



# NF- $\kappa$ B plays a key role in microcystin-RR-induced HeLa cell proliferation and apoptosis

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## ARTICLE INFO

### Article history:

Received 11 February 2014

Received in revised form 30 May 2014

Accepted 5 June 2014

Available online 14 June 2014

### Keywords:

Microcystin-RR

NF- $\kappa$ B

Cell proliferation

Apoptosis

Hormesis

HeLa cells

## ABSTRACT

Microcystins (MCs) are well-known cyanobacterial toxins produced in eutrophic waters and can act as potential carcinogens and have caused serious risk to human health. However, pleiotropic even paradoxical actions of cells exposure to MCs have been reported, and the mechanisms of MC-induced tumorigenesis and apoptosis are still unknown. In this study, we performed the first comprehensive *in vitro* investigation on carcinogenesis associated with nuclear factor kappa B (NF- $\kappa$ B) and its downstream genes in HeLa cells (Human cervix adenocarcinoma cell line from epithelial cells) exposure to MC-RR. HeLa cells were treated with 0, 20, 40, 60, and 80  $\mu$ g/mL MC-RR for 4, 8, 12, and 24 h. HeLa cells presented dualistic responses to different doses of MCs. CCK8 assay showed that MC-RR exposure evidently enhanced cell viability of HeLa cells at lower MCs doses. Cell cycle and apoptosis analysis revealed that lower MCs doses promoted G<sub>1</sub>/S transition and cell proliferation while higher doses of MCs induced apoptosis, with a dose-dependent manner. Electrophoretic mobility shift assay (EMSA) revealed that MC-RR could increase/decrease NF- $\kappa$ B activity at lower/higher MC-RR doses, respectively. Furthermore, the expression of NF- $\kappa$ B downstream target genes including c-FLIP, cyclinD1, c-myc, and c-IAP2 showed the same variation trend as NF- $\kappa$ B activity both at mRNA and protein levels, which were induced by lower doses of MC-RR and suppressed by higher doses. Our data verified for the first time that NF- $\kappa$ B pathway may mediate MC-induced cell proliferation and apoptosis and provided a better understanding of the molecular mechanism for potential carcinogenicity of MC-RR.

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## 1. Introduction

Cyanobacteria frequently form algae blooms with progressive eutrophication of water bodies all over the world and evoke profound concerns. Microcystins (MCs), a family

of cyclic heptapeptide cyanotoxins, are the secondary metabolites produced by several cyanobacteria species and are responsible for illness and death of human and animals (Dias et al., 2010; Žegura et al., 2011). Our previous study has also confirmed the presence of MCs in serum samples of fisherman who were naturally chronic exposure to cyanotoxins by oral route (Chen et al., 2009). Up to now, there have been reported over 80 analogues of MCs, among which MC-LR, MC-RR are most commonly present. MC toxicity is primarily governed by its covalently binding to and inhibition of eukaryotic protein serine/threonine

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phosphatases 1 and 2A (PP1 and PP2A), leading to the disruption of the dynamic equilibrium of protein phosphorylation/dephosphorylation and thereby disruption of many cellular processes (Mackintosh et al., 1990; Žegura et al., 2011). But there is also substantial evidence that MCs can enhance intracellular production of reactive oxygen species (ROS) and induce oxidative stress (Guzman and Solter, 1999; Campos and Vasconcelos, 2010).

On one hand, MCs have potential tumor-promoting activity in humans. Epidemiologic studies have suggested that people consuming drinking water contaminated MCs have higher incidences of primary liver cancer (Ueno et al., 1996; Svirčev et al., 2009) and colorectal cancer (Zhou et al., 2002). Cell proliferation may contribute to carcinogenesis at stages of initiation, promotion, or progression (Butterworth and Goldsworthy, 1991). Recent studies have also found that MCs induce cell proliferation, which was closely linked to activation of Akt, p38, c-Jun N-terminal kinase (JNK) (Zhu et al., 2005), extracellular signal-regulated kinase 1/2 (ERK1/2) of mitogen-activated protein kinase (MAPK) pathway (Dias et al., 2010), and nuclear factor erythroid 2-related factor 2 (Nrf2) (Gan et al., 2010).

On the other hand, many studies revealed that MCs could induce apoptosis in a variety of cell types, including hepatocytes (McDermott et al., 1998; Hooser, 2000), neurons (Feurstein et al., 2011; Li et al., 2012a), kidney epithelial cells (Menezes et al., 2013), and lymphocytes (Lankoff et al., 2004), etc. It can be concluded that MCs produce non-monotonic effects on cells in a dose-dependent manner: higher concentrations of MCs promote cell death or apoptosis, whereas lower concentrations increase cell survival and proliferation (Rymuszka et al., 2007; Dias et al., 2010), and can cause cancer (Gehring, 2004; Herfindal and Selheim, 2006). Our previous study also indicated that MC-LR induced both death and survival signals in the human hepatoma cell line HepG2 (Zhang et al., 2013b). However, the mechanisms of the confusing effects of MCs on cellular biochemistry that it is involved in cell proliferation and carcinogenesis and yet is also involved in the conflicting response of apoptosis are still not clarified.

Nuclear factor kappa B (NF- $\kappa$ B), a transcription factor, is best known for its key roles in immune, inflammation, and acute phase responses, but it also controls several other cellular processes including cell survival, proliferation, apoptosis and oncogenesis (Karin and Lin, 2002; Kucharczak et al., 2003). By a variety of biochemical and cell treatments, the activation of NF- $\kappa$ B occurs as it is transported from the cytoplasm to the nucleus where it binds to specific  $\kappa$ B sites on the DNA ( $\kappa$ B DNA consensus sequences) and mediates the expression of multiple target genes involved in tumorigenesis (Piva et al., 2006; Chaturvedi et al., 2011). Several studies have reported that MC-LR could activate NF- $\kappa$ B in HepG2 cells (Feng et al., 2011), rat insulinoma cells (INS-1) (Ji et al., 2011), melanoma cells (MDA-MB-435) (Zhang et al., 2012), human hepatoma cells (Huh7) (Christen et al., 2013), and mice hepatocytes (Zhang et al., 2013a). NF- $\kappa$ B mediates the induction of apoptosis by MC-LR in HepG2 (Feng et al., 2011) and INS-1 cells (Ji et al., 2011). Christen et al. (2013) demonstrated that MC-LR activates NF- $\kappa$ B, induces IFN- $\alpha$  and TNF- $\alpha$  expression and may contribute to tumor

development and induction of apoptosis in Huh7 cells. However, the effects of MCs on cell proliferation and apoptosis via NF- $\kappa$ B pathway have not been elucidated clearly yet. As we know, both MC-LR and MC-RR act as an inhibitor of PP1 and PP2A (Ito et al., 2002; Chen et al., 2006), and can induce oxidative stress (Prieto et al., 2006; Pavagadhi et al., 2012) and cytokine secretion (Huguet et al., 2013). However, most of present studies focused on the toxicity of MC-LR, possibly due to its higher toxicity than MC-RR (Gupta et al., 2003; Chen et al., 2006). The toxicity of MC-RR has been studied less extensively; also, whether MC-RR could modulate NF- $\kappa$ B is unknown yet, despite of their wide distribution and abundance in the environment.

