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微囊藻毒與蛋白質磷酸酵素 PP1 與 PP2A 的活性抑制研究

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Comparison of Protein Phosphatase Inhibitory Activities and Acute Toxicity of Microcystins

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[1]Abbreviations: MC(s), microcystin(s); Adda, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; MeAsp, methylaspartic acid; Mdha , methyldehydroalanine; MAP kinase, mitogen-activated protein kinase; PP-1(c), (catalytic subunit of) protein phosphatase 1; PP-2A (c), (catalytic subunit of) protein phosphatase 2A; *p*-NPP, *para*-nitrophenyl phosphate; SAR, structure-activity relationship.

Abstract

Eight naturally purified microcystins (MCs), including MC-LR, MC-FR, MC-WR, MC-RR, [D-Asp^3] MC-FR, [D-Asp^3] MC-WR, [D-Asp^3] MC-RR and [Dha^7] MC-RR were utilized to determine the effects of amino acid substitutions and modifications on the MC-induced phosphatase inhibitory activity and animal toxicity. It was found that the replacement of the non-polar amino acid L-leucine at the second position of these heptacyclic peptide toxins by a polar L-arginine greatly reduced their animal toxicities and inhibitory activities against protein phosphatase 1 (PP-1) and 2A (PP-2A). Demethylation of methyldehydroalanine at the seventh amino acid position of MC-RR showed the least animal toxicity and phosphatases inhibition. The loss of methyl group on the common methylaspartic acid (MeAsp) at the third position of MCYST-FR, MCYST-WR and MCYST-RR did not alter their toxicity levels, but significantly reduced their activities in PP-1 inhibition. It suggests that the methyl group on MeAsp is essential in PP-1 inhibition for MCs. However, such a tendency was not observed in the assay of PP-2A activity. Comparing the LD_{50} of the mouse toxicity assay and IC_{50} of the PP-1 and PP-2A inhibition assay of eight forms of microcystins by linear correlation, it was clearly demonstrated that the MC-induced toxicity is much more related to the inhibition of PP-2A than PP-1. These results suggest that PP-2A inhibition plays a major role in the MC-induced toxicity.

Introduction

Microcystins (MCs) are cyclic peptides produced by cyanobacteria genera of *Microcystis, Anabaena, Nostoc and Planktothrix* (*1*). They shared a general structure of cyclic [-D-Ala[1]-L-**X**[2]-MeAsp[3]-L-**Z**[4]-Adda[5]-D-Glu[6]-*N*-Mdha[7]] (Fig. 1.), where X and Z represent two variable L-amino acid, and MeAsp, Mdha, Adda are abbreviations of methylaspartic acid, methyldehydroalanine and 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, respectively. MCs are generally named as microcystin with the suffix of a single-letter abbreviation of X and Z amino acids. For example, MC-LR contains a leucine and an arginine for the X and Z amino acid, respectively. In addition, minor modifications, such as demethylation or isomerization along with the numbering of amino acids affected are prefixed (*2*). To date more than 70 forms of MCs have been identified and documented (*3*).

MCs are potent hepatotoxins against mammals, birds and fishes (*4*). They are not only causing acute toxicity of animals due to liver dysfunction and hemorrhage, but are also triggering a chronic effect of tumor promotion (*5*, *6*). The mechanisms of the MCs-induced liver toxicity are generally regarded to be mediated via the inhibition of both protein phosphatases 1 (PP-1) and 2A (PP-2A), since PP-1 and PP-2A are the primary targets of MCs (7, *8*). In addition, the Ser/Thr phosphatases have been demonstrated to be the important regulators of keratin intermediate filament assembly, which is central to the liver cytoskeleton integrity (*9*). In contrast, the mechanism for the MC-induced tumor promotion has been focused primarily on the PP-2A, since it is the major regulator for the mitogen-activated protein kinase (MAP kinase) signaling pathways, which regulate the transcriptional onset of genes activated during cells proliferation (*9, 10*). PP-1 and PP-2A have different structures and distinct biological functions (*11, 12*). Although, both PP-1 and PP-2A can be inhibited by MCs, but the relative importance between PP-1A and PP2-A to the animal toxicity-induced by different forms of MCs has not be elucidated.

