and eosin for evaluation of liver injury and hemorrhage. TUNEL staining was performed according to manufacturer's suggested protocol (Roche, Basel, Switzerland). TUNEL positive cells were counted in five separate high powered fields. Generally, cells with nuclear staining were considered to be apoptotic, and those with cytoplasmic and nuclear staining were deemed necrotic as our group and others have shown previously (Grasl-Kraupp et al., 1995; Jaeschke et al., 2011).

2.1.4 Western Blotting

Western blots for caspase-3 were performed as described previously (Woolbright et al., 2013). In brief, 30µg of protein was loaded into a Miniblot electrophoresis apparatus (Invitrogen, Carlsbad, CA). The gel was transferred to nitrocellulose membrane and immunoblotted using an anti-caspase-3 antibody (Ab# 9665), or β-actin antibody (Ab#4970) (Cell Signaling, Danvers, MA).

2.1.5 Cell Death Analysis *in vitro*

Cell death *in vitro* was assessed by lactate dehydrogenase (LDH) release, as described previously in detail (Bajt et al., 2004). ALT activities were measured using a kinetic ALT assay kit according to manufacturer's instructions (Pointe Scientific, Pointe, WI). Caspase-3 activity was measured using a fluorescent substrate as described (Jaeschke et al., 1998). In brief, liver homogenate was incubated with caspase-3 substrate Ac-DEVD-fmk according to manufacturer's suggested instructions (Enzo Biochem, Farmingdale, New York), either with or without z-VAD-fmk, a potent caspase inhibitor, to allow for the elimination of caspase-independent background fluorescence. Fluorescence was measured using a SpectraMax fluorescence plate reader and normalized to mg protein (Jaeschke et al., 1998).

2.1.6 In Vitro Fluorescent Staining

Primary human hepatocytes were seeded on 35mm glass dishes and treated with the indicated concentration of MC-LR for up to six hours. Propidium iodide (Invitrogen, Carlsbad, CA) or DAPI dye were added for the final five minutes of the treatment, before washing the cells twice with PBS and replacing the media with fresh PBS. TUNEL staining was performed according to manufacturer's protocol (Roche, Basel, Switzerland). Phalloidin stain was performed using an Oregon-488 phalloidin antibody according to manufacturer's instruction (Thermo, Waltham, MA). The live cells were imaged on a Zeiss Axiovert inverted microscope to assess PI uptake or DAPI staining. Imaging was done using the same fields at the same time and superimposed later using Image J software.

2.1.7 Glutathione (GSH and Glutathione Disulfide (GSSG) Measurements

GSH was measured using a modified Tietze assay described in detail elsewhere (McGill and Jaeschke, 2015).

2.1.8 Statistical Analysis

All data represent the mean ± the standard error. Population sizes for experiments are listed in the figure legend, but were typically between 4–5 animals per group and the average of minimum 3 repeats for cell culture experiments. Comparisons between multiple groups were done using one-way ANOVA with a Student-Neuman-Keuls post hoc test. Direct comparisons between two groups were performed using a *t*-test. If the data were not normally distributed, the Mann-Whitney test was applied for comparison of 2 groups instead. $p < 0.05$ was considered significant.