Our previous study showed that high MCs not only accumulated in the hepatopancreas but also in the gonad, suggesting that the reproductive systems are the second important target organ of MCs (Chen and Xie, 2005). And the study of Chen and Xie (2005) has raised questions and great concerns on the probable reproductive toxicity of MCs. Our previous and others' studies found that MCs induce morphological damages, and result in significant decrease of gamete quality and disturbance of sex hormones in rats (Li et al., 2008; Chen et al., 2013), mice (Chen et al., 2011; Wang et al., 2012; Wu et al., 2014), rabbits (Liu et al., 2010), and zebrafish (Zhao et al., 2012; Qiao et al., 2013b). Similar to the hepatocytes, MCs also exert tumor-promoting activity by up-regulating proto-oncogenes (c-jun, c-fos, and c-myc) in testis (Li et al., 2009) and can also lead to cellular apoptosis in gonads (Li et al., 2008; Chen et al., 2013; Qiao et al., 2013b; Zhou et al., 2013). HeLa cervical adenocarcinoma cell, originated from a cervical cancer tumor, is the first human cell line established in culture, and has since become the most widely used model cell line in cellular and molecular biology research (Landry et al., 2013). Recently, HeLa cell has been used to investigate responses to environmental perturbations, and the induction of apoptosis in HeLa cells occurs in response to various stimuli such as antimycin A (Park et al., 2007), pyrogallol (Kim et al., 2008), gallic acid (You et al., 2010), and butylated hydroxyanisole (Moon and Park, 2011). However, little is known about the toxic effect of microcystin, the well-known cyanobacterial toxins that can act as potential carcinogen and cause serious risk to human health, on HeLa cells.

By focusing on the results described above, here we investigated the NF- $\kappa$ B pathway as a possible mechanism by which MC-RR promoted cell proliferation and apoptosis *in vitro* using HeLa cells. To the best of our knowledge, this is the first comprehensive *in vitro* investigation on MC-induced tumorigenesis associated with NF- $\kappa$ B and its downstream genes (c-FLIP, cyclinD1, c-myc, and c-IAP2). It will help us better understand the molecular mechanisms of MC-RR induced toxicity and potential carcinogenicity and will also guide us to protect human health efficiently.

## 2. Materials and methods

### 2.1. Chemicals

MC-RR was isolated and purified from the freeze-dried surface blooms (mainly *Microcystis aeruginosa*) collected

from Lake Dianchi (Yunnan, China), following the method described by Wang et al. (2008). MC-RR was separated by semi-performance preparative liquid chromatography system (Waters 600E, USA) and pure MC-RR was obtained. MC-RR was analyzed for MCs content via a reverse-phase high performance liquid chromatography (HPLC) (LC-10A, Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan). MC-RR (purity >95%) concentration was determined by the UV spectra and retention time, and by using a commercial standard MC-LR, -RR (Wako Pure Chemical Industries, Japan) to compare the peak areas of the test samples. All reagents obtained from various commercial sources were analytical or higher grades.

## 2.2. Cell culture and exposure

HeLa cells were obtained from professor Wuhan Xiao (Institute of Hydrobiology, Chinese Academy of Sciences) and were cultured in Dulbecco's modified eagle's medium (DMEM; Hyclone, USA) with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin (Invitrogen, USA) in a 5% carbon dioxide (CO<sub>2</sub>) humidified incubator at 37 °C. Whenever cells reached about 90% confluence, they were detached, reconstituted with medium, and split into the required portions for the next seeding stage. HeLa cells were treated with 0, 20, 40, 60, and 80 µg/mL MC-RR for 4, 8, 12, and 24 h. Each treatment was repeated 3 times.

## 2.3. Cell proliferation assay

The cell proliferation status was assessed by Cell Counting Kit-8 (CCK8) Kit (Beyotime, China). Briefly, HeLa cells were seeded in 96-well plates at the density of 2000 cells per well with 100 µL culture medium. After adhesion for 24 h, MC-RR was added to the medium to the final concentrations (0, 20, 40, 60, and 80 µg/mL MC-RR). The cells were then cultured for another 48 h. Then, 20 µL of CCK8 solution was added to each well, and the culture was incubated for another 1 h at 37 °C. The optical density (OD) values were read at 450 nm (Li et al., 2012b) by a microplate reader (Thermo, USA).

## 2.4. Cell cycle and apoptosis analysis

HeLa cells were seeded in six-well plates and incubated for 48 h, before the cells were treated with different solutions of MC-RR (0, 20, 40, 60, and 80 µg/mL) for 24 h. After treatment, the cells were collected by trypsinization and washed with cold phosphate buffered saline (PBS). The cells (1 × 10<sup>6</sup> cells/mL) were fixed in 70% ethanol at 4 °C overnight. Fixed cells were washed with PBS and incubated with RNase A (100 µL) at 37 °C for 30 min and stained with propidium iodide (PI, 400 µL) for another 30 min at 4 °C in the dark. The distribution of cells in the cell cycle was measured at 488 nm by a flow cytometer (Becton Dickinson, San Jose, CA), followed by data analyses using Cell Quest software (Becton Dickinson). The cells with sub-G<sub>0</sub>/G<sub>1</sub> peak were evaluated as DNA degradation caused by apoptosis.

**Table 1**

Quantitative real-time PCR primer sequences used in this study.