Although more and more MCs have been identified and documented, very few of them have been analyzed for their phosphatase inhibitory activities in correlation to the animal toxicities (*13*). For those MCs that have been analyzed for their phosphatase inhibitory activities and/or the animal toxicities, it is still difficult to make a fair comparison among them, since no standard methods were applied in those studies. To systematically study the relationship between the MC-induced phosphatase inhibition and animal toxicities, we used eight forms of MCs isolated and purified previously in our laboratory (*14, 15*), to measure their relative inhibition on

PP-1 and PP-2A as well as the lethal toxicity in mice via intraperitoneal injection. We demonstrated that (1) MC-LR is the most potent phosphatase inhibitor (with equal potency to PP-1 and PP-2A) and the strongest toxin for inducing lethality in mice. (2) The substitution of leucine (L) with either arginine (R), phenylalanine (F) or tryptophan (W) at the X amino acid of MC greatly reduced the inhibitory effects on PP-1 and PP-2A to different extents. (3) The demethylation of the 3rd methylaspartic acid (MeAsp) or the 7th methyldehydroalanine alanine (Mdha) further decreases the relative inhibition on phosphatases. (4) The changes of MC toxicity by X-amino acids substitution or other amino acids modifications are somehow different from the changes in the inhibitory effects on PP-1 or PP-2A. However, it appears that the change in MC toxicity is more correlative to the changes in PP-2A than PP-1.

Experimental Procedures

Caution: MCs are hazardous due to their potent hepatotoxicity and tumor-promoting activity and should be handled carefully.

Preparation of MCs

Eight MCs, including MC-LR, MC-FR, MC-WR, MC-RR, [D-Asp^3] MC-FR, [D-Asp^3] MC-WR, [D-Asp^3] MC-RR and [Dha^7] MC-RR were isolated from strains of *Microcystis aeruginosa* indigenous to Taiwan. The identity, structure and purity of different MCs were analyzed by nuclear magnetic resonance as described by Lee et al. (*14*, *15*). The concentrations of different MCs used were designated in each individual experiment.

Phosphatase Inhibition Assay

Catalytic subunits of the native PP-1 and PP-2A (PP-1c and PP-2Ac) were purified from the rabbit skeletal muscle according to Huang et al. (*16*). These enzymes were diluted to 1 U ml^{-1} with 50 mM Tris·HCl buffer containing 0.1 mM EDTA, 5 mM dithiothreitol, 0.2 mM $MnCl_2$, and 0.2 mg ml^{-1} bovine serum albumin at pH 7.0. One unit (U) was defined as the phosphatase activity that hydrolyzed 1 nmol of *para*-nitrophenyl phosphate (*p*-NPP) min^{-1}. *p*-NPP was used as substrate and prepared at 250 mM in the Tri-HCl buffer with additives as described above. Various amounts of MCs were dissolved in 30 μl methanol and mixed with 970 μl Tris-HCl buffer as the toxin solution. The phosphatase activity assay was performed according to the protein phosphatase 1 (#539493, Calbiochem) activity assay by the

standard protocol of Calbiochem-Novabiochem Corp. Briefly, phosphatase assay was carried out in a 96-well transparent microplate. For each reaction, 100 μl Tris-HCl buffer was added, and then mixed with toxin solution with or without different amounts of MC designated and enzyme solution of 50 μl each. The reactions were pre-incubated for 10 minutes and then 50 μl of substrate solution was added and kept at 30^{o}C for an additional one hour. To account for the change in absorbance due to the *p*-NPP de-composition, a blank reaction without toxins and enzymes was prepared for each experiment. The absorbance of each reaction mix was determined at 405 nm using an ELISA reader (Tecan, Grodig, Australia). All treatments were run in twice and an average was taken. The inhibitory activity of each toxin was calculated by the change of absorbance, and expressed as percentage activity of control.

Mouse Toxicity Assay

Purified MC (1.5-36 μg) was dissolved in 3 ml saline solution (0.95 % NaCl), and then an aliquot of 1 ml toxin solution was injected into the peritoneal cavity of a male mouse (ICR strain, body weights were about 20 ± 1 g), which was purchased from the National Taiwan University Hospital. Each treatment was performed in triplicates. At 4 hrs post injection, the mortality of mice was recorded (*17*) and the dosage that killed half of the mice tested (LD_{50}) was calculated accordingly using the probit analysis (*18*). All the animal handling procedures were under the regulation of laboratory animals' usage at the National Taiwan University.

