Altered cellular metabolism of HepG2 cells caused by microcystin-LR

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Abstract

This study aimed to evaluate the possible effects of microcystin-LR (MC-LR) exposure on the metabolism and drug resistance of human hepatocellular carcinoma (HepG2) cells. For this purpose, we first conducted an experiment to make sure that MC-LR could penetrate the HepG2 cell membrane effectively. The transcriptional levels of phase I (such as CYP2E1, CYP3A4, and CYP26B1) and phase II (such as EPHX1, SULTs, and GSTM) enzymes and export pump genes (such as MRP1 and MDR1) were altered by MC-LR-exposure for 24 h, indicating that MC-LR treatment may destabilize the metabolism of HepG2 cells. Further research showed that the CYP inducers omeprazole, ethanol, and rifampicin inhibited cell viability, in particular, ethanol, a CYP2E1 inducer, induced ROS generation, lipid peroxidation, and apoptosis in HepG2 cells treated with MC-LR. The CYP2E1 inhibitor chlormethiazole inhibited ROS generation, mitochondrial membrane potential loss, caspase-3 activity, and cytotoxicity caused by MC-LR. Meanwhile, the results also showed that co-incubation with the ROS scavenger L-ascorbic acid and MC-LR decreased ROS levels and effectively prevented apoptosis. These findings provide an interesting mechanistic explanation of cellular metabolism associated with MC-LR, i.e., MC-LR-exposure exerted toxicity on HepG2 cells and induced apoptosis of HepG2 cells via promoting CYP2E1 expression and inducing excessive ROS in HepG2 cells.

Capsule abstract

The linkage between CYP2E1-dependent oxidative stress and mitochondrial injury contributes to the toxic action of MC-LR on HepG2 cells.

Keywords: Microcystin-LR; HepG2; OATPs; metabolism-associated enzyme; drug resistance gene

1. Introduction

In recent years, cyanobacterial blooms have frequently occurred in eutrophic waterbodies worldwide, and the resulting release of cyanotoxins by some toxic cyanobacterial blooms not only leads to water pollution but also poses a potential health hazard to aquatic organisms and humans (O'Neil et al., 2012; Rastogi et al., 2014). Among these cyanotoxins, microcystins (MCs) are the most common of the toxins widely distributed in fresh water. They are produced by some cyanobacteria such as *Anabaena*, *Aphanizomenon*, *Microcystis*, *Planktothrix*, *Oscillatoria*, and *Nostoc* (Carmichael et al., 2001; van Apeldoorn et al., 2007). MCs are a group of cyclic heptapeptide toxins with the general structure cyclo (D)-Ala- X- (D)- erythro- b- methyl- iso- Asp- Y- Adda- (D)- iso- Glu- N- meth- yldehydro- Ala, where X and Y represent positions occupied by the variable L-amino acids (Welker and von Döhren, 2006), and now more than 80 MC variants have been identified (Funari and Testai, 2008). Among these MCs, MC-LR is the most ubiquitous and frequently studied and it has been found to have hepatotoxicity in humans (Feng et al., 2011), other

mammals (Li et al., 2009), birds (Peckova et al., 2009), and aquatic animals (Li et al., 2013; Ma et al., 2015a). Due to its high toxicity, the World Health Organization has recommended a drinking water guideline imposing an upper limit of $1.0 \mu\text{g L}^{-1}$ MC-LR for human daily consumption (WHO, 1998). Unfortunately, increasing studies have demonstrated that the concentrations of MCs can be much higher than this standard in cyanobacterial bloom-infested waters, such as $6.69 \mu\text{g L}^{-1}$ of MCs in the summer periods (Song et al., 2007) and $15.6 \mu\text{g L}^{-1}$ in the wintertime (Xu et al., 2008) in Lake Taihu, China, $48.6 \mu\text{g L}^{-1}$ in the blooming season in Argentina (Giannuzzi et al., 2011), and 10 to $500 \mu\text{g L}^{-1}$ MCs in eutrophic lakes in America (Backer et al., 2010). Therefore, the MCs could be a health hazard to humans through drinking water or due to exposure via the food chain.

It has been demonstrated that acute intoxication with MCs can lead to human death (Azevedo et al., 2002), while chronic exposure to MCs has been verified to be one of the risk factors for human primary hepatocellular cancer (Nishiwaki-Matsushima et al., 1992). It is now well recognized that MCs are highly potent and specific inhibitors of serine/threonine protein phosphatases 1 and 2A (PP1 and PP2A) (Runnegar et al., 1993). Due to PP2A inhibition, the intracellular balance of phosphorylation/dephosphorylation is destroyed, and this causes hyperphosphorylation of some key structural or functional proteins and signaling molecules or pathways, for example, the mitogen-activated protein kinases (MAPKs) (Daily et al., 2010), which is directly related to the tumor-promoting activity of MCs (Campos and Vasconcelos, 2010). Simultaneously, excess intracellular reactive

oxygen species (ROS) and oxidative stress caused by MCs have also been considered as another important mechanism of MCs toxicity (Li et al., 2003). However, studies regarding the effects of MCs on the cellular metabolism and drug resistance are limited.

It is well known that liver is the main target organ of MC toxicity (Nishiwaki-Matsushima et al., 1992). Therefore, hepatocytes or hepatoma cell line have been increasingly used as in vitro models to evaluate the toxicity of MCs. Human hepatocellular carcinoma (HepG2) cells can display the morphology and biochemical activities of healthy hepatocytes (Javitt, 1990) and hence have been widely used to evaluate the toxic effects of various toxicants on hepatocytes for many years (Van et al., 1997; Carvalho et al., 2014).

