



Microcystin-leucine arginine blocks vasculogenesis and angiogenesis through impairing cytoskeleton and impeding endothelial cell migration by downregulating integrin-mediated Rho/ROCK signaling pathway

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Abstract

The main characteristic of eutrophication is cyanobacteria harmful algae blooms. Microcystin-leucine arginine (MC-LR) is considered to be the most toxic and most commonly secondary metabolite produced by cyanobacteria. It has been reported that MC-LR had potential vascular toxicity. However, the mechanism that MC-LR-induced vascular toxicity is very limited and remains to be clarified. The aim of this study was to evaluate the toxic hazard toward the vasculogenesis and angiogenesis of MC-LR. Its effects on vasculogenesis, sprouting angiogenesis, and endothelial cell tube formation were studied. The study showed that MC-LR exposure blocked vasculogenesis in zebrafish embryos, sprouting angiogenesis from rat aorta, and tube formation of human umbilical vein endothelial cells (HUVECs). In addition, MC-LR exposure also induced the disruption of cytoskeletal structures and markedly inhibited endothelial cell (EC) migration from caudal hematopoietic tissue in zebrafish and HUVEC migration. Western blot analysis showed that MC-LR exposure downregulated the expressions of integrin β 1, FAK, Rho, and ROCK. Combined with these results, MC-LR could induce disruption of cytoskeleton via downregulating integrin-mediated FAK/ROCK signaling pathway, leading to the inhibition of EC migration, which finally blocked vasculogenesis and angiogenesis.

Keywords Cell migration · Cytoskeleton · Microcystin-leucine arginine · Vasculogenesis · Angiogenesis · Responsible Editor: Lotfi Aleya

Introduction

Owing to climate change and irrational anthropogenic activities, eutrophication in freshwaters, such as ponds, streams, swamps, lakes, and rivers, and marine ecosystems have an increasing trend worldwide (O'Neil et al. 2012; Preece et al. 2017). The main characteristic of eutrophication is cyanobacteria harmful algae blooms (cyano-HABs). The

overgrowth of harmful algae causes deterioration of water quality and emits an unpleasant smell, leading to the loss of recreational and property values of aquatic ecosystems (Zhang et al. 2021). In cyano-HABs, several cyanobacterial genera such as *Anabaena*, *Microcystis*, *Nostoc*, and *Planktothrix* can produce toxic secondary metabolites (Dziga et al. 2013), which can inhibit the growth of other taxa and increase the competitive and defensive abilities (Liu et al. 2020). The existence of these toxins in water bodies for recreation, drinking water, and irrigation can pose serious human health risks and thus become an increasingly concerned public health topic (Preece et al. 2021).

In freshwater, among the multifarious cyanobacteria (blue-green algae), microcystis aeruginosa is the most common genera producing microcystins. So far, a family of more than 200 different structurally monocyclic heptapeptide toxins, with microcystin-leucine arginine (MC-LR) is considered to be the most toxic and most commonly encountered microcystin analogues (Puddick et al. 2014; Zhou et al. 2018). It has been

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reported that MC-LR can damage the liver, kidneys, lungs, brain, heart, blood vessels, intestines, pancreatic islets, gonads, and reproductive system (Wang et al. 2019; Zhang et al. 2021). A large quantity of data has shown that MC-LR causes morphological injuries by inducing the abnormal condensation of microtubules, the shortening and loss of actin filaments, and the reorganization of intermediate filaments, suggesting that MC-LR has cytoskeletal-specific effects containing reorganization of all three essential cytoskeletal structures (Wickstrom et al. 1995; Zhou et al. 2015).

The vascular system is constructed and maintained by vasculogenesis and angiogenesis (Benson and Southgate 2021). Vasculogenesis is the development of a primary blood system via the de novo formation of blood vessels during embryogenesis, which is that endothelial precursor cells in the embryonic mesenchyme form an aggregate and then mature into small, single-layered endothelial tubes (Kubis and Levy 2003; Semenza 2007). Angiogenesis is the formation of new blood vessels by sprouting or intussusception from pre-existing vessels, which is vital to a range of processes containing embryonic or fetal development, organ growth, wound tissue healing, skeletal development, pregnancy, or during the menstrual cycle (Carmeliet 2005; Carmeliet and Jain 2011). Angiogenesis also is related to various kinds of pathological conditions, such as cancer, diabetic retinopathy, autoimmune disorders, arthritis, infectious diseases, and so on (Chung and Ferrara 2011). Sprouting angiogenesis is the expansion of existing vessels. Once the vessel has fully expanded, endothelial cell (EC) proliferation and migration are inhibited by anti-angiogenic factors. Therefore, in the development of vascular networks, the proliferation and migration of ECs are critical processes, which are influenced by the activity of small Rho GTPases (Benson and Southgate 2021).

The cytoskeleton consists of three major structural elements: microfilaments, microtubules, and intermediate filaments, which plays a central role in a myriad of cellular processes such as the establishment and maintenance of cell architecture, cell shape, motility, growth, division, differentiation, apoptosis, and the transport or positioning of organelles (Ding et al. 2000; Henty et al. 2011). Contraction of the actin cytoskeleton is one necessary element of individual cell migration, which facilitates both forward movement of the cell body and detachment of the trailing edge (Schwarzbauer 1997). Furthermore, the contraction has been proposed to generate tension within three-dimensional matrices that conduces to lead the migration of ECs toward one another to promote tube formation (Davis and Camarillo 1995). Thus, the perturbation of the cytoskeleton could involve not only direct inhibition of cell motility but also possibly interfere with guidance mechanisms for triggering cell-cell interaction networks.

Zebrafish (*Danio rerio*) is frequently used as a general fish and vertebrate model for investigating chemical toxicity and mechanism, due to its high fecundity, rapid development,

short life cycle, transparency of the eggs and embryos, and ease of maintenance in the laboratory (Wang et al. 2020). Therefore, zebrafish embryos were used to evaluate vasculogenesis and angiogenesis. The rat aortic ring model has been proven to be the most useful method for the evaluation of the angiogenic process (Nicosia and Ottinetti 1990; Blacher et al. 2001).

