RESEARCH ARTICLE

Protein expression profiling in the zebrafish (*Danio rerio*) embryos exposed to the microcystin-LR

Guangyu Li[1], Jun Chen[1], Ping Xie[1], Yan Jiang[1], Laiyan Wu[1] and Xuezhen Zhang[2]

[1] Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Wuhan, P. R. China
[2] Fisheries College of Huazhong Agricultural University, Wuhan, P. R. China

Received: July 17, 2010
Revised: January 22, 2011
Accepted: February 1, 2011

Microcystin-leucine-arginine (MCLR) is the most toxic and the most commonly encountered variant of microcystins (MCs) in aquatic environment, and it has the potential for developmental toxicity. A number of previous studies have described the developing toxicity of MCLR based on conventional toxicological indices. However, the molecular mechanisms by which it expresses its toxicity during the early development remain largely unknown. To further our understanding of mechanisms of action and identify the potential protein biomarkers for MCLR exposure, a proteomic analysis was performed on developing zebrafish embryos exposed to 0.5 mg/L MCLR until 96 hours post-fertilization. 2-DE combined with MS was employed to detect and identify the protein profiles. Results showed that 75 spots from the 0.5 mg/L MCLR condition showed a significant increase or decrease in abundance compared with the control. In total, 40 proteins were identified. These proteins were mainly included in process related to oxidative stress, energetic metabolism, and the cytoskeleton assembly. MCLR exposure also affects the expression of the subunits of protein phosphatases 2A. Furthermore, the proteomic and transcriptional analysis of nine proteins was determined by Western blot and quantitative real-time PCR due to their correlation with the known MCLR toxic mechanisms. The consistent and discrepant results between protein and mRNA levels indicated complicated regulatory mechanisms of gene expression in response to MCLR exposure.

Keywords:
Animal proteomics / Developmental toxicity / Gene expression / Microcystin-leucine-arginine / Zebrafish

1 Introduction

In recent decades, public health concerns about toxic cyanobacteria have increased due to the frequent occurrence of cyanotoxins in both drinking and recreational waters. Among cyanotoxins, microcystins (MCs) are the most common all over the world, with molecular weights ranging between 900 and 1000 Da. So far, more than 80 different structural analogues of MCs have been identified [1], with microcystin-leucine-arginine (MCLR) being the most common variant and toxic [1, 2]. MCs potently inhibited protein phosphatases 1 and 2A [3] and induced production of reactive oxygen species (ROS) [4, 5], followed by destruction of the cytoskeleton, leading to liver apoptosis, necrosis, and hemorrhage [6]. MCs have been shown to have a far-reaching impact on the aquatic organisms [7, 8]. Both field and laboratory studies indicated that MCs accumulated mainly in liver, but also in intestine, gill and kidney, subsequently resulting in damages to these organs [9–14].

Correspondence: Dr. Ping Xie, Donghu Experimental Station of Lake Ecosystems, State Key Laboratory for Freshwater Ecology and Biotechnology of China, Institute of Hydrobiology, The Chinese Academy of Sciences, Donghu South Road 7, Wuhan 430072, P. R. China
E-mail: xieping@ihb.ac.cn
Fax: +86-27-68780622

Abbreviations: CK, creatine kinase; **CKmb,** creatine kinase, muscle b; **DRP2,** dihydropyrimidinase-like 2; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **hpf,** hour(s) post-fertilization; **IF,** intermediate filament; **IPI,** International Protein Index; **MC,** microcystin; **MCLR,** microcystin-leucine-arginine; **MT,** microtubule; **QPCR,** quantitative real-time PCR

Colour Online: See the article online to view Figs. 4, 5 in colour.

Recently, both laboratory and field studies demonstrated the accumulation of MCs in the gonads of rats, water bird, duck, fish, and even the eggs of shrimps [13, 15–17], which indicates the maternal transfer of MCs to offspring. Therefore, the embryonic developing toxicity of MCs is the cause for concern.

The embryos have been shown to be more susceptible to MCs than juvenile and adult fish, as described for many xenobiotics [18]. In recent decades, increasing studies that examine the toxic effect of MCs on the larval growth and embryonic development of aquatic organisms have been reported [14, 19–26]. Due to lower mobility, the early life stage of fish was believed to be more vulnerable than juveniles and adults [20]. Toxic effects of MCs on embryonic development were stage specific [22, 27], which might cause low hatching, retarded development, and high malformation [14, 19, 21–23, 27]. However, acute endpoints, such as survival rate, hatching, and morphological disorders cannot provide enough information to reveal the mechanism of MC-induced effects. Overall, the molecular mechanisms of MC-induced toxicity during the embryonic development remain largely unknown. This hampers the risk assessment for the developing embryos when they are environmentally exposed to MCs.

Proteomic-based approaches, which examine the expressed proteins of a tissue or cell type, complement the genome initiatives and are increasingly being used to address biomedical questions. Proteins are the main functional output, and the genetic code cannot always indicate which proteins are expressed, in what quantity, and in what form. For example, post-translational modifications of proteins, such as phosphorylation or glycosylation, are very important in determining protein function. In recent studies, such approaches have been employed to gain a better understanding of the mechanisms of toxicity induced by MCs and several other toxicants, such as MCLR in mouse, medaka fish (*Oryzias latipes*) and adult zebrafish (*Danio rerio*) [28–31]; MC-RR in human amnion FL cells [32]; perfluorooctane sulfonate in zebrafish [33]; *Corbicula fluminea* exposed to a *Microcystis aeruginosa* toxic strain [34]; tetrabromobisphenol-A in zebrafish liver [35]. Proteomic-based methods have been approved to be effective in identifying early responses to the MCs and, simultaneously identifying the toxicity and mechanisms involved in MC-induced effects on fish [29–31].

To further our understanding with respect to the toxic effects and modes of action of MCLR on early-life development, we first examined endpoints, such as deformation, hatching rate, body length, heart beat as well as the toxin content accumulating in the embryo exposed to MCLR. Second, we performed 2-DE to delineate the expressed protein patterns in the embryo of zebrafish following MCLR treatment. Seventy-five spots were found to be altered in abundance ($\geq$2-fold or $\leq$0.5-fold; $p<0.05$) and subsequently analyzed with sensitive and accurate MALDI-TOF-TOF MS, coupled with database interrogation. Forty proteins were successfully identified. Western blot and quantitative real-time PCR (QPCR) was then used to assay the protein expression of nine selected altered protein spots due to their correlation with the known MCLR toxic mechanisms. Based on the proteomic analysis together with the Western blot and transcriptional data, the main purpose of the present study is to provide the basis for understanding the underlying mechanisms of MCLR-induced developmental toxicity.

2 Materials and methods

2.1 Chemicals

The cyanobacterial toxin MCLR was obtained from Express (Taiwan), with a purity of $\geq$95%, confirmed by a high-performance liquid chromatography (HPLC, LC-10A, Shimadzu, Nakagyo-ku, Kyoto, Japan) following the method by Moreno et al. [36]. The chemical was dissolved in deionized water. All of the other chemicals utilized in this study were of analytical grade and the chemicals used for electrophoresis were obtained from Amersham Biosciences (Piscataway, NJ, USA).

2.2 Maintenance of zebrafish and embryo toxicity test

Zebrafish maintenance and embryo collection was performed according to the protocol described by Shi et al. [33]. Briefly, WT (AB strain) zebrafish were cultured in a closed flow-through system with charcoal-filtered tap water at 28$\pm$0.5°C in a 14:10 h light:dark cycle. The fish were fed with *Artemia nauplii* twice daily. The spawning adults in groups of about 20 males and 10 females in tanks were prepared for collecting fertilized eggs. At 0.5–1 hour(s) post-fertilization (hpf), normally developed embryos were selected and randomly distributed in beakers (30 embryos in each beaker) containing different concentrations of MCLR (0, 0.2, 0.5, 2, and 5 mg/L) and a 50 mL solution comprising $Ca(NO3)_2$ (0.2 mM), $MgSO_4$ (0.13 mM), NaCl (19.3 mM), KCl (0.23 mM), and HEPES (1.67 mM) [37]. The exposure time was selected at 96 hpf, because most organs of the embryos are well developed at 96 hpf. Based on earlier range-finding studies, the environmentally relevant or high concentrations were selected in the present study. The control group received no MCLR. Each concentration had three replicates and each replicate was consisted of a glass beaker containing 50 mL of the respective treatment solutions and 30 viable embryos. Acute endpoints such as hatching success, mortality, embryo malformation, and heart rate were monitored (Figs. 1 and 2). Mortality was identified under a stereomicroscope according to Shi et al. [33]. All the larvae were measured for the whole body length with digital images produced using the Image Pro Plus

software (Media Cybernetics, Bethesda, MD, USA). The larvae that had developed normally were selected for the following experiments.

2.3 Determination of MCLR concentration in larvae

Extraction and quantitative analysis of the MCLR content in zebrafish larvae (0.2 g lyophilized sample [about 6000 larvae] for each MCLR-exposed group) exposed to MCLR (0, 0.2, 0.5, 2, and 5 mg/L) followed the method of our previous study [15].

2.4 Proteomic analysis

2.4.1 Protein extraction

The embryos exposed to 0.5 mg/L MCLR were selected for 2-DE analysis. The concentrations of MCLR were selected on the basis of endpoints measured in the present study. Protein extraction was performed basically according to the method reported by Tay et al. [38] with minor modification by Shi et al. [33]. Briefly, about 20 frozen zebrafish larvae were homogenized in 800 μL lysis buffer (2 M thiourea, 7 M urea, 2% DTT, 20 mM Tris base, 4% CHAPS, 1% protease inhibitor cocktail, 20 μL/mL Bio-Lytes 3/10, 0.5 μL benzonase). The solution was then centrifuged at $12\,000 \times g$ for 20 min at 4°C, after which the supernatant was collected. The protein content was quantified using a 2-DE Quant Kit (GE Healthcare, Piscataway, NJ, USA).