In recent years, gene expression alterations have been increasingly used in toxicological studies to provide an early and global answer regarding the molecular initiating events of the effects of toxicants (Brown and Botstein, 1999), and the utilization of transcriptional patterns is also a rational tool in molecular toxicology (Schmidt, 2002). Moreover, monitoring the transcriptional level of a panel of genes may help to reveal the role of genes in the response to toxicological stress and to elucidate the toxicological mechanism involved (Nuwaysir et al., 1999). In the present study, HepG2 cells were adopted to determine the transcriptional levels of organic anion transporter polypeptides (OATP1B1 and OATP1B3), metabolism-related enzymes such as cytochrome P450 (e.g., CYP2E1, CYP3A4, and CYP26B1), phase II (such as EPHX1, SULTs, NAT1 and GSTM) enzymes, and export pump genes (such

as MRP1 and MDR1) using quantitative real-time PCR to reveal the possible destabilization of cellular metabolism in HepG2 cells caused by MC-LR exposure. The selected genes from phase I and phase II enzymes, and export pump genes were play important role in the cellular metabolism or detoxification processes in the mammalian cells. For example, among phase I CYP450 superfamily, subfamilies CYP1, CYP2, and CYP3 are account for the metabolization and detoxification of more than 80% xenobiotics in animals and humans (Nelson, 2003; Singh et al., 2011), and in subfamilies CYP3, CYP3A subfamily is major components in human liver microsomes (Murray and Reidy 1990; Cantoni et al., 2003), and CYP3A plays a key role in the metabolism of about 50% of all pharmaceuticals in use at present (Nelson, 2003).

Moreover, the CYP inducers omeprazole, ethanol, and rifampicin and the CYP inhibitor chlormethiazole were used to investigate the role of CYPs in MC-LR cytotoxicity. The results of the present study might provide valuable information on the mechanisms of action of MC-LR on mammalian cells.

2. Materials and methods

2.1. Cell culture and MC-LR exposure

HepG2 cells were purchased and cultured as described previously (Ma et al., 2016). MC-LR ($C_{49}H_{74}N_{10}O_{12}$, $\geq 95\%$ purity, CAS 101043-37-2) were purchased from Express Technology Co., Ltd. China.

A HepG2 culture at the logarithmic growth phase was trypsinized with 0.25%

trypsin, and then 5×10^3 or 1.5×10^4 cells per well were inoculated in 96- or 6-well plates in RPMI-1640 medium supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS), 100 U mL⁻¹ of penicillin, and 100 µg mL⁻¹ of streptomycin in a 5% CO₂ humidified incubator at 37 °C. After 24 h of culture, MC-LR was added to each well at the concentrations specified below for each assay. The cells were cultured for an additional period of time as specified bellow for each test, and the medium and toxin concentrations were refreshed daily to maintain an approximately constant MC-LR concentration.

For the cell viability assay (96-well culture plate), (1) the culture medium was replaced with complete RPMI-1640 medium containing 0.1-10 000 nM MC-LR, and the cells cultured in complete medium without MC-LR served as the control for another 24 h; (2) treatment groups containing 5 µM MC-LR were grown with and without 10 µM omeprazole (OME), 50 mM ethanol (ETH), and 15 µM rifampicin (RIF), and the cells cultured in complete medium without MC-LR served as the control for another 72 h; (3) cultures containing 0-10 µM MC-LR were grown with and without 50 mM ETH for another 24 h; (4) cultures containing 5 µM MC-LR were grown with and without 10 µM chlormethiazole (CMZ) for another 72 h; (5) cultures containing various concentrations of MC-LR (0-100 µM) were grown for another 96 h; (6) cultures containing 30 µM MC-LR were grown with and without 10 µM CMZ for another 96 h; (7) cultures containing 30 µM MC-LR and 10 µM L-ascorbic acid / 30 µM MC-LR were grown for another 72 h.

For the flow cytometric assay, as described above, the treatment groups received

0.1-10 000 nM MC-LR exposure, and the cells cultured without MC-LR served as the control for another 24 h.

For the western blots and the quantitative real-time PCR (qPCR) assay, as described above, the culture medium in a 6-well culture plate was replaced with complete RPMI-1640 medium containing 0.1-10 000 nM MC-LR in the treatment groups or no MC-LR in the control for another 24 h.

For measurement of intracellular ROS level, as described above, (1) the culture medium was replaced with complete RPMI-1640 medium containing 5 μ M MC-LR with and without 50 mM ETH, and the cells were cultured for another 72 h; (2) cultures containing 30 μ M MC-LR with and without 10 μ M CMZ were grown for another 72 h; and (3) cultures containing 30 μ M MC-LR with and without 10 μ M L-ascorbic acid were grown for another 72 h.

For the lipid peroxidation assay, as described above, the culture medium was replaced with fresh complete RPMI-1640 medium. (1) Cultures containing 5 μ M MC-LR with and without 50 mM ETH were grown for another 72 h, and (2) cultures containing 30 μ M MC-LR with and without 10 μ M CMZ were grown for another 48 h.

For the caspase-3 activity assay, the culture medium was replaced with fresh complete RPMI-1640 medium containing 30 μ M MC-LR with and without 10 μ M CMZ, and the cells were cultured for another 48 h.

For Hoechst 33342 Staining, the culture medium was replaced with fresh complete RPMI-1640 medium containing 5 μ M MC-LR with and without 50 mM

ETH, and the cells were cultured for another 48 h.

For the mitochondrial membrane potential (MMP) assay, in a 6-well culture plate, the culture medium was replaced with complete RPMI-1640 medium containing 30 μ M MC-LR with and without 10 μ M CMZ, and the cells were cultured for another 48 h.

