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Comparison of protein phosphatase inhibition activities and mouse toxicities of microcystins

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Abstract

Eight naturally purified microcystins (MCs), including MC-LR, -FR, -WR, -RR, [D-Asp^3]MC-FR, -WR, -RR, and [Dha^7]MC-RR were utilized to determine the effects of amino acid substitutions and modifications on MC-induced protein phosphatase inhibition activity and mouse toxicity. Catalytic subunits of protein phosphatase 1 (PP-1) and 2A (PP-2A) were purified and subjected to the inhibition assays, and intraperitoneal injection was used to administer MCs into mice for the toxicity assay. It is 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 reduces their mouse toxicities and inhibitory activities against PP-1 and PP-2A to different extends. Demethylation of methyldehydroalanine (Mdha) at the seventh amino acid of MC-RR exhibits the least mouse toxicity and phosphatase inhibition. The loss of a methyl group on the common methylaspartic acid (MeAsp) at the third position of MC-FR, -WR, and -RR does not alter their toxicity levels, but dominantly reduces their activities in PP-1 inhibition compared to other substitutions or modifications. This suggests that the methyl group on MeAsp is also important for MCs inhibition. However, such a tendency is not observed for PP-2A. By comparing the LD_{50} values of the mouse toxicity assay and IC_{50} values of the PP-1 and PP-2A inhibition assay of eight MCs using linear regression, it is evident that the MC-induced toxicity is much more related to the inhibition of PP-2A than PP-1, which suggests that PP-2A inhibition may play a major role in the MC-induced mouse toxicity.

Keywords: Microcystin (MC); Mouse assay; Protein phosphatase 1 (PP-1); Protein phosphatase 2A (PP-2A)

1. Introduction

Microcystins (MCs) are cyclic peptides produced by cyanobacterial genera of *Microcystis, Anabaena, Nostoc, and Planktothrix* (Falconer, 1999). They share a general structure of cyclic [-D-Ala^1-L-X^2-MeAsp^3-L-Z^4-Adda^5-D-Glu^6-*N*-Mdha^7], where X and Z represent two variable L-amino acids. 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 (MC) with the suffix of a single-letter abbreviation of X or Z amino acids. For example, MC-LR contains a leucine and an arginine for the X and Z amino acids, respectively. Additionally, minor modifications, such as demethylation

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or isomerization along with the position of amino acids affected are prefixed (Carmichael et al., 1988). To date, more than 70 forms of MCs have been identified and documented (Lawton and Edwards, 2001).

MCs are potent hepatotoxins in mammals, birds and fish (Kaya, 1996). They not only cause acute toxicity of animals due to liver dysfunction and hemorrhage, but also trigger the chronic effect of tumor promotion (Nishiwaki-Matsushima et al., 1992; Dawson, 1998). The mechanisms of the MC-induced liver toxicity are generally regarded to be mediated via the inhibition of protein phosphatases 1 (PP-1) and 2A (PP-2A), two major serine/threonine protein phosphatases in eukaryotic cells (Cohen, 1989), since PP-1 and PP-2A are the primary targets of MCs (Honkanen et al., 1990; Runnegar et al., 1995). The Ser/Thr phosphatases have been demonstrated to be the important regulators of keratin intermediate filament assembly, which is central to the integrity of hepatocyte (Toivola and Eriksson, 1999). In contrast, the mechanism for the MC-induced tumor promotion has been focused primarily on 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 cell proliferation (Toivola and Eriksson, 1999; Janssens and Goris, 2001). PP-1 and PP-2A have different structures and distinct biological functions (Cohen, 1989; Wera and Hemmings, 1995). Although both PP-1 and PP-2A can be inhibited by MCs and can be linked to the acute toxicity, the relative importance between PP-1 and -2A to the animal toxicity induced by different MCs has not been clarified.