2.4.2 2-DE analysis

2-DE analysis was performed according to the method described by Shi et al. [33], with slight modification. In brief, the prepared pooled protein samples (600 μg protein on preparative gels or 120 μg protein on analytical gels) were mixed with rehydration buffer to a volume of 450 μL. The IPG strips (pH 4–7, 24 cm, GE Healthcare) for the first dimension were used to isolate the altered proteins, and the running condition was set at 20°C, step 1: 300 V for 0.5 h, step 2: 700 V for 0.5 h, step 3: 1500 V for 1.5 h, step 4: 9900 V for 3 h, step 5: 9900 V for 6.5 h, step 6: 600 V for 20 h, step 7: 8000 V constant for a total of 56 000 Vh. After completion of the IEF program, the strips were equilibrated in two steps: 15 min in an IPG equilibration buffer: 6 M urea, 2% SDS, 30% glycerol, 0.375 M Tris (pH 8.8), 20 mg/mL DTT, and a trace of bromophenol blue, and then alkylated for 15 min. Subsequently, a 12.5% SDS-PAGE 2-DE was performed. Electrophoresis was carried out at 20 mA per gel for 40 min and then at 30 mA per gel until the dye front reached the bottom. The protein spots were visualized via either silver staining or Coomassie Brilliant Blue G-250 staining. Triplicate 2-DE gels were performed for each group.

2.4.3 Image acquisition and analysis

Triplicate gels from MCLR treated (0.5 mg/L) and untreated larvae (control) were analyzed for spot intensity using Image Master 2D Platinum software (GE Healthcare) according to the protocols provided by the manufacturer. The criterion for significant changes in protein expression was differences more than or equal to two-fold calculated from the treated and control groups.

2.4.4 Protein identification

The protein identification was performed according to the method described by Shi et al. [33], with slight modification. In brief, gel spots showing significant changes were excised from 2-DE gels. Gel spots were washed and then digested with sequencing-grade trypsin (Promega, Madison, WI, USA). MALDI-TOF MS and TOF/TOF tandem MS were performed on a MALDI-TOF-TOF mass spectrometer (4800 Proteomics Analyzer, Applied Biosystems, Foster City, CA, USA). The instrument was set in reflector mode. Peptide mass fingerprints coupled with peptide fragmentation patterns were used to identify the protein in the International Protein Index (IPI) (http://www.ebi.ac.uk/IPI/IPIhelp.html) database (Version v3.67) using the MASCOT search engine (http://www.matrixscience.com). The functions and specific processes of the identified proteins were matched by searching Gene Ontology (www.geneontology.org).

2.5 Gene expression

Isolation, purification, and quantification of total RNA, first-strand cDNA synthesis, and QPCR were performed using our previously described protocols [39]. Seventeen differentially expressed important proteins were examined to detect the corresponding mRNA levels by QPCR to validate the protein expression. The QPCR was performed as described in our previous study [39]. Gene names, accession numbers, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. PCR amplification was conducted on a Chrom 4^{TM} detector (BioRad, USA) in sterile, 96-well PCR plates (Applied Biosystems). Every sample was analyzed individually and processed in triplicate. On the basis of the results of our previous studies, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression levels were not significantly different between control and treated groups following the treatment with MCLR, and it was chosen as an internal control to normalize the data. After verifying that the amplification efficiencies of the selected genes and GAPDH were approximately equal, differences in expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [40, 41].

Table 1. Primers for QPCR analysis

Gene	Accession no.	Forward primer (from 5′ to 3′)	Reverse primer (from 5′ to 3′)	Amplicon size (bp)
GAPDH	NM_001115114	CTGGTGACCCGTGCTGCTT	TTTGCCGCCTTCTGCCTTA	150
PP2aA	NM_213376	AGTTCTGCTTGCCCTTGCTG	GACTCCACTGCCTTGTCCC	141
PP2aC	NM_200911	AGGAAACCACGAAAGCAGG	TCTACCAGGGCAGTGAGGG	135
Hsp90b1	NM_198210	ATGAGGCGGCTGTGGATTAT	AGGCATCGGAAGCATTAGAG	334
CKmb	NM_001105683	TCACCCTGCCTCCTCACAA	TGCCCTTGAACTCACCATCC	96
Tuba1	NM_194388	CTATCCTCGTATCCACTTCCC	CACCACGGTACAGCAGACA	182
LaminB2	NM_131002	ACTCGCCAAGGCTGAAGATG	CCACCTCCACCATACGCTT	185
keratin4	NM_131509	TTCCTCAGGGCAGTCTACGA	CACGAACTTCAGCCACGAT	127
Tpma	NM_131105	AGGAGAACGCCTTGGACAG	CAGGGCCTCGGAGTATTTG	149
Bactin1	AF057040	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC	102

2.6 Western blot analysis

The zebrafish larvae exposed until 96 hpf were first washed twice with PBS (pH 7.4), and then homogenized for extract proteins in ice-cold protein extraction buffer (Wuhan Boster Biological Technology, China). Each set of 150 larvae was pooled for protein preparation, such that $n = 1$ refers to protein from these 150 embryos. The homogenates were centrifuged for 10 min at 12 000 × *g* and supernatants were collected. The concentrations of protein were determined by Bradford method.

Western blotting analysis was performed as described in our previous study [39] with some modifications. About 20 μg of protein from each sample was denatured, electrophoresed, and transferred onto a PVDF membrane. The membrane was blocked and blots were incubated in specific antibody against PP2A A, PP2A C, Hsp90b1, Bactin1, Tuba1, Tpma, Lmnb2, Krt4, Ckmb, and GAPDH (Abcam, UK), and then secondary antibodies following the manufacturer's instructions. NBT/BCIP system was used to evaluate the protein signal. The results of Western blot were quantified with Gene Snap software (Syngene, America).

2.7 Statistical analysis

The homogeneity of variance was checked by using Levene's test. If the data failed to pass the test, then a logarithmic transformation was used. The differences were evaluated by a one-way ANOVA followed by a Tukey's test using SPSS 13.0 (SPSS, Chicago, IL, USA). Significant differences between treatments and corresponding control were identified by a *p*-value of < 0.05.

3 Results

3.1 General impact on animals and level of MCLR in tissues

Zebrafish embryos were exposed statically to MCLR from 0.5–1 hpf until the time of observation. MCLR exposure caused malformation in a concentration-dependent manner, with malformation rates being recorded as 4.4 ± 1.9, 6.7 ± 3.3, 15.6 ± 5.0, 27.8 ± 1.9, and $42.2 \pm 5.1\%$ at concentrations of 0, 0.2, 0.5, 2, and 5 mg/L, respectively (Fig. 1A). The survival rate for 0.2 and 0.5 mg/L MCLR exposed groups was not affected, but was significantly reduced in other exposure groups at 76.6 ± 1.7 and $75.2 \pm 1.1\%$ relative to $82.2 \pm 1.9\%$ in the control group (Fig. 1B). The developmental abnormalities included tail malformations, heart malformations, skeletal malformations, spinal curvature, yolk sac, and pericardial edema, epiboly deformities and swim bladder inflation. The heart rates of the larvae were significantly reduced after exposure to MCLR for 96 hpf in all of the exposure groups (0.2, 0.5, 2, and 5.0 mg/L) at 178 ± 1.4, 169.9 ± 1.0, 163.7 ± 3.4, and 146.5 ± 1.5 beats/min relative to 197.6 ± 3.0 beats/min in the control group (Fig. 2A). Growth was also recorded until exposed to 96 hpf. Body length was not significantly affected by exposure to 0.2 mg/L MCLR, but was significantly reduced in the other exposure groups at 3.74 ± 0.10, 3.73 ± 0.09 and 3.70 ± 0.07 mm compared with 3.91 ± 0.06 mm in the control group (Fig. 2B). The hatching rate in the control group was $92.4 \pm 1.4\%$, and in the 0.2, 0.5, and 2 mg/L MCLR-treated groups was 91.2 ± 1.9, 89.6 ± 2.6, and $88.2 \pm 2.9\%$ with no significant difference. A reduced hatching rate ($87.4 \pm 2.1\%$) was observed only in the 5.0 mg/L MCLR-exposure group.

Levels of MCLR in larvae exposed to 0.5, 2, and 5 mg/L MCLR increased in a dose-dependent manner and were as high as 58.5, 172.5, and 478.5 ng/g DW, respectively (Fig. 3). No MCLR was detected in tissues of untreated control and 0.2 mg/L MCLR-treated group.