Gene name	Primer sequences (5'–3')	
cyclinD1	Forward	CTGTCGCTGGAGCCCGTGAA
	Reverse	TGGCACAGAGGGCAACGAAG
c-FILP	Forward	GACTTGGCTGAAGTCTCTA
	Reverse	GTCCGAAACAAGGTGAGGGT
c-myc	Forward	GGAGGCTATTCTGCCCATTT
	Reverse	GTCGAGGTCATAGTCTCTGTTG
c-IAP2	Forward	CATGCAGCCCGCTTTAAAACATTC
	Reverse	CATTTCCACGGCAGCATTAAATCAC
GAPDH	Forward	TGGACCTGACCTGCCGTCTAG
	Reverse	AGTGGGTGTCGCTGTGAAGTC

## 2.5. Electrophoretic mobility shift assay (EMSA)

Cells treated with different doses of MC-RR for 12 h were separately collected by centrifugation, and nuclear proteins were extracted using Nuclear Protein Extraction Kit (Viagene, USA) and kept at –80 °C. Protein concentration was determined in the supernatant using Bradford method (Beyotime, China) and 5 µg nuclear protein was used for EMSA. The DNA-binding activity of NF-κB was detected using EMSA Kit (Viagene, USA) according to the manufacturer's protocol.

## 2.6. RNA extraction and quantitative real-time PCR (QRT-PCR)

Total RNA was isolated by use of Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Concentrations of total RNA were estimated by use of 260 nm reading value using a spectrophotometer. The RNA purity was verified by measuring the 260/280 nm ratios with values between 1.8 and 2.0 (Fleige and Pfaffl, 2006; Li et al., 2012b). The 1% agarose-formaldehyde gel electrophoresis with ethidium bromide (EB) staining was used to further verify the quality of total RNA. Reverse transcription was performed with oligo-dT primer using a First Strand cDNA Synthesis Kit (TOYOBO, Japan) following the manufacturer's instructions.

The sequences of primers used in the study were designed with Primer Premier 5.0 (Premier, Canada) and the primers were listed in Table 1. Specification of each pair of primers was tested by randomly sequencing three clones, and further confirmed by the melting curve analysis using QRT-PCR. The amplification efficiency of each pair of primers was tested by constructing corresponding plasmid, and only primers with similar amplification efficiency were used in this experiment. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was analyzed in samples, and the level was stable and unaffected by MC-RR exposure, similar to the results reported in previous study (Huang et al., 2011), therefore GAPDH was used as the endogenous assay control.

SYBR Green QRT-PCR kit (TOYOBO, Japan) was used as the fluorescent dye for QRT-PCR on a Chromo4 96-well reactor with optical caps (MJ Research, Cambridge, MA, USA). The reactions were performed in a 20 µL volume mix containing 10 µL SYBR Green I mixture, 1 µL primers, 1 µL

cDNA template, and 1  $\mu$ L sterile, distilled-deionized water. The thermal cycle was set as follows: pre-denaturation at 95 °C for 3 min, followed by 35–45 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 20 s, and elongation at 72 °C for 20 s. Melt curve analysis of amplification products was performed at the end of each PCR reaction to confirm that a single PCR product was detected. Each sample was run in 3 tubes, and PCR reactions with distilled-deionized water instead of the addition of the template were used as blanks. After completion of the PCR amplification, data were analyzed with the Option Monitor software Version 2.03 (MJ Research, Cambridge, MA, USA). The relative expression levels of the genes were calculated using the formula  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001).

### 2.7. Western blotting

Cells were washed with PBS, and lysed in a buffer containing 50 mM Tris–HCl (pH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA; pH 8.0), 1 mM NaF, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM  $\text{Na}_3\text{VO}_4$  and protease inhibitor for 1 h on ice. Samples were then centrifuged at 12,000 rpm for 10 min at 4 °C and supernatants were collected. Bradford method was used to determine the concentration of proteins. All samples were stored at –80 °C prior to electrophoresis.

Aliquots from supernatant containing 20  $\mu$ g of proteins were mixed with equal volume of  $2\times$  loading buffer and 5%  $\beta$ -mercaptoethanol. The sample was boiled for 5 min and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the resolved proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany) using an electro blotting apparatus (Bio-Rad, America). Membranes were blocked in Tris-Buffered Saline and Tween 20 (TBST; 50 nmol/L Tris–Cl, pH 7.6, 150 mmol/L NaCl, 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature to prevent non-specific binding of reagents, and then incubated with anti-cyclinD1 (Santa Cruz, MA, USA; 1: 500), anti-c-IAP2 (Santa Cruz, MA, USA; 1: 500), and anti-GAPDH (Santa Cruz, CA, USA; 1: 1000) for 1 h at room temperature. The membranes were then washed in TBST for 10 min, repeated 3 times and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce, USA; 1: 5000) for 1 h at room temperature. The membranes were then washed with TBS 4 times for 5 min and the membrane was revealed with chemiluminescent substrates (Pierce, Rockford, IL, USA). Each sample was measured in triplicate. The protein signal of immunoblot analysis was developed using NBT/BCIP system. The quantification of the relative expression of cyclin D1 and c-IAP2 was performed by using Quantity one (Bio-Rad, USA). The GAPDH was analyzed in samples, and the protein level was stable and unaffected by MC-RR exposure, similar to the results reported in human amnion FL cells (Fu et al., 2005), mice (Huang et al., 2011) and zebrafish (*Danio rerio*) (Zhao et al., 2012) exposed to MC-RR. Thus, the present study chose GAPDH as internal control gene.

### 2.8. Statistical analysis

All values were presented as the mean  $\pm$  standard deviation (SD). The Kolmogorov–Smirnov test and Levene's test were employed to check the normality and homogeneity of variances in the data, respectively. The values were then analyzed using one-way ANOVA and Turkey's multiple comparison tests with SPSS package 16.0 (SPSS, Chicago, IL, USA). Statistical differences between data of the control and MC-RR treatment groups were determined at the  $p < 0.05$  or  $p < 0.01$  levels for all analyses and indicated with \* and \*\*, respectively.

