

Why mammals more susceptible to the hepatotoxic microcystins than fish: evidences from plasma and albumin protein binding through equilibrium dialysis

Wei Zhang · Gaodao Liang · Laiyan Wu ·
Xun Tuo · Wenjing Wang · Jun Chen ·
Ping Xie

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Abstract To elucidate the interspecies variation of susceptibility to microcystins (MCs), fresh plasma and purified albumin from six kinds of mammals and fish were used in toxins-substances binding test. Protein contents in the test plasma were analyzed and the binding characteristics to MCs were compared. Two kinds of widely observed MCs, microcystin-LR (MC-LR) and microcystin-RR (MC-RR) were tested and data were collected through the method of equilibrium dialysis. It was found that total plasma protein and albumin content in mammals were nearly two times and four times higher than that in fish, respectively. In the test range of 0–100 µg/mL, binding

rates of fish plasma to MCs were considered significant lower ($p < 0.01$) than that of mammals. And human plasma demonstrated the highest binding rate in mammals. In all the test species, plasma protein binding rates of MC-RR were significantly higher than MC-LR ($p < 0.01$). Besides, binding profiles of albumin were acquired under the protein content of 0.67 mg/mL. Human serum albumin demonstrated the highest affinity to MCs throughout the six species and differences among the other five species were considered not significant ($p > 0.05$). From the view of protein binding, it is concluded that both the variation of plasma protein composition and albumin binding characteristic could influence the existing form of MCs in circulation, change MCs utilization, alter MCs half-life and further contribute to the difference of susceptibility between mammals and fish.

W. Zhang · G. Liang · L. Wu · X. Tuo · W. Wang ·
J. Chen · P. Xie (✉)
Donghu Experimental Station of Lake Ecosystems, State Key
Laboratory of Freshwater Ecology and Biotechnology of China,
Institute of Hydrobiology, Chinese Academy of Sciences,
Wuhan 430072, People's Republic of China
e-mail: xieping@ihb.ac.cn

W. Zhang
e-mail: zhangwei0203@yahoo.cn

G. Liang
e-mail: lianggd@126.com

L. Wu
e-mail: wulaiyan@yahoo.com.cn

X. Tuo
e-mail: tuoxun@163.com

W. Wang
e-mail: wenjing-325@163.com

J. Chen
e-mail: chenjun@ihb.ac.cn

W. Zhang
Graduate University of Chinese Academy of Sciences,
Beijing 100049, People's Republic of China

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Introduction

Since the world's first scientific report of poisoning livestock by toxic cyanobacteria in the nineteenth century (Francis 1878), there have been plenty of descriptions of fish, reptile, bird and mammal mortalities associated with exposure to cyanobacteria (Nasri et al. 2008; Chen et al. 2009; Carbis et al. 1994). Some species of cyanobacteria can produce several toxic metabolites. Among them, microcystins (MCs) are considered to be the most common and dangerous group (Chorus and Bartram 1999). MCs are known to be potent hepatotoxin (Dawson 1998) and tumor promoter (Nishiwaki-Matsushima et al. 1992). So far, more than 80 analogues have been identified (Dietrich and

Hoeger 2005). Among them, MC-LR (Leucine and Arginine) and MC-RR (Arginine and Arginine) are the most commonly detected, and the provisional guideline value for drinking water was 1 µg/L for MC-LR proposed by WHO.