3.2 Proteome analysis

To understand how MCLR could affect fish development, the effects of MCLR on the protein expression pattern in early developing zebrafish larvae were investigated. 2-DE technique was used to compare embryos from the untreated control group with embryos exposed to 0.5 mg/L MCLR during 96 h. On average, more than 2800 protein spots were detected in

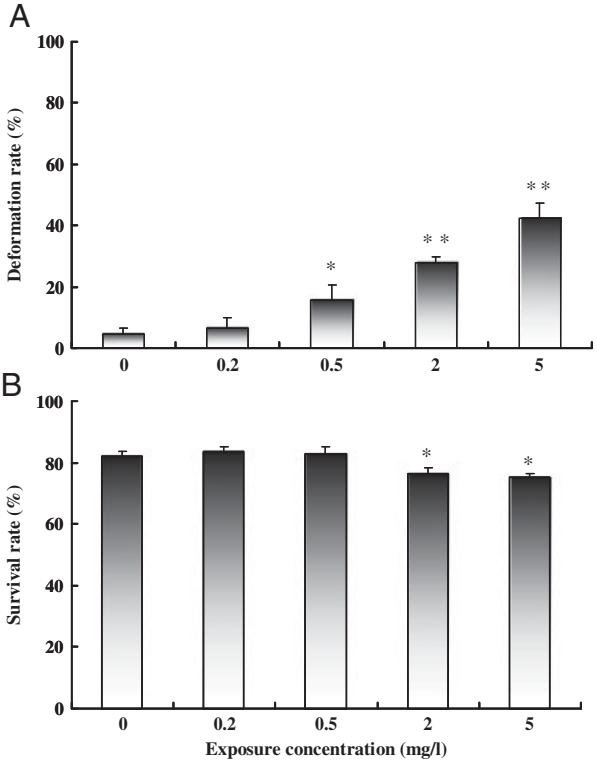

Figure 1. Cumulative malformation rate (A) and survival rate (B) in zebrafish embryos exposed to different MCLR concentrations (0, 0.2, 0.5, 2.0, and 5.0 mg/L) until 96 h. The values are presented as the mean ± SEM. Values that are significantly different from the control are indicated by asterisks (one-way ANOVA, followed by a post-hoc test: LSD: $^{*}p<0.05$; $^{**}p<0.01$).

each gel. Compared with the 2-DE gels of the nonexposed zebrafish larvae, altered expression of 75 protein spots (≥2-fold or ≤0.5-fold; $p<0.05$) were detected. In total, the analysis of 75 protein spots allowed the identification of 40 different proteins (Table 2). Among these altered proteins, 16 protein spots were significantly upregulated and 24 protein spots were noticeably downregulated in the 0.5 mg/L MCLR exposed zebrafish larvae (Table 1 and Fig. 4). These altered protein spots were excised for identification analysis using MALDI-TOF-TOF MS. All of the protein spots were successfully identified with confidence interval % (C.I. %) values greater than 95% (Table 2) and the matched proteins were obtained from the IPI database for zebrafish. Of the identified proteins, 20 proteins were characterized as cell cytoskeleton proteins, corresponding to cytoskeleton-microtubule (MT), microfilament, intermediate filament (IF), dynein, and nuclear matrix-related proteins. Four proteins (spots A19, A29, A49, and B63) were involved in metabolism, and four proteins (spots A1, A7, A53, and A55) in response to stimulus. Two proteins (spots A9 and A57) were characterized as PP2A complex, classified as PP2A A and PP2A C subunit. The other ten proteins were categorized into structure formation, signal transduction, regulation of translational initiation, and other functional proteins.

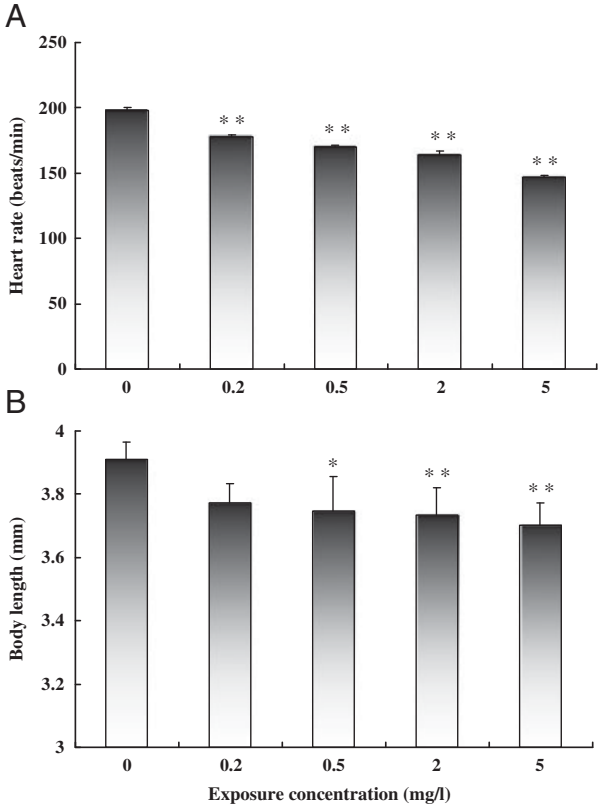

Figure 2. Heart rate (A) and growth (B) of zebrafish embryos exposed to different MCLR concentrations (0, 0.2, 0.5, 2.0, and 5.0 mg/L) until 96 h. The values are presented as the mean ± SEM. Values that are significantly different from the control are indicated by asterisks (one-way ANOVA, followed by a post-hoc test: LSD: $^{*}p<0.05$; $^{**}p<0.01$).

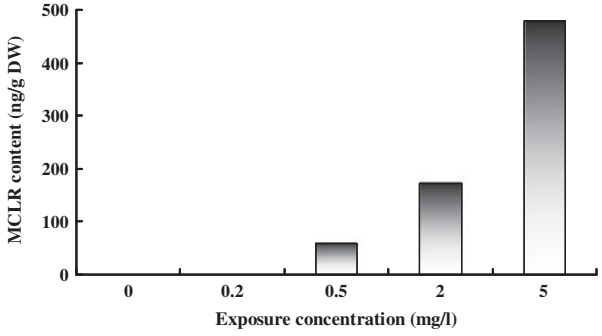

Figure 3. MCLR contents accumulated in zebrafish larvae after 96 h of MCLR exposure (0, 0.2, 0.5, 2.0, and 5.0 mg/L).

3.3 Verification of differentially expressed protein by Western blot analysis and gene expression

From the candidates, PP2A A, PP2A C subunit, Hsp90b1, Bactin1, Tuba1, Tpma, Lmnb2, Krt4, and Ckmb were selected for Western blot analysis due to their correlation with the known MCLR toxic mechanisms. As shown in

Table 2. A detailed list of protein spots identified by MALDI-TOF/TOF MS from the zebrafish larvae following 0.5 mg/L MCLR exposure

No. on gel	Identification	Fold change[a]	Accession no.	p[b]	Mw (KDa)/ p/ theoretical	Mw (KDa)/ p/ actual	Protein score	Total ion score	MS/MS peptide sequence	SC (%)[c]	Functional category
Cytoskeleton											
	Cytoskeleton-MT										
B16	α-Tubulin (Tuba1)	0.454	IPI00503192	0	49.93/4.97	47.52/6.15	202	101	ELIDLVLDR	34.74	Constituent of cytoskeleton; MT-based movement
B51	Tubulin, α 8 like 3 (Tuba8/3)	0.460	IPI00503192	0	50.03/4.93	28.03/6.12	97	68	NLDIERPTYTNLNR	17.82	Constituent of cytoskeleton; MT-based movement
B75	Tubulin β (Tubb2c)	0.346	IPI00851337	0	6.87/9.86	19.24/4.36	101	29	DMQFHQR	86.67	Cell motion; MT; MT-based movement
B32	zgc:55461	0.451	IPI00494039	0	49.76/4.79	38.63/5.26	319	145	IREEYPDR	48.31	Protein polymerization; MT-based movement
	Cytoskeleton microfilament										
B19	Actin, α, cardiac muscle like (Actc1a)	0.398	IPI00507230	0.0211	41.94/5.22	45.02/5.57	63	40	GYSFVTTAER	15.12	Embryonic heart tube development; structural constituent of cytoskeleton
B20	Actin, α 1, skeletal muscle (Actc1b)	0.431	IPI00504207	0	41.94/5.23	44.13/6.74	316	213	AGFAGDDAPR	45.09	Cytoskeleton; embryonic heart tube development
B35	Actin, α 1b, skeletal muscle (Acta1b)	0.422	IPI00504207	0	41.94/5.22	36.45/5.46	347	267	GYSFVTTAER	57.29	Structural constituent of cytoskeleton; myosin binding; muscle contraction
B46	Actin, α 2, smooth muscle, aorta (Acta2)	0.460	IPI00551966	0	41.96/5.23	31.44/5.08	254	192	AGFAGDDAPR	39.79	Constituent of cytoskeleton; MT-based movement
B58	β-Actin (Bactin1)	0.432	IPI00920131	0	14.23/5.46	26.31/5.26	147	71	AVFPSIVGRPR	64.34	Cytoskeleton; cytoplasm; ATP binding
A32	Desmin (Desm) cytoskeleton-IF	2.081	IPI00774314	0	54.01/5.50	54.20/4.65	197	NA[d]	NA[d]	43.97	Muscle contraction
B40	Keratin 4 (Krt4)	0.341	IPI00834037	0	54.24/5.42	34.65/4.82	108	26	AVYEAELR	19.08	Cell adhesion
B28	Keratin 5 (Krt5)	0.360	IPI00861872	0.0009	58.55/5.34	40.24/4.85	76	27	VDSLQDEINFLR	16.85	Cell adhesion; signal transduction
B12	Novel keratin family protein zgc:136930 (zgc:136930)	0.477	IPI00851554	0	49.48/4.69	50.02/4.34	323	102	ALQQENAALEAK	53.78	Structural molecule activity
B3	LOC572200 protein (Fragment) (LOC572200)	0.445	IPI00493493	0	47.09/5.00	71.87/6.77	240	103	GYIEEEVEVWR	60.29	Vimentin protein binding; IF-based process
A35	zgc:92533	2.469	IPI00489480	0	49.96/5.31	48.69/6.61	323	104	NHEEELLAAR	51.50	Enveloping layerIF; cell migration
	Cytoskeleton-dynein										
B43	Tropomyosin α-1 chain (Tpma)	0.437	IPI00508152	0	32.70/4.70	33.18/4.73	208	74	LVIVEGELER	36.27	Cortical cytoskeleton; growth cone; actin binding
B68	Myosin, heavy peptide2, fast muscle specific (Myhz2)	0.192	IPI00860402	0	22.17/5.55	23.21/5.08	122	80	GGSMQTVSSQFR	21.70	Actin binding; striated muscle contraction