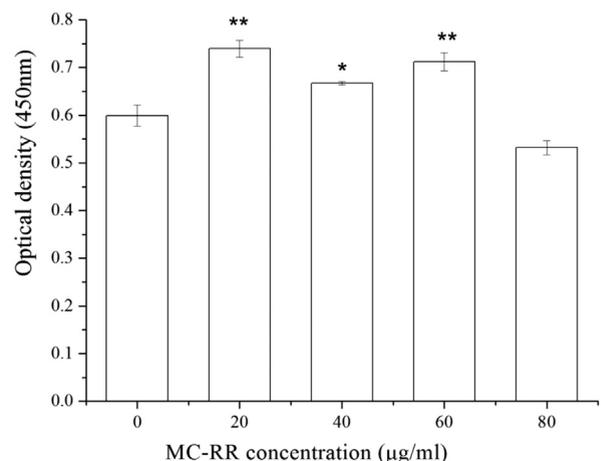
## 3. Results

### 3.1. Cell proliferation

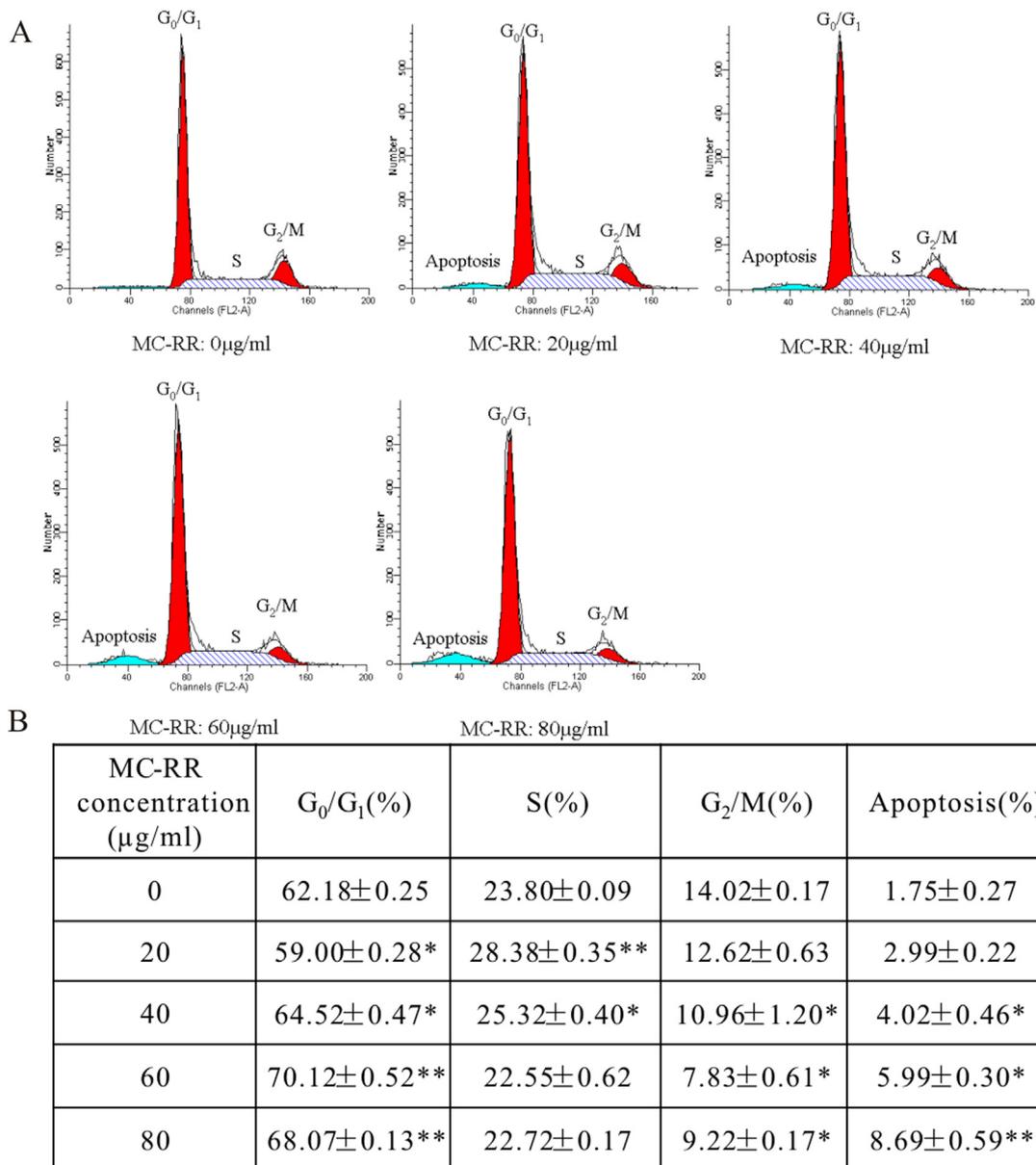
The *in vitro* effects of MC-RR on HeLa cell proliferation measured by CCK8 are presented in Fig. 1. Compared with the control, MC-RR significantly increased cell proliferation in the 20, 40, 60  $\mu$ g/mL group ( $p < 0.05$ ). However, the proliferative activity was decreased in the 80  $\mu$ g/mL group, although without showing statistical difference from the control ( $p > 0.05$ ).

### 3.2. Cell cycle distribution and apoptosis

As shown in Fig. 2, MC-RR exposure significantly increased S-phase cell population in the 20, 40  $\mu$ g/mL group ( $p < 0.05$ ), while caused a drastic accumulation of cells in  $G_0/G_1$ -phase of the cell cycle in 40, 60, 80  $\mu$ g/mL group ( $p < 0.05$ ), accompanied by a reduction of cells in the  $G_2/M$  phases of the cell cycle in 40, 60, 80  $\mu$ g/mL group ( $p < 0.05$ ). Interestingly, the flow cytometric cell cycle distribution analysis also showed that the ratio of cells with apoptotic nuclei to total cells significantly increased in a dose-dependent manner following treatment with MC-RR at the dose of 40, 60, 80  $\mu$ g/mL ( $p < 0.05$ ).



**Fig. 1.** Cell viability of HeLa cells incubated with different microcystin-RR concentrations. Values are presented as the mean  $\pm$  standard deviation (SD). Shown are the results of three independent experiments. \* indicates significant difference at  $p < 0.05$  versus control, and \*\* indicates significant difference at  $p < 0.01$  versus control.



**Fig. 2.** Effect of microcystin-RR on cell cycle distribution and apoptosis. (A) Flow cytometric analysis of cell cycle distribution. One representative of the three independent experiments is shown. (B) The percentage of cells in each cell cycle phase and apoptotic cells. Values are presented as the mean  $\pm$  standard deviation (SD). \* indicates significant difference at  $p < 0.05$  versus control, and \*\* indicates significant difference at  $p < 0.01$  versus control.