Results

To investigate the structure-activity relationship (SAR) of MCs, the inhibitory activities on PP-1 or PP-2A by eight forms of MCs were determined by the phosphatase activity assay. In general, both PP-1 and PP-2A activities were decreased dose-dependently with the increase of MC concentration. The correlation between % activity of control (Y) and the concentration of MC in pM (X) was derived as a linear regression equation of $\mathbf{Y} = a\mathrm{Ln}\mathbf{X} + b$, where $a < 0$ and $b > 0$, for both PP-1c and PP-2Ac (Fig. 2 and 3). Using these formulas, the IC_{50}s of various MCs for PP-1c and PP-2Ac were calculated and listed at Table 1. The inhibitory activities of various MCs on PP-1c and PP-2Ac were compared according to their respective IC_{50}. MC-LR was found to be the most potent PP-1 and PP-2A inhibitor that both their IC_{50}s were 0.3 nM. With the substitutions of leucine (L) with either arginine (R), phenylalanine (F) or tryptophan (W) at the X amino acid, the inhibitory effects on phosphatases of

MC were greatly reduced to different extents with respect to PP-1c and PP-2Ac. The IC_{50}s of the arginine substituted MC-RR were increased to 1.7 and 58.1 nM, which was a 5.7 and 193.7 fold rise to that of MC-LR, against PP-1c and PP-2Ac, respectively. It is apparent that the arginine at X amino acids significantly reduced the potency of MC against PP-2Ac, but less severely on PP-1c. The IC_{50}s of the phenylalanine substituted MC-FR were increased to 1.1 and 3.8 nM which was a 3.7 and 12.7 fold rise to that of MC-LR, against PP-1c and PP-2Ac, respectively. The IC_{50}s of the tryptophan substituted MC-WR were increased to 1.0 and 4.9 nM, which was a 3.3 and 16.3 fold rise to that of MC-LR, against PP-1c and PP-2Ac, respectively. Although, the rises in IC_{50}s over MC-LR in MC-FR and MC-WR were still higher in PP-2Ac than PP-1c, but the extent of difference was much less significant than the MC-RR. [Dha^7]MC-RR with a proton instead of a methyl group at the 7^{th} Mdha of MC-RR slightly increased the IC_{50} to 2.2 and 84.4 nM, which was a 1.3 and 1.5 fold rise to that of MC-RR against PP-1c and PP-2Ac, respectively. Demethylation on the 3^{rd} MeAsp of MCs also increased the IC_{50} to PP-1c and PP2Ac to various extents in MC-RR, MC-FR and MC-WR. It appeared that the demethylation on the 3^{rd} MeAsp of MCs negatively regulated the inhibition on PP-1c by MCs and was decreased more than that of PP-2Ac.

To assay the animal toxicity induced by different MCs, we injected pure MCs at 1.5-36 μg into the peritoneal cavity of male mice, recorded the mortality of mice at 4 hours post injection and determined the LD_{50} for each MC. It showed that MC-LR has the lowest LD_{50} (0.05 mg/kg), which coincides very well to its strongest inhibitory activities against both PP-1c and PP-2Ac. Surprisingly, the toxicity [D-Asp^3]MC -FR and [D-Asp^3]MC -WR were only next to the MC-LR with a LD_{50} of 0.09 mg/kg. MC-FR and MC-WR appeared to be comparable to their [D-Asp^3] modified counterparts with a LD_{50} of 0.1 and 0.14 mg/kg, respectively. The least toxic MCs were MC-RR and its derivatives whose LD_{50}s were from 0.32 to 0.42 mg/kg.

For those mice given the lethal dosages of different MCs died within 0.5 - 4 hr. Upon necropsy, grossly enlarged livers engorged with blood were observed in treated-mice, which were similar to previous reports (*19*). The effects were similar among different MCs used (data not shown).

To elucidate the relationship between toxicity of MCs and inhibitory activities against PP-1c and PP-2Ac, the LD_{50} and IC_{50} of MCs were compared using the linear correlation. It was found that the toxicity of MCs examined has little correlation with inhibition activity against PP-1c (Fig. 4A). Although, the toxicities of MC-LR, -RR, -FR, -WR and [Dha^7] MC-RR increased as the inhibitory activity against PP-1c increased, but, the three [^{3}D-Asp] modified MCs showed much lower inhibition

against PP-1c compared with other MCs with the same toxicity level (Fig. 4A). In contrast, the toxicities of MCs were significantly correlated with inhibitory activity against PP-2Ac as shown in Fig. 4B.