Table 2. Continued

No. on gel	Identification	Fold change[a]	Accession no.	p[b]	Mw (KDa)/ p/ theoretical	Mw (KDa)/ p/ actual	Protein score	Total ion score	MS/MS peptide sequence	SC (%)[c]	Functional category
B54	Myosin, heavy polypeptide 1.2, skeletal muscle (Myhz1.2)	0.27	IPI00860402	0.0003	51.56/5.54	27.55/5.65	81	30	DAQLHLDDAVR	21.70	Actin binding; striated muscle contraction
B1	Novel myosin family protein (Myhz1.3)	0.476	IPI00858466	0	222.07/5.56	88.02/5.33	240	121	DAQLHLDDAVR	23.18	Actin binding; striated muscle contraction;
	Cytoskeleton-nuclear matrix										
A6	Lamin B2 (Lmnb2)	3.452	IPI00513243	0	65.86/5.18	102.29/5.42	130	67	SMFDEEVR	28.99	Lamin filament; nuclear matrix
Metabolism											
A49	CKmb	2.26	IPI00508395	0	42.82/6.29	30.96/6.11	139	79	ELFDPVISDR	27.89	Phosphocreatine biosyntheic process
A19	ATP synthase, H+ transporting mitochondrial F1 complex, β subunit (ATP5B)	2.045	IPI00897805	0	55.09/5.14	66.52/4.73	1000	815	IMNVIGEPIDER	56.29	Polypeptide hydrogen-exporting ATPase activity; hydrogen ion transporting ATP synthase activity
B63	ATPase, H+ transporting, lysosomal V1 subunit A, like (ATP6v1al)	0.451	IPI00484500	0	68.33/5.42	24.26/5.91	103	75	WDFTPINNLR	15.07	Calcium ion homeostasis; ATP metabolic process
A29	Mitochondrial aldehyde dehydrogenase 2b (Aldh2b)	3.13	IPI00920751	0	56.51/6.06	55.74/4.55	105	54	TIPIDGNYFCYTR	16.28	Cellular aldehyde metabolic process
PP2A complex											
A9	Protein phosphatase 2A, regulatory subunit A, β iso (PP2A A)	2.009	IPI00632161	0	65.38/4.91	83.21/4.99	152	89	LASGDWFTSR	25.64	Phosphatase activity; protein phosphatase type 2A complex
A57	Protein phosphatase 2a, catalytic subunit, α (PP2A C)	3.483	IPI00934381	0	31.58/5.55	26.63/5.73	121	32	RGEPHVTR	44.93	Phosphoprotein phosphatase activity; hydrolase activity
Response to oxidative stress											
A1	Heat shock protein 90, β (grp94), member 1 (Hsp90b1)	5.601	IPI00506057	0	91.22/4.77	160.08/4.91	283	186	NKEIFLR	21.56	Response to stress
A7	Heat shock 70 kDa protein 9 (Hspa9)	5.936	IPI00506014	0	73.90/6.72	87.34/5.63	152	68	DAGQIAGLNVLR	16.28	Response to stress
A53	Annexin A1a (Anxa1a)	2.199	IPI00817700	0	25.36/5.38	27.19/5.39	189	113	GVDEPTIIDTLVHR	26.52	Response to stress;
A55	14-3-3 Protein β/α-2 (14-3-3b)	2.088	IPI00498898	0	27.37/4.68	26.97/6.41	176	72	MKGDYYR	30.17	Signal transduction calcium; phospholipid binding

Table 2. Continued

No. on gel	Identification	Fold change[a)]	Accession no.	p[b)]	Mw (KDa)/ p*I* theoretical	Mw (KDa)/ p*I* actual	Protein score	Total ion score	MS/MS peptide sequence	SC (%)[c)]	Functional category
Other functions											
B62	Crystallin, β A2b (Cryba2b)	0.358	IPI00513173	0.0197	23.55/5.79	25.19/6.03	63	43	GYQYIFER	57.87	Structural constituent of eye lens; sensory organ development
B66	Crystallin, β B1 (Crybb1)	0.492	IPI00502990	0	26.76/6.44	23.66/5.30	330	270	IFLFDQENFQGR	76.29	Structural constituent of eye lens; sensory organ development
B74	Crystallin, β B1 (Crybb1)	0.435	IPI00502990	0	26.76/6.44	19.53/6.62	286	203	DMQFHQR	58.62	Structural constituent of eye lens; sensory organ development
A17	DRP2	2.223	IPI00493073	0.0004	62.36/6.05	67.71/5.82	80	19	IFNLYPR	20.59	Signal transduction
B23	Corticotropin-lipotropin (pro-opiomelanocortin) (POMC)	0.385	P01193	1E-05	74.53/8.09	43.59/6.52	117	109	RFPPLVEKFVYPNGAEDESAEA	8.24	Neuropeptide signaling pathway
A47	Eukaryotic translation initiation factor 3, subunit B (Eif3b)	3.862	IPI00801770	0.018	79.74/5.31	32.86/4.38	63	36	WTETYVR	6.30	Regulation of translational initiation
A38	zgc:92533 40 kDa protein	2.981	IPI00864129	0	40.28/5.08	44.26/4.80	149	NA[d)]	NA[d)]	37.19	Novel krab box and zinc c2h2 type domain containing protein
A31	Si:dkeyp-113d7.4	2.456	IPI00886613	0	49.90/4.94	54.30/5.52	149	73	FTLQNLNDR	40.38	Cell migration involved in gastrulation
B57	26 kDa protein	0.481	IPI00931604	0	26.35/4.93	26.42/5.10	108	92	LQCLEIPILSDR	18.60	ARF guanyl-nucleotide exchange factor activity
B30	LOC100149534 hypothetical protein	0.215	IPI00931756	0	41.94/5.18	39.89/5.24	149	59	SYELPDGQVITIGNER	45.89	Unknown

a) The fold changes (mean values $\pm$SD, $n=3$) are indicated as compared with the controls. Only the fold changes ($\geq$2-fold or $\leq$0.5-fold) are shown. Values $\geq$2 indicate upregulations, and $\leq$0.5 downregulations.
b) p indicates p-values for comparison between groups.
c) SC indicates the sequence coverage of the protein in percentage obtained by MS/MS identification.
d) NA, not available.

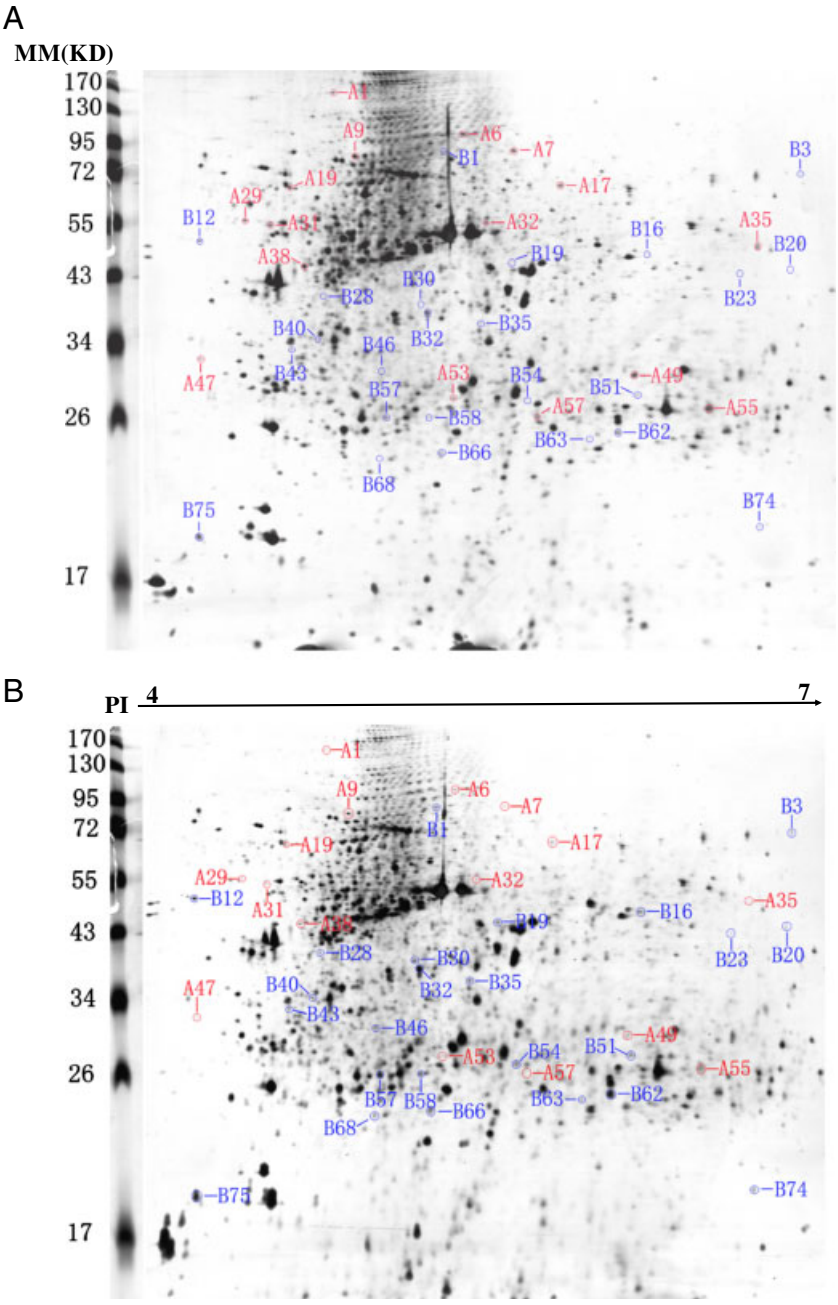

Figure 4. Representative 2-DE gels of the proteins from the zebrafish larvae of the control- and MCLR-treated groups (the 0.5 mg/L treatment was selected). (A) 2-DE gel image with proteins expressed in the 0.5 mg/L MCLR exposure condition; (B) 2-DE gel image with proteins expressed in the control condition. The proteins of the samples were separated by 2-DE and visualized by silver staining. Protein spots that were altered by MCLR exposure are labeled by characters. The molecular weights (MW) and p*I* scales are indicated. Each gel is representative of three independent replicates.