The goal of the present study was to test whether MC-LR blocked vasculogenesis and angiogenesis by inhibiting EC migration. Toward this goal, studies were undertaken with the objective of evaluating the toxic effects of MC-LR on vessel networks in rat aorta rings, EC migration and vasculogenesis in zebrafish, and migration and tube formation of human umbilical vein endothelial cells (HUVECs). In addition, actin cytoskeleton and Rho/ROCK signaling pathways in HUVEC progression and dissemination were examined.

Materials and methods

Rat aorta ring assay

Adult healthy Sprague-Dawley (SD) rats specific pathogen-free (SPF) grade, weighing 200–220 g, were purchased from the Experimental Animal Center of Army Military Medical University (Chongqing, China). Rat aorta was obtained from normal adult SD rats in accordance with the protocol approved by the ethical committee of the college of bioengineering of Chongqing University. All procedures complied with the China Council on Animal Care and Use protocol.

On the day of experimentation, the adult SD rats were euthanized by an overdose of phenobarbital (50 mg/kg body weight). After being rapidly dissected and isolated, the thoracic aorta was placed in preheated (37 °C) phosphate-buffered saline (PBS). Following being carefully cleaned of adhering connective and fat tissues, the aorta was washed thrice with preheated PBS and cut into ring segments of 1 mm in length. Exposure of the gel in a solution of sulfo-SANPAH with a UV light source at 365 or 320 nm covalently links the sulfo-SANPAH to the polyacrylamide hydrogel. After adding an appropriate amount of rat tail tendon type I collagen (Solarbio, Beijing, China), the solution with the gel was incubated overnight at 37 °C. Rat thoracic aortic rings were plated on the top of rat tail tendon type I collagen incubating in M199 medium from Gibco (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Gibco, California, USA), 50 ng/mL vascular endothelial growth factor 165 (VEGF 165), and designated concentrations of MC-LR (100 and 1000 nM) for 2 weeks, changing the spent medium with fresh medium every other day. Vascular sprouting from each ring was examined with an Olympus microscope (20× magnification; Olympus,

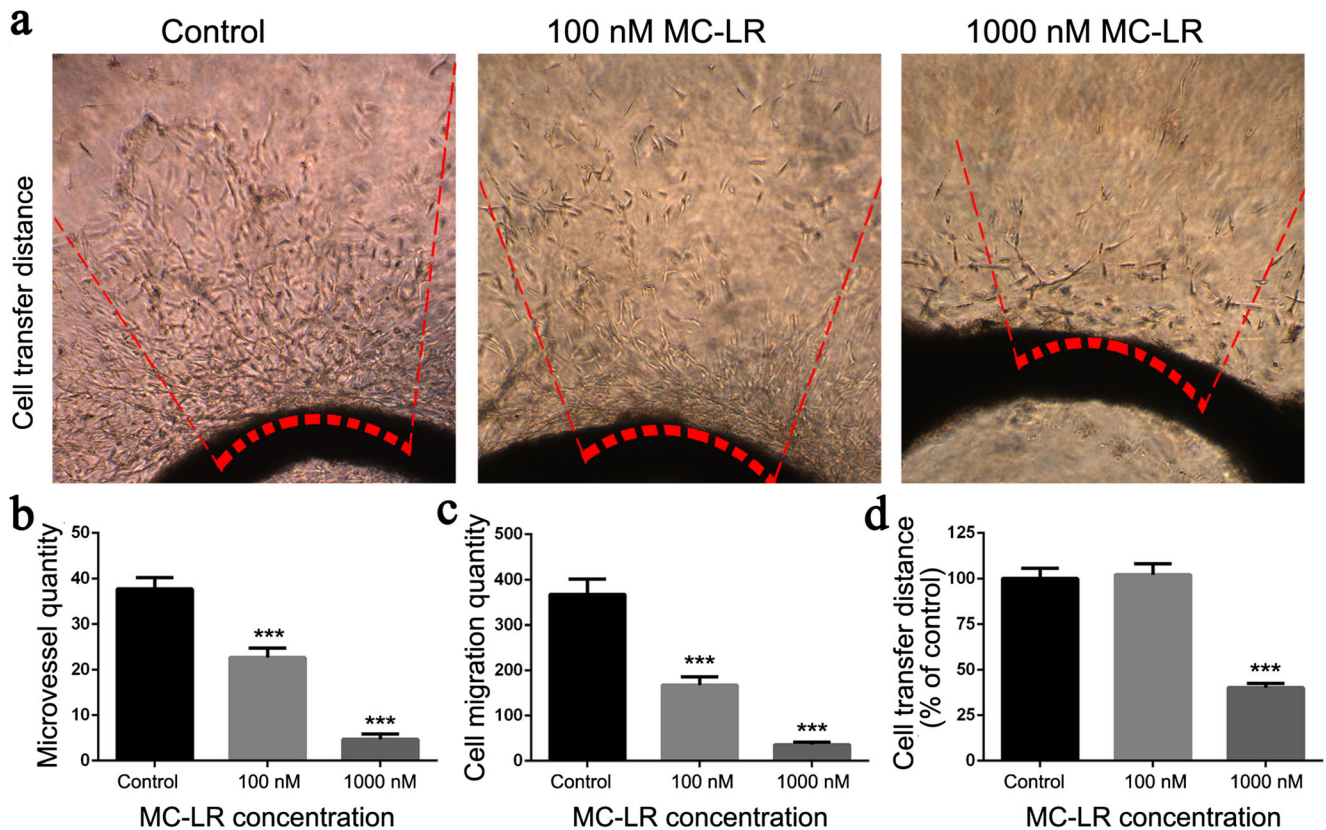


Fig. 1 MC-LR inhibits sprouting angiogenesis in aorta rings. **a** Aortic ring assay. **b** Microvessel quantity. **c** Cell migration quantity. **d** Cell transfer distance. Aorta rings were treated with MC-LR for 2 weeks.