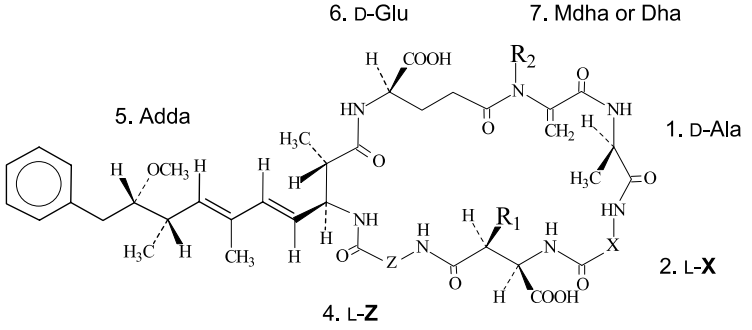
Although more and more MCs have been identified and reported, very few of them have been analyzed for their phosphatase inhibition activities in correlation to their animal toxicities (Harada, 1996). For those MCs that have been analyzed for their phosphatase inhibition activities and/or the animal toxicities, 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 MC-induced phosphatase inhibition and animal toxicities, we used eight MCs isolated and identified previously in our laboratory (Lee et al., 1998; Lee and Chou, 2000) to measure their relative inhibition on PP-1 and PP-2A as well as the toxicity in mice via intraperitoneal injection. In addition, the structure–activity relationship (SAR) rules for MCs are summarized.

2. Materials and methods

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

2.1. Preparation of MCs

Eight MCs, including MC-LR, -FR, -WR, -RR, [D-Asp^3]MC-FR, -WR, -RR and [Dha^7]MC-RR (Fig. 1.) were isolated from strains of *M. aeruginosa* indigenous to Taiwan. The identity, structure and purity of different MCs were analyzed mainly by NMR and MS spectra as described in our previous reports (Lee et al., 1998; Lee and Chou, 2000). The range of concentrations of different MCs used were different in each individual experiment, depending on toxicity or inhibition activity.



	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

Fig. 1. Chemical structures of MCs used in the study.

2.2. Phosphatase inhibition assay

Catalytic subunits of the native PP-1 and PP-2A were purified from the rabbit skeletal muscle according to Huang et al. (1997). 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) is defined as the phosphatase activity that hydrolyzes 1 nmol of *para*-nitrophenyl phosphate (*p*-NPP) min^{-1}. *p*-NPP was used as the substrate and prepared at a concentration of 250 mM in the Tri–HCl buffer with the additives 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 (catalog number 539493) activity assay using the standard protocol of Calbiochem-Novabiochem Corp. (San Diego, CA, USA). The phosphatase assay was carried out in a 96-well transparent microplate. For each reaction, 100 μL Tris–HCl buffer was added, then mixed with the toxin solution with different amounts of the MC and 50 μL of enzyme solution each. The reactions were pre-incubated for 10 min, then 50 μL of substrate solution was added and kept at 30 °C for one hour. To account for the change in absorbance due to the *p*-NPP decomposition, a blank reaction without toxins and enzymes was prepared for each experiment. The absorbance of each reaction mixture was determined at 405 nm using an ELISA reader (Tecan, Grodig, Australia), and all treatments were analyzed in duplicate. The inhibitory activity of each toxin was calculated by the change in absorbance, and expressed as percentage activity of the control.

2.3. Mouse toxicity assay

Pure MC (1.5–36 μg) was dissolved in saline solution (0.95% NaCl) at different concentrations. 1 mL toxin solution was injected intraperitoneally into a male mouse (ICR strain, 20 ± 1 g), purchased from the National Taiwan University Hospital. Each treatment was performed in triplicate. The mortality of mice was recorded and the dosage that killed half of the mice in four hours (LD_{50}) was calculated accordingly, using probit analysis (Finney, 1963). All the animal handling procedures were performed under the regulation of laboratory animals' usage at the National Taiwan University.

