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Structure-Function Relationships of Microcystins, Liver Tumor Promoters, in Interaction with Protein Phosphatase

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Microcystins, isolated from toxic blue-green algae, are potent inhibitors of protein phosphatases 1 and 2A. Recently, we have reported that microcystin LR has a potent tumor-promoting activity on rat liver initiated with diethylnitrosamine. The structure of microcystins is unique in having an unusual amino acid, 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4(*E*),6(*E*)-dienoic acid (Adda), which is thought to be significant for the activity. Geometrical isomers at C-7 in the Adda portion of microcystins, 6(*Z*)-Adda microcystins LR and RR, have been isolated from cyanobacteria. To estimate their tumor-promoting activities and to understand the importance of the Adda portion for activity, the maternal microcystins LR and RR and their isomers were subjected to examination of their interaction with protein phosphatases 1 and 2A and the release of glutamic pyruvic transaminase from rat liver. 6(*Z*)-Adda microcystins LR and RR bound to protein phosphatases 1 and 2A, inhibited their activities and released glutamic pyruvic transaminase from rat liver into serum, ten to one hundred times more weakly than the maternal microcystins LR and RR. These results indicated that the conjugated diene with 4(*E*),6(*E*) geometry in the Adda portion is important in the interaction with protein phosphatases.

Key words: Microcystin — 6(*Z*)-Adda microcystin — Protein phosphatase 2A — Liver tumor promoter

Microcystins, isolated from blue-green algae, such as *Microcystis, Oscillatoria* and *Anabena,* induce severe intrahepatic hemorrhages and liver necrosis at low concentrations in rats and mice.[1-4] Microcystins are structurally monocyclic heptapeptides, which consist of two variable L-amino acids, that is, leucine and arginine for LR and arginine and arginine for RR, three D-amino acids and the two unusual amino acids, *N*-methyldehydroalanine and 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4(*E*),6(*E*)-dienoic acid (Adda)[5,6] (Fig. 1). Recently, we found that microcystin LR has a potent tumor-promoting activity in rat liver initiated with diethylnitrosamine.[7] Microcystins bind to protein phosphatases 1 and 2A, and strongly inhibit their activities. The resultant increase of phosphoproteins, which was referred to the apparent "activation" of protein kinases, is assumed to be involved in tumor-promoting activity in the liver.[7-9]

Although the structures of fourteen microcystins have been elucidated so far, all these microcystins commonly contain the Adda molecule. Therefore, it is thought that the Adda molecule is necessary for their activities and liver tumor-promoting activities. Interestingly, geometrical isomers of microcystins LR and RR have been separated as minor components together with the maternal microcystins LR and RR from field samples containing *Microcystis* species by high-performance liquid chromatography.[10] They are 4(*E*),6(*Z*) isomers of the diene of the Adda portion in microcystins LR and RR, and have been tentatively named 6(*Z*)-Adda microcystins LR and RR (Fig. 1).[11]

To test the interaction of geometrical isomers of microcystins with protein phosphatases and to estimate their tumor-promoting activity in the liver, two 6(*Z*)-Adda microcystins LR and RR were subjected to our assay systems, namely inhibition of the specific [^{3}H]-okadaic acid binding to a cytosolic fraction of mouse skin containing protein phosphatases 1 and 2A, inhibition of protein phosphatase 2A activity and release of glutamic pyruvic transaminase (GPT) from the rat liver into serum. The potencies of 6(*Z*)-Adda microcystins were ten to one hundred times weaker than those of the maternal microcystins, due to the structural changes in the Adda portion. Thus, the 4(*E*), 6(*Z*) configuration of the Adda portion reduces interaction with protein phosphatases. This might result in a low tumor-promoting activity, compared with that of the maternal microcystins.