2.2. Cell viability assay

After the treatment time, the culture medium was replaced with 200 μ L of fresh medium containing 0.5 mg mL⁻¹ MTT (Sigma, USA), and the cells were cultured for an additional 4 h at 37 °C. Then, the cells were washed thrice with PBS, and the generated formazan was dissolved with 200 μ L DMSO. The cell viability measurement was performed as the described previously (Li et al., 2015).

2.3. Flow cytometric analysis

The cell cycle and apoptosis of HepG2 cells were evaluated using the PI/RNase Kit (Becton Dickinson, USA) and the FITC Annexin V Apoptosis Detection Kit (Becton Dickinson, USA), respectively, by flow cytometry (Becton Dickinson) as described previously (Li et al., 2015).

2.4. Western blot

MC-LR uptake and entrance into HepG2 cells was determined by western blotting with antibodies against MC-LR as described by Xing et al. (2008). Briefly,

the cells were washed twice with ice-cold PBS to remove MC-LR after 24 h of incubation with MC-LR (0-10 000 nM) in a 6-well culture plate and then collected by digestion using 0.25% of trypsin and centrifugation at 4 000 g for 8 min. Western blot analysis of cellular lysates was performed as previously described (Ma et al., 2016). The following primary antibodies were employed: anti-MC-LR (1:300 dilution) (Alexis Biochemicals, Lausen, Switzerland) and anti-actin (1:3 000) (Proteintech, Wuhan, China). Goat anti-mouse IgG-alkaline phosphatase (1:20 000) was used as the secondary antibody. Immunoreactive protein bands were detected with a BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime, Haimen, China). The figures presented were representative of at least three independent assays.

2.5. RNA isolation and qPCR

After 3, 6, 12, and 24 h of MC-LR exposure in a 6-well culture plate, the cells were collected as described above and stored at -80 °C for the following RNA isolation.

Total RNA isolation, synthesis of first-strand cDNA, and qPCR were performed with an RNAiso Plus kit (Takara, Dalian, China), a PrimeScriptTM Reagent Kit (Takara), and a SYBR Premix Ex Taq II Kit (Takara), respectively, using methods described previously (Ma et al., 2015b). The expression of *CYP1A1*, *CYP1A2*, *OATP1B1*, *CYP2B6*, *CYP2E1*, *CYP3A4*, *CYP4A11*, *CYP26B1*, *GSTM4*, *EPHX1*, *UGT1A1*, *NAT1*, *SULT1A1*, *MRP1*, *MRP2*, *MRP3*, and *MDR1* at the mRNA level was

determined. The amount of target mRNA was normalized to GAPDH mRNA and was determined by the method of Livak and Schmittgen (2001). The primers designed for the qPCR reaction are listed in table 1.

2.6. Measurement of intracellular ROS level

The ROS level in HepG2 cells was measured according to the method of measuring the oxidative conversion of 2', 7'-dichlorofluorescein diacetate (DCFH-DA) to the fluorescent compound dichlorofluorescein (DCF) (Jia et al., 2006) as described previously by Ma et al. (2016).

2.7. Lipid peroxidation assay

Lipid peroxidation was determined by the method of thiobarbituric acid (TBA) (Ohkawa et al., 1979) as described previously (Li et al., 2015).

2.8. Caspase-3 activity assay

After MC-LR exposure described as above, the activity of caspase-3 was measured using a caspase activity kit (Beyotime, Haimen, China) according to the manufacturer's instructions as described previously by Ma et al. (2016).

2.9. Hoechst 33342 staining

After MC-LR exposure, the HepG2 cells were collected and washed twice with PBS, then stained with Hoechst 33342 (Sigma, USA) at a final concentration of 10 µg

mL⁻¹ for 30 min at 37 °C in darkness. Cells were then washed twice with PBS and immediately observed using a fluorescence microscope.

2.10. Mitochondrial membrane potential assay

The MMP was determined using the JC-1 probe by the manufacturer's instructions (Beyotime, Haimen, China). After exposure, the medium was discarded and the cells were then washed twice with PBS, after which 1 mL JC-1 working solution per well was added and incubated for 20 min at 37 °C. Then, the JC-1 working solution was removed and washed twice with JC-1 staining buffer. Later, 2 mL of medium was added and MMP was assessed using a fluorescence microscope.

2.11. Statistical analysis

Data were analyzed using one-way analysis of variance (Tukey) with a least significant difference determination by SPSS 13.0, and the results level was considered statistically significant was set at *P* value < 0.05.

3. Result

3.1. Effect of MC-LR exposure on the viability, cell cycle, and apoptosis of HepG2 cells

The viability of HepG2 cells determined after exposure to MC-LR is depicted in [Supplemental Fig. 1](#), which shows that MC-LR did not induce cytotoxicity in HepG2 cells exposed for 24 h. The results of flow cytometric analysis with PI and annexin

V/PI staining showed that the 0.1-10 000 nM MC-LR-exposure for 24 h did not alter the cell cycle (Supplemental Fig. 2) and apoptosis (Supplemental Fig. 3) of HepG2 cells, respectively.

3.2. Detection of MC-LR entrance into HepG2 cells

The result of the western blot assay showed that MC-LR entered HepG2 cells in a concentration-dependent pattern (Fig. 1). Indeed, our results from qPCR showed that HepG2 cells expressed *OATP1B1* and *OATP1B3*, which are known to transport MC-LR. The expression levels of both *OATP1B1* and *OATP1B3* in HepG2 cells were promoted in the MC-LR-treated groups when compared to the control groups except for *OATP1B3* in the 5 000 nM group at 12 or 24 h and 10 000 nM at 24 h (Supplemental Fig. 4 A, B).