Fig. 5, the expression changes of the selected proteins were consistent with the 2-DE and silver-staining results. Such results demonstrated that the proteomic analysis of response of zebrafish larvae to MCLR was convincing.

Consistent and discrepant results between mRNA and proteins for nine genes were obtained (Fig. 5). The protein levels of these genes were changed in abundance by MCLR exposure and the corresponding mRNA levels, which had identical annotations with the proteins. Five protein (PP2A A subunit, PP2A C subunit, creatine kinase, muscle b [CKmb], keratin 4, and Tpma) expressions of zebrafish larvae showed the same regulated trends in mRNA and protein levels. The other four genes (Hsp90b1, Tuba1, Bactin1, and Lamin B2) exhibited an increase or decrease in protein levels, whereas the mRNA expressions were virtually inconsistent following MCLR exposure.

4 Discussion

The molecular mechanisms by which MCLR induce their toxicity during the development of zebrafish remain largely unknown. In recent studies, the importance of engaging in zebrafish proteomics to reveal the potential mechanisms of

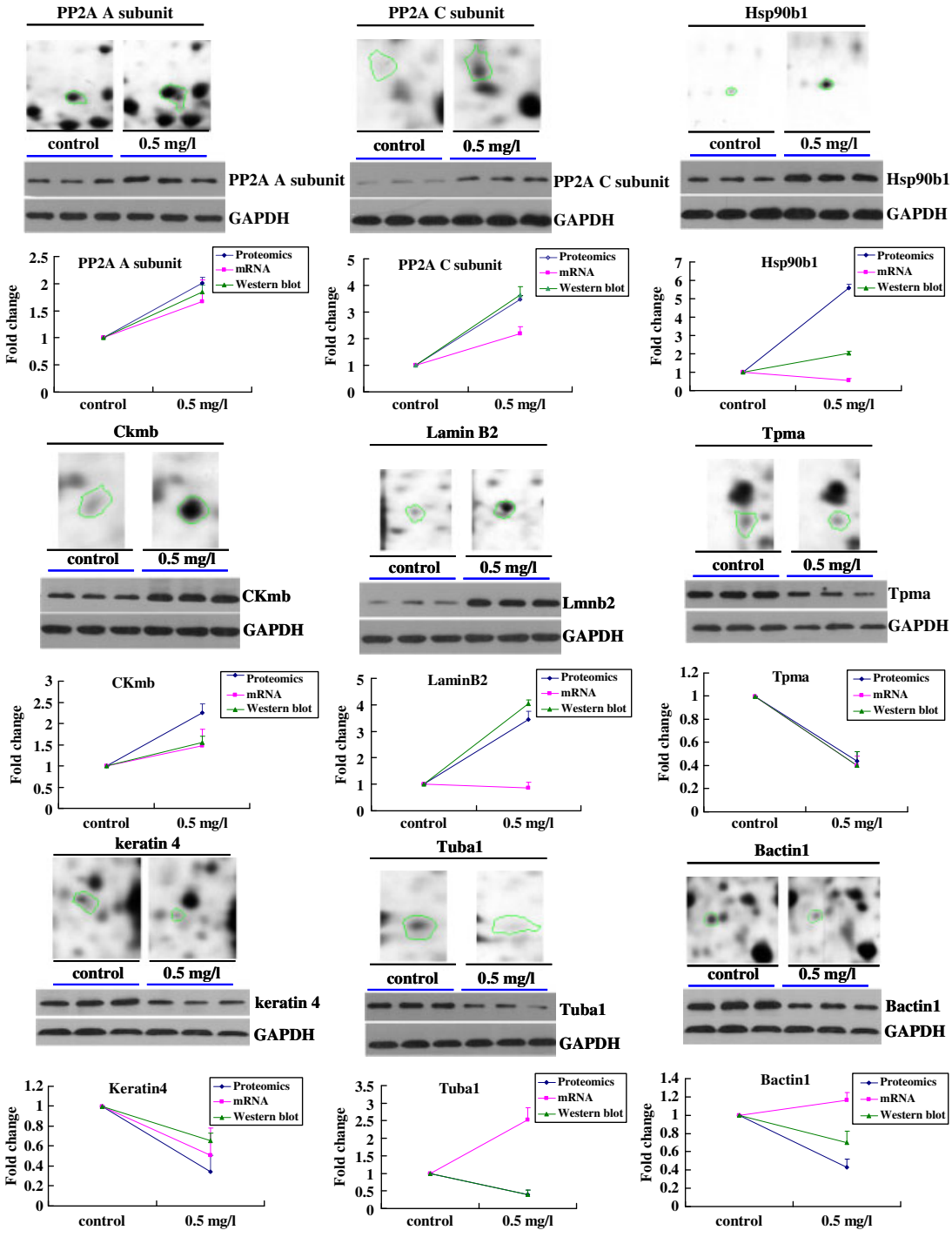

Figure 5. The alteration of proteomic, Western blot, and mRNA analysis of selected altered proteins in the zebrafish larvae following 0.5 mg/L MCLR treatments. The magnified images of protein spots from the 2-DE gels are shown in the upper part of each panel. The line charts show the protein levels based on 2-DE, Western blot results, and mRNA levels using QPCR analysis. The values represent the average fold changes. The values of protein abundance are the average %Vol of spots in three replicated gels.

the model of action has been highlighted [38, 42]. A proteomic approach to zebrafish embryos covers many different aspects of the changes in proteins during the early embryonic stages. The present study is the first to investigate the potential effects of MCLR on the protein expression profiles and transcriptional level of developing zebrafish larvae. As we known, concentration of MCLR in the aquatic environment could reach as high as 1.8 mg/L [43], MCs

could also bioaccumulate in fish and mammals including human through the food web [44, 45], which lead to the higher MC level in aquatic organisms than surface water. The average content of MCLR in the muscle of the 16 species of aquatic animals was 33.1 ng/g DW in Taihu Lake of China [45]. In the present study, the level of MCLR in larvae exposed to 0.5 mg/L MCLR was 58.5 ng/g DW, the content of which could be treated as environmentally relevant. Therefore, the results obtained in the present study could be applied to species living with environmental exposure of MCLR to a certain extent.

In the present study, MCLR exposure through submersion caused developmental toxicity, such as malformation, growth delay and also depressed heart rates in the zebrafish embryos. The sensitivity to MCs exposure is variable in developmental stages among fish species. Oberemm et al. [19, 20] demonstrated that zebrafish embryos and larvae were insensitive when exposed to MCLR at concentrations of 0.5, 5, and 50 μg/L. Although no acute effects were observed during embryonic development, a reduced survival rate and a retarded growth were reported in MCLR and MCRR pre-exposed larvae at 21 days of age. However, Zhang et al. [14] reported that exposure of eggs with medium and high doses of crude extracted MCs (10 and 100 μg/L) not only retarded egg and larval development but also significantly reduced hatching rates of southern catfish. The possible explanations for the observed variations could be attributed to (i) the difference in membrane permeability of MCs between different fish species; and (ii) toxic effects of crude extracted MCs are much more evident than pure MCLR, probably due to the facts that substances (as-yet-unidentified components) of the crude extracts might increase the uptake rate of toxins [19, 20, 46].

PP2A comprises a family of ubiquitously expressed serine-threonine phosphatases implicated in regulation of many signaling pathways. PP2A holoenzymes are composed of three subunits, a 36 kDa catalytic C subunit, a 65 kDa structural A or PR65 subunit, and a variable regulatory B subunit. Each of these subunits is encoded by several distinct genes, which are assembled to create numerous ABC holoenzymes [47]. It is generally believed that MC is a potent inhibitor of the protein PP2A in vitro [48, 49] and in vivo [50] which in turn leads to an increase in protein phosphorylation and subsequently influencing the cell cycle, cytoskeleton and tumor promotion activity. However, the protein expression level of the PP2A exposed to MCs is little known. One important finding of this study is that MCLR can upregulate the expression of the PP2A A and PP2A C subunit in zebrafish larvae exposed to MCLR. Western blot and QPCR analysis also confirm it. In recent studies, Fu et al. [51] observed that MCRR can upregulate the expression of the PP2A A subunit in human amniotic epithelial cells at 12 and 24 h after exposure, which was in agreement with our results. They also concluded that the expression level of the A subunit can modulate the activity of PP2A rather than serving the previously predicted structural roles as a molecular scaffold. Wang et al. [52] demonstrated that the mutations of the β-isoform of PR65 are associated with lung and colon tumors. Wera et al. [53] found that PR65 α overexpressing rat embryo fibroblasts (REF-52 cells) become multinucleated, and these data suggest the multiplicate roles of PP2A A subunit. Constitutively overexpressing the PP2A C subunit was accompanied by cardiac hypertrophy, by a depressed contractile function [54]. In the present study, the heart rates of zebrafish larvae exposed to MCLR were significantly reduced. This effect was possibly due to the high expression of PP2A C subunit in the heart, and may partly explain the observed heart malformations. The underdevelopment of the heart may also affect the cardiac function which could induce an abnormal circulation failure and heart rates, subsequently causing body growth retardation due to insufficient nutrients [55]. Our results together with the previous studies show that the PP2A subunits may have their unique role in biological progress and increased expression is not merely a compensatory effect to counteract the inhibition of PP2A hypothesized by Fu et al. [51]. To our knowledge, this is the first report showing the upregulation of PP2A A and C subunit in zebrafish larvae exposed to MCLR. Further studies are needed for a better understanding of the relationship between upregulation of PP2A subunit and inhibition of PP2A activity.