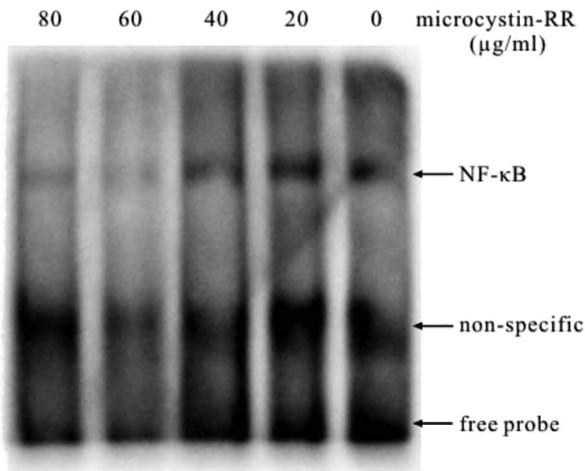
### 3.3. The DNA-binding activity of NF- $\kappa$ B

Fig. 3 shows the NF- $\kappa$ B electrophoretic mobility shift assay at 12 h after MC-RR stimulus. NF- $\kappa$ B binding ability was enhanced in the 20, 40  $\mu$ g/mL group, and the binding ability of cells treated with 20  $\mu$ g/mL MC-RR was higher. However, the NF- $\kappa$ B DNA binding ability of cells in 60, 80  $\mu$ g/mL group was inhibited compared with the control.

### 3.4. Quantitative real-time PCR (QRT-PCR)

The transcriptional changes of NF- $\kappa$ B downstream genes including c-FLIP, cyclinD1, c-myc, and c-IAP2 were shown in

Fig. 4. The c-FLIP transcription was markedly induced by 20, 40, 60  $\mu$ g/mL MC-RR at 4, 8, 24 h ( $p < 0.05$ ), whereas significantly suppressed by 80  $\mu$ g/mL MC-RR at 12, 24 h ( $p < 0.05$ ). The cyclinD1 mRNA levels were up-regulated by 20  $\mu$ g/mL MC-RR at 4 h ( $p < 0.01$ ), 60  $\mu$ g/mL MC-RR at 8, 12 h ( $p < 0.01$ ), and 20, 40, 60  $\mu$ g/mL MC-RR at 24 h ( $p < 0.05$ ); however, significant lower expression was detected in 80  $\mu$ g/mL MC-RR group at 4, 8 h ( $p < 0.05$ ), but it recovered to the normal level at 12, 24 h. Compared with the control, the transcriptional level of c-myc was significantly increased in most cases after MC-RR treatment ( $p < 0.05$ ), while it was down-regulated in 80  $\mu$ g/mL group at 4 h ( $p < 0.01$ ) and 40  $\mu$ g/mL group at 12 h ( $p < 0.05$ ),

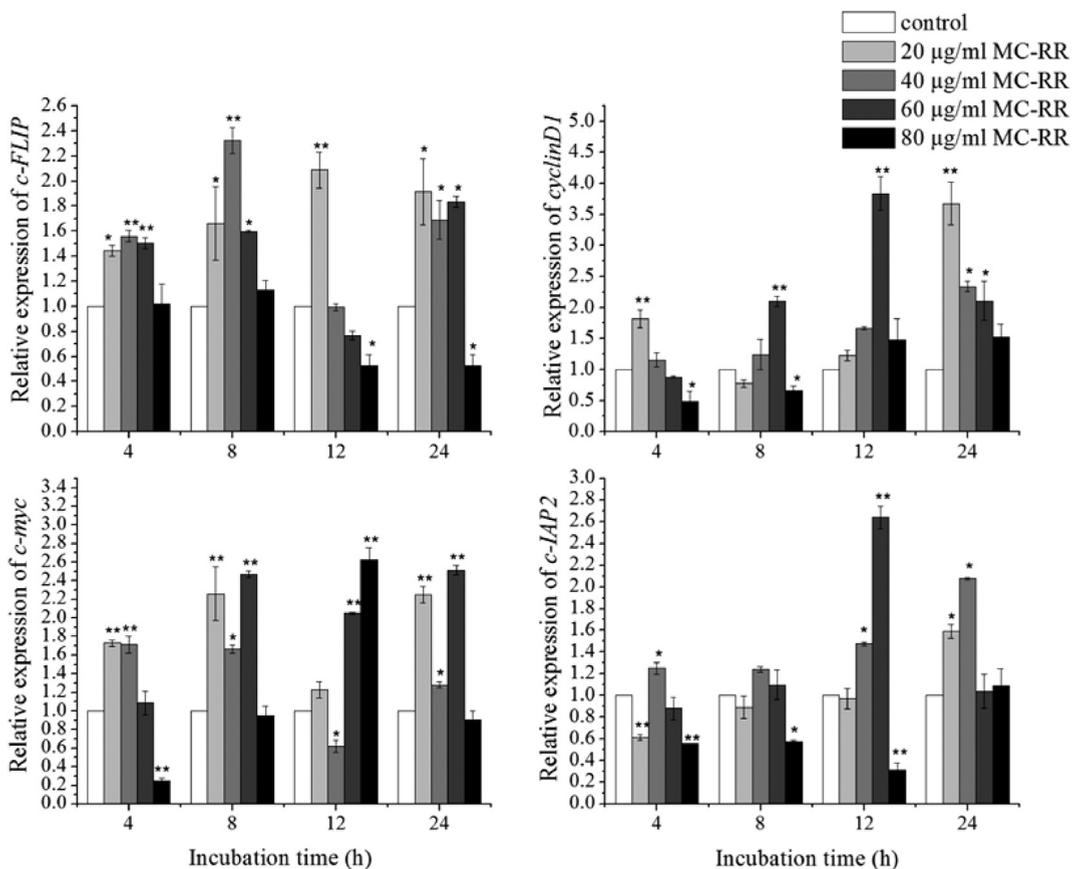


**Fig. 3.** NF- $\kappa$ B DNA-binding activity of HeLa cells incubated with microcystin-RR by electrophoretic mobility shift assay (EMSA). Nuclear proteins were extracted before the detection. The first band is NF- $\kappa$ B-probe specific band, the second band is non-specific band, and the bottom band is free probe.

respectively. The expression of c-IAP2 was obviously elevated by 40, 60  $\mu$ g/mL MC-RR at 12 h ( $p < 0.05$ ) and 20, 40  $\mu$ g/mL MC-RR at 24 h ( $p < 0.05$ ); conversely, the transcription was inhibited by 80  $\mu$ g/mL MC-RR exposure at 4, 8 and 12 h ( $p < 0.05$ ), and it recovered to the normal level finally at 24 h.