3. Results

Activities of both PP-1 and -2A decrease with the increase of MC concentration. The correlation between percentage activity of control (Y) and the concentration of MC in pM (X) is derived as a linear regression equation of $Y = a\mathrm{Ln}X + b$, where $a < 0$ and $b > 0$, for both PP-1 and PP-2A. Using these formulas, the IC_{50} values of various MCs for PP-1 and PP-2A are calculated and listed in Table 1. The inhibition activities of various MCs on PP-1 and -2A are compared according to their respective IC_{50} values. Among them, MC-LR is found to be the strongest, with similar IC_{50} values of 0.3 and 0.4 nM toward PP-1 and -2A, respectively. With the substitutions of leucine (L) by arginine (R), phenylalanine (F) or tryptophan (W) at the X amino acid, the inhibitory effects on phosphatases by MCs reduce to different extents with respect to PP-1 and -2A. The IC_{50} values of the arginine substituted MC-RR increase to 1.7 and 58 nM, which are a 6 and 145-fold higher than those of MC-LR. It is apparent that the replacement of the arginine at X position significantly reduces the potency of MC against PP-2A, but less severely for PP-1. The IC_{50} values of the phenylalanine substituted MC-FR increase to 1.1 and 4 nM, which are a 4 and 10-fold higher than those of MC-LR. The IC_{50} values of the tryptophan substituted MC-WR increase to 1.0 and 5 nM, which are a 3- and 13- fold higher than those of MC-LR. The increases in IC_{50} values of MC-FR and MC-WR are also higher in PP-2A than in PP-1. However, the increase is less significant. [Dha^7]MC-RR with a proton instead of a methyl group at the 7th Mdha compared to MC-RR slightly increases the IC_{50} to 2.2 and 86 nM, which are a 1.3 and 1.5 fold increase to those of MC-RR. Demethylation on the 3rd MeAsp of MCs also increases the IC_{50} to PP-1 and -2A to various extents in MC-RR, -FR and -WR. It appears that this modification negatively regulates the inhibition on PP-1 by MCs more than that of PP-2A.

Table 1
IC_{50} and LD_{50} values of MCs

MCs	IC_{50} (nM)		LD_{50} (μg/kg) (Ranking)
	PP-1 (Ranking)	PP-2A (Ranking)	
MC-LR	0.3 ± 0.1 (1)	0.4 ± 0.1 (1)	50 ± 5 (1)
MC-RR	1.7 ± 0.1 (4)	58 ± 3 (6)	320 ± 10 (6)
MC-FR	1.1 ± 0.1 (3)	4 ± 1 (2)	100 ± 10 (4)
MC-WR	1.0 ± 0.1 (2)	5 ± 1 (3)	140 ± 20 (5)
[Dha^7]MC-RR	2.2 ± 0.4 (5)	86 ± 18 (8)	420 ± 30 (8)
[D-Asp^3]MC-RR	4.8 ± 0.2 (8)	59 ± 13 (7)	350 ± 10 (7)
[D-Asp^3]MC-FR	3.6 ± 0.2 (7)	9 ± 1 (4)	90 ± 10 (2)
[D-Asp^3]MC-WR	3.1 ± 0.2 (6)	12 ± 1 (5)	95 ± 10 (3)

For mouse toxicity assays, the mice given the lethal dosages of different MCs died between 0.5–4 h. Upon necropsy, grossly enlarged livers engorged with blood were observed in dead mice, which were similar to previous reports (Theiss et al., 1988). Using the mortality rate, the LD_{50} values were calculated (Table 1), which shows that MC-LR has the lowest LD_{50} value (0.05 mg/kg). This result coincides very well to it having the strongest inhibition activities against both PP-1 and -2A. Surprisingly, the toxicities of [D-Asp^3]MC -FR and -WR are close to the MC-LR with a LD_{50} values near 0.09 mg/kg. MC-FR and -WR appear to be comparable to their [D-Asp^3] modified counterparts with a LD_{50} values of 0.1 and