3.1 Results

3.1.1 Microcystin causes rapid and progressive destruction of the liver

In order to better understand microcystin-induced toxicity *in vivo*, C57BL/6J mice were treated with 120µg/kg MC-LR intraperitoneally, or PBS as a control, and toxicity was assessed after 45 and 90 minutes. The dose and route of injection were based on a pilot study, and designed to mimic clinical findings (Pouria et al., 1998). These time points were chosen as previous poisonings in humans have indicated that microcystin toxicity occurs very rapidly in clinical settings in patients (Pouria et al., 1998). Consistent with this assessment, mice dosed with microcystin had severe liver injury after 90 minutes (Figure 1). Histologically, there was obvious liver damage and hemorrhage in mice after MC-LR administration (Figure 1A). MC-LR administration led to significant increases in plasma ALT as early as 45 minutes after administration and these values were further elevated after 90 minutes (Figure 1B). In order to quantify the hemorrhage, hemoglobin was measured in liver samples of MC-LR treated mice or controls (Figure 1C). Hemoglobin, which is typically restricted to red blood cells, was substantially elevated as early as 45 minutes after MC-LR administration, and was further increased after 90 minutes confirming extensive vascular leakage. To further support the toxicity we also assessed hepatic glutathione levels. MC-LR treatment resulted in significant depletion of GSH within 45 minutes after exposure (Figure 1D). In conclusion, the release of ALT combined with increased liver levels of hemoglobin and histological assessments indicate MC-LR treatment results in rapid and progressive hemorrhagic necrosis in the mouse.

Given the rapid destruction of the liver, we next wanted to assess whether or not this occurred through apoptosis. While the TUNEL assay is commonly used as a way to assess apoptosis, our group and others demonstrated that this assay marks both apoptotic and necrotic cells when DNA fragmentation is present (Grasl-Kraupp et al., 1995; Jaeschke et al., 2011). TUNEL staining is largely limited to the nucleus in the case of apoptotic cells, whereas necrotic cells will leak nicked DNA into the cytosol, and thus the entire cell will stain darkly. We performed TUNEL stains on MC-LR treated mice, as well as mice treated with 700mg/kg galactosamine and 100µg/kg endotoxin (Gal/LPS), as a well-defined control for apoptosis (Jaeschke et al., 1998). Cytoplasmic inclusion of TUNEL stain was readily apparent in MC-LR treated mice and predominated over nuclear only stains (Figure 2A). This was in sharp contrast to the Gal/LPS model where numerous nuclei stained positive for TUNEL without any cytoplasmic inclusion (Figure 2A). Quantification of TUNEL positive cells yielded an increase that paralleled the increase in ALT observed previously (Figure 2B) indicating that the ALT release was likely a result of necrosis. These data suggest necrosis is the primary form of cell death in mice.

3.1.2 Caspase inhibition fails to protect against MC-LR toxicity

Caspase inhibition with the pan-caspase inhibitor z-VAD-fmk is protective against a number of different liver diseases that are primarily apoptotic in nature, and is effective both *in vitro* (Woolbright et al., 2015) and *in vivo* (Jaeschke et al., 1998; Bajt et al., 2000). To confirm the apoptosis-independent cell death in the mouse model, mice were pre-treated with 5mg/kg z-VAD-fmk and then received either MC-LR or Gal/LPS. Pre-treatment with the caspase

inhibitor failed to provide any protective effect in MC-LR treated mice (Figure 3A–C). Histology indicated widespread liver injury in both MC-LR and Gal/LPS treated mice; however, while Gal/LPS mice were strongly protected against liver injury indicated by histology (Figure 3A), ALT release (Figure 3B), and caspase-3 activity (Figure 3C), there was no protection against any of these parameters in MC-LR treated mice. Moreover, there was no significant elevation in caspase-3 activity in MC-LR treated mice, which strongly argues against any persistent apoptotic injury in the model (Figure 3B). Gal/LPS mice were partially protected against GSH depletion, and almost fully protected against formation of oxidative stress as indicated by reduction of GSSG formation (Figure 4A,B). In contrast, MC-LR treated mice had depletion of GSH and formation of GSSG, yet there was no protection with z-VAD-fmk pretreatment (Figure 4A and Figure 4B). As such, it can be concluded that oncotic necrosis predominates the injury process during MC-LR toxicity in the murine model.