3.3. The transcriptional alteration of phase I and phase II genes in HepG2 cells after 24 h of MC-LR exposure

More than 90% of drugs and xenobiotic compounds are metabolized and detoxified by phase I and phase II enzymes; consequently, we wanted to determine whether phase I and phase II played a role after 24 h of MC-LR exposure. The results of qPCR showed that the transcription levels of *CYP2E1* and *CYP26B1* were generally up-regulated in the MC-LR-treated groups in comparison with the control (Fig. 2 A, Supplemental Fig. 5 G). However, *CYP3A4* transcription was suppressed in higher concentration groups (5 000 and 10 000 nM) in the early periods of MC-LR

exposure (from 3 to 6 h), but soon afterwards it was promoted (Supplemental Fig. 5 E). Moreover, transcriptional levels of *CYP1B1*, *CYP2B6*, and *CYP4A11* were also found to be altered in HepG2 cells after MC-LR exposure (Supplemental Fig. 5 C, D, and F). However, MC-LR did not affect the mRNA levels of CYP 1A1 or CYP1A2 (Supplemental Fig. 5 A, B).

The mRNA levels of *GSTM4* are shown in Fig. 2 B, in which the transcription as a whole was suppressed in the MC-LR-exposed cells. Moreover, the transcription suppression was intense with the increase of MC-LR concentrations absorbed by in HepG2 cells. The altered *EPHX1* (Supplemental Fig. 6 A) and *SULT1A1* (Supplemental Fig. 6 B) transcriptional levels were similar to that of *GSTM4*, while there was no statistically significant difference in UGT1A1 between the treatment groups and control group (Supplemental Fig. 6 C). The NAT1 expression rate in the 10 000 nM group was remarkably up-regulated after MC-LR exposure. In contrast, mRNA levels of NAT1 in the lower concentration groups were significantly lower than that of the control group, as seen in 1 000 nM group at 12 or 24 h (Supplemental Fig. 6 D).

3.4. The transcriptional alteration of export pump genes in HepG2 cells after 24 h of MC-LR exposure

The expression of both *MRP1* and *MDR1* at the mRNA level was mainly promoted in the MC-LR-exposed cells at higher concentrations when compared to that of control cells, for example, 1 000, 5 000, or 10 000 nM of MC-LR as shown in

Supplemental Fig. 7 A and D, while MRP2 (except for 10 000 nM at 24 h) and MRP3 transcription level were generally down-regulated in the MC-LR-treated groups (Supplemental Fig. 7 B, C).

3.5. Effects of CYP inducers on MC-LR-induced cytotoxicity and damage in HepG2 cells

The viability of HepG2 cells exposed to 5 μ M MC-LR with and without CYP induction is depicted in Supplemental Fig. 8 A. Cell viability significantly decreased in OME/MC-LR groups after 48 h of exposure and in RIF/MC-LR groups after 24 h of exposure, and ETH/MC-LR groups significantly decreased in cell viability from 24 to 72 h. After 24 h of exposure, the 5 and 10 μ M MC-LR with ETH groups significantly decreased in cell viability, but no changes occurred in the MC-LR treatment groups (Fig. 3 A). Further research showed that using ETH along with 5 μ M MC-LR led to a significant increase of ROS generation in HepG2 cells (Fig. 3 B), and significantly increased levels of MDA after 48 h were also found (Supplemental Fig. 8 B). Moreover, the Hoechst 33342 staining assay revealed that ETH/MC-LR increased the apoptosis of HepG2 cells compared with the cells treated with MC-LR alone (Supplemental Fig. 8 C). These results indicate that the CYPs inducers plus MC-LR can make HepG2 cells more sensitive to MC-LR exposure.

3.6. Effects of CMZ on MC-LR-induced cytotoxicity and damage in HepG2 cells

Cell viability was generally not affected at CMZ/5 μ M MC-LR (Supplemental Fig.

9 A). Therefore, we chose 30 μ M MC-LR to determine the effects of CMZ on MC-LR-induced cytotoxicity in the cells through the cell activity assay by MTT (Supplemental Fig. 9 B). Cell viability increased more than 18% in CMZ/ 30 μ M MC-LR compared with the cells treated with MC-LR alone after 96 h exposure (Supplemental Fig. 9 C). The intracellular ROS levels in the 30 μ M MC-LR-treated HepG2 cells were higher than those of the CMZ/30 μ M MC-LR-treated cells (Fig. 4 A). Moreover, in cultures co-incubated with the ROS scavenger L-ascorbic acid and MC-LR, the ROS levels decreased compared with the cells treated with MC-LR alone, and cellular toxicity could be partly diminished by L-ascorbic acid (Supplemental Fig. 9 D, E). 30 μ M MC-LR significantly increased HepG2 cells MDA levels; however, CMZ partially counteracted these effects (Fig. 4 B). MMP was higher in CMZ/30 μ M MC-LR-treated HepG2 cells than in 30 μ M MC-LR-treated cells (Fig. 4 C). To determine whether a CYP2E1 inhibitor is able to block MC-LR-induced apoptosis in HepG2 cells, a caspase-3 activity assay was performed. The results showed that treatment with CMZ and MC-LR exposure significantly reduced caspase-3 activity compared with the cells treated with MC-LR alone (Fig. 4 D).