Plenty of toxicokinetic studies have been performed with MCs, using different subjects, doses and administration routes (Carbis et al. 1996; Wang et al. 2008; Tencalla et al. 1994). It has been shown that the toxicokinetic profile of the toxin varies considerably among different species, and mammals seem to be more susceptible to MCs than fish. For example, half lethal dose (LD₅₀) of MC-LR via intraperitoneal injection (i.p.) is approximately 50–60 µg/kg body weight (BW) in mice (Botes et al. 1984; Carmichael et al. 1988), but increases to 300–550 µg/kg BW in common carp (*Cyprinus carpio*) (Råbergh et al. 1991), to 400–500 µg/kg BW in rainbow trout (*Oncorhynchus mykiss*) (Tencalla et al. 1994; Kotak et al. 1996) and even to 1,500 µg/kg BW in perch (*Perca fluviatilis*) (Ibelings et al. 2005). The use of radioactive labeled MCs as a tracer through i.p. clearly confirmed that a faster rate of MCs accumulation with higher proportion was observed in the liver of mice than that of fish (Brooks and Codd 1987; Williams et al. 1995). Besides, fish manifested a significantly lower whole body reservation of radioactive materials than mice (Robinson 1990; Williams et al. 1997). These results suggest that the tissue distribution and clearance rates of MCs in fish may substantially different from those in mammals.

To our knowledge, the binding of drug to plasma proteins has been long shown to have significant effects on its distribution and elimination (Meuldermans et al. 1982; Levy and Yacobi 1974). Only the unbound drug is available for diffusion or transport across cell membranes, and interaction with specific targets (e.g. receptor, ion channel, transporter and enzyme) (Wright et al. 1996; Baranczewski et al. 2006). As a result, a full characterization of the binding property to plasma proteins has become essential for understanding the pharmacokinetic and toxicological profile of a drug. Among various plasma proteins, serum albumin is the predominant plasma protein, comprising more than half of the total protein in normal serum (Kanakakis et al. 2006; Ascoli et al. 2006). It often contributes significantly towards the total binding of basic drugs in plasma (Kurtzhals et al. 1995; Dennis et al. 2002; Yuwiler et al. 2006). So far as we have concerned, there is lack of report about the plasma protein binding of MC-RR and MC-LR as well as the specific binding of serum albumin across species. Thus, we specifically aimed to investigate the plasma and albumin binding property in different animal species so as to provide possible explanation for the substantial differences of tolerance to MCs between mammals and fish on the bases of protein binding profile.

Material and methods

Ethics statement

This study received specific approval from the Blood Management Center of Hubei Province, PR China (approval ID: 2011-10). Local institutional review board of Wuhan Hygiene Bureau approved the collection of human plasma for the binding test. Chengxin[®] animal cultivation company (Wuhan) carried out contract experimental work on our behalf to prepare porcine and bovine plasma. Institutional review boards of Bureau of Commerce and Bureau of Food and Drug administration approved for the blood collection and further usage.

Fish were commercially available from the market of Wuhan University. To prevent the contamination of fresh blood, fish were anaesthetized through dipping in ice cold water. Fish were sacrificed after blood collection through spinal transection in accordance with the ethical guidelines and written protocols mandated by Experimental Animal Management Ordinance, Hubei Province, PR China. Blood collection and sacrifice of test fish were conducted under the permission of Expert Committee of Biomedical Ethics, IHB/CAS (Institute of Hydrobiology of the Chinese Academy of Sciences) (approval ID: Y11309-1-201).

For the surface blooms collection, no specific permits were required and the location is not privately-owned or protected in any way. We confirm that the field studies did not involve endangered or protected species.

Materials

Standards (Purity >95 %, by HPLC) of MC-LR (catalog number: 136-12241) and MC-RR (catalog number: 133-12251) used in this study were purchased from the Pure Chemical Industries (Osaka, Japan). Toxins used in biodegradation experiments were extracted and purified (>95 %) from the freeze-dried surface blooms collected from Lake Dianchi in Yunnan of China according to the method of Ramanan et al. (2000).

Human serum albumin (HSA) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (NY, USA). Healthy human plasma was bought from the Blood Center of Wuhan, PR China. Heparinized porcine and bovine plasma were prepared from fresh and healthy animal blood. Healthy silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys Nobilis*) and crucian carp (*Carassius auratus*) were commercially available. In order to prevent contamination of fresh blood, test fish were anaesthetized through dipping in ice cold water and hypothermia (Hovda and Linley 2000) instead of using exogenous anesthesia. Blood were taken by caudal venipuncture with heparinized disposable syringes. Then

centrifuged at 3,000g for 15 min at 4 °C and plasma was stored at −20 °C until analysis. For fish, pooled plasma was used in plasma protein binding test.