3.1.3 MC-LR causes cell death in primary human hepatocytes

Primary human hepatocytes remain the gold standard for human toxicity testing. As such, we wanted to determine which model, the *in vivo* mouse model, or the *in vitro* rat model, more closely approximated the human response to MC-LR. Furthermore, to confirm our data *in vivo* in the mouse, we wanted to examine the same factors in primary murine hepatocytes. Primary human hepatocytes were isolated from normal resection material and primary mouse hepatocytes were isolated as described previously (Xie et al., 2014; Woolbright et al., 2015). Exposure to increasing concentrations of MC-LR indicated primary human hepatocytes were acutely susceptible to MC-LR (Figure 5A). Concentrations above 5nM produced toxicity in as little as 3 hours in human hepatocytes (Figure 5A). Concentrations of 1nM and below failed to produce any increase in LDH release even when these cells were incubated for up to 72 hours (data not shown). Mouse hepatocytes had a minimal increase in cell death when exposed to the same concentrations of MC-LR (Figure 5B). Of note, there was obvious toxicity in human samples at doses at and above 50nM, but no toxicity in murine samples at 3 hours (Figures 5A and 5B); however, toxicity was similar at later time points and higher doses (doses above 200nM). Propidium iodide staining was used to ascertain whether or not membrane instability indicative of necrosis occurred in primary human hepatocytes exposed to MC-LR. Significant propidium iodide staining was present in both murine and human samples treated with 200nM MC-LR for 3 hours, indicating membrane instability and necrosis. Thus, oncotic necrosis appears to be the primary modality of cell death in MC-LR exposed primary human and mouse hepatocytes.

3.1.4 Caspase inhibition fails to protect against MC-LR induced toxicity in primary human hepatocytes

To more conclusively determine if MC-LR toxicity occurred through necrosis or apoptosis in primary human hepatocytes, or murine hepatocytes, cells were treated with 200nM MC-LR for 3 hours, with or without 10μM z-VAD-fmk, and LDH release and caspase-3 activity were assessed. Pre-treatment with z-VAD-fmk failed to protect against LDH release, in MC-LR human hepatocytes (Figure 6A) or murine hepatocytes (Figure 6B), although z-VAD-fmk pre-treatment did protect against TNF-α induced apoptosis *in vitro* in primary human hepatocytes (Figure 6C). There was no increase in caspase-3 activity in either primary

human hepatocytes or murine hepatocytes after MC-LR (Figure 6C,D). To confirm these results, a western blot for caspase-3 activation was performed (Figure 6E and Figure 6F). MC-LR treated hepatocytes displayed no activation of caspase-3 by western blot though. These data indicate that these hepatocytes likely undergo necrosis primarily after exposure to MC-LR. MC-LR is known to cause significant cytoskeletal disruption (Eriksson et al., 1989). We wanted to further determine if necrosis happened before or after the well characterized cytoskeletal disruption. As the propidium iodide staining was largely present in cells with what appeared to be normal cytoskeletal architecture in primary human hepatocytes, we co-stained cells with either DAPI and phalloidin to stain the nucleus and actin, respectively, or DAPI and propidium iodide to assess cell death (Figure 7). Co-staining between propidium iodide and phalloidin was not achievable as all cells become positive to PI after fixation and permeabilization. Regardless, obvious actin breakdown occurred at 6 hours post 200nM MC-LR treatment (Figure 7). Of note, this occurred significantly after obvious increases in overall cell death as evidenced by both PI staining (Figure 7) and LDH release (Figure 5, 6). As such, a portion of cells undergo necrosis before the onset of cytoskeletal disruption in human hepatocytes.

Given the entirety of this data, it can be concluded that both in the *in vivo* murine model, and in primary human and murine hepatocytes, apoptosis plays a very limited role in hepatocyte cell death and disease progression. In contrast, a majority of cell death occurs through necrosis in the tested models.