The cytoskeleton is the basic structural element of all cell types and plays key roles in the maintenance of cell architecture, adhesion, migration, differentiation, division, and organelle transport. It is known that cytoskeleton disruption is one of the first striking cytotoxicities caused by MCLR. Our study demonstrated that MCLR induced the varied expression of cytoskeleton and its associated proteins in early life stage of zebrafish. We found that the abundances of four MTs proteins: Tuba1, Tuba8l3, Tubb2c, zgc:55461; six microfilament (MFs) proteins: Actc1a, Actc1b, Acta1b, Acta2, Bactin1, Desm; and five IFs proteins; keratin 4, keratin 5, zgc:136930, LOC572200 and zgc:92533 were significantly altered in the present study. Among these 15 proteins, the expression of 13 proteins was significantly suppressed after exposure to 0.5 mg/L MCLR. In recent years, MCs-induced morphological changes of MFs, MTs, and IFs have been described. The disruption of the IFs could be attributed to MCLR-induced hyperphosphorylation of keratins 8 and 18 [56, 57]. Several studies have reported that MCLR could induce changes in hepatocytes, fibroblasts and kidney cells, first occurred in IFs, followed by MTs and MFs [58, 59]. Fu et al. [51] showed that some cells lose MTs after MC treatment except the reorganization and aggregation of MTs. Together with these results, we may assume that the suppression of MFs, MTs, and IFs may lead to the morphological changes of zebrafish embryos, and also cause damage to the cytoskeleton of developing embryo/larvae.

In the present study, the average body length of zebrafish was significantly reduced by the exposure to MCLR which is in agreement with other studies, highlighting the inter-

ferences of MCLR with the growth of fish larvae [14, 19, 23]. The explanation of the observed body length reduction of this study might be that MCLR could affect muscle development by interfering with normal myogenesis, as actin α, myosin heavy chain α, and myosin, heavy peptide were downregulated in zebrafish larvae exposed to 0.5 mg/L of MCLR in the present study. Chen et al. [45] showed that MCs could accumulate in the muscle of various vertebrates (fish, turtle, duck, and water bird) from a large eutrophic Chinese lake, Lake Taihu, with toxic microcystis blooms. This suggested that developing muscles could also be influenced by MCLR. Though the global effect of MCLR on the muscle development regulation has been poorly studied at the molecular level, it has been well studied that other toxicants such as Aroclor 1254 could reduce the weight of tadpoles by inhibition the expression of myogenesis-related proteins such as actin α, and myosin heavy chain α [60]. Wang et al. [31] also reported the suppression of α actin in adult zebrafish exposed to MCLR which was consistent with our study. Therefore, the present study showed that MCLR affected MTs, MFs, Ifs, and other cytoskeleton-related proteins, indicating that the cytoskeleton disruption can be a marker in MCLR-induced developmental toxicity. Also, the downregulation of some cytoskeleton-related proteins may contribute a lot to the reduction of body length and have to be taken into account while estimating the developmental toxicity of MCLR.

Another explanation of the observed body length/weight reduction in developing larvae might be linked to an impairment of the energetic pathways in response to an increased energy acquirement with stress [33, 60]. After exposure to 0.5 mg/L MCLR, zebrafish larvae displayed upregulation of CKmb and ATP synthase, H+ transporting mitochondrial F1 complex, β subunit (ATP5B) (also known as the β subunit of ATP synthase), the enzymes of which involved in energy metabolism. Biologically, creatine kinases (CKs) are important enzymes that catalyze the conversion of creatine, and their abundance is commonly correlated with muscle injury. Qiu et al. [61] reported that the increased CK activity was linked to the heart injury in MC-exposed rat, suggesting that the upregulation of CKmb may involve in MCLR-induced cardiotoxicity in developing larvae. On the other hand, CKs are also crucial enzymes for high-energy consuming tissues such as the brain, heart, and muscle. In the reported cases, the upregulation of CK had been hypothesized to be correlated with the increased requirements energy pathway [60]. This hypothesis is strengthened by the fact that ATP5B, which directly participates in the process of energy production, is upregulated in the study condition. However, MCLR has been shown to be able to bind the β subunit of ATP synthase [62], which indicates that the increased expression may also be merely a compensatory effect to counteract the inhibition of ATP5B. Similarly, mitochondrial aldehyde dehydrogenase 2 (Aldh2) described as MC sensitive by a proteomic study [28] also displays an increase in expression in this study. Additionally, this is the first report showing the downregulation of V-ATPase subunit A (ATP6v1a) in zebrafish larvae exposed to MCLR. The *atp6v1a* knockdown embryos revealed several abnormalities, including suppression of acid secretion from skin, growth retardation, trunk deformation, and loss of internal Ca^{2+} and Na^{+} [63]. This result suggested that the observed length reduction and trunk deformation in the present study could also be attributed to the suppression of ATP6v1a.

MC is also known to produce oxidative stress [4, 64]. Four proteins can be assigned to the oxidative stress response in the present study. The heat shock proteins (HSP) are a group of proteins that have a high degree of identity at the amino acid level of different organisms. HSP induction has been suggested as early marker of oxidative stress [65, 66]. The expression of Hsp90b1and Hspa9 was significantly upregulated in the zebrafish larvae exposed to MCLR in the present study. The increased expression of Hsp90b1and Hspa9 is consistent with other researches, which found that the expression levels of various HSPs were increased in vitro or in vivo after exposure to MCs [51, 67, 68]. The dramatically increased expression of Hsp90b1and Hspa9 following exposure to MCLR may indicate their important roles as molecular chaperones under oxidative stress caused by MCLR in developing larvae. MCLR also increased the abundance of Annexin A1a in exposed larvae. Annexin genes are found to be expressed in a wide range of tissues in zebrafish during the embryonic and larval stages. The Annexin complexes have been suggested to be involved in calcium-signaling pathways [69], and play an important role in cells during their response to oxidative stress [70–72]. A recent study showed that the upregulation of Annexin 4 might be associated with MCLR-induced oxidative stress via peroxidase activity [31]. Therefore, the increased expression of Annexin A1a may play a protective role in fighting against stress response. In this study, the abundance of 14-3-3 protein β/α-2 was remarkably enhanced in MCLR-exposed larvae, which was consistent with a previous study that overexpressed 14-3-3 protein was observed in human amnion FL cells after exposure to MCs [32]. The 14-3-3 proteins are a family of serine/threonine-binding proteins that have anti-apoptotic functions and exert their anti-apoptotic apoptotic activity through the Bad protein, a proapoptotic member of the Bcl-2 family which discovered as a heterodimeric partner for Bcl-2 and Bcl-XL, and inhibit Bad's proapoptotic activity [73, 74].

We also observed that MCLR exposure affected the expression of a few proteins such as crystallin, dihydropyrimidinase-like 2 (DRP2) (which is also known as collapsin-response mediator protein 2) that are known to be associated with structure formation, and signal transduction. Crystallins are a large family of genes expressed primarily in the lens and constitute approximately 90% of the soluble proteins in vertebrate lens fiber cells [75, 76]. In a recent study, the downregulation of β-crystallins was identified in

eyeless masterblind zebrafish [77]. Thus, the decreased expression of crystallin, β A2b and crystallin, β B1 protein in MCLR-exposed zebrafish larvae of this study appears to affect lens development. These findings together with our research provide the prospects of identifying new toxic mechanisms. In this study, we observed the increased expression of DRP2 which is implicated in axonal outgrowth, neuronal growth, cell migration, and path finding through the transmission and modulation of extracellular signals [78]. Decreased expression of DRP2 has been observed in the Alzheimer disease, the Down syndrome, schizophrenia, and affective disorders [79, 80]. Overexpression of DRP2 induces the formation of supernumerary axons in dorsal root ganglion neurons, whereas the expression of a DRP2 dominant negative deletion mutant suppresses axon formation [81]. These reports and the present results altogether suggest a potential involvement of DRP2 in MC-induced neurotoxicity. However, the research needs to be further studied.

We followed up in proteomic analysis with Western blot and QPCR to investigate the protein expression and transcriptional pattern of nine genes that encode the identified proteins. Consistent and discrepant results between mRNA and proteins for the target genes were obtained (Fig. 5). The Western blot results correlated well with the proteomic analysis, indicating that the proteomic analysis was convincing. However, among the nine proteins selected for the gene expression study, only five gene/protein samples (55.6%) showed consistent expression in both mRNA and protein expressions. As was shown in recent articles performing parallel proteomic/gene expression studies on the effects of perfluorooctanoic acid on rare minnow [82] or dioxin on rats [83], the relationship between the transcription of mRNA and the abundance of protein is not always a direct one as there are many regulatory mechanisms that can affect these processes. Our results suggest that the gene expression in response to MCLR involves diverse regulatory mechanisms from transcription of mRNA to the formation of functional proteins.

To summarize, we used a proteomic approach to elucidate protein profiles of zebrafish embryos/larvae following MCLR exposure. A number of proteins were altered in abundance and some of them were successfully identified. These proteins were involved in multiple functions and took part in a variety of biological processes. The results not only further our knowledge of the effects of MCLR on organisms, but also provide the basis for predicting the underlying mechanisms of developing toxicity as well as the identification of protein biomarkers for MCLR exposure. The results that the expression changes of the selected proteins were consistent with the 2-DE and silver-staining results demonstrated that the proteomic analysis was convincing. The discrepancy between mRNA and protein expression complicates the responses of organisms to MCLR which should be highlighted in future studies.