4. Discussion

Cellular viability generally represents the toxicity degree of toxicants and thus it is a key index that should be determined in cytotoxicity tests (Li et al., 2015). In this study, the results showed that 0.1-10 000 nM MC-LR failed to induce cytotoxicity in HepG2 cells after 24 h of exposure and no difference in the cell cycle and apoptosis

was found between the MC-LR-treated groups and the control group assayed by flow cytometry ([Supplemental Fig. 1](#), [Fig. 2](#), and [Fig. 3](#)). It is known to us that MCs themselves cannot readily diffuse through the plasma membrane and enter cells without the help of any transporter located in the cell membrane due to their relatively higher molecular weight (900-1100 Da) and moderate hydrophobicity ([Komatsu et al., 2007](#); [Bieczynski et al., 2014](#)). Previous studies have found that MC-LR is usually transported into cells via OATPs; for example, OATP1B1 and OATP1B3 are the most efficient MC-LR transporters in human hepatocytes ([Hagenbuch and Gui, 2008](#); [Kalliokoski and Niemi, 2009](#)). However, Abe et al. ([2001](#)) reported that OATP1B1 and OATP1B3 had no transporting activity in HepG2 cells. Therefore, first it is essential for the present study to determine whether MC-LR entered HepG2 cells and whether expression of OATP1B1 and OATP1B3 could be detected in the cells. The result of a western blot assay revealed that MC-LR could enter HepG2 cells as shown in [Fig. 1](#). Furthermore, it was observed that many protein bands could be detected by the antibody against MC-LR in HepG2 cells exposed to 1 000, 5 000, and 10 000 nM of MC-LR for 24 h, suggesting that there may be other new target molecules for MC-LR in the cell aside from PP2A. For a long time, it has been generally believed that the combination of MCs with PP2A is the molecular initiating event of MC hepatotoxicity ([Dawson, 1998](#)). Therefore, these new molecules should be identified in future study because this information may be valuable for completely uncovering the molecular initiating events of MC hepatotoxicity. Meanwhile, qPCR results revealed that OATP1B1 and OATP1B3 were assuredly expressed and their

transcription was promoted by MC-LR exposure in HepG2 cells ([Supplemental Fig. 4 A, B](#)). This result was in accordance with reports by Hilgendorf et al. (2007) and Roegner and Puschner (2014), in which they found that OATP1B1 and OATP1B3 were expressed in HepG2 cells.

CYP450s are the largest and most important heme monooxygenases among the intracellular Phase I metabolic enzymes, and they play a key role not only in catalyzing a variety of reactions and converting xenobiotics to potentially reactive products as well as making them less toxic (Juchau, 1990; Isin and Guengerich, 2007) but also in the metabolic activation of carcinogens and may be further implicated in tumor initiation, promotion, and progression (Nelson et al., 1993). Within this superfamily, the subfamilies CYP1, CYP2, and CYP3 account for the metabolism and detoxification of more than 80% of xenobiotics in animals and humans (Nelson, 2003; Singh et al., 2011). Hudder et al. (2007) noted that the mRNA levels of CYP1A2 could be induced in mice after MC exposure, and this might be specifically in response to MC-LR. However, in the present study, no significant changes were found in CYP1A1 and CYP1A2 mRNA levels in the HepG2 cells exposed to MC-LR, which is in accordance with the report by Sun et al. (2011) in which they found no changes of CYP1A1 and CYP1A2 mRNA levels in male BALB/c mice after MC-LR exposure. The CYP1B1 gene is involved in tumorigenesis (Bruno and Njar, 2007), and it is overexpressed in a variety of cancers (McKay et al., 1995; Murray et al., 1997). However, CYP1B1 gene expression has not been previously analyzed in relation to MC-LR. In this study, CYP1B1 transcription was significantly altered by

MC-LR, indicating that CYP1B1 may potentially be one of the factors responsible for the MC-LR-related increase in hepatoma risk.

CYP2B6 is strongly expressed in human liver, where it constitutes approximately 6% of total CYP450s (Stresser and Kupfer, 1999). The transcriptional regulation of CYP2B6 is important in drug metabolism and xenobiotic toxicity (Hodgson and Rose, 2007; Wang and Tompkins, 2008). In this study, CYP2B6 expression in HepG2 cells of the treatment groups was down-regulated, thus, we presumed that down-regulation of CYP2B6 by MC-LR exposure may disturb hepatocyte metabolism and hence cause damage to HepG2 cells. Additionally, the function of CYP2B6 in MC-LR hepatotoxicity must be further investigated.

CYP2E1 plays a pivotal role in the oxidative metabolism of numerous therapeutic and environmentally important chemicals, and CYP2E1 gene expression can be a pivotal determinant of some human cancers (Feierman and Cederbaum, 1987; Umeno et al., 1988). Previous studies have noted that the CYP2E1 mRNA level could be induced in mouse liver after MC exposure and that the induction of CYP2E1 may increase the generation of ROS (Sun et al., 2011; Zhang et al., 2015). In the present study, qPCR detection showed that CYP2E1 expression was significantly increased by MC-LR exposure in HepG2 cells. This result is consistent with the report by Nong et al. (2007) in which they observed the induction of CYP2E1 by MC-LR exposure in HepG2 cells. Similarly, we also found that transcription of CYP3A4 was also promoted in HepG2 cells by MC-LR. This result is in accordance with our previous study in zebrafish (Li et al., 2013), in which MC exposure altered the transcription of

CYP3A65 in zebrafish liver. However, it is notable that CYP3A4 transcription is suppressed in higher concentration groups at early periods of exposure (from 3 to 6 h). CYP4A11 converts arachidonic acid to 20-hydroxyeicosatetraenoic acid, which has a crucial role in the modulation of cardiovascular homeostasis (Fu et al., 2012), and CYP26B1 is a novel candidate gene for carcinoma (Chen et al., 2011). In the present study, CYP4A11 and CYP26B1 transcription levels were generally up-regulated in the MC-LR-treated groups in comparison with the control. To the best of our knowledge, this is the first report regarding MC-LR effects on CYP4A11 and CYP26B1, and we supposed that CYP4A11 and CYP26B1 might be involved in the metabolism of MC-LR, which might disturb the normal biological function of HepG2 cells. In short, our result indicates that MC-LR exposure alters the expression of Phase I enzymes and may disturb the metabolism of HepG2 cells.