4.1 Discussion

Critical to preventing microcystin-induced liver injury is understanding the molecular basis behind microcystin-induced hepatocyte cell death. We used the model microcystin toxin MC-LR in this study. The current literature largely focuses on apoptosis as a form of cell death after MC-LR induced liver injury (Ding et al., 2000; Kleppe et al., 2015; Chen and Xie, 2016). While this hypothesis has been extensively tested in primary rat hepatocytes, and transfected cell line models (Ding et al., 2000; Komatsu et al., 2007; Takumi et al., 2010), there has been limited testing in the mouse. Previous studies have indicated that there is a minor amount of caspase activation in ICR mice treated with microcystin (Wei et al., 2008); however, whether or not direct inhibition of caspase activity protects against the injury was not tested. To address this potential issue, we assessed apoptosis in three models of MC-LR induced liver injury, including the use of primary human hepatocytes. Our data strongly indicate future studies should be focused on hepatic necrosis after MC-LR toxicity.

4.1.1 Cell death in liver injury

It should be noted that both necrotic and apoptotic hepatocytes were identified in necropsies and biopsies from patients exposed to microcystin via their dialysis solution in the Carauru, Brazil poisoning (Pouria et al., 1998). Biopsies from these patients were obtained significantly after the initial exposure, and thus apoptosis of hepatocyte might be seen in patients that are also under palliative care and ongoing treatment. Despite this, the initial reaction to MC-LR in the absence of other effects is likely necrosis which is corroborated by the elevation in ALT seen in patients with MC-LR toxicity (Pouria et al., 1998).

Understanding modality of cell death during liver disease is imperative, as apoptotic and necrotic cell death pathways, despite some potential overlap, will require different therapeutic approaches. Our group has recently questioned the prevalence of apoptosis in human disease in other models such as acetaminophen hepatotoxicity (McGill et al., 2012) and bile acid toxicity (Woolbright et al., 2015). Even in murine and cell culture models with demonstrable apoptosis (Jones et al., 1998; Antoine et al., 2010), the clinical analogues of these diseases rarely present with substantial apoptotic cell death (McGill et al., 2012; Woolbright et al., 2015). Clearly in the case of MC-LR toxicity, avenues addressed at preventing further necrosis should be explored more thoroughly.

Apoptosis is a controlled, and tightly regulated, form of cell death that is characterized by cellular shrinkage, chromatin condensation, DNA fragmentation by caspase-activated DNases, and formation of apoptotic bodies that are rapidly phagocytosed (reviewed in Green and Llambi, 2015). Necrosis can occur through a number of different mechanisms, but is typically associated with cellular swelling, mitochondrial membrane disruption, DNA fragmentation by mitochondrial endonucleases and cellular lysis (Jaeschke and Lemasters, 2003). Despite this, models of both necrosis and apoptosis present with many similar cellular and biochemical changes. In the acetaminophen (APAP) overdose model, for example, cell death occurs almost exclusively through necrosis in mice (Gujral et al., 2002) and humans (McGill et al., 2012), yet many of the same events occur as in classical apoptosis such as bid (El-Hassan et al., 2003) and bax (Bajt et al., 2008) translocation, mitochondrial permeability transition pore formation (Kon et al., 2004), and release of mitochondrial proteins (Bajt et al., 2006). It is not understood why these similarities occur, yet the cells do not undergo apoptosis (Gujral et al., 2002). The strong similarity between these two pathways can lead to misinterpretation of data though, and thus studies specifically addressing the role of caspases and other mutually exclusive events are usually required for full delineation (McStay and Green, 2014). MC-LR toxicity results in obvious DNA fragmentation *in vivo* as evidenced by considerable TUNEL staining (Figure 2). Other reports have indicated alterations in Bcl-2 proteins including changes in the expression of Bid and Bcl-2 (Wei et al., 2008). Despite these changes typically being associated with apoptosis, a caspase inhibitor with near 100% efficacy *in vivo* failed to produce any obvious changes in MC-LR toxicity in mice *in vivo* or *in vitro* (Figure 3–6).