Protein content and MCs concentration determination

Total plasma protein was determined by biuret reaction according to the method of Doumas (1975). Albumin was determined by bromocresol green reaction according to the method of Doumas et al. (1971). For total plasma protein and albumin content determination, BSA was used as the standard.

Extraction of MCs in plasma were basically followed the method of Xie et al. (2004). Qualitative and quantitative analysis of MCs was performed with Thermo Finnigan LCQ Advantage LC-ESI-MS system (MA, USA). The instrument control, data processing, and analysis were conducted by using Xcalibur software as previously described by Zhang et al. (2009).

Albumin purification

Ion-exchange chromatography was employed to purify albumin from plasma samples using similar method as previous described by Metcalf et al. (1998). Plasma were dialyzed overnight against 0.02 M potassium phosphate buffer (pH 5.2) at 4 °C then applied to a 20 × 2.0 cm column of DEAE Sepharose FF (GE Healthcare, USA) in the same buffer. The column was eluted at 1 mL/min using a pH gradient of pH 5.2–4.2. Fractions were collected and analyzed by spectrophotometry method described above. The albumin-containing components (≥95 %) were dialyzed overnight against HPLC water at 4 °C then freeze-dried and stored at −20 °C until use.

Equilibrium dialysis

Membrane (Biosharp, USA) for the equilibrium dialysis had a MWCO of 8 kDa. Prior to use, the membrane was soaked in HPLC water for 1 h and thereafter rinsed twice with HPLC water then cut into unified dialysis units and conserved in isotonic phosphate buffer (pH 7.4) at 4 °C. In preliminary test, the time to reach equilibrium was evaluated by collecting samples both from protein chamber and buffer chamber at intervals of 6, 12, 20, 24 and 40 h and an equilibrium time of 24 h was set. Dialysis experiments were performed at 25 °C and an agitation rate of 100 rpm/min.

Freeze-dried albumins were dissolved in pH 7.4 isotonic phosphate buffer to make a final concentration of 0.67 mg/mL. In the study of protein binding of MCs, an aliquot of 50 µL MC-RR or MC-LR was injected into 1,950 µL plasma or albumin solution in the dialysis units and

dialyzed against an equal volume of isotonic phosphate buffer (pH 7.4). After 24 h of incubation, plasma/albumin and buffer were withdrawn from each side of the dialysis units and analyzed as described above. For each test, a duplicated was included.

Statistics

Interspecies differences of total plasma and albumin concentration were tested by One-way ANOVA. Binding rate variations of MC-RR and MC-LR were tested by Two-way ANOVA respectively. Duncan's test was introduced for further comparison. Significance was determined at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. To decide best-fit curve, nonlinear regression was used. Scatchard transform were introduced in the albumin binding test to determine the maximum number of binding sites (B_{\max}) and the ligand concentration that binds to half the receptor sites at equilibrium (K_d).

Results

Plasma from the six species used in this study initially did not contain detectable amounts of MCs.

Protein content

Total plasma protein and albumin content of the six species were analyzed and the results were shown in Table 1. The highest content of total plasma protein was detected in porcine with the concentration of 79.45 ± 2.18 g/L, followed by human 72.14 ± 2.44 g/L, bovine 70.82 ± 1.94 g/L, crucian carp 40.72 ± 1.52 g/L, silver carp 38.12 ± 2.12 g/L, and the lowest was present in bighead carp with the concentration of 36.32 ± 1.35 g/L. As for albumin, the highest content was detected in human with the concentration of 46.36 ± 1.49 g/L, followed by bovine 40.89 ± 1.02 g/L, porcine 37.56 ± 1.84 g/L, silver carp 12.31 ± 0.58 g/L, big head carp 11.44 ± 0.92 g/L and the lowest was present in crucian carp with the concentration