MATERIALS AND METHODS

Chemicals Microcystins LR and RR, and 6(*Z*)-Adda microcystins LR and RR were isolated from a blue-

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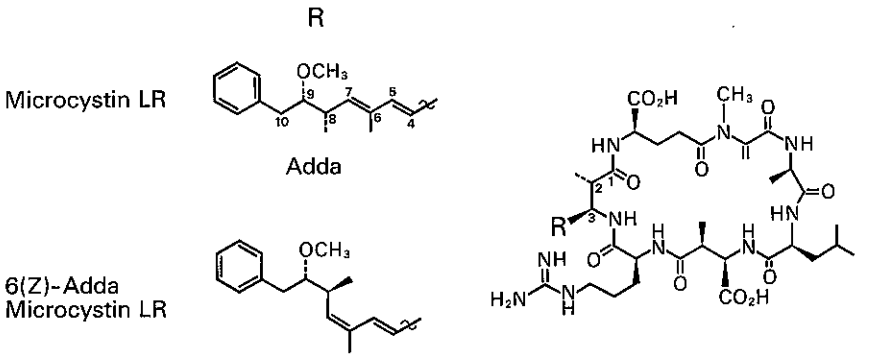

Fig. 1. Structures of microcystin LR and 6(Z)-Adda microcystin LR. 6(Z)-Adda microcystin LR is a geometrical isomer of microcystin LR at the 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyl-deca-4,6-dienoic acid (Adda) portion. The structure on the right is the common structure of the two compounds.

green alga *Microcystis viridis* as described previously.[10, 12] [27-^{3}H]Okadaic acid (14 Ci/mmol) was synthesized as reported previously.[13]

Inhibition of specific [^{3}H]okadaic acid binding Specific [^{3}H]okadaic acid binding to a cytosolic fraction of mouse skin containing protein phosphatases 1 and 2A was measured by a method described previously.[13] For the inhibition test, various concentrations of a microcystin or a 6(Z)-Adda microcystin were incubated with a reaction mixture, as described previously.[13]

Inhibition of protein phosphatase 2A activity Protein phosphatase 2A was partially purified from a cytosolic fraction of mouse brain, which was subjected to DEAE-cellulose column chromatography. The fraction eluted with 0.2 *M* NaCl was used as the enzyme fraction containing protein phosphatase 2A.[14] Protein phosphatase activity was measured in terms of the amount of ^{32}P released from the ^{32}P-phosphorylated histone H1, incubated with 8.8 mU/ml of protein phosphatase 2A. Inhibition of protein phosphatase activity was determined by incubation with various concentrations of a microcystin, as described previously.[14] One unit of protein phosphatase activity was defined as the release of 1 nmol phosphate/min from the substrate.

Measurement of serum transaminase level Two different doses, 50 or 500 μg/kg, of microcystins LR or RR or the corresponding 6(Z)-Adda isomers, dissolved in 500 μl of saline were intraperitoneally (i.p.) injected into male F344 rats. In the control group, saline alone was i.p. injected into the rats. Three, six, nine, and 24 h after injection, blood serum was collected and serum transaminase levels were measured using the Transaminase C Test (Wako Chemical Company, Osaka).

RESULTS

Inhibition of specific [^{3}H]okadaic acid binding We have reported that microcystins and okadaic acid interact similarly with protein phosphatases 1 and 2A, although they are structurally unrelated.[8, 9] Microcystins LR and RR inhibited the specific [^{3}H]okadaic acid binding to a cytosolic fraction of mouse skin containing protein phosphatases 1 and 2A dose-dependently. The ED_{50} values (the effective dose for 50% inhibition) of microcystins LR and RR were 8.0 and 7.0 n*M*, respectively. 6(Z)-Adda microcystins LR and RR showed about ten-times-weaker binding activity to a cytosolic fraction than did their maternal microcystins. The ED_{50} values were 68.0 and 130.0 n*M* for 6(Z)-Adda microcystins LR and RR, respectively (Fig. 2).

Inhibition of protein phosphatase 2A Microcystins LR and RR showed dose-dependent inhibition of the activity of protein phosphatase 2A, which was partially purified from a cytosolic fraction of mouse brain. The ED_{50} values (the effective dose for 50% inhibition) of microcystins LR and RR were 0.28 and 0.78 n*M*, respectively. 6(Z)-Adda microcystins LR and RR inhibited protein phosphatase 2A activity about 100 times more weakly than the maternal microcystins. The ED_{50} values were 80.0 n*M* for both 6(Z)-Adda microcystins LR and RR (Fig. 3).