The dotted line in Fig. 1a is the statistical calculation area. All values are represented as mean \pm SD of three replicates ($n=3$). ***, $p < 0.001$ vs control

Tokyo, Japan), and the captured images were analyzed by using NIH Scion Image software (Scion Corp., Frederick, MD).

Breeding and exposure of zebrafish

Adult zebrafish including Tg (*flkl1*: EGFP) expressed enhanced green fluorescent protein (GFP) in ECs and Tg (*fli1a*: NGFP) expressed GFP in the nucleus of ECs was provided by Prof. Anming Meng of Tsinghua University. Zebrafish embryos were maintained and exposed according to the method described by Wang et al. (2019). Tg (*flkl1*: EGFP) and Tg (*fli1a*: NGFP) embryos at 2 h post-fertilization (hpf) were collected and were placed into 5 mL medium containing various concentrations of MC-LR (100 and 1000 nM). The Tg (*flkl1*: EGFP) embryos were taken at 24 hpf to evaluate vasculogenesis. The Tg (*fli1a*: NGFP) transgenic zebrafish line embryos were taken at 72 hpf to evaluate the numbers of EC migration from caudal hematopoietic tissue (CHT). The embryos were examined using a stereoscopic microscope (Stemi 2000 C, Carl Zeiss, Jena, Germany) and analyzed by ImageJ software.

EC tube formation assay

Cell tube formation assay was performed as follows (Shi et al. 2017). Each well of the 96-well plate was coated with 80 μ L BD matrigel (BD Biosciences, San Jose, CA) at 37 $^{\circ}$ C for 1 h. After starvation treatment of HUVECs in the logarithmic phase, HUVECs were suspended in M199 medium containing 2% FBS and designated concentrations of MC-LR (100 and 1000 nM). Then, the HUVECs were plated onto the matrigel layer with a density of 1×10^5 cells/well and cultured with 5% CO₂ at 37 $^{\circ}$ C for 12 h. The tube networks were visualized by using an Olympus microscope.

HUVEC migration assay

Cell migration was measured adopting a monolayer scratch injury assay as described by Wang et al. (2020). Briefly, the HUVECs were seeded on glass chamber slides and cultured until confluence. Then, a uniform straight scratch was created in the confluent monolayer with a plastic Cell Scraper (Corning, USA). The cell monolayers were washed gently and examined using an inverted microscope (Nikon Eclipse TE2000-Y system, Japan). All images were analyzed with the ImageJ software.

Cytoskeleton observation

The cytoskeleton of HUVECs was assessed according to the method described by Wang et al. (2020). After being cultured with fresh cell medium containing the different concentrations of MC-LR (1, 10, 100, and 1000 nM) for 24 h, the cytoskeleton was observed and images were captured using a confocal laser-scanning microscope (CLSM; TCS SP8, Leica, Germany).

Western blot

The cell culture, toxin treatment, and western blot analysis were as described by Wang et al. (2019). After 24 h exposure to 1, 10, 100, and 1000 nM MC-LR, the western blot analysis of integrin $\beta 1$, FAK, Rho, and ROCK was carried out.

Statistical analysis

Prism 8 statistical software (GraphPad Software, La Jolla, CA, USA) and IBM SPSS Statistics 22.0 (IBM Corp., Armonk, NY) were used for the statistical analysis. All the experimental data are from three independent experiments. The data are shown as mean \pm standard deviation (SD) and differences with $p < 0.05$ were considered to be statistically significant.

Results

MC-LR inhibited vessel growth in aorta rings

The rat aortic ring assay was used to verify that MC-LR led to inhibition of angiogenesis is a direct effect of MC-LR. The aorta rings were embedded in type I collagen and cultured in M199 medium containing 10% FBS for microvascular endothelial cells to generate branching microvessels (Fig. 1a). Compared with the control group, the number of microvessels and migrated cells in 100 and 1000 nM MC-LR-treated groups decreased significantly (Fig. 1 b and c). No significant difference in cell transfer distance was observed between the 100 nM MC-LR-treated group and the control group, while the transfer distance of cell in the 1000 nM MC-LR-treated group reduced significantly, which was 56.67% of the control group (Fig. 1d). The findings showed that the sprouting angiogenesis from the rat aorta was significantly blocked by MC-LR.

MC-LR inhibited vasculogenesis in zebrafish embryos

To investigate the effects of MC-LR on zebrafish vasculogenesis, Tg (*flkl1*: EGFP) embryos at 2 hpf were