Cytoskeletal disruption has previously been reported around, or before, the onset of cell death associated histological features such as membrane blebbing, after MC-LR toxicity in the rat model (Hooser et al., 1991) and in human hepatocytes (Batista et al., 2003). Our own data indicate higher doses of MC-LR resulted in cell death before the initiation of cytoskeletal disruption (Figure 7). The fact that cell death occurs before the onset of cytoskeletal disruption in human hepatocytes only serves to increase the likelihood this plays a role in the clinical disease state (Figure 7). Additional studies should be aimed at understanding the biochemical pathways underlying the signaling pathways leading to DNA fragmentation during MC-LR induced necrosis.

4.1.2 MC-LR toxicity in man

Clinical cases of human microcystin poisoning have indicated that microcystin is a potent human hepatotoxin that can result in acute liver failure, although poisonings in general are rare (Pouria et al., 1998). As such, patient samples are limited, and other models and tools, are necessary for understanding microcystin toxicity in human patients. Primary human hepatocytes are an exceptionally useful model for understanding how toxins affect human hepatocytes as they retain the expression of drug metabolizing enzymes and hepatic transporters such as OATP1B1/1B3, and their functional activities for extended periods during culture (Szabo et al., 2013). Moreover, studies using patient samples in other models of liver injury are largely supported by data gained in primary human hepatocytes, indicating primary human hepatocytes retain similar biological profiles to the intact human liver (Xie et al., 2014, Woolbright et al., 2015). Previous work from our laboratory has indicated that while some rodent models, e.g. APAP toxicity, accurately model human exposure (Xie et al., 2014), other models, e.g. bile acid toxicity, may be inherently more sensitive and have entirely different mechanisms (Woolbright et al., 2015). This is supported by the fact that humans seem to undergo significantly less hemorrhage than other mammalian exposures (Pouria et al., 1998; van der Merwe et al., 2012; Figure 1). As mice expire from hemorrhagic shock shortly after toxic MC-LR exposure, it is imperative to understand MC-LR toxicity in primary human hepatocytes due to their fidelity with the human condition, and the established role of cell death in MC-LR toxicity in humans.

One important facet of this study is the dose response of toxicity of MC-LR in human hepatocytes. Surprisingly, doses of MC-LR only five times higher than the current WHO drinking standards produced toxicity in human hepatocytes (Figure 5). Of note, doses of 1nM, the current WHO drinking standard, failed to produce toxicity even after 72 hours of exposure time in a single test (data not shown). Oral exposure through contaminated drinking water is by far the most likely scenario of microcystin poisoning in humans. Oral exposure to microcystin typically has an LD50 between 50 and 150 fold higher than *i.p.* administration in animal models, and thus, a much higher toxicity threshold (Yoshida et al., 1997). While oral exposure has a higher acceptable margin of safety, the fact that exposure to concentrations of microcystin less than one order of magnitude higher than the current minimum standard of MC-LR can induce LDH release in hepatocytes indicates that current margins of safety should be re-evaluated. Recreational activity and incidental consumption of water with even only moderately elevated MC-LR could potentially lead to toxicity in vulnerable populations, including children, genetically predisposed populations with enhanced OATP1B1 activity (Li et al., 2012), and patients with ongoing liver disease. Given that patients rapidly proceed to ALF after acute MC-LR intoxication, exposure to even low levels could result in unacceptably high morbidity and mortality rates in the event of civilian exposure to a contaminated water source, especially given the likelihood that multiple microcystin species are commonly present in water simultaneously.

In conclusion, there are readily apparent differences between rats (Ding et al., 2000, Hooser et al., 2000) and mice or humans, as shown in this study. Mice appear to model the human response more closely and should be the model organism used in future studies. Further studies examining mechanisms of MC-LR induced necrosis are warranted in both the murine

model and in primary human hepatocytes to investigate downstream mechanisms of protein phosphatase inhibition and the subsequent DNA fragmentation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Microcystin induced liver injury causes significant necrosis and hemorrhage in mice.

Caspase inhibition fails to protect against microcystin induced liver injury in mice.

There is limited caspase activation in human hepatocytes exposed to microcystin-LR.

Caspase inhibitors do not protect against microcystin in human hepatocytes.