Measurement of serum transaminase level Administration of 500 μg/kg microcystins LR and RR released GPT from the liver into blood serum rapidly and showed high GPT levels of 500 IU/liter and 562 IU/liter, respectively. As a result, the rats died within 3 h after i.p. injection (data not shown). The time-course of GPT level was followed for 24 h after administration of 50 μg/kg microcystins LR and RR. The maximal GPT levels were in the range of 16 IU/liter to 19 IU/liter between 3–6 h after injection. The amounts of 500 μg/kg 6(Z)-Adda microcystins LR and RR showed a similar release of GPT to those of 50 μg/kg of maternal microcystins LR and RR (Fig. 4). Release of glutamic oxaloacetic transaminase was also induced by microcystins, showing the same pattern as GPT (data not shown).

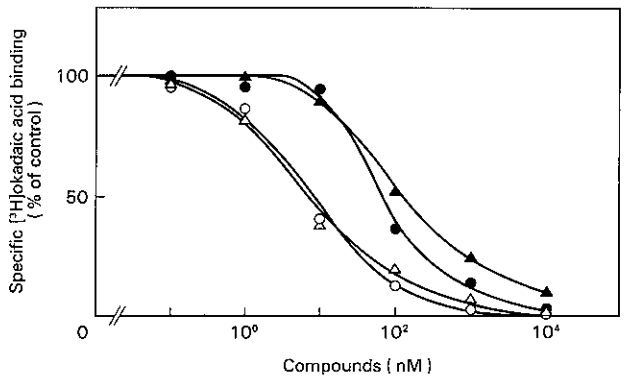

Fig. 2. Inhibition of specific [^{3}H]okadaic acid binding to a cytosolic fraction of mouse skin. Microcystin LR (○), microcystin RR (△), 6(*Z*)-Adda microcystin LR (●) and 6(*Z*)-Adda microcystin RR (▲).

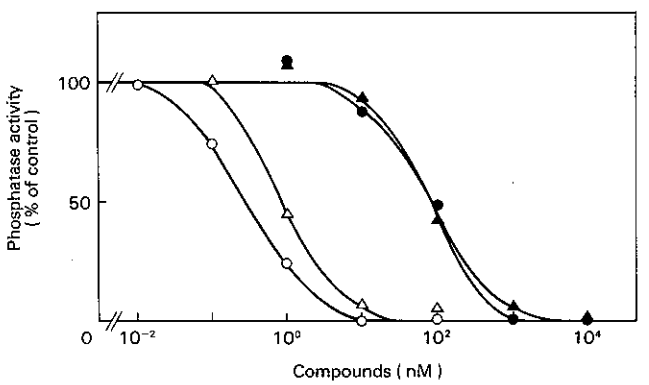

Fig. 3. Inhibition of protein phosphatase 2A activity by microcystin LR (○), microcystin RR(△), 6(*Z*)-Adda microcystin LR (●) and 6(*Z*)-Adda microcystin RR (▲).

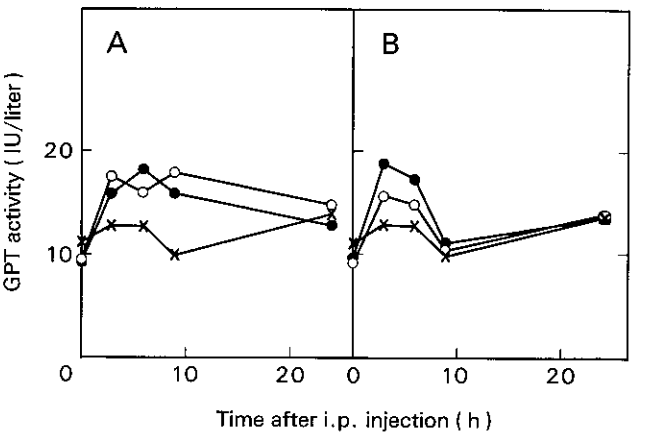

Fig. 4. Effects of microcystins LR, RR and 6(*Z*)-Adda microcystins LR, RR on release of GPT into rat serum. A, control (saline only) (×), 50 μg/kg microcystin LR (○), 500 μg/kg 6(*Z*)-Adda microcystin LR (●). B, control (×), 50 μg/kg microcystin RR (○), 500 μg/kg 6(*Z*)-Adda microcystin RR (●).