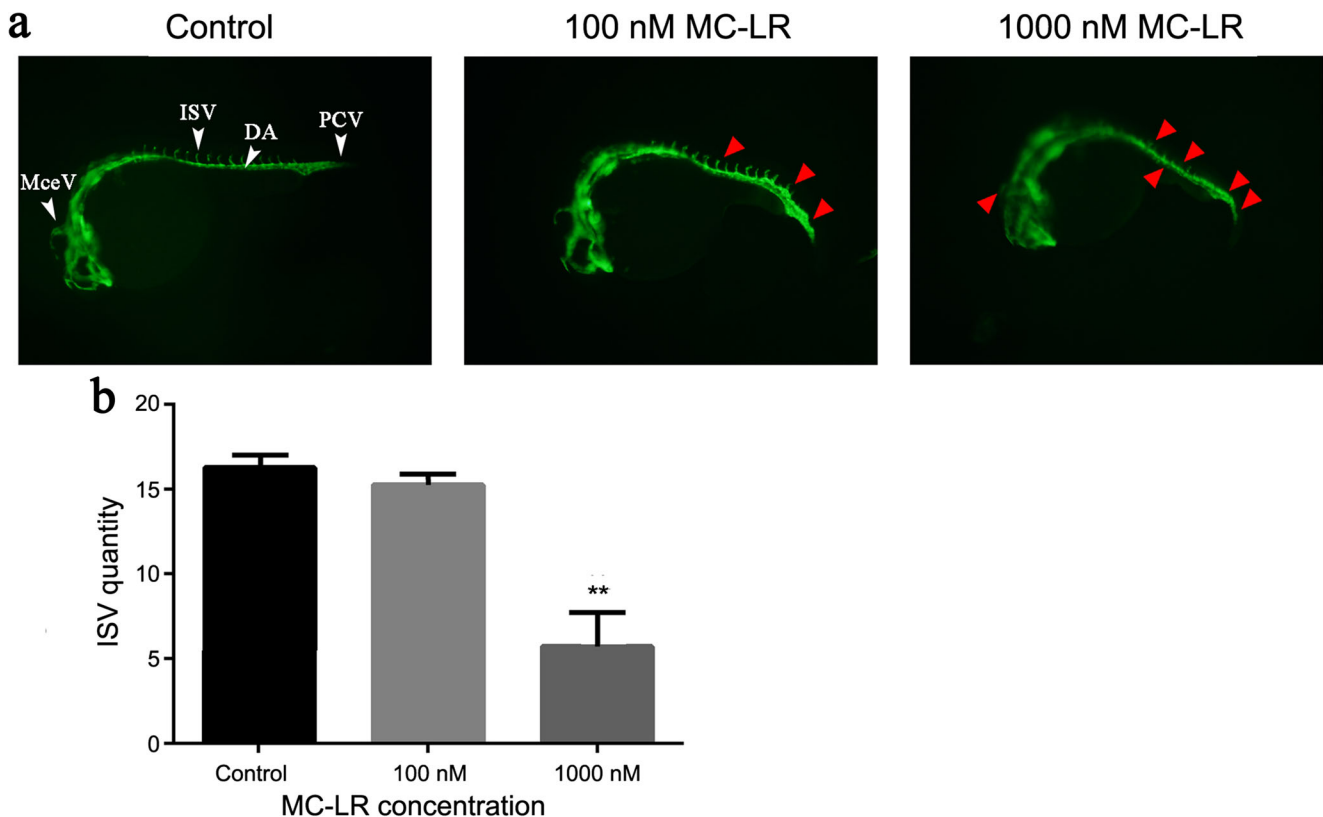


Fig. 2 MC-LR inhibits vasculogenesis in zebrafish embryos. **a** The phenotype of vessels at 24 hpf. **b** ISV statistics based on Fig. 2a. Arrows in 100 and 1000 nM MC-LR-treated groups indicate vascular

deletion. All values are represented as mean \pm SD of three replicates ($n=3$, 30 embryos larvae/replicate). **, $p < 0.01$ vs control

exposed to MC-LR. As shown in Fig. 2, compared with the control group, the dorsal aorta (DA) in 100 and 1000 nM MC-LR-treated groups at 24 hpf has shown rougher appearance and curved shape, a curved posterior cardinal vein (PCV) has been observed accompanying growth inhibition, and the midcerebral veins (MceV) was suppressed. Moreover, the developmental absence of intersegmental vessel (ISV) has also been observed. Statistical analysis showed that ISV sprouting in the 1000 nM MC-LR-treated group was significantly blocked (Fig. 2b). The results showed that MC-LR blocked vasculogenesis in zebrafish embryos.

MC-LR inhibited EC migration in zebrafish

After exposure to MC-LR, the number of migrating ECs was quantified at 72 hpf. The percentage of EC migration from CHT was significantly dropped to 94.26% and 73.87% at the concentrations of 100 and 1000 nM MC-LR relative to the control, respectively, which showed that exposure to MC-LR markedly inhibited EC migration from CHT (Fig. 3).

MC-LR inhibited tube formation of HUVECs

To evaluate whether MC-LR was involved in the process of angiogenesis, a matrigel tube formation assay was performed. As shown in Fig. 4, compared with the control group, the number of the enclosed lumen, the number of the common node of the enclosed lumen, the circumference of the enclosed lumen, and total lumen area in 100 and 1000 nM MC-LR-treated groups decreased significantly following 12 h of incubation. The results showed that MC-LR inhibited tube formation of HUVECs.

MC-LR inhibited HUVEC migration

The adverse effect of MC-LR on cell migration is shown in Fig. 5. After exposure of HUVECs to MC-LR at 37 °C for 12 and 24 h, the distance of HUVEC migration in 1, 10, 100, and 1000 nM MC-LR-treated groups reduced significantly in a dose-dependent manner compared with the control group. Especially in the highest concentration of MC-LR (1000 nM) treatment group, the distance of cell migration is the shortest, which is only 41.62% of the control after being