### 3.5. Western blot analysis

The effects of MCs on protein expressions of cyclinD1 and c-IAP2 in HeLa cells were shown in Fig. 5. CyclinD1 protein level was induced obviously in 20  $\mu$ g/mL MC-RR group at all the time-points ( $p < 0.05$ ), 40  $\mu$ g/mL MC-RR group at 4, 12 h ( $p < 0.01$ ); 60  $\mu$ g/mL MC-RR significantly decreased the cyclinD1 expression at 4 h ( $p < 0.01$ ), but increased at 12 h ( $p < 0.05$ ) and decreased at 24 h afterward ( $p < 0.05$ ); however, the protein expression of cyclinD1 was inhibited in 80  $\mu$ g/mL MC-RR group at all the time-points ( $p < 0.05$ ). The expression of c-IAP2 protein was significant up-regulated in 20, 40  $\mu$ g/mL MC-RR group, especially at 24 h ( $p < 0.05$ ); but it was down-regulated in 60  $\mu$ g/mL MC-RR group at 12 h ( $p < 0.01$ ), and 80  $\mu$ g/mL MC-RR group at all the time-points ( $p < 0.05$ ).



**Fig. 4.** Transcriptional changes of NF- $\kappa$ B downstream genes c-FILIP, cyclinD1, c-myc, and c-IAP2 in HeLa cells incubated with microcystin-RR. Quantitative real-time PCR was used to test the expression levels of NF- $\kappa$ B downstream genes. GAPDH was used as an internal control. The experiments were conducted in triplicate. Values are presented as the mean  $\pm$  standard deviation (SD). \* indicates significant difference at  $p < 0.05$  versus control, and \*\* indicates significant difference at  $p < 0.01$  versus control.

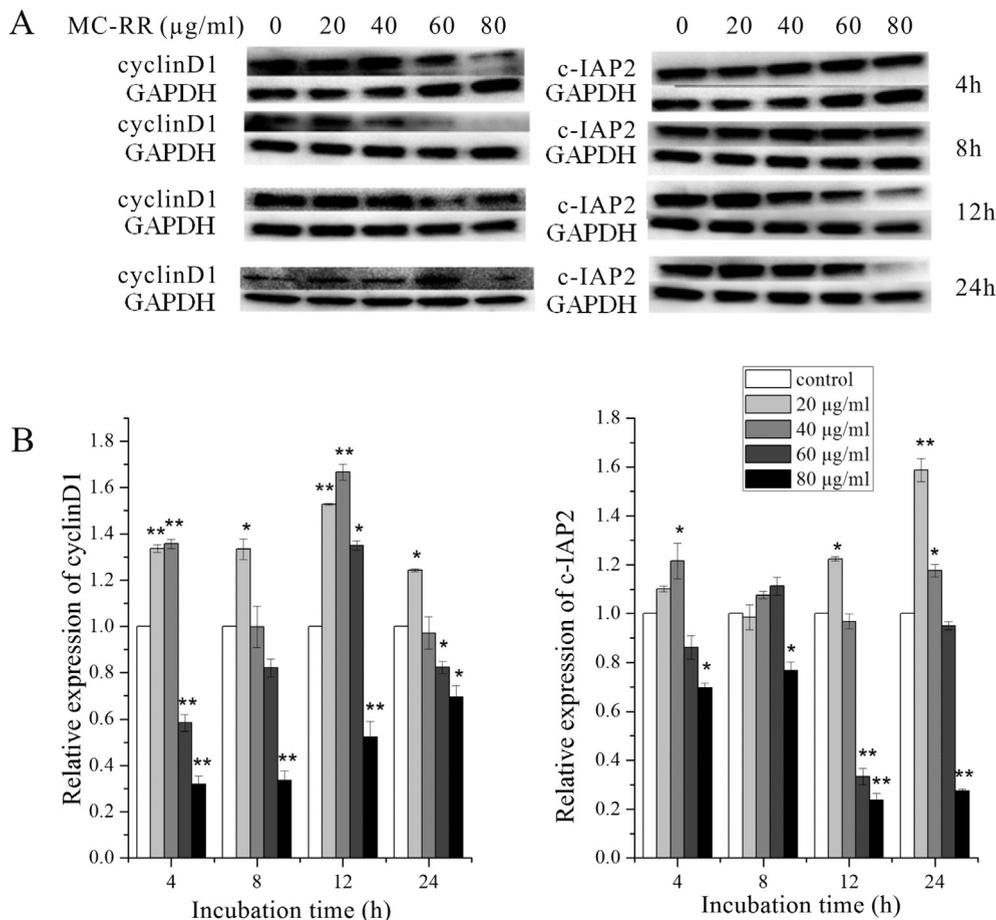
#### 4. Discussion

In the present study, we showed for the first time that microcystin-RR (MC-RR) promoted cell proliferation by activating NF- $\kappa$ B DNA-binding activity and its target genes expression. Moreover, the G<sub>1</sub>/S transition and cell proliferation stimulated at lower MCs doses while apoptosis induced at higher MCs doses were both mediated by NF- $\kappa$ B pathway in HeLa cells. These findings indicated that NF- $\kappa$ B contributed to MC-induced cell proliferation and apoptosis.

Eukaryotic cell cycle, a highly conserved and ordered set of events, has checkpoints between its four phases G<sub>0</sub>/G<sub>1</sub>, S, G<sub>2</sub> and M, to regulate accurate cell division and growth (Vermeulen et al., 2003). The major control sites include DNA damage checkpoints (G<sub>1</sub>/S, intra-S, G<sub>2</sub>/M) and spindle checkpoints. Deregulation of the cell cycle checkpoints could lead to aberrant cell proliferation, apoptosis and cancer development (Nakanishi et al., 2006). Relative lower MCs concentrations could induce cell cycle through G<sub>1</sub> into S phase in human hepatoma cell lines HepG2 and Hep3B (Gan et al., 2010), human normal hepatic cell line WRL-68

(Xu et al., 2012), and monkey kidney-derived cell line Vero-E6 (Dias et al., 2010). The present study also found that MC-RR induced G<sub>1</sub>/S progression and cell proliferation at lower doses; while led to G<sub>1</sub>/S arrest and apoptosis of HeLa cells at higher MC-RR doses. This dualistic response was also reflected by several studies before (Rymuszka et al., 2007; Dias et al., 2010). MCs promoted cell proliferation at lower concentrations and induced apoptosis/cell death at higher concentrations in liver cells (Humpage and Falconer, 1999; Herfindal and Selheim, 2006), lymphocytes (Rymuszka et al., 2007), and kidney epithelial cells (Dias et al., 2010).