Discussion

Microcystins (MCs) are cyclopeptides with more than 70 different members, which differ in the amino acids X and Z as well as other modifications on the amino acid functional groups (*3*). Different MC may process differential potency in inhibiting their major targets, PP-1 and PP-2A, which may correlate to the animal toxicity exerted by MC. By analyzing and comparing the inhibitory effects on PP-1c and PP-2Ac as well as the acute toxicity to mice of eight different forms of MCs isolated and purified previously in our laboratory, we have clearly demonstrated that a leucine at the X amino acid is essential for both high PP-1c and PP-2Ac inhibitory activity and animal toxicity. Demethylation of the 3rd MeAsp or 7th Mdha further deteriorates MC activities. More importantly, we demonstrated that the MC-induced animal toxicity is correlated more closely to its PP-2Ac inhibitory effects than that of PP-1c.

The correlation between MCs' toxicity and their inhibitions against two structural and functional distinct phosphatase PP-1 and PP-2A has long been puzzled. Our present findings reveal that PP-2A is significantly more important to exert the MC-induced acute toxicity by using MCs with modifications at three different amino acid sites. Similarly, Ito et al. (*20*) also found that the more toxic MC-LR exerts stronger inhibitory activities against PP-2Ac than MC-RR does, but no difference on the inhibitory activity against PP-1c compared with MC-RR. These results strongly suggest that the PP-2A inhibition not only plays a major role in tumor promotion induced by MCs (*9, 10*), but also is more important in mediating the acute toxicity of MCs. It also implies that toxicity of MC-containing samples could be better estimated by using the PP-2Ac inhibition assay.

With the results presented, the SAR rules of MCs can be summarized as: (1) the replacement of amino acid at the 2nd position of MCs with L-Arg has the most significant inhibition on MCs' activities (2) the demethylation on the 7th amino acid Mdha of MCs also makes the MC toxicity and phosphatase inhibitory activities decrease. (3) the demethylation on the 3rd amino acid MeAsp of MCs had little influence on both toxicity and inhibitory activities against PP-2Ac, but significantly reduce inhibitory activities against PP-1c.

The SARs of MCs related to PP-1c inhibition could be partly explained by the interaction model established by Goldberg et al (*21*). It was described that when

MC-LR interacts with PP-1c, the side chain on L-Leu of MC-LR forms a hydrophobic interaction with the aromatic ring on Tyr272 of PP-1c; carbonyl and carboxyl group on MeAsp of MC formed two hydrogen bonds with Arg96 and Tyr134 of PP-1c; side chain of Adda formed hydrophobic interaction with hydrophobic groove near active site of PP-1c. The carboxyl group of D-Glu formed hydrogen bonding with metal ions in the active site, and finally, the methylidene group of Mdha formed covalent bonding with Cys273 of PP-1c. According to the model, the change of inhibitory activity against PP-1c when functional groups on the 2^{nd}, 3^{rd}, 5^{th}, 6^{th}, and 7^{th} amino acids of MCs were changed could be predicted. It is safe to predict that the decrease of inhibition activity against PP-1c when L-X amino acid is replaced by a more hydrophilic amino acid L-Arg is due to the loss of hydrophobic interaction. However, the model could not interpret why the loss of methyl group on MeAsp and Mdha decreased inhibitory activity against PP-1c. It is tempting to speculate that the two methyl groups may be important for the formation of hydrogen bond or covalent bond occurred nearby, or they may have interactions with PP-1 that did not found in the previous studies.

The different SARs of MCs for PP-1c and PP-2Ac inhibitions implies that the MCs interacted with PP-1c and PP-2Ac in a different way. By comparing the SARs of MC against PP-1 and PP-2A, it is suggested that interactions between the second amino acid and seventh amino acid of MC and the two phosphatases were quite similar, since the changes on the two positions of MCs have the same influences on inhibitory activities against PP-1 and PP-2A. A previous study showed that esterification on the D-Glu, and the change of conformation on Adda side chain from *Z* to *E* form also had the same influence on phosphatase inhibitions, which caused the total loss of inhibitory activities on both PP-1c and PP-2Ac (*3*). It revealed that the interactions on the 5^{th} and 6^{th} amino acid MC to both phosphatases should be also similar. Furthermore, since the amino acids equivalent to the Tyr272 and Cys273 of PP-1c in PP-2Ac were also Tyr (Tyr265) and Cys (Cys266) (*22*), the side chain of L-X amino acid could form a hydrophobic interaction with Tyr265 of PP-2Ac, and the methylidene group at Mdha of MCYST-LR could also form the covalent bonding with Cys266 of PP-2Ac. The covalent bonding between Mdha of MCYST-LR and Cys266 of PP-2Ac has been demonstrated previously (*7*). The interaction on the 3^{rd} amino acid of MCs to both phosphatases, however, should be quite different, since the loss of methyl group on MeAsp showed little influence on PP-2Ac inhibition, but dramatically decreased inhibitory activities on PP-1c.