Table 1 Total plasma protein and albumin content of the subjects

Plasma	Total (g/L)	Albumin (g/L)	Subject
Bovine	70.82 ± 1.94	40.89 ± 1.02	n = 3
Porcine	79.45 ± 2.18	37.56 ± 1.84	n = 5
Human	72.14 ± 2.44	46.36 ± 1.49	n = 5
Bighead carp	36.32 ± 1.35	11.44 ± 0.92	n = 6
Silver carp	38.12 ± 2.12	12.31 ± 0.58	n = 6
Crucian carp	40.72 ± 1.52	10.80 ± 0.79	n = 6

Data are presented as the mean \pm SD

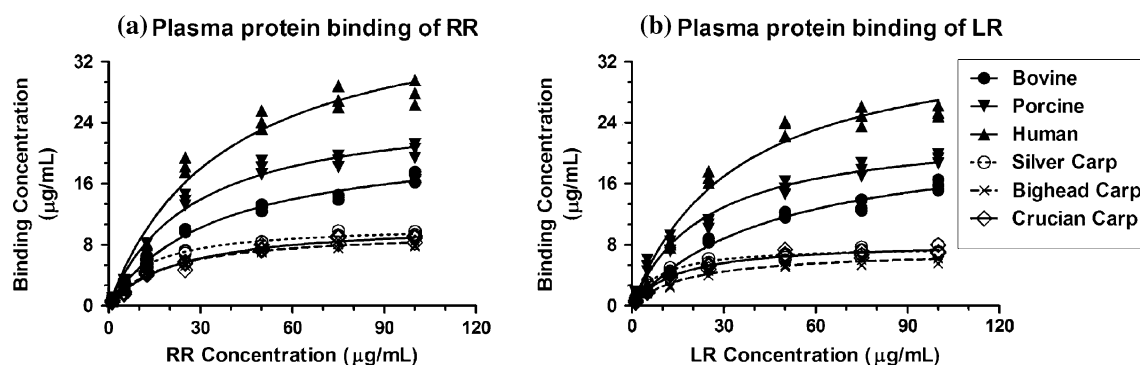


Fig. 1 Plasma protein binding of MC-RR (a) and MC-LR (b) in bovine, porcine, human, silver carp, bighead carp and crucian carp. Data represent the amount of bound MCs to the plasma proteins as a

function of the initial MCs in the dialysis unit ($n = 3$). MCs concentrations were estimated after incubating at 25 °C for 24 h

of 10.80 ± 0.79 g/L. Significant difference ($p < 0.01$) of percentage of albumin to the total plasma protein was observed between mammals and fish ($p < 0.01$). In mammals, albumin account for around 50 % of the total plasma protein while only about 30 % in fish. Either total plasma protein or albumin concentration, mammals were substantially higher than fish ($p < 0.01$).

Plasma protein binding of MC-RR and MC-LR

Binding curve of MC-RR and MC-LR for the six species was shown in Fig. 1. The concentrations of MCs in plasma chamber and buffer chamber were estimated after incubating at 25 °C for 24 h. The concentration of the bound form was obtained by subtracting the MCs concentration in the buffer chamber from the total concentration in the plasma chamber.

In the test range of 0–100 µg/mL, binding rate of MC-RR and MC-LR were in the order of human > porcine > bovine > silver carp > crucian carp > bighead carp. MCs binding rates were significantly higher in the plasma of mammals than that of fish ($p < 0.01$), and among the mammals, binding rate of human plasma were significantly higher than that of porcine and bovine ($p < 0.05$). The amounts of bound MCs in mammals were in the range of 16–28 % at 100 µg/mL and fish were around 4 %. At MCs concentration of 1.25 µg/mL, bound MCs accounted for over half of the total MCs in mammals while <20 % in fish. In all the test species, the binding rates of MC-RR were significantly higher than MC-LR ($p < 0.01$) and the differences in fish ($p < 0.001$) turn to be more obvious than that of mammals.