The authors express their thanks to Professor Michael Dunn and Professor He, Fuchu and two anonymous reviewers for their very useful comments and suggestions on our manuscript. This work was financially supported by the National Basic Research Program of China (973 Program) (Grant No. 2008CB418101); and National Natural Science Foundation of China (31070457).

The authors have declared no conflict of interest.

5 References

[1] Fastner, J., Codd, G. A., Metcalf, J. S., Woitke, P. et al., An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. *Anal. Bioanal. Chem.* 2002, *374*, 437–444.

[2] Mirura, G. A., Robinson, N. A., Geisbert, T. W., Bostian, K. A. et al., Comparison of in vivo and in vitro toxic effects of microcystin-LR in fasted rats. *Toxicon* 1989, *27*, 1229–1240.

[3] Falconer, I. R., Yeung, D. S. K., Cytoskeletal changes in hepatocytes induced by *Microcystis toxins* and their relation to hyperphosphorylation of cell proteins. *Chem. Biol. Interact.* 1992, *81*, 181–196.

[4] Ding, W. X., Shen, H. M., Ong, C. N., Microcystic cyanobacteria extract induces cytoskeletal disruption and intracellular glutathione alteration in hepatocytes. *Environ. Health Persp.* 2000, *108*, 605–609.

[5] Ding, W. X., Shen, H. M., Ong, C. N., Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. *J. Toxicol. Environ. Health* 2001, *64*, 507–519.

[6] Gehringer, M. M., Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett.* 2004, *557*, 1–8.

[7] Falconer, I. R., An overview of problem caused by toxic bluegreen algae (Cyanobacteria) in drinking and recreational water. *Environ. Toxicol.* 1999, *14*, 5–12.

[8] Codd, G. A., Cyanobacterial toxins: occurrence, properties and biological significance. *Water Sci. Technol.* 1995, *32*, 149–156.

[9] Kotak, B. G., Semalulu, S., Fritz, D. L., Prepas, E. E. et al., Hepatic and renal pathology of intraperitoneally administered microcystins-LR in rainbow trout (*Oncorhynchus mykiss*). *Toxicon* 1996, *34*, 517–525.

[10] Xie, L., Xie, P., Ozawa, K., Dynamics of microcystins-LR and -RR in the phytoplanktivorous silver carp in a subchronic toxicity experiment. *Environ. Pollut.* 2004, *127*, 431–439.

[11] Soares, R. M., Magalhães, V. F., Azevedo, S. M. F. O., Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in *Tilapia rendalli* (*Cichlidae*) under laboratory conditions. *Aquat. Toxicol.* 2004, *70*, 1–10.

[12] Li, L., Xie, P., Chen, J., *In vivo* studies on toxin accumulation in liver and ultrastructural changes of hepatocytes of the

phytoplanktivorous bighead carp i.p.-injected with extracted microcystins. *Toxicon* 2005, *46*, 533–545.

[13] Ding, X. S., Li, Y. Y., Duan, H. Y., Chung, I. K., Lee, J. A., Toxic effects of *Microcystis* cell extracts on the reproductive system of male mice. *Toxicon* 2006, *48*, 973–979.

[14] Zhang, X., Xie, P., Li, D., Shi, Z., Haematological and plasma biochemical responses of crucian carp (*Carassius auratus*) to intraperitoneal injection of extracted microcystins with the possible mechanisms of anemia. *Toxicon* 2008, *49*, 1150–1157.

[15] Wang, Q., Xie, P., Chen, J., Liang, G. D., Distribution of microcystins in various organs (heart, liver, intestine, gonad, brain, kidney and lung) of Wistar rat via intravenous injection. *Toxicon* 2008a, *52*, 721–727.

[16] Chen, J., Xie, P., Tissue distributions and seasonal dynamics of the hepatotoxic microcystins-LR and -RR in two freshwater shrimps. *Palaemon modestus* and *Macrobrachium nipponensis*, from a large shallow, eutrophic lake of the subtrophical China. *Toxicon* 2005, *45*, 615–625.

[17] Lei, H. H., Xie, P., Chen, J., Liang, G. D., Zhang, X. Z., Distribution of toxin in various tissues of crucian carp intraperitoneally injected with hepatotoxic microcystins. *Environ. Toxicol. Chem.* 2008, *27*, 1167–1174.

[18] Von Westerhagen, H., in: Hoar, W. S., Randall, D. J. (Eds.), *Fish Physiology: Hoar, vol. 11*, Academic Press, San Diego 1988, pp. 253–346.

[19] Oberemm, A., Fastner, J., Steinberg, C. E. W., Effects of microcystin-LR and cyanobacterial crude extracts on embryolarval development of zebrafish (*Danio rerio*). *Water Res.* 1997, *31*, 2918–2921.

[20] Oberemm, A., Becker, J., Codd, G. A., Steinberg, C., Effects of cyanobacterial toxins and aqueous crude extracts of cyanobacteria on the development of fish and amphibians. *Environ. Toxicol.* 1999, *14*, 77–88.

[21] Wiegand, C., Pflugmacher, S., Oberemm, A., Meems, N. et al., Uptake and effects of microcystins-LR on detoxication enzymes of early life stages of the zebra fish (*Danio rerio*). *Environ. Toxicol.* 1999, *14*, 89–95.

[22] Liu, Y. D., Song, L. R., Li, X. Y., Liu, T. M., The toxic effects of microcystins-LR on embryo–larval and juvenile development of loach, *Misgurnus mizolepis Gunthe*. *Toxicon* 2002, *40*, 395–399.

[23] Wang, P. J., Chien, M. S., Wu, F. J., Chou, H. N., Lee, S. J., Inhibition of embryonic development by microcystins-LR in zebrafish, *Danio rerio*. *Toxicon* 2005, *45*, 303–308.

[24] Wright, A., Olaf Papendorf, O., König, G., Oberemm, A., Effects of cyanobacterium *Fischerella ambigua* isolates and cell free culture media on zebrafish (*Danio rerio*) embryo development. *Chemosphere* 2006, *65*, 604–608.

[25] Malbrouck, C., Kestemont, P., Effects of microcystins on fish. *Environ. Toxicol. Chem.* 2006, *25*, 72–86.

[26] Palíková, M., Krejci, R., Hilscherova, K., Babica, P. et al., Effects of different cyanobacterial biomasses and their fractions with variable microcystin content on embryonal development of carp (*Cyprinus carpio L.*). *Aquat. Toxicol.* 2007, *81*, 312–318.

[27] Jacquet, C., Thermes, V., de Luze, A., Puiseux-Dao, S. et al., Effects of microcystins-LR on development of medaka fish embryos (*Oryzias latipes*). *Toxicon* 2004, *43*, 141–147.

[28] Chen, T., Wang, Q., Cui, J., Yang, W. et al., Induction of apoptosis in mouse liver by microcystin-LR: A combined transcriptomic, proteomic, and simulation strategy. *Mol. Cell. Proteomics* 2005, *4*, 958–974.

[29] Mezhoud, K., Bauchet, A. L., Château-Joubert, S., Praseuth, D. et al., Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (*Oryzias latipes*) following oral gavage with microcystin-LR. *Toxicon* 2008, *51*, 1431–1439.

[30] Mezhoud, K., Praseuth, D., Puiseux-Dao, S., François, J. C. et al., Global quantitative analysis of protein expression and phosphorylation status in the liver of the medaka fish (*Oryzias latipes*) exposed to microcystin-LR[1]. Balneation study. *Aquat. Toxicol.* 2008, *86*, 166–175.

[31] Wang, M. H., Chan, L. L., Si, M. Z., Hong, H. S., Wang, D. Z., Proteomic analysis of hepatic tissue of zebrafish (*Danio rerio*) experimentally exposed to chronic microcystin-LR. *Toxicol. Sci.* 2010, *113*, 60–69.

[32] Fu, W. Y., Xu, L. H., Yu, Y. N., Proteomic analysis of cellular response to microcystin in human amnion FL cells. *J. Proteome Res.* 2005, *4*, 2207–2215.

[33] Shi, X. J., Yeung, L. W. Y., Lam, P. K. S., Wu, R. S. S., Zhou, B. S., Protein profiles in zebrafish (*Danio rerio*) embryos exposed to perfluorooctane sulfonate. *Toxicol. Sci.* 2009, *110*, 334–340.

[34] Martins, J. C., Leão, P. N., Vasconcelos, V., Differential protein expression in *Corbicula fluminea* upon exposure to a *Microcystis aeruginosa* toxic strain. *Toxicon* 2009, *53*, 409–416.

[35] De Wit, M., Keil, D., Remmerie, N., van der Ven, K. et al., Molecular targets of TBBPA in zebrafish analysed through integration of genomic and proteomic approaches. *Chemosphere* 2008, *74*, 96–105.

[36] Moreno, I., Pereira, P., Franca, S., Camean, A., Toxic cyanobacteria strains isolated from blooms in the Guadiana river (Southwest of Spain). *Biol. Res.* 2004, *37*, 405–417.

[37] Westerfield, M., *The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 3rd Edn, University of Oregon Press, Eugene, OR, USA 1995.

[38] Tay, T. L., Lin, Q., Seow, T. K., Tan, K. H. et al., Proteomic analysis of protein profiles during early development of the zebrafish, *Danio rerio*. *Proteomics* 2006, *6*, 3176–3188.

[39] Li, G. Y., Xie, P., Li, H. Y., Hao, L. et al., Involment of p53, Bax, and Bcl-2 pathway in microcystins-induced apoptosis in rat testis. *Environ. Toxicol.* 2009 (DOI 10.1002/tox.20532).

[40] Livak, K. J., Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001, *25*, 402–408.

[41] Li, G. Y., Xie, P., Fu, J., Hao, L. et al., Microcystin-induced variations in transcription of GSTs in an omnivorous freshwater fish, goldfish. *Aquat. Toxicol.* 2008, *88*, 75–80.