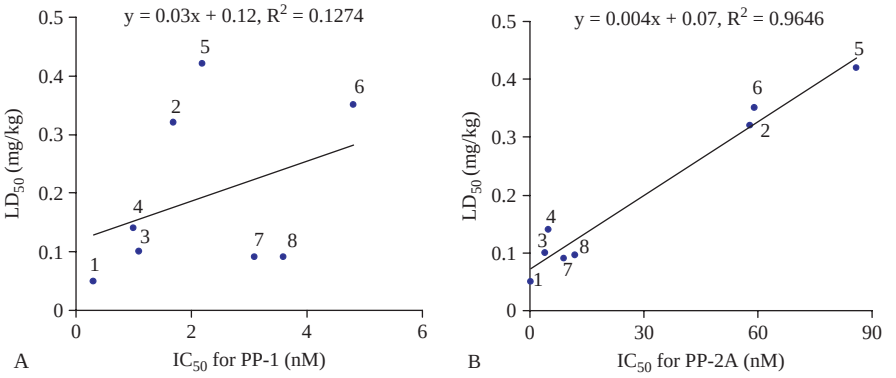


Fig. 2. Correlations between toxicity and inhibition activity against PP-1 (A) and PP-2A (B) of various MCs i.e. MC-LR (1), MC-RR (2), MC-FR (3), MC-WR (4), [Dha^7]MC-RR (5), [D-Asp^3]MC-RR (6), [D-Asp^3]MC-FR (7), and [D-Asp^3]MC-WR (8).

0.14 mg/kg, respectively. The least toxic MCs are MC-RR and its derivatives, with LD_{50} values ranging from 0.32 to 0.42 mg/kg.

To elucidate the relationship between toxicity of MCs and inhibition activities against PP-1 and PP-2A, the LD_{50} and IC_{50} values of MCs were analyzed using linear regression. It is found that the toxicity of MCs examined has little correlation with inhibition activity against PP-1 (Fig. 2A). Although the toxicities of MC-LR, -RR, -FR, -WR and [Dha^7] MC-RR increase with the inhibition activity of PP-1, the three [D-Asp^3] modified MCs show lower inhibitory activities against PP-1 compared to other MCs with the same toxicity level (Fig. 2A). In contrast, the toxicities of MCs show very good correlation with the inhibitory activity against PP-2A, as shown in Fig. 2B.

4. Discussion

Microcystins (MCs) are cyclopeptides with more than 70 different members. Different MCs may have different inhibition activities on PP-1 and PP-2A, which may correlate to the animal toxicity: By analyzing and comparing the inhibitory effects on PP-1 and PP-2A as well as the acute toxicity to mice of eight different MCs, we have clearly demonstrated that a leucine at the X amino acid is important for both high PP-1 and -2A inhibition 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-2A inhibitory effects than that of PP-1.

The correlation between MCs' toxicity and their inhibitions of two structurally and functionally distinct phosphatases PP-1 and -2A has long been puzzled. Our present findings reveal that PP-2A can be more important for MCs to induce acute toxicity. Similarly, Ito et al. (2002) found that the more toxic MC-LR exerted stronger inhibition activities against PP-2A than MC-RR does, but no difference on the inhibition activity against PP-1. These results suggest that the PP-2A inhibition may not only plays a major role in tumor promotion induced by MCs (Toivola and Eriksson, 1999; Janssens and Goris, 2001), but also be more important for mediating the acute toxicity of MCs. It is evident that the toxicity of MC-containing samples can be better estimated by using the PP-2A inhibition assay.

With the results presented, the SAR rules of MCs can be summarized as follows: (1) the replacement of the amino acid at the second position of MCs has the greatest influence on phosphatase inhibitions; (2) the demethylation on the 7th amino acid (Mdha) of MCs makes the MC toxicity and phosphatase inhibitory activities decrease, but less important; (3) the demethylation on the 3rd amino acid (MeAsp) of MCs has little influence on either toxicity or inhibition activities against PP-2A, but dominantly reduce inhibition activities against PP-1 compared to other substitutions or modifications.

The SARs of MCs related to PP-1 inhibition can be partly explained by the interaction model established by Goldberg et al. (1995). According to the model, when MC-LR interacts with the catalytic subunit of PP-1 (PP-1c), the side chain on L-Leu of MC-LR forms a hydrophobic interaction with the aromatic ring on Tyr272 of PP-1c; the carbonyl and carboxyl group on MeAsp of MC form two hydrogen bonds with Arg96 and Tyr134; the side chain of Adda forms a hydrophobic interaction with the hydrophobic groove near the PP-1c active site; the carboxyl group of D-Glu forms hydrogen bonding with metal ions in active site, and the methylidene group of Mdha forms covalent bonding with Cys273 near the C-terminal. The change of inhibition activity against PP-1 resulting from the modifications on the 2nd, 3rd, 5th, 6th, and 7th amino acids of MCs can be partly predicted by this model. It is safe to say that the decrease of inhibition activity against PP-1 results from the replacement of L-X amino acid which decreases the of hydrophobic interaction. However, the model cannot interpret the loss of inhibitory activity resulting from demethylation on MeAsp and Mdha. It is tempting to speculate that the two methyl groups may be important for the formation of hydrogen bonding or covalent bonding

occurred nearby, or they may have interactions with PP-1 that did not found in the previous studies.

The different SAR rules of MCs for PP-1 and PP-2A inhibitions imply that MCs interact with PP-1 and -2A in a different way. By comparing the SARs of MC against PP-1 and -2A, it is suggested that interactions between the second amino acid and seventh amino acid of MC and catalytic subunits of the two phosphatases are quite similar, since the changes on the two positions of MCs have the same influence on inhibition activities against PP-1 and -2A. A previous study showed that esterification on the D-Glu, and the change of conformation on Adda side chain from *Z* to *E* cause the total loss of inhibition activities on both PP-1 and PP-2A (Kaya, 1996). It revealed that the interactions of the 5th and 6th amino acid of MC to both phosphatases should be similar. Furthermore, since the amino acids equivalent to the Tyr272 and Cys273 of PP-1c in the catalytic subunit of PP-2A are also Tyr (Tyr265) and Cys (Cys266) (MacKintosh, 1995), the side chain of L-X amino acid may form a hydrophobic interaction with Tyr265 in the catalytic subunit of PP-2A, and the methylidene group at Mdha of MC-LR can also form covalent bonding with PP-2A at Cys266. The covalent bonding between Mdha of MC-LR and Cys266 of PP-2A has been demonstrated (Runnegar et al., 1995). The interaction of the third amino acid of MCs to both phosphatases, however, should be quite different, since the loss of a methyl group on MeAsp shows little influence on PP-2A inhibition compared to other modifications, but has a dominant influence for inhibition activities on PP-1.

In summary, we have provided evidence to show that different amino acids and their side chain modifications that occur in natural microcystins could differentially affect the PP-1 and -2A activities as well as their acute animal toxicity. Furthermore, we have demonstrated nicely that the acute toxicity of MC is more dependent on the inhibition of PP-2A rather than PP-1.

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References

Carmichael, W.W., Beasly, V., Bunner, D.L., Eloff, J.N., Falconer, I., Gorham, P., Harada, K.I., Krishnamurthy, T., Yu, M.-J., Moore, R.E., Rinehart, K., Runnegar, M., Skulberg, O.M., Watanabe, M., 1988. Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26, 971–973.

Cohen, P., 1989. The structure and regulation of protein phosphatase. Annu. Rev. Biochem. 58, 453–508.

Dawson, R.M., 1998. The toxicology of microcystins. Toxicon 36, 953–962.

Falconer, I.R., 1999. An overview of problems caused by toxic blue-green algae (Cyanobacteria) in drinking and recreational water. Environ. Toxicol. 14, 5–12.