Cell death via necrosis precedes cytoskeletal disruption.

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Figure 1.

Microcystin induces rapid liver destruction. C57BL/6J mice were given 120µg/kg microcystin-LR for 45 or 90 minutes or given PBS as a control and hematoxylin and eosin stain (A) was performed to assess liver health. ALT was assessed to measure cell death (B) and liver hemoglobin levels (C) were assessed to measure hemorrhage. Glutathione levels were also assessed in liver tissue (D). n=5. *p<0.05.

Figure 2.

TUNEL staining indicates widespread DNA fragmentation and necrosis in microcystin-LR treated mice. TUNEL staining was performed to measure DNA fragmentation in mice treated with 120 μ g/kg microcystin-LR or 700mg/kg galactosamine and 100 μ g/kg lipopolysaccharide (Gal/LPS) (A). Microcystin-LR treated mice had a diffuse staining pattern consistent with leakage of TUNEL stain into the cytoplasm indicating widespread necrosis (black arrows). Gal/LPS treated mice had both diffuse and nuclear staining for

TUNEL indicating both apoptosis (red arrows) and necrosis (black arrows). Five high powered fields (HPF) were quantified (B) for microcystin-LR treated mice. n=5. *p<0.05

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Figure 3.

Inhibition of caspase-3 fails to protect against microcystin-LR induced liver injury. Mice were pretreated with 5mg/kg z-VAD-fmk or DMSO as a control, and then treated with 120 μ g/kg microcystin-LR or 700mg/kg galactosamine and 100 μ g/kg lipopolysaccharide (Gal/LPS) and hematoxylin and eosin stain (A), ALT (B) and caspase-3 activity were assessed (C). n=4. *p<0.05

Figure 4.

Inhibition of caspase-3 fails to inhibit ROS formation in microcystin-LR induced liver injury. Mice were pretreated with 5mg/kg z-VAD-fmk or DMSO as a control, and then treated with 120μg/kg microcystin-LR or 700mg/kg galactosamine and 100μg/kg lipopolysaccharide (Gal/LPS) and glutathione (GSH) or glutathione disulfide (GSSG) was measured in liver homogenates. Pretreatment with z-VAD-fmk protected Gal/LPS mice almost completely against GSSG formation but had no effect on microcystin-LR treated mice. n=4. *p<0.05

Figure 5.

Primary human hepatocytes are acutely susceptible to microcystin-LR induced liver toxicity. Primary human hepatocytes (A) or primary murine hepatocytes (B) were exposed to increasing concentrations of microcystin-LR via the media for the indicated time and lactate dehydrogenase release was measured in media. Propidium iodide staining was used to identify necrotic cells (C). Primary human hepatocytes, n=4. Primary murine hepatocytes, n=3. *p<0.05.

Figure 6.

Inhibition of caspase-3 fails to protect primary human hepatocytes or primary murine hepatocytes against microcystin-LR induced liver toxicity. Primary human hepatocytes (A, C, E) or primary murine hepatocytes (B, D, F) were pre-treated for 30 minutes with DMSO or 10 μ M z-VAD-fmk and then treated with either 200nM microcystin-LR or 5mM galactosamine and 250ng/mL TNF- α and LDH release (A, B) and caspase-3 activity (C, D) were measured. Cleaved caspase-3 was measured by western blot (E,-F). Liver homogenate

from Gal/LPS treated mice was included as an additional positive control for apoptosis. Primary human hepatocytes, n=3–4. Primary murine hepatocytes, n=3. *p<0.05.

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Figure 7.

Actin breakdown in primary human hepatocytes. Human hepatocytes were treated with 200nM MC-LR for up to 6 hours. Actin breakdown was assessed by phalloidin staining (left) and cell death was assessed by PI staining (right). Nuclear staining was assessed by DAPI staining in both cases. Co-staining was not possible as phalloidin staining requires permeabilization of membranes and thus would result in 100% PI positive cells.