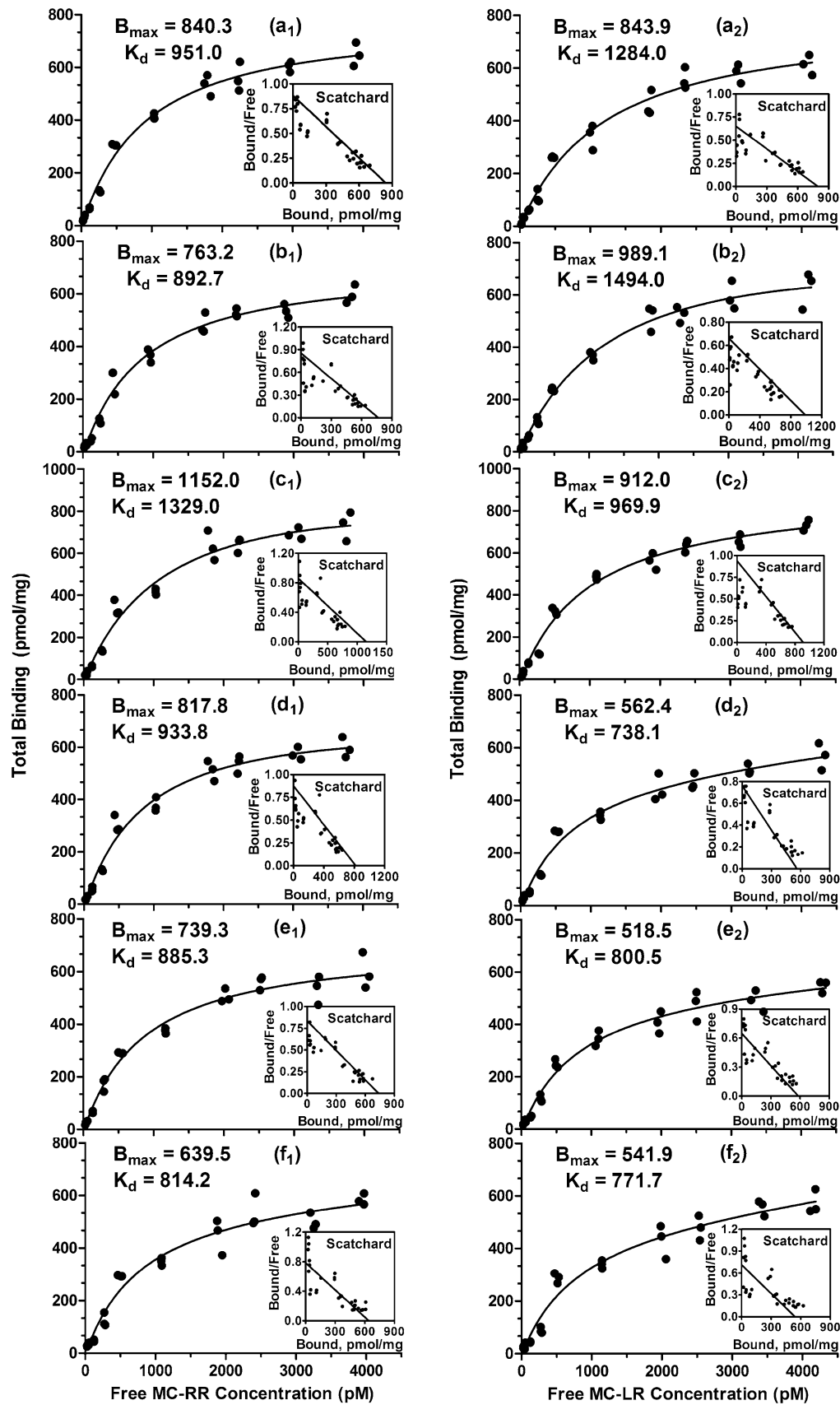
Albumin binding of MC-RR and MC-LR

For further comparison of interspecies difference, purified albumin was used in binding test and results were shown in Fig. 2. Bound form MCs concentrations were obtained by

subtracting the MCs concentration in the buffer chamber from the total concentration in the albumin chamber at 24 h.