GST and SULTs are the key Phase II metabolic enzymes that play a pivotal role in the detoxification of xenobiotics by biotransformation. It is already known that GST is a key enzyme responsible for the detoxification of MCs in animal liver or hepatocytes (Ding et al., 2001; Takenaka, 2001). However, there has been no report about the possible function of SULTs in the detoxification or metabolism of MCs until now. In this study, we observed that the transcription levels of both GSTM4 and SULTs were significantly down-regulated in MC-LR-treated HepG2 cells at higher concentrations (e.g., 1 000, 5 000, and 10 000 nM) when compared to the control, indicating that MC-LR could suppress the expression of GSTM4 and SULTs and that GSTM4 and SULTs might play key roles in the detoxification of MCs in HepG2 cells

([Ding et al., 2001](#); [Takenaka, 2001](#)).

Microsomal epoxide hydrolase (mEH) encoded by the EPHXs gene is involved in the metabolism of numerous xenobiotics and mediates the sodium-dependent transport of bile acids ([von Dippel et al., 1993](#); [Decker et al., 2009](#)). Naturally occurring mutations of EPHX1 have been shown to result in dysfunction of cholesterol metabolism, signal transduction pathways, and excretion of numerous metabolized xenobiotics ([Zhu et al., 2003](#); [Monte et al., 2009](#)). In the present study, EPHX1 expression was significantly down-regulated in MC-LR-treated HepG2 cells ([Supplemental Fig. 6 A](#)). It has been reported that the inhibition of EPHX1 expression can lead to a decrease in the transport capacity of bile acids ([Zhu et al., 2003](#)). Our result suggests that MC-LR may interfere with the metabolism of bile acid in HepG2 cells, therefore, EPHX1 might be serving to protect cells from the toxic effects of MC-LR. However, the transcriptional levels of EPHX1 were not the same as those of OATP1B1 and OATP1B3, which might be associated with the complex regulation of cellular signaling networks, and further studies are needed to determine the phenomenon. EPHX1 is localized mainly in the microsomal fraction of the endoplasmic reticulum of eukaryotic cells ([Václavíková et al., 2015](#)), the decreased expression of EPHX1 caused by MC-LR may affect the function of endoplasmic reticulum, which confirms that the endoplasmic reticulum was involved in MC-LR-mediated toxicity in HepG2 cells ([Menezes et al., 2013](#)). NAT1 is a ubiquitously expressed human arylamine N-acetyltransferase (NAT) enzyme that biotransforms various aromatic, heterocyclic amines, drugs, or environmental

carcinogens ([Dairou et al., 2005](#); [Minchin et al., 2007](#)). In this study, the NAT1 expression rate was also changed after MC-LR exposure, indicating that MC-LR may activate the expression of NAT1, and NAT might play an important role in the activation and detoxification of environmental carcinogens ([Sim et al., 2008](#); [Butcher and Minchin, 2012](#)), such as MCs. UGT1A1 is a critical enzyme (encoded by the UGT1A1 gene) that is responsible for metabolism and detoxification of small lipophilic molecules such as bilirubin, hormones, and drugs ([Liu et al., 2010](#)). Our results reveal that there were no changes of UGT1A1 mRNA level at different MC-LR concentrations in HepG2 cells, suggesting that MC-LR or products might not be the ligand or substrate of UGT1A1.

Transporters such as P-glycoprotein (Pgp, MDR1, ABCB1) and multidrug resistance proteins (MRPs, ABCC) are members of the ATP-binding cassette (ABC) superfamily, and they have been identified in tumor cells to resist drugs or chemicals by immediate discharge and therefore prevention of cell abundance of the drugs or chemicals ([Bolhuis et al., 1997](#); [Keppler et al., 2011](#); [Bieczynski et al., 2014](#)). Particularly, MRP1 is involved not only in multidrug resistance of tumor cells but also in drug distribution and hepatic detoxification ([Conseil et al., 2005](#); [Kathawala et al., 2015](#)). However, there is no literature available regarding the effect of MCs on MRPs. Our result showed that the MRP1 mRNA level was remarkably up-regulated in HepG2 cells exposed to higher concentrations of MC-LR (from 1 000 to 10 000 nM) in comparison with the control cells ([Supplemental Fig. 7 A](#)). In addition, a very similar result was obtained in MDR1 encoding P-glycoprotein, i.e., higher

concentrations of MC-LR up-regulated MDR1 expression in HepG2 cells as shown in [Supplemental Fig. 7 D](#). The above results indicate that expressions of multidrug resistance genes *MRP1* and *MDR1* can be promoted by MC-LR exposure in HepG2 cells, suggesting that they may take part in cell resistance to MC-LR poisoning. However, MRP2 (except for 10 000 nM at 24 h) and MRP3 transcription levels were generally down-regulated in the MC-LR-treated groups. In 2007, de Souza Votto et al. reported that MDR was partly responsible for MC insensitivity in human tumoral cells ([de Souza Votto et al., 2007](#)). Although MC-LR could enter HepG2 cells ([Fig. 1](#)) and drive ROS production in the 5 μ M MC-LR-treated group at 6 h ([Fig. 3 B](#)), MC-LR may have not reached the mitochondria or perhaps was being metabolized or eliminated through export pumps prior to resulting in further toxic effects to HepG2 cells. Our results suggest that altered transcription of export pump genes may be involved in the insensitivity of HepG2 cells towards MC-LR-toxicity. However, it is not characterized which transporters are involved with MC-LR efflux, further research is needed.