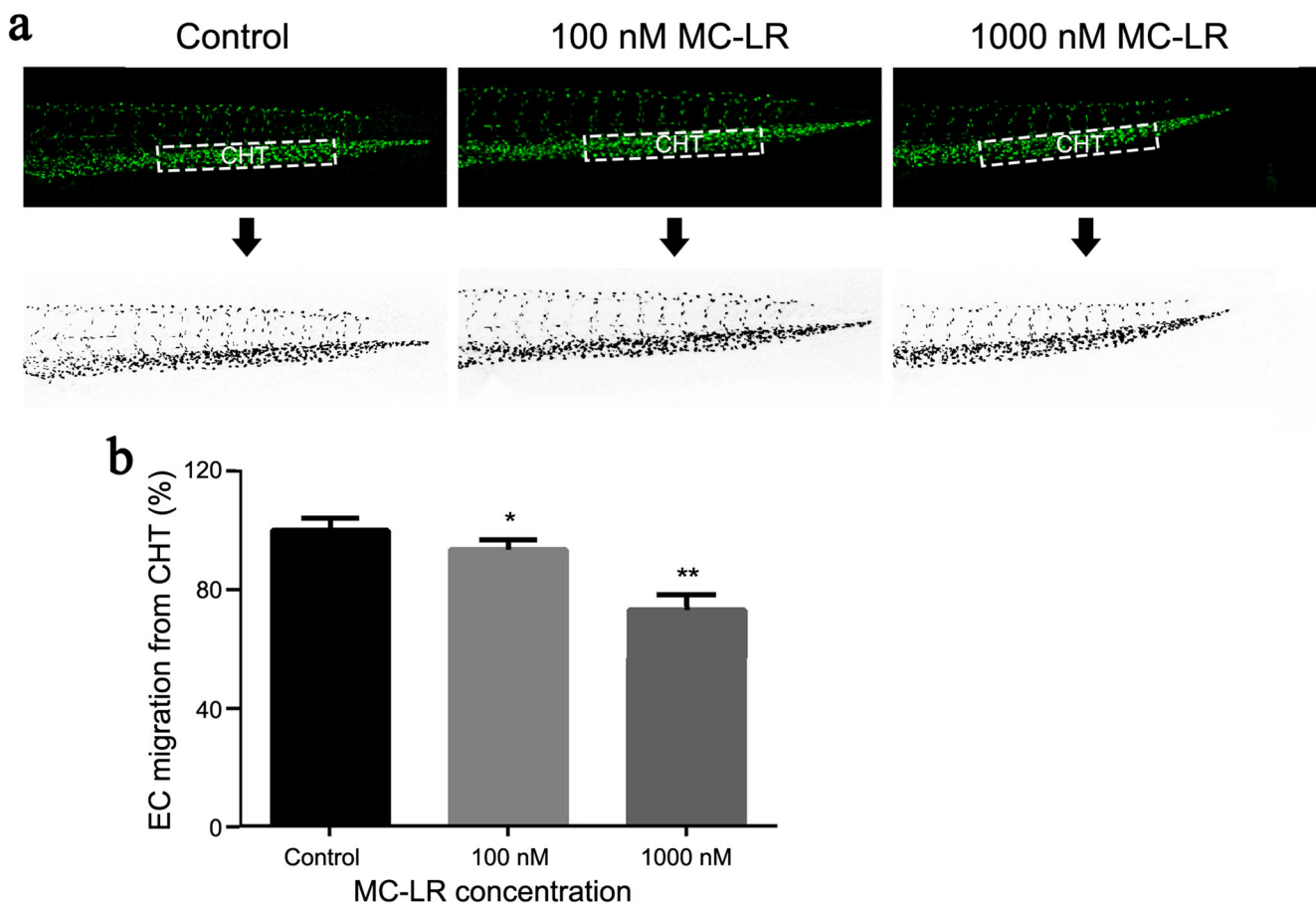


Fig. 3 Effects of MC-LR on EC migration. **a** EC migration from CHT at 72 hpf. **b** The statistics of the cell migration from CHT based on Fig. 3a. All values are represented as mean \pm SD of three replicates ($n=3$). *, $p < 0.05$ and **, $p < 0.01$ vs control