Because molecular weight of albumin varied significantly among species, the concentrations of albumin were prepared at 0.67 mg/mL by reference to BSA. Binding data were collected under the original MC-RR or MC-LR concentration of 0.05, 0.1, 0.3, 0.6, 1.2, 2.4, 4.6, 5.8, 7.8 and 10.0 µg/mL. Through Duncan's test, binding rates of MC-RR and MC-LR could be divided into 2 groups respectively. HSA demonstrated the highest binding rate in both MC-RR and MC-LR. Binding rates of MC-RR or MC-LR in the other 5 species were considered not significant ($p > 0.05$). Apart from human ($p = 0.326$) and porcine albumin ($p = 0.289$), the variation of binding rates between MC-RR and MC-LR in the other four animals were significantly different ($p < 0.001$). Nonlinear regressions were used to find the best fit curves. The data were transformed by Scatchard assay to determine the intercept on the X axis which represents B_{\max} and the slope of the line which represent $1/K_d$. B_{\max} and K_d of mammals were generally higher than that of fish. For MC-RR, B_{\max} of fish ranged from 639.5 to 817.8 and K_d ranged from 814.2 to 933.8. As to mammals, B_{\max} ranged from 763.2 to 1,152.0 and K_d ranged from 892.7 to 1,329.0. For MC-LR, B_{\max} and K_d of fish decreased slightly with the value ranged from 518.5 to 562.4 and 738.1 to 800.5 respectively. B_{\max} and K_d of mammals ranged from 843.9 to 989.1 and 969.9 to 1,494.0 respectively.

Fig. 2 Albumin (0.67 mg/mL) binding of MC-RR and MC-LR in bovine (a_1 & a_2), porcine (b_1 & b_2), human (c_1 & c_2), silver carp (d_1 & d_2), bighead carp (e_1 & e_2) and crucian carp (f_1 & f_2). Data represent the amount of bound MCs to the albumin as a function of the free MCs in the buffer ($n = 3$). MCs concentrations were estimated after incubating at 25 °C for 24 h. Scatchard transform were introduced to determine the maximum number of binding sites (B_{\max}) and the ligand concentration that binds to half the receptor sites at equilibrium (K_d)



Discussion

On ascending of the evolutionary scale, plasma protein diversity and albumin concentration have been found to increase remarkably (Desmet 1978; Doolittle 1987) which indicated the importance of albumin concentration in terms of interspecies variation on binding of drugs. Similar results were observed in present study, where total plasma protein and albumin concentration in mammals were substantially higher than that in fish, meanwhile, MCs binding rates were significantly higher in the plasma of mammals than that of fish.

Previous studies pointed out that plasma protein binding could be an effective means of improving the pharmacokinetic properties of short lived molecules (Dennis et al. 2002). As drug enters into the body, it is usually transported by bloodstream and distributed throughout the body. In the blood, various plasma proteins are able to bind with certain drug, increase the molecule weight, make it difficult to across the cell membrane and loss activity temporarily (Buxton 2006). Thereby, plasma protein provides a reservoir that regulates the equilibrium of bound and unbound form of the drug in circulation (Day and Mysza 2002). For a certain drug, variation of plasma protein composition could obviously influence its binding capacity. For instance, Hagelberg et al. (1989) compared the plasma protein binding rate of Ochratoxin A in different species through equilibrium dialysis, they found that binding rate revealed in the order of mammals > quail > fish. Galtier et al. (1981) considered the dissimilar persistence of Ochratoxin A in different animals had been attributed to the different affinities of the toxin to serum albumins of these species. In the present study, total plasma protein and albumin content of mammals were nearly two times and four times higher than that of fish, respectively. Correspondingly, plasma protein binding rates in mammals were evidently higher than that of fish. Different binding rates may subsequently affect the distribution and absorption of MCs in the body. High binding rate of MCs in mammals' plasma would temporarily decrease the free concentration but prolong the duration of MCs in body. On the contrary, low level of binding in fish plasma would increase the availability of MCs, while reduce metabolism half-life through transformation and excretion. It is generally acknowledged that liver is the primary target organ of MCs (Dawson 1998; Robinson et al. 1991). Acute toxicity test using tritium-labeled MCs proved that the combination of mammals' liver to MCs were more effective than that of fish (Brooks and Codd 1987), while clearances of MCs in either fish liver or whole body were proved to be much faster than mammal (Williams et al. 1995; Robinson 1990; Robinson et al. 1989). This indicated that mammals are more affinitive to MCs than that of fish. Apparently, the