Taken together, we have provided evidences to show that different amino acids and their side chain modifications occurred in natural microcystins could

differentially affect the PP1 and PP2A activities as well as its acute animal toxicity. Furthermore, we have demonstrated nicely that the acute toxicity of MC is more dependent on the inhibition of PP-2A than PP-1A.

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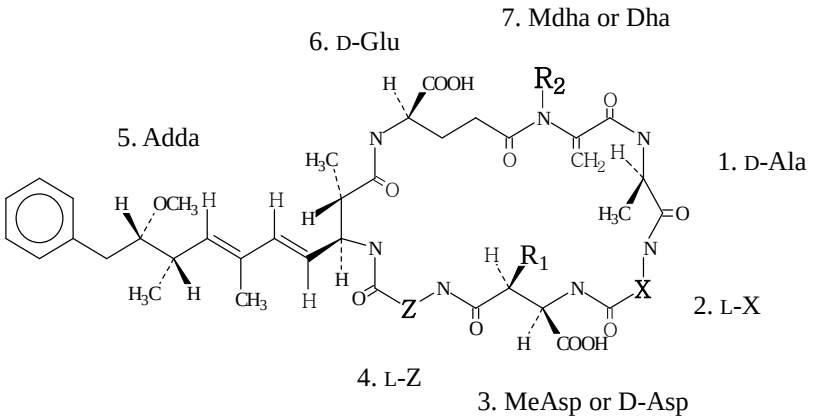

	X	Z	R_1	R_2
MC-LR	Leu	Arg	CH_3	CH_3
-RR	Arg	Arg	CH_3	CH_3
-FR	Phe	Arg	CH_3	CH_3
-WR	Trp	Arg	CH_3	CH_3
[Dha^7] MC-RR	Arg	Arg	CH_3	H
[D-Asp^3] MC-RR	Arg	Arg	H	CH_3
-FR	Phe	Arg	H	CH_3
-WR	Trp	Arg	H	CH_3