DISCUSSION

We have demonstrated that microcystins LR, YR and RR bind to and inhibit protein phosphatases 1 and 2A with the same potencies as okadaic acid.[8,9] In the present experiments, 6(*Z*)-Adda microcystins LR and RR, which are geometrical isomers at the Adda portion, clearly showed weaker activities than maternal microcystins in receptor binding, inhibition of protein phosphatase 2A activity and release of transaminase into blood serum. From these results, 6(*Z*)-Adda microcystins are thought to be weaker tumor promoters in rat liver than their maternal microcystins. Nodularin is a hepatotoxic monocyclic pentapeptide, and contains the 4(*E*),6(*E*)-Adda portion.[6,15] Nodularin inhibits protein phosphatase 2A activity as potently as microcystins.[8] Therefore, these results further support our idea that the configuration and conformation of the 4(*E*),6(*E*)-Adda portion are important for inhibition of protein phosphatases activities, release of serum transaminase from the liver and tumor-promoting activity of microcystins and nodularin.

One potent inhibitor of protein phosphatases 1 and 2A, okadaic acid, shows tumor-promoting activity on mouse skin and rat glandular stomach.[16] Microcystin LR is a liver tumor promoter.[7] Inhibition of protein phosphatases 1 and 2A seems to be a general pathway of tumor promotion in various organs.

Thus far, we have found 18 okadaic acid class compounds which bind to protein phosphatases 1 and 2A and inhibit their activities.[17-19] The structures of okadaic acid class compounds are very heterogeneous. We are interested in receptor modeling of these ligands. Such work should provide clues to the relationship between protein phosphatases and tumor promotion.