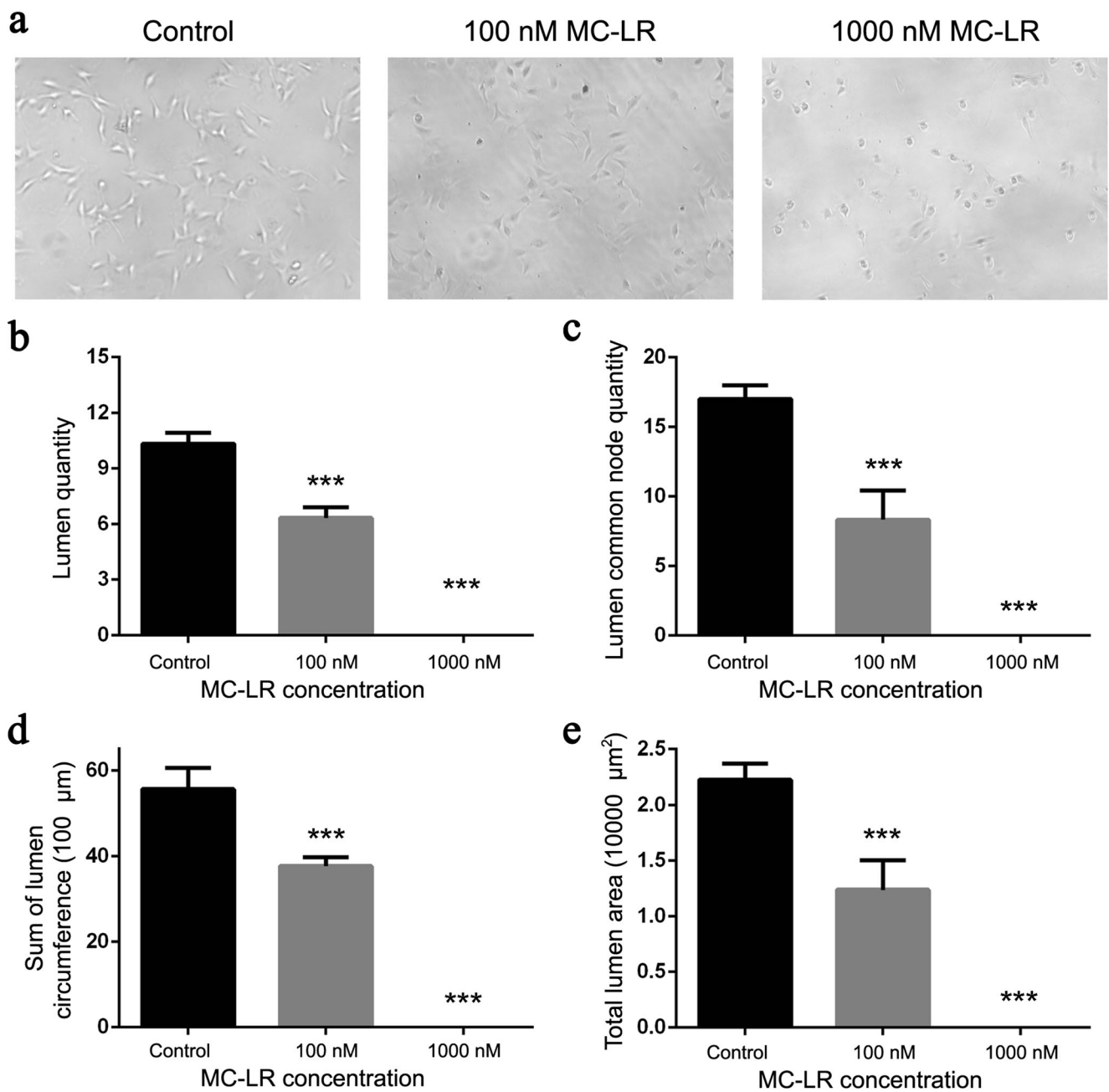


Fig. 4 MC-LR inhibition of angiogenesis. **a** EC tube formation assay. **b** The number of the enclosed lumen. **c** The number of the common node of the enclosed lumen. **d** The circumference of the enclosed lumen. **e** Total

lumen area. All values are represented as mean ± SD of three replicates ($n=3$). ***, $p < 0.001$ vs control

exposed to MC-LR for 24 h (Fig. 5b). Therefore, MC-LR could inhibit HUVEC migration in a dose- and time-dependent manner.

MC-LR induced HUVEC cytoskeleton damage

To determine whether MC-LR induced disruption of the endothelial actin-myosin cytoskeleton, the changes in cytoskeleton were measured following 24 h of MC-LR exposure. The HUVEC cytoskeleton was shown in Fig. 6. In the control

group, the cells exhibited a normal and clear distribution of actin microfilaments. The nuclei were widely surrounded by highly organized microfilaments with normal morphology. However, in contrast to the control group, the microfilaments morphology in MC-LR-treated groups changed obviously.

Fig. 5 MC-LR inhibited HUVEC migration. **a** The microscope images of cell migration. **b** The distance of cell migration. All values are represented as mean ± SD of three replicates ($n=3$). **, $p < 0.01$, ***, $p < 0.001$ vs control

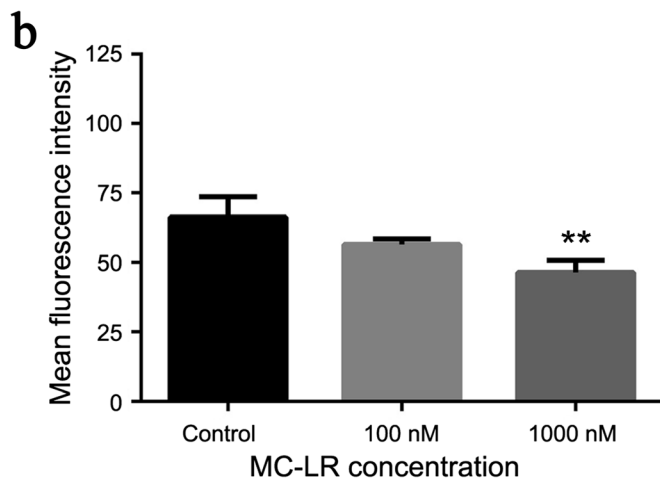
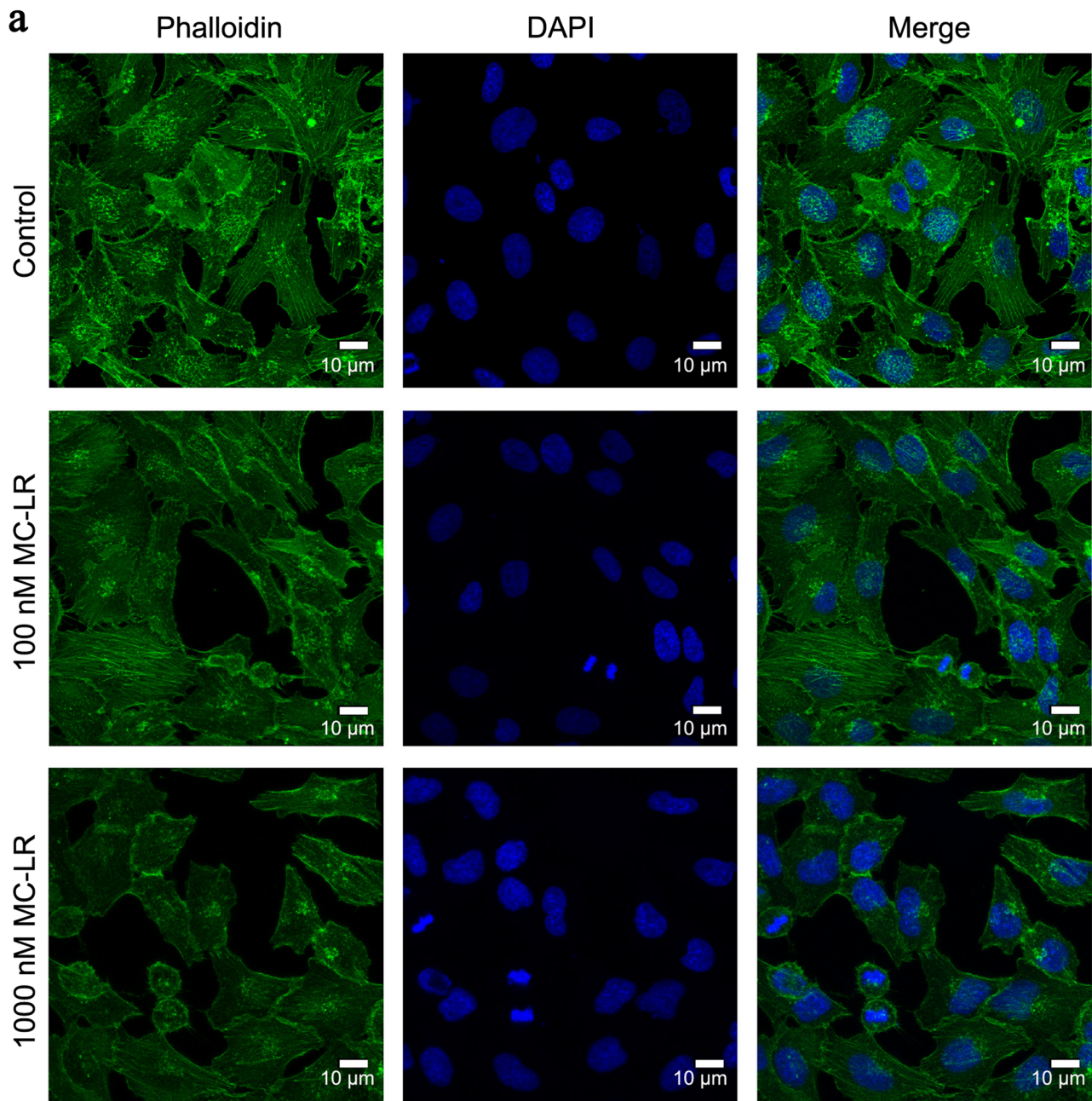