The altered transcriptional levels of Phase I, Phase II, and export pump genes suggest that MC-LR-exposure may destabilize the metabolism of HepG2 cells, thus, we hypothesize that the detoxification enzyme systems play important role in the resistance of HepG2 cells to MC-LR. Our results revealed that 0.1-10 000 nM MC-LR could enter HepG2 cells but did not induce cytotoxicity upon exposure for 24 h, this might be explained by the low levels of biotransformation enzymes in HepG2 cells ([Liu and Zeng, 2009](#)). Therefore, the next step is to utilize CYPs inducers to

506 verify whether biotransformation enzymes play a role in the metabolic processes of
507 MC-LR in the HepG2 cells. In this study, the viability of HepG2 cells is influenced by
508 5 μ M MC-LR after exposure to CYP inducers, for example, the cell viability
509 significantly decreased in OME/MC-LR groups after 48 h of exposure and
510 RIF/MC-LR groups after 24 h of exposure, and ETH/MC-LR groups significantly
511 decreased in cell viability from 24 to 72 h, suggesting that CYP-induced HepG2 cells
512 are more sensitive to MC-LR exposure. Comparison of OME, ETH, and RIF revealed
513 that in ETH incubated cells MC-LR exposure could reduce cell viability in
514 a time-dependent fashion ([Supplemental Fig. 8 A](#)). In cells, ETH has proved to be a
515 very effective inducer of CYPs, such as CYP1A1, CYP1A2, CYP3A4, and
516 particularly CYP2E1 ([Roymans et al., 2005](#)). Based on the present results, the study
517 proceeded using ETH to further investigate the mechanisms by which HepG2 cells are
518 more sensitive to MC-LR. The MTT assay showed that MC-LR was not toxic to cells
519 after 24 h of exposure except in ETH/MC-LR groups at MC-LR concentrations above
520 5 μ M ([Fig. 3 A](#)). Interestingly, we found that in comparison with MC-LR exposure
521 alone, ETH/MC-LR treatment significantly triggered ROS generation in HepG2 cells
522 ([Fig. 3 B](#)). One could make a reasonable case that the overexpression of CYP1A1,
523 CYP1A2, and CYP2E1 increased ROS generation ([Puntarulo and Cederbaum, 1998](#)).
524 Several studies have shown that oxidative stress is considered an important
525 mechanism of MC-LR toxicity, which is known to produce a large amount of ROS
526 modifying intracellular antioxidant enzymes and inducing lipid peroxidation ([Li et al.,](#)
527 [2003](#); [Žegura et al., 2004](#)). Moreover, our results also showed that MDA levels in

ETH/MC-LR groups (48 and 72 h) significantly increased compared to the control and MC-LR-treatment-alone groups ([Supplemental Fig. 8 B](#)). These results confirm the production of excessive ROS in ETH/MC-LR-treated HepG2 cells, which may induce apoptosis. Importantly, the Hoechst 33342 staining assay proved that ETH/MC-LR increased the apoptosis of HepG2 cells ([Supplemental Fig. 8 C](#)). ETH is an effective inducer of CYP2E1 ([Kostrubsky et al., 1995](#)), and CYP2E1 exhibits NADPH oxidase activity and is the most active form of ROS-generating CYP450 ([Nong et al., 2007](#)). Therefore, we hypothesize that CYP2E1 plays an important role in cellular metabolic changes of HepG2 cells caused by MC-LR.

To further investigate the role of CYP2E1 in MC-LR-induced cytotoxicity, we chose CMZ, which has been shown to a specific inhibitor of CYP2E1, to inhibit the induction of CYP2E1 at the mRNA and enzyme levels ([Tindberg et al., 1996](#)) to understand the relationship between CYP2E1 and MC-LR-induced ROS. The results showed that the cell viability was generally not affected at CMZ/5 μ M MC-LR, but it increased more than 18% in CMZ/30 μ M MC-LR compared with the cells treated with MC-LR alone for 96 h ([Supplemental Fig. 9 C](#)), suggesting that CMZ may play a role in protection against MC-LR-induced cytotoxicity. Co-incubation with the ROS scavenger L-ascorbic acid and MC-LR showed that the ROS scavenger appeared to be protective against MC-LR-induced ROS and cell death ([Supplemental Fig. 9 D, E](#)). Further study showed that CMZ decreased the elevated intracellular levels of ROS after 24, 48, and 72 h ([Fig. 4 A](#)), and inhibited MCLR-induced MDA levels in 24 and 48 h ([Fig. 4 B](#)), suggesting the involvement of CYP2E1 in MCLR-induced

cytotoxicity in HepG2 cells. The mitochondria are the most pivotal subcellular site of superoxide production and play an important role in apoptosis (Guzy et al., 2005), and MMP generally represents the integrity of mitochondria membrane (Eaton and Klaassen, 2001). Caro and Cederbaum (2004) found that MMP was decreased in the CYP2E1-expressing HepG2 cells, and the decrease shared similar characteristics with the developing toxicity. In the present study, MMP was lower in MC-LR group than in CMZ/MC-LR group (Fig. 4 C), indicating that CMZ suppresses CYP2E1 expression and neutralizes damage to mitochondria. Caspase-3 has been shown to be a pivotal regulator of apoptosis and is usually cleaved and activated during the final step of apoptosis (Martin and Green, 1995). We found that CYP2E1 inhibitor CMZ inhibited caspase-3 activity induced by MC-LR in the present study (Fig. 4 D). The result of this study indicates that CMZ protects cells from MC-LR-induced apoptosis through inhibition of CYP2E1.

5. Conclusion

In conclusion, the results of our study showed that MC-LR exposure promoted the altered transcriptions of Phase I and Phase II metabolic enzymes and export pump genes in HepG2 cells. This result suggests that MC-LR-exposure may destabilize the metabolism of HepG2 cells. We believe that the linkage between CYP2E1-dependent oxidative stress and mitochondrial injury contributes to the toxic action of MC-LR on HepG2 cells via our experiments using the CYP2E1 inducer ETH and the CYP2E1 inhibitor CMZ. MC-LR-induced cytotoxicity is a multifactorial process. Because

ETH is not the specific inducer for CYP2E1, and the OME and RIF exposure also affected the cell viability caused by MC-LR, further studies are necessary to investigate the function of other metabolic enzymes and export pump genes in MC-LR metabolism.