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REFERENCES

1) Carmichael, W. W. Toxins of Freshwater Algae. *In* "Handbook of Natural Toxins," ed. A. Tu, Vol. 3, pp. 121–147 (1988). Marcel Dekker, New York.
2) Slatkin, D. N., Stoner, R. D., Adams, W. H., Kycia, J. H. and Siegelman, H. W. Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. *Science,* **220,** 1383–1385 (1983).
3) Hooser, S. B., Beasley, V. R., Lovell, R. A., Carmichael, W. W. and Haschek, W. M. Toxicity of microcystin LR, a cyclic heptapeptide hepatotoxin from *Microcystis aeruginosa. Vet. Pathol.,* **26,** 246–252 (1989).
4) Watanabe, M. F., Oishi, S., Harada, K-I., Matsuura, K., Kawai, H. and Suzuki, M. Toxins contained in *Microcystis* species of cyanobacteria (Blue-green algae). *Toxicon,* **26,** 1017–1025 (1988).
5) Botes, D. P., Tuinman, A. A., Wessels, P. L., Viljoen, C. C., Kruger, H., Williams, D. H., Santikarn, S., Smith, R. J. and Hammond, S. J. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa. J. Chem. Soc. Perkin Trans.,* **1,** 2311–2318 (1984).
6) Rinehart, K. L., Harada, K-I., Namikoshi, M., Chen, C., Harvis, C. A., Munro, M. H. G., Blunt, J. W., Mulligan, P. E., Beasley, V. R., Dahlem, A. M. and Carmichael, W. W. Nodularin, microcystin, and the configuration of Adda. *J. Am. Chem. Soc.,* **110,** 8557–8558 (1988).
7) Fujiki, H., Matsushima, R., Yoshizawa, S., Suganuma, M., Nishiwaki, S., Ishikawa, T. and Carmichael, W. W. Liver tumor promotion through the okadaic acid pathway, inhibition of protein phosphatases 1 and 2A. *Abstr. 1991 AACR Meet.,* 157 (1991).
8) Yoshizawa, S., Matsushima, R., Watanabe, M. F., Harada, K-I., Ichihara, A., Carmichael, W. W. and Fujiki, H. Inhibition of protein phosphatases by microcystin and nodularin associated with hepatotoxicity. *J. Cancer Res. Clin. Oncol.,* **116,** 609–614 (1990).
9) Matsushima, R., Yoshizawa, S., Watanabe, M. F., Harada, K-I., Furusawa, M., Carmichael, W. W. and Fujiki, H. *In vitro* and *in vivo* effects of protein phosphatase inhibitors, microcystins and nodularin on mouse skin and fibroblasts. *Biochem. Biophys. Res. Commun.,* **171,** 867–874 (1990).
10) Harada, K-I., Matsuura, K., Suzuki, M., Watanabe, M. F., Oishi, S., Dahlem, A. M., Beasley, V. R. and Carmichael, W. W. Isolation and characterization of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). *Toxicon,* **28,** 55–64 (1990).
11) Harada, K-I., Ogawa, K., Matsuura, K., Murata, H., Suzuki, M., Watanabe, M. F., Itezono, Y. and Nakayama, N. Structural determination of geometrical isomers of microcystins LR and RR from cyanobacteria by two-dimensional NMR spectroscopic techniques. *Chem. Res. Toxicol.,* **3,** 473–481 (1990).
12) Harada, K-I., Matsuura, K., Suzuki, M., Oka, H., Watanabe, M. F., Oishi, S., Dahlem, A. M., Beasley, V. R. and Carmichael, W. W. Analysis and purification of toxic peptides from cyanobacteria by reversed-phase high-performance liquid chromatography. *J. Chromatogr.,* **448,** 275–283 (1988).
13) Suganuma, M., Suttajit, M., Suguri, H., Ojika, M., Yamada, K. and Fujiki, H. Specific binding of okadaic acid, a new tumor promoter in mouse skin. *FEBS Lett.,* **250,** 615–618 (1989).
14) Sassa, T., Richter, W. W., Uda, N., Suganuma, M., Suguri, H., Yoshizawa, S., Hirota, M. and Fujiki, H. Apparent "activation" of protein kinases by okadaic acid class tumor promoters. *Biochem. Biophys. Res. Commun.,* **159,** 939–944 (1989).
15) Carmichael, W. W., Eschedor, J. T., Patterson, G. M. L. and Moore, R. E. Toxicity and partial structure of a hepatotoxic peptide produced by the cyanobacterium *Nodularia spumigena* Mertens emend. L575 from New Zealand. *Appl. Environ. Microbiol.,* **54,** 2257–2263 (1988).
16) Fujiki, H., Suganuma, M., Nishiwaki, S., Yoshizawa, S., Yatsunami, J., Matsushima, R., Furuya, H., Okabe, S., Matsunaga, S. and Sugimura, T. Specific mechanistic aspects of animal tumor promoters: the okadaic acid pathway. *In* "Relevance of Animal Studies to Evaluate Human Cancer Risk," ed. R. D'Amato, T. J. Slaga, W. Farland and C. Henry, in press, John Wiley and Sons, New York.
17) Suganuma, M., Fujiki, H., Suguri, F. H., Yoshizawa, S., Yasumoto, S., Kato, Y., Fusetani, N. and Sugimura, T. Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin. *Cancer Res.,* **50,** 3521–3525 (1990).
18) Nishiwaki, S., Fujiki, H., Suganuma, M., Suguri, F. H., Matsushima, R., Iida, Y., Ojika, M., Yamada, K., Uemura, D., Yasumoto, T., Schmitz, F. J. and Sugimura, T. Structure-activity relationship within a series of okadaic acid derivatives. *Carcinogenesis,* **11,** 1837–1841 (1990).
19) Magae, J., Osada, H., Fujiki, H., Saido, T. C., Suzuki, K., Nagai, K., Yamasaki, M. and Isono, K. Morphological changes of human myeloid leukemia K562 cells by a protein phosphatase inhibitor, tautomycin. *Proc. Jpn. Acad.,* **66,** Ser. B, 209–212 (1990).