Actually, the opposing cell fate induced by lower and higher doses of MC in the present study and previous studies (Gehring et al., 2004; Rymuszka et al., 2007; Dias et al., 2010) are termed hormesis, which is a biphasic dose–response phenomenon that is characterized by low-dose stimulation and high-dose inhibition resulting in either a J-shaped or an inverted U-shaped dose–response, which is a non-monotonic response (Calabrese, 2008). Hormetic dose responses may occur via a direct stimulatory response or



**Fig. 5. Effect of microcystin-RR on the cyclinD1 and c-IAP2 protein expression in HeLa cells.** (A) The representative immunoblot result of protein. Western blot analysis was performed with antibody against cyclinD1, c-IAP2, and GAPDH. The blots are representative of three independent experiments. (B) The quantization of protein expression levels. Mean protein expression in each treated groups is shown as a fold increase compared with mean expression in control groups which has been ascribed an arbitrary value of 1. The value represents mean  $\pm$  standard deviation (SD) from three independent experiments. \* indicates significant difference at  $p < 0.05$  versus control, and \*\* indicates significant difference at  $p < 0.01$  versus control.

via an overcompensation to a disruption of homeostasis, i.e., activating existing cellular and molecular pathways that will enhance the ability of the cell and organism to withstand more severe stress (Calabrese, 2008; Luna-López et al., 2013). Being independent of biological model (cellular, organ, individual, and population levels; microbial, animal, and plant species), endpoint measured, chemical class, and inter-individual variability, hormesis is regarded as the most fundamental dose response and corresponds to traditional concepts in toxicology, in particular the dogma of “the dose makes the poison”. However, it's difficult for toxicologists to assess hormetic dose-time-response and its mechanisms, because it may be hard to detect, especially when study designs have too few doses, limited statistical power, and only one time point (Calabrese, 2008). Hormesis of MCs has also been reported in human amniotic epithelial (FL) cells (Liang et al., 2011), common carp pronephros phagocytes (Rymuszka et al., 2010), crucian carp (Qiao et al., 2013a), and even plants, rape and cabbage (Liu et al., 2008). Liang et al. (2011) found that a low-dose treatment of MC-LR induced an increase in PP2A activity, and a high-dose treatment of MC-LR decreased the activity of PP2A in FL cells. Considering that PP2A is the primary target of MCs, the pattern of MCs effects on PP2A and associated toxic mechanisms are much more complicated than previously thought. Further investigations on dose-dependent PP2A activity modulation responded to MCs exposure will be required.

Since the discovery of NF- $\kappa$ B and its paradoxical but important role in the regulation of cell proliferation, apoptosis and hormesis, it has been considered as a critical transcription factor associated with cancer development and progression (Karin, 2006; Luna-López et al., 2013). Several studies have demonstrated that MCs could activate NF- $\kappa$ B (Feng et al., 2011; Ji et al., 2011; Zhang et al., 2012; Christen et al., 2013). In the present study, we also found that MC-RR significantly increased NF- $\kappa$ B DNA-binding activity at lower MC-RR doses but decreased the binding activity at higher doses in the nuclear fraction of HeLa cells. The role of NF- $\kappa$ B in regulating the cell cycle and apoptosis was previously implicated (Kucharczak et al., 2003). For example, B cells from NF- $\kappa$ B knockout mice show defective proliferative response to mitogenic stimulation and result in G<sub>1</sub> phase arrest (Hsia et al., 2002; Pohl et al., 2002; Kucharczak et al., 2003). Taken together with the cell cycle and apoptosis analysis, we suppose that the apoptosis or the increased cellular proliferation in HeLa cells appears to be dependent on the NF- $\kappa$ B activity, which is responded to the concentration of MC-RR. The role of NF- $\kappa$ B on the MC-mediated cell cycle regulation and its ultimate contribution to the proliferation and apoptosis will significantly improve the knowledge of the mechanisms underlying MCs tumor-promoting activity.

To confirm our hypothesis, we then conducted quantitative real-time PCR (QRT-PCR) as well as western blotting to detect the expression of NF- $\kappa$ B downstream target genes including c-FLIP, cyclinD1, c-myc, and c-IAP2. Interestingly, both mRNA level of these 4 genes and protein level of cyclinD1 and c-IAP2 in HeLa cells were significantly affected by MC-RR and showed the similar variation trend as NF- $\kappa$ B activity, which were induced by lower doses of

MC-RR and suppressed by higher doses, indicating that NF- $\kappa$ B signaling indeed mediate cell fate regulation in HeLa cells exposed to MCs.

Cyclin D1 is a crucial regulator of the G<sub>1</sub>/S phase transition, can also function as a proto-oncogene and its deregulation and overexpression are frequently linked to various types of human cancer (Shan et al., 2009; Witzel et al., 2010). C-myc gene is the best-studied member of myc oncogene family, which encodes a transcription factor involved in cell proliferation and carcinogenesis (O'Donnell et al., 2005; Li et al., 2009). In the present study, MC-RR up-regulated cyclin D1, c-myc, and promoted cell cycle following MCs exposure associated with NF- $\kappa$ B activation. Induction of the expression of cyclin D1 by MCs was also described in human colorectal crypt epithelial cells by Zhu et al. (2005), and c-myc was induced in rat liver, kidney and testis of rats exposed to MCs in our previous study (Li et al., 2009). Therefore, the abundance of these two proteins, which promote cell proliferation by accelerating the course of the cell through G<sub>1</sub> phase, might be one of the molecular events related to the proliferative and apoptotic activity of MCs in HeLa cells. However, Takumi et al. (2010) reported that the mRNA level of cyclin D1 did not change after treatment with MC-LR, but c-myc was considerably up-regulated in HEK293-OATP1B3 cells. In fact, the cyclin D1 and c-myc promoter is targeted not only by NF- $\kappa$ B but also by multiple other transcription factors; also, in additional direct transcriptional regulation of cyclin D1 and c-myc, some other mechanisms, such as co-transcriptional processing, splicing, RNA stability as well as protein degradation could further influence MC-induced cyclin D1 and c-myc expression (Vervoorts et al., 2006; Barré and Perkins, 2007; Witzel et al., 2010). Therefore, analysis of the mechanism by which cyclin D1 and c-myc abnormal expression induced by MCs remains to be investigated.