Fig. 6 MC-LR induced actin-myosin cytoskeleton reorganization. **a** Immunofluorescence images of cytoskeleton. **b** Quantitative analysis based on Fig. 6a. HUVECs were treated with MC-LR for 24 h. All values are represented as mean \pm SD of three replicates ($n=3$). **, $p < 0.01$ vs control

Microfilaments were condensed around the nucleus. HUVECs exhibited apoptotic properties and had a hollow nucleus with condensed chromatin (Fig. 6a). The mean fluorescence intensity of HUVECs in MC-LR-treated groups also reduced significantly compared with the control group (Fig. 6b). This finding suggests that MC-LR induces the dysfunction of the HUVEC cytoskeleton system.

MC-LR downregulated expression of integrin $\beta 1$, FAK, Rho, and ROCK

To verify whether MC-LR inhibits HUVEC migration by integrin-mediated FAK/ROCK signaling pathway, integrin $\beta 1$, FAK, Rho, and ROCK were analyzed by western blot analysis (Fig. 7a). The data showed that the expression of integrin $\beta 1$, FAK, Rho, and ROCK downregulated significantly by MC-LR in a dose-dependent manner (Fig. 7b–e).

Discussions

MC-LR is reported to be a group of well-known hepatotoxic cyclic peptides and is the main culprit for giving rise to serious cellular damage, as evident by various MC-LR concentrations associated with DNA injury, cytoskeleton disruption, mitochondria dysfunction, endoplasmic reticulum disturbance, and cell cycle deregulation, all of which can contribute to apoptosis/programmed cell death (Brzuzan et al. 2009; Zhou et al. 2015; Faltermann et al. 2016). A tube formation assay has been typically used to demonstrate the angiogenic activity of vascular ECs in vitro (Shao and Guo 2004). EC migration is an essential component of angiogenesis that requires a tight regulation of the contractile and non-contractile states of the cell (Lamallice et al. 2007). Previous research work has proved that angiogenesis exhibits a positive correlation with cell motility (Robinet et al. 2005). In the present study, MC-LR inhibited vasculogenesis in zebrafish embryos, sprouting angiogenesis from rat aorta and tube formation of HUVECs. MC-LR also markedly inhibited EC migration from CHT in zebrafish and HUVEC migration. Therefore, it is plausible that MC-LR can inhibit vasculogenesis and angiogenesis by inhibiting EC migration, leading to structural and functional damage to the vascular system.

The cytoskeleton is a complex association of detergent-soluble fractions of the cytoplasm, which plays vital roles in