Figure 1. Chemical structures of MCs used in the study

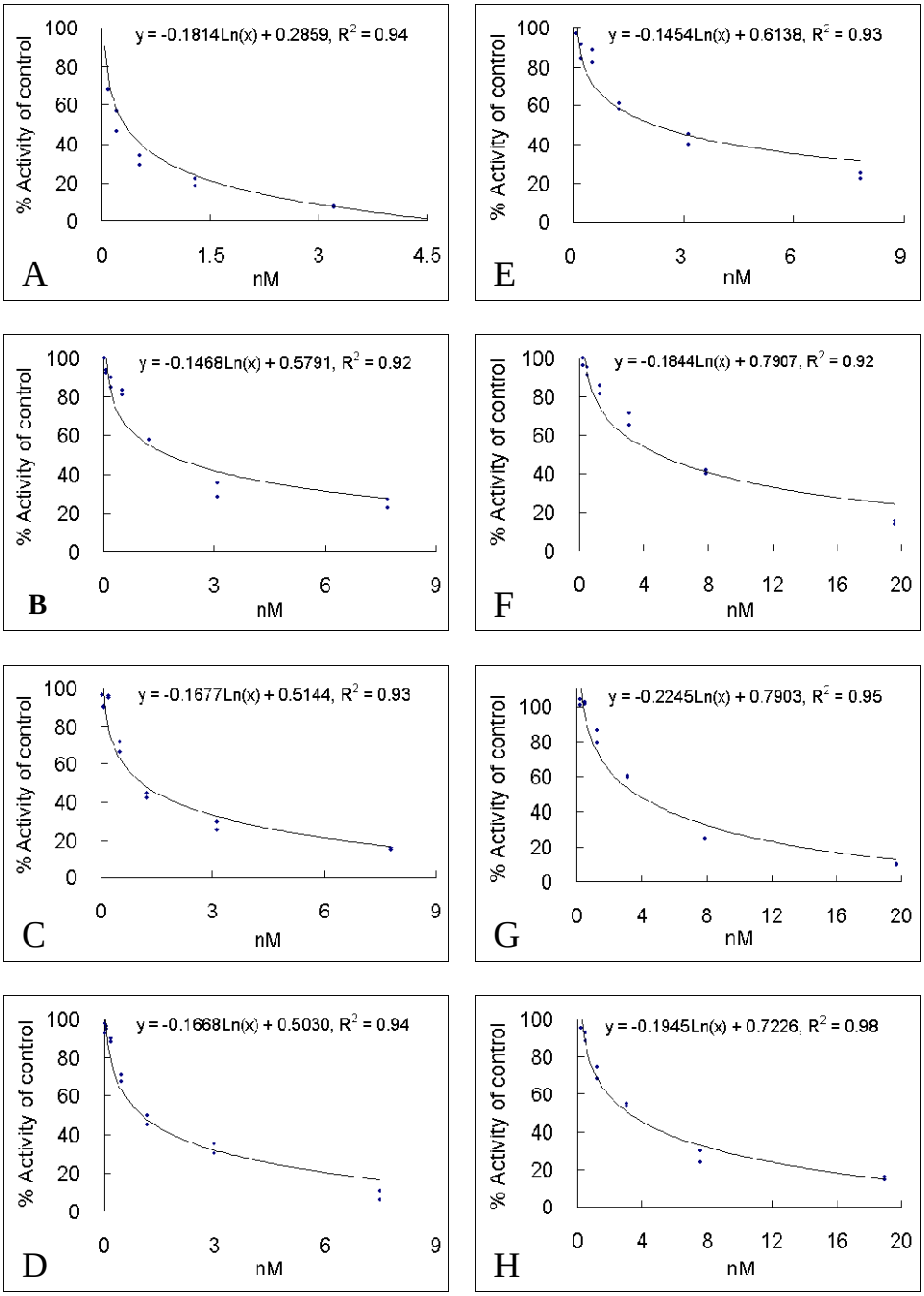

Fig. 2. Correlation analysis between PP-1c activity and concentration of MCs. including A. MC-LR, B. MC-RR, C. MC-FR, D. MC-WR, E. [Dha^7]MC-RR, F. [D-Asp^3]MC-RR, G. [D-Asp^3]MC-FR, and H. [D-Asp^3]MC-WR.