Cellular FLICE-inhibitory protein (c-FLIP) is a catalytically inactive procaspase-8/10 homologue and considered as an anti-apoptotic, pro-survival factor (Bagnoli et al., 2010). In current study, we found for the first time that MCs promoted cell proliferation with concomitant elevated c-FLIP transcription, suggesting that c-FLIP played a key role in cell fate determination. At the same time, another anti-apoptotic protein cellular inhibitor of apoptosis 2 (c-IAP2) (Mahoney et al., 2008), also displayed its function in MC-induced cell survival and apoptosis. It is notable that the induction of c-IAP2 by MCs starts at transcription level since the results showed the induction of c-IAP2 at both mRNA and protein levels. However, the expression of c-FLIP and c-IAP2 was suppressed by MC-RR at relative higher doses, leading to cell apoptosis eventually. This similar effect of MC-LR on c-IAP2 protein expression was also reported in HepG2 cells in our previous study (Zhang et al., 2013b). These suggest that MCs could activate both death and survival signals, and the death signal was stronger than survival signal induced by higher doses of MC-RR.

The main effect of a carcinogen is its ability to induce a disorder of gene expression, resulting in aberrant proliferation and apoptosis through a wide variety of integrative mechanisms. An abnormal activation of the NF- $\kappa$ B pathway, controlling cell growth and apoptosis, is common in tumors and this could promote cell proliferation (Piva

et al., 2006; Chaturvedi et al., 2011). In this study, NF- $\kappa$ B activation coincided with its target genes cyclinD1, c-myc, c-FLIP, and c-IAP2 expression, suggesting that NF- $\kappa$ B signaling pathway was indeed involved in MC-RR-induced cell proliferation and apoptosis. However, the underlying mechanisms how MCs regulate NF- $\kappa$ B DNA-binding activity and further influence the cyclinD1, c-myc, c-FLIP, and c-IAP2 expression are uncertain in our model, which should be made clear in future works. NF- $\kappa$ B exists as a latent and inactive cytoplasmic complex, whose predominant form is a heterodimer composed of p50 (NF- $\kappa$ B1) and p65 (RelA) subunits, bound to inhibitory proteins of the I $\kappa$ B family (Piva et al., 2006). Actually, some unique features of MC may contribute to its effect on NF- $\kappa$ B activation (Fig. 6) (Feng et al., 2011). First, PP1 and PP2A are the primary targets of MCs (MacKintosh et al., 1990). Liang et al. (2011) demonstrated that PP2A activity and PP2Ac mRNA and protein levels were markedly increased at low concentrations of MC-LR in human amniotic epithelial (FL) cells; however, exposure to high concentrations of MC-LR significantly decreased the activity of PP2A. Inhibition of PP1 and PP2A can promote I $\kappa$ B phosphorylation and degradation, ultimately leading to the activation of NF- $\kappa$ B (Kucharczak et al., 2003), and Akt may be involved in the I $\kappa$ B phosphorylation, allowing for translocation of the released NF- $\kappa$ B to the nucleus (Zhang et al., 2012). What's more, MCs can generate a large amount of ROS and induce oxidative stress (Guzman and Solter, 1999; Campos and Vasconcelos, 2010). ROS have various inhibitory or stimulatory roles in NF- $\kappa$ B signaling; a relative low oxidative stress can activate NF- $\kappa$ B; however, too much ROS may inhibit NF- $\kappa$ B and cells will undergo apoptosis (Román et al., 1999; Gloire et al., 2006; Morgan and Liu, 2011; Luna-López et al., 2013). Additionally, MCs can also activate NF- $\kappa$ B indirectly by endoplasmic reticulum (ER)

stress induction (Christen et al., 2013). Further studies may be required to understand how MC-RR regulates NF- $\kappa$ B activation.

In conclusion, we have shown for the first time that MC-mediated NF- $\kappa$ B signaling has a binary effect (hormesis) in HeLa cells: the stimulation of cell proliferation at lower MC-RR doses and the induction of apoptosis at higher doses. Fig. 6 summarizes the modes of MC action of previous studies (Feng et al., 2011; Zhang et al., 2012; Christen et al., 2013) and our observations in a schematic way, and at the same time, serves as a working hypothesis for further investigations. The activation/inactivation of NF- $\kappa$ B is responded to the concentration of MC-RR and can lead to the induction/suppression of its downstream genes, resulting in cell proliferation/apoptosis. More information is required to further elucidate the biphasic toxic effects and tumorigenesis mechanism of MCs.

### Acknowledgments

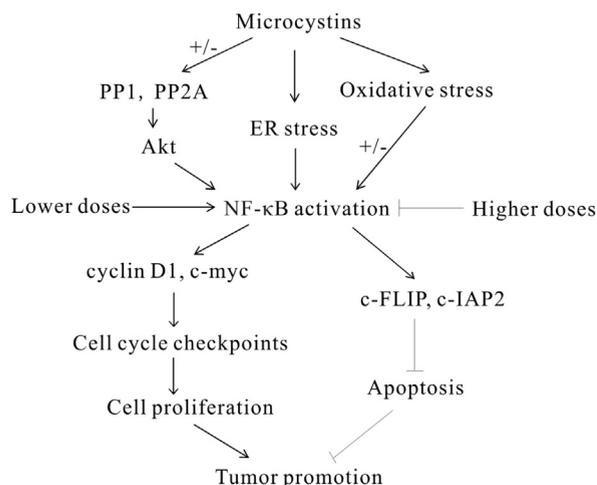
We greatly appreciate the constructive and useful comments and suggestions by Dr. Alan Harvey and anonymous reviewers. We would also like to thank professor Wuhan Xiao for offering HeLa cells and technical assistance on cell culture. This work was supported by the National Natural Science Foundations of China [grant number 31070457; 31322013]. This work was also supported by the State Key Laboratory of Freshwater Ecology and Biotechnology [grant number 2014FBZ02].

### Conflict of interest

The authors declare that there are no conflicts of interest.

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**Fig. 6. A possible molecular basis underlying the dualistic responses triggered in cells exposed to microcystins.** NF- $\kappa$ B is activated exposure to lower doses of microcystins (MCs) resulting in expression of its downstream target genes including c-FLIP, cyclinD1, c-myc, and c-IAP2, and promoted cell proliferation and tumor development; while higher doses of MCs inactivated NF- $\kappa$ B signaling pathway and induced apoptosis. PP1 and PP2A: phosphatases 1 and 2A; ER stress: endoplasmic reticulum stress.

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