[42] Love, D. R., Pichler, F. B., Dodd, A., Copp, B. R., Greenwood, D. R., Technology for high-throughput screens: the present

and future using zebrafish. *Curr. Opin. Biotechnol.* 2004, *15*, 564–571.

[43] Chorus, I., Bartram, J., *Toxic Cyanobacteria in Water. A Guide to Public Health Consequences, Monitoring and Management*, E and FN Spon on behalf of WHO, London 1999, p. 416.

[44] Sahin, A., Tencalla, F. G., Dietrich, D. R., Naegeli, H., Biliary excretion of biochemically active cyanobacteria (blue-green algae) hepatotoxins in fish. *Toxicology* 1996. *106*, 123–130.

[45] Chen, J., Xie, P., Li, L., Xu, J., First identification of the hepatotoxic microcystins in the serum of a chronically exposed human population together with indication of hepatocellular damage. *Toxicol. Sci.* 2009, *108*, 81–89.

[46] Buryskova, B., Hilscherova, K., Babica, P., Vrskova, D. et al., Toxicity of complex cyanobacterial samples and their fractions in *Xenopus laevis* embryos and the role of microcystins. *Aquat. Toxicol.* 2006, *80*, 346–354.

[47] Janssens, V., Goris, J., Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* 2001, *353*, 417–439.

[48] Honkanen, R. E., Zwiller, J., Moore, R. E., Daily, S. L. et al., Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. *J. Biol. Chem.* 1990, *265*, 19401–19404.

[49] MacKintosh, C., Beattie, K. A., Klumpp, S., Cohen, P., Codd, G. A., Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. *FEBS Lett.* 1990, *264*, 187–192.

[50] Runnegar, M. T., Kong, S., Berndt, N., Protein phosphatase inhibition and in vivo hepatotoxicity of microcystins. *Am. J. Physiol.* 1993, *265*, 224–230.

[51] Fu, W. Y., Yu, Y. N., Xu, L. H., Identification of temporal differentially expressed protein responses to microcystin in human amniotic epithelial cells. *Chem. Res. Toxicol.* 2009, *22*, 41–51.

[52] Wang, S. S., Esplin, E. D., Li, J. L., Huang, L. et al., Alterations of the PPP2R1B gene in human lung and colon cancer. *Science* 1998, *282*, 284–287.

[53] Wera, S., Fernandez, A., Lamb, N. J., Turowski, P. et al., Deregulation of translational control of the 65-kDa regulatory subunit (PR65 alpha) of protein phosphatase 2A leads to multinucleated cells. *J. Biol. Chem.* 1995, *270*, 21374–21381.

[54] Gergs, U., Boknik, P., Buchwalow, I., Fabritz, L. et al., Overexpression of the catalytic subunit of protein phosphatase 2A impairs cardiac function. *J. Biol. Chem.* 2004, *279*, 40827–40834.

[55] Yamauchi, M., Kim, E. Y., Iwata, H., Tanabe, S., Molecular characterization of the aryl hydrocarbon receptors (AhR1 and AhR2) from red seabream (*Pagrus major*). *Comp. Biochem. Physiol. C* 2005, *141*, 177–187.

[56] Toivola, D. M., Goldman, R. D., Garrod, D. R., Eriksson, J. E., Protein phosphatases maintain the organization and structure interactions of hepatic keratin intermediate filaments. *J. Cell Sci.* 1997, *110*, 23–33.

[57] Toivola, D. M., Omary, M. B., Ku, N. O., Peltola, O. et al., Protein phosphatase inhibition in normal and keratin 8/18 assembly-incompetent mouse strains supports a functional role of keratin intermediate filaments in preserving hepatocyte integrity. *Hepatology* 1998, *28*, 116–128.

[58] Khan, S. A., Wickstrom, M. L., Haschek, W. M., Schaeffer, D. J. et al., Microcystin-LR and kinetics of cytoskeletal reorganization in hepatocytes, kidney cells, and fibroblasts. *Nat. Toxins* 1996, *4*, 206–214.

[59] Wickstrom, M. L., Khan, S. A., Haschek, W. M., Wyman, J. F. et al., Alterations in microtubules, intermediate filaments, and microfilaments induced by microcystin-LR in cultured cells. *Toxicol. Pathol.* 1995, *23*, 326–337.

[60] Gillardin, V., Silvestre, F., Dieu, M., Delaive, E. et al., Protein expression profiling in the African clawed frog *Xenopus laevis* tadpoles exposed to the polychlorinated biphenyls mixture aroclor 1254. *Mol. Cell. Proteomics* 2009, *8*, 596–611.

[61] Qiu, T., Xie, P., Liu, Y., Li, G. et al., The profound effects of microcystin on cardiac antioxidant enzymes, mitochondrial function and cardiac toxicity in rat. *Toxicology* 2009, *257*, 86–94.

[62] Mikhailov, A., Härmälä-Braskén, A. S., Hellman, J., Meriluoto, J., Eriksson, J. E., Identification of ATP-synthase as a novel intracellular target for microcystin-LR. *Chem. Biol. Interact.* 2003, *142*, 223–237.

[63] Horng, J. L., Lin, L. Y., Huang, C. J., Katoh, F. et al., Knockdown of V-ATPase subunit A (*atp6v1a*) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2007, *292*, 2068–2076.

[64] Ding, W. X., Shen, H. M., Zhu, H. G., Ong, C. N., Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. *Environ. Res.* 1998, *781*, 12–18.

[65] Polla, B. S., Kantengwa, S., Francois, D., Mitochondria are selective targets for the protective effects of heat shock against oxidative injury. *Proc. Natl. Acad. Sci. USA* 1996, *93*, 6458–6463.

[66] Peng, J., Graham, J., Kenneth, W., Stress proteins as biomarkers of oxidative stress: effects of antioxidant supplements. *Free Rad. Biol. Med.* 2000, *28*, 1598–1606.

[67] Malécot, M., Mezhoud, K., Marie, A., Praseuth, D. et al., Proteomic study of the effects of microcystin-LR on organelle and membrane proteins in medaka fish liver. *Aquat. Toxicol.* 2009, *94*, 153–161.

[68] Li, G., Xie, P., Fu, J., Hao, L. et al., Microcystin-induced variations in transcription of GSTs in an omnivorous freshwater fish, goldfish. *Aquat. Toxicol.* 2008, *88*, 75–80.

[69] Gerke, V., Moss, S. E., Annexins: from structure to function. *Physiol. Rev.* 2002, *82*, 331–371.

[70] Rhee, H. J., Kim, G. Y., Huh, J. W., Kim, S. W., Na, D. S., Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. *Eur. J. Biochem.* 2000, *267*, 3220–3225.

[71] Sacre, S. M., Moss, S. E., Intracellular localization of endothelial cell annexins is differentially regulated by oxidative stress. *Exp. Cell Res.* 2002, *274*, 254–263.

[72] Tanaka, T., Akatsuka, S., Ozeki, M., Shirase, T. et al., Redox regulation of annexin 2 and its implications for oxidative stress-induced renal carcinogenesis and metastasis. *Oncogene* 2004, *23*, 3980–3989.

[73] Subramanian, R. R., Masters, S. C., Zhang, H., Fu, H., Functional conservation of 14-3-3 isoforms in inhibiting Bad-induced apoptosis. *Exp. Cell Res.* 2001, *271*, 142–151.

[74] Masters, S. C., Subramanian, R. R., Truong, A., Yang, H. et al., Survival-promoting functions of 14-3-3 proteins. *Biochem. Soc. Trans.* 2002, *30*, 360–365.

[75] Wistow, G. J., Piatigorsky, J., Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. *Annu. Rev. Biochem.* 1988, *57*, 479–504.

[76] Bloemendal, H., de Jong, W., Jaenicke, R., Lubsen, N. H. et al., Ageing and vision: structure, stability and function of lens crystallins. *Prog. Biophys. Mol. Biol.* 2004, *86*, 407–485.

[77] Wang, H., Kesinger, J. W., Zhou, Q. C., Wren, J. D. et al., Identification and characterization of zebrafish ocular formation genes. *Genome* 2008b, *51*, 222–235.

[78] Poon, H. F., Vaishnav, R. A., Getchell, T. V., Getchell, M. L., Butterfield, D. A., Quantitative proteomics analysis of differential protein expression and oxidative modification of specific proteins in the brains of old mice. *Neurobiol. Aging* 2006, *27*, 1010–1019.

[79] Lubec, G., Nonaka, M., Krapfenbauer, K., Gratzer, M. et al., Expression of the dihydropyrimidinase related protein 2 (DRP-2) in Down syndrome and Alzheimer's disease brain is downregulated at the mRNA and dysregulated at the protein level. *J. Neural Transm. Suppl.* 1999, *57*, 161–177.

[80] Johnston-Wilson, N. L., Sims, C. D., Hofmann, J. P., Anderson, L. et al., Disease-specific alterations in frontal cortex brain proteins in schizophrenia, bipolar disorder, and major depressive disorder: the Stanley Neuropathology Consortium. *Mol. Psychiatry* 2000, *5*, 142–149.

[81] Inagaki, N., Chihara, K., Arimura, N., Menager, C. et al., CRMP-2 induces axons in cultured hippocampal neurons. *Nat. Neurosci.* 2001, *4*, 781–782.

[82] Wei, Y., Chan, L. L., Wang, D., Zhang, H. et al., Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. *J. Proteome Res.* 2008, *7*, 1729–1739.

[83] Pastorelli, R., Carpi, D., Campagna, R., Airoldi, L. et al., Differential expression profiling of the hepatic proteome in a rat model of dioxin resistance: correlation with genomic and transcriptomic analyses. *Mol. Cell. Proteomics* 2006, *5*, 882–894.