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858 Table 1. Specific primers used for qPCR.

Primer	Forward primer	Reverse primer
OATP1B1	GAATGCCCAAGAGATGATGCTT	AACCCAGTGCAAGTGATTTCAAT
OATP1B3	GTCCAGTCATTGGCTTTGCA	CAACCCAACGAGAGTCCTTAGG
CYP1A1	CCACCAAGAACTGCTTAGCC	CAGCTCCAAAGAGGTCCAAG
CYP1A2	TCGTAAACCAGTGGCAGGT	GGTCAGGTCGACTTTTCACG
CYP1B1	CGGCTGGATTTGGAGAACGTA	TGATCCAATTCTGCCTGCACT
CYP2B6	ATGGGGCACTGAAAAAGACTGA	AGAGGCGGGGACACTGAATGAC
CYP2E1	GCAAGAGATGCCCTACATGGA	GGGCACGAGGGTGATGAA
CYP3A4	TCTTCACCGTGACCCAAAGTA	CATTGGCATGAGGTTTGCTC
CYP4A11	ATGAAGTGTGCCTTCAGCCA	AAGGCATTCCTCACACGGG
CYP26B1	CACTCGCGACAAGAGCTG	GCCTCCTGGTACACGTTGAT
GST M4	TTTCCTCGCCTATGATGTCC	CAGACAGCCACCCTTGTGTA
EPHX1	TGCACATCCAGTGCACCAA	CCAGACCCACAGGAGAGTCATT
UGT1A1	AACTTTCTGTGCGACGTGGTT	GTCACCTCTCTCTGAAGGAATTCTG
NAT1	TGGGAGGATTGCATTCAGTCT	TGCTTCTTCCTGGCTTGAATTC
SULT1A1	GTCACCGAGCTCCCATCTTC	GTCTCCATCCCTGAGGGAATC
MRP1	ACCCATGAGCTTCTTTGAGC	CACCAATGACGTTGAACAGG
MRP2	TGAGCAAGTTTGAAACGCACAT	AGCTCTTCTCCTGCCGTCTCT
MRP3	GTCCGCAGAATGGACTTGAT	TCACCACTTGGGGATCATT
MDR1	GCCAAAGCCAAAATATCAGC	TTCCAATGTGTTTCGGCATT
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC

Fig.1. Detection of MC-LR entrance into cells exposed to various concentrations of MC-LR for 24 h.

Fig.2. The transcriptional alteration of CYP2E1 and GSTM4 genes in HepG2 cells after 24 h of MC-LR-exposure.

Fig.3. Effects of CYP inducers on MC-LR-induced cytotoxicity and damage in HepG2 cells.

Fig.4. Effects of CMZ on MC-LR-induced cytotoxicity and damage in HepG2 cells.

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Figure Legends

Fig.1. Detection of MC-LR entrance into cells exposed to various concentrations of MC-LR for 24 h.

HepG2 cells were treated with MC-LR at various concentrations for 24 h and western blot assay were described in the Materials and methods. All experiments were performed in triplicate. M: marker.

Fig.2. The transcriptional alteration of CYP2E1 and GSTM4 genes in HepG2 cells after 24 h of MC-LR-exposure.

MC-LR exposure and qPCR performance were described in the Materials and methods. All experiments were performed in triplicate, and the data are shown as the mean $\pm$ SD. Asterisks denote a response that is significantly different from the control (* p < 0.05, ** p < 0.01). (A) *CYP2E1* and (B) *GSTM4*.

Fig. 3. Effects of CYP inducers on MC-LR-induced cytotoxicity and damage in HepG2 cells.

MC-LR-exposure and research assay were described in the Materials and methods. All experiments were performed in triplicate, and the data are shown as the mean $\pm$ SD. Asterisks denote a response that is significantly different from the control (* p < 0.05, ** p < 0.01) and hash symbol denote a response that is significantly different from the MC-LR treated alone ([#] p < 0.05, ^{##} p < 0.01). (A) Viability of HepG2 cells exposed to 0.5, 1, 5, and 10 μ M MC-LR with and without ETH for 24 h. (B)

Intracellular ROS levels in HepG2 cells exposed to 5 μ M MC-LR with and without
ETH for 72 h.

Fig. 4. Effects of CMZ on MC-LR-induced cytotoxicity and damage in HepG2 cells.

MC-LR-exposure and biochemical assays were described in the Materials and
methods. All experiments were performed in triplicate, and the data are shown as the
mean $\pm$ SD. Asterisks denote a response that is significantly different from the control
(* p <0.05, ** p <0.01) and hash symbol denote a response that is significantly different
from the MC-LR treated alone ([#] p <0.05, ^{##} p <0.01). (A) Intracellular ROS levels in
HepG2 cells exposed to 30 μ M MC-LR with and without CMZ for 72 h. (B)
Intracellular contents of MDA in HepG2 cells exposed to 30 μ M MC-LR with and
without CMZ for 48 h. (C) MMP changes in HepG2 cells incubated with 30 μ M
MC-LR with and without CMZ for 48 h. Red fluorescence represents the
mitochondrial aggregate form of JC-1 with high MMP and green fluorescence
represents the monomeric form of JC-1 with low MMP. a. control, b. CMZ (20 μ M), c.
MC-LR (30 μ M), d. CMZ (20 μ M)/MC-LR (30 μ M). (D) Caspase-3 activity in
HepG2 cells exposed to 30 μ M MC-LR with and without CMZ for 48 h.

Graphical abstract