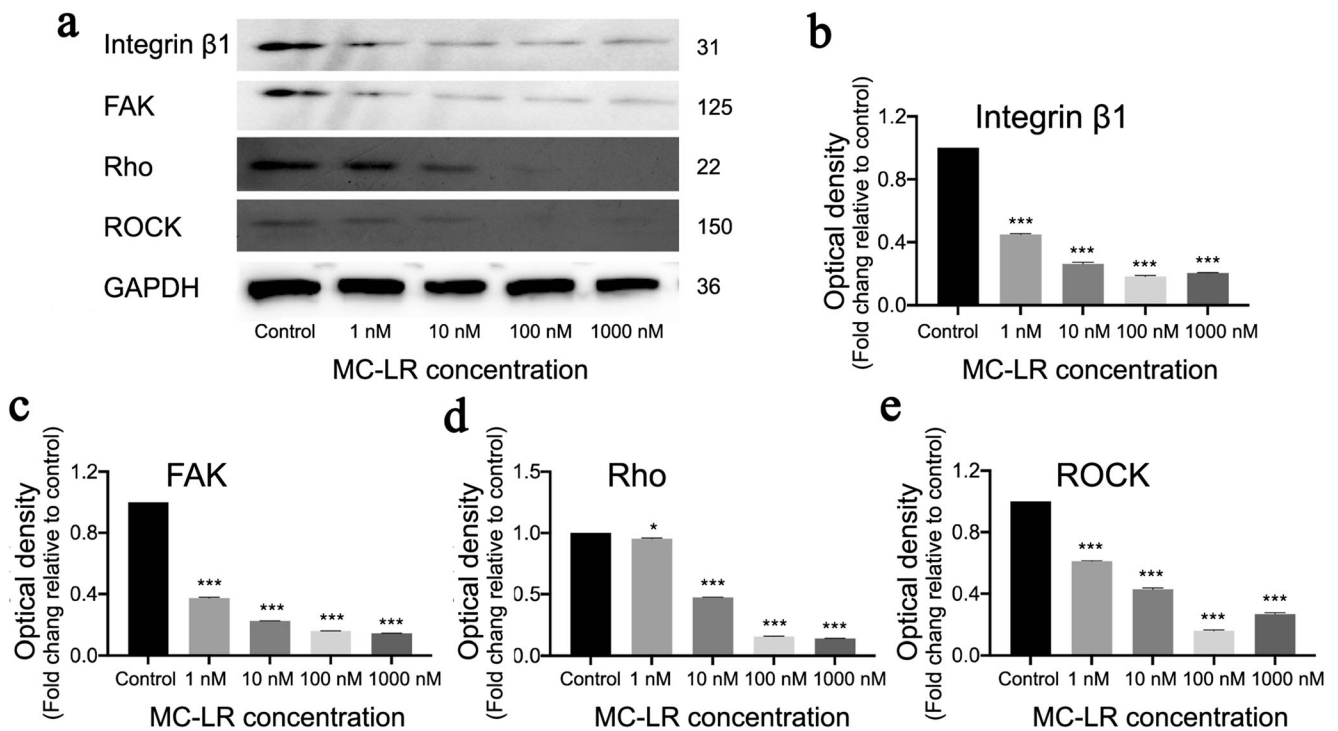


Fig. 7 Western blot analysis of integrin $\beta 1$, FAK, Rho, and ROCK in HUVECs. **a** Western blot. **b–e** The quantitative analysis based on Fig. 7a. HUVECs were treated with MC-LR for 24 h. The vertical value in Fig. 7b–e is a ratio of the gray value of target protein/gray value of internal

reference protein (GAPDH) based on Fig. 7a. All values are represented as mean \pm SD of three replicates ($n=3$). *, $p < 0.05$, ***, $p < 0.001$ vs control

cell motility and shape formation of a cell. The perturbation of the cytoskeleton is important in vascular pathophysiology, which could involve not only direct inhibition of cell motility but also possibly interferes with guidance mechanisms for triggering cell-cell interaction networks (Thomas and Advani 2006; Hu et al. 2017). The toxic effects of MC-LR on the cytoskeletal structures have been widely investigated (Wickstrom et al. 1995; Zhou et al. 2015). Some studies have shown that exposure of human, mouse, or rat hepatocytes to MC-LR causes the rearrangement or collapse of the three components of the cytoskeleton. Multiple cytoskeletal and cytoskeleton-associated proteins were affected by MC-LR (Zhou et al. 2015). MC-LR can induce aggregation and collapse of microfilaments and microtubules in CIK cells and even loss of some cytoskeleton structure (Huang et al. 2015). Relatively short-term exposure to MC-LR induced ROS-mediated cytoskeletal disruption in the liver of carp (Jiang et al. 2013). However, there has been little concentrate on the endothelial cytoskeletal effects of MC-LR. In our study, MC-LR induced the change of microfilaments morphology. HUVECs had a hollow nucleus with condensed chromatin and exhibited apoptotic properties. Microfilaments were condensed around the nucleus. Hence, our study showed that the inhibiting effect of MC-LR on EC migration attributed to the changes of the cytoskeleton, which was essential to angiogenesis.

We also investigated the mechanism of cytoskeletal disruption induced by MC-LR. Adhesion, migration, and invasion are important for HUVEC metastasis. The cytoskeleton confers the extent of the integrin junction. The key part of the adhesive function of integrins is the ability that connects to the actin cytoskeleton (Delon and Brown 2007). The integrin $\beta 1$ and FAK are important for the focal adhesion complex formation, which controls cell adhesion and migration in human glioblastoma (Piao et al. 2009). The focal adhesion complex is directly related to ROCK pathways (Schofield and Bernard 2013). The Rho family of small monomeric GTPases is the monomeric GTP-binding protein, which contains Rho and Rac and is related to an abundance of cellular events, including cell cycle progression, cytoskeletal structure, and cell motility. It has been proved that ROCK stimulates actin-myosin contractility and focal adhesion heavily involved in cell migration (Zohrabian et al. 2009). Integrin $\beta 1$ -mediated FAK-Rho-ROCK-myosin signaling transduction pathway plays an important role in cell shape change, adhesion, and migration (Hu et al. 2017). In the present study, western blot analysis showed that MC-LR down-regulated expressions of integrin $\beta 1$, FAK, Rho, and ROCK in a dose-dependent manner, which suggested that MC-LR could induce cytoskeletal disruption, leading to the inhibition of EC migration by integrin-mediated FAK/ROCK signaling pathway.

Conclusions

In summary, the present study demonstrated that MC-LR blocked vasculogenesis in zebrafish embryos, sprouting angiogenesis from rat aorta, and the tube formation of HUVECs. MC-LR also markedly inhibited EC migration from CHT in zebrafish embryos and HUVEC migration. Therefore, the effect of MC-LR on the vascular system was attributed to the inhibition of EC migration. MC-LR could also induce the disruption of the cytoskeleton by integrin $\beta 1$ -mediated FAK/ROCK signaling pathway. These differences might be attributed to factors affecting both in vivo and in vitro data including physiological differences in animal models, which might influence MC-LR absorption (Juhasz et al. 2014). Furthermore, the integrin $\beta 1$ -mediated FAK-Rho-ROCK pathway played important roles in cell migration and vascular remodeling by regulating actin filament cytoskeleton and focal adhesion (Kogata et al. 2009). Taken together, MC-LR could inhibit EC migration by inducing disruption of cytoskeleton via integrin-mediated FAK/ROCK signaling pathway, leading to the inhibition of vasculogenesis and angiogenesis. Therefore, it is plausible that MC-LR holds a great adverse effect on vascular phylogeny. Our study helps to understand MC-LR risk to human cardiovascular health.

Author contribution Tingzhang Hu conceived and designed the study. Qilong Wang was involved in methodology. Guoliang Chen contributed to original draft preparation. Qian Zhang and Mingxing Wang were associated with review and editing. Guixue Wang and Tingzhang Hu provided supervision. All authors have read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate We all declare that manuscript reporting studies do not involve any human participants, human data, or human tissue.

Consent for publication All authors approved the final manuscript as submitted for publication.

Competing interest The authors declare no competing interests.

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