synergistic effects of high plasma protein binding and liver affinity would slow down MCs excretion and intensify the overall utilization of MCs in mammals. In fish, however, low plasma protein binding coupled with low target organ affinities would reduce the whole body conservation of MCs and strengthen their tolerance to the toxic effect of MCs. To sum up, plasma protein binding test suggest that the coaction of plasma protein binding and target organ affinity in mammals is likely to make them more susceptible to the toxic effects of MCs than fish.

In order to further discuss the role of plasma protein in animals' susceptibility, purified albumin were involved in the MCs binding test in the present study. As the predominant protein in blood, albumin binds non-specifically and reversibly of many drugs, contributes significantly towards the total binding of basic drugs in plasma (Dennis et al. 2002). Through Scatchard transformation, our data revealed that binding rate of HSA was considered to be significantly higher than the other five species. This suggested that HSA may obtain a higher capacity of MCs and could effectively retain MCs in circulation through binding. Lack of approach makes it difficult to calculate the LD₅₀ of human, but some inference could be concluded from the dialysis events in Brazil. An estimated MCs concentration of 19.5 µg/L of water used during dialysis treatment from 13 to 17 February 1996 was suggested by Carmichael et al. (2001). If we consider the amount (120 L) of water necessary for each individual hemodialysis were used, that is about 2,340 µg for each patient and 33.4 µg/kg BW for a 70 kg individual. For healthy human, tolerance could be a little higher, but not necessarily to exceed mice (50–60 µg/kg BW), not to mention fish. As human beings own the highest albumin content, plasma and albumin binding rate, we could be quite fragile to the toxic effect of MCs. And these variation could be more apparent in low-dose and chronic exposure. As to the comparison of other five albumins, no significant binding rates were detected. These results indicated that in addition to the binding characteristic of albumin, plasma albumin content may play a more important role in the aspect of interspecies MCs binding comparison. The variation of albumin content and albumin binding characteristic provide a direct evidence for the evident difference of plasma protein binding between mammals and fish.

Inhibition of protein phosphatase activity was considered to be the main mechanisms of MCs toxicity (MacKintosh et al. 1990; Honkanen et al. 1990). In vitro study of Yoshizawa et al. (1990) reported that the half effective dose of inhibiting protein phosphatase activity for MC-LR and MC-RR were 1.6 and 3.4 nM respectively. But i.p. study in mice found that the LD₅₀ of MC-LR was less than one fifth that of MC-RR (Gupta et al. 2003). Considering of these disagreements, in addition to the structure difference caused by

a single amino acid, the existing form of MCs in circulation could also exert certain influence on their toxicity. For LD₅₀ were acquired in short-term experiments, availability of unbound toxins becomes the critical factor that determines the acute toxic effects. As it proved that plasma protein binding rate of MC-LR was lower than MC-RR, more unbound MC-LR molecules were accessible in the blood. To a large extent, lower plasma protein binding rate would in favor of the potent inhibition effects of MC-LR.

In conclusion, the present work for the first time examined and compared the plasma and albumin binding properties of mammals and fish. From the view of protein binding, the dissimilar susceptibility to MCs in mammals and fish has been attributed to the significant different plasma protein composition and albumin binding characteristic. As HSA demonstrated a higher binding rate, human may be more susceptible to the toxic effect of MCs than other kinds of mammals. Besides the protein content and albumin binding characteristic of MCs, other properties could also affect the behavior of MCs (e.g. age, gender, body temperature, blood pressure, perfusion rate etc.). For a more comprehensive understanding, further study is still required.

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Conflict of interest The authors declare that all of the authors are acknowledged and there is no conflict of interest.

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