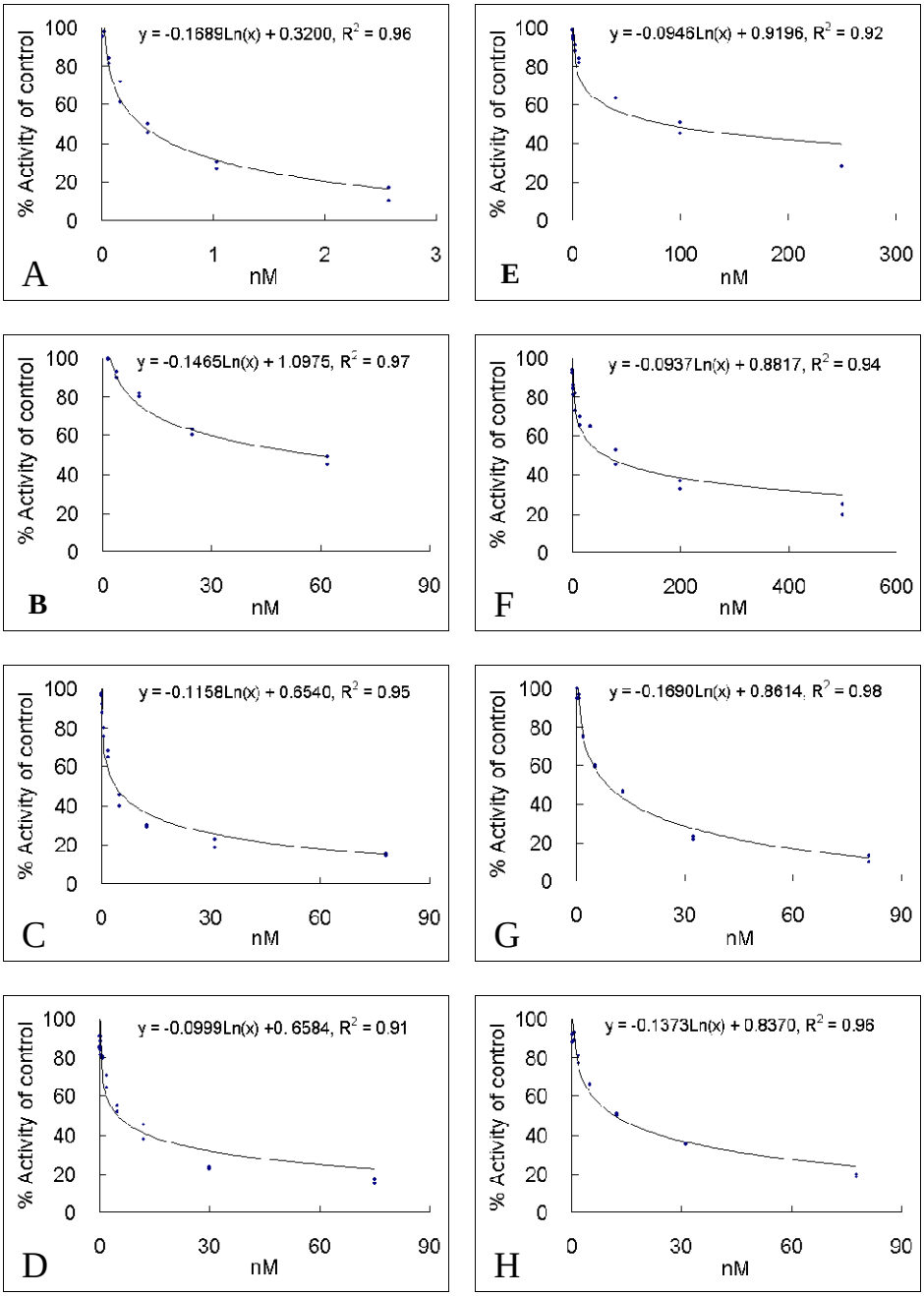

Fig. 3. Correlation analysis between PP-2Ac activity and concentration of MCs. including A. MC-LR, B. MC-RR, C. MC-FR, D. MC-WR, E. [Dha^{7}]MC-RR, F. [D-Asp^{3}]MC-RR, G. [D-Asp^{3}]MC-FR, and H. [D-Asp^{3}]MC-WR.

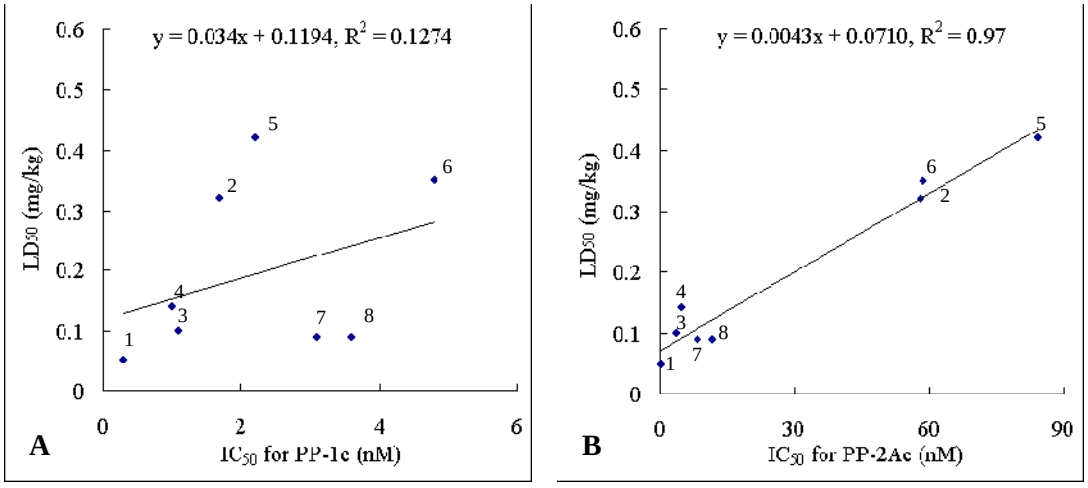

Fig. 4. Correlations between toxicity and inhibition activity against PP-1c (A) and PP-2Ac (B) of various MCs i.e. MC-LR (1), MC-RR (2), MC-FR (3), MC-WR (4), [Dha^7]MC-RR (5), [D-Asp^3]MC-RR (6), [D-Asp^3]MC-FR (7), and [D-Asp^3]MC-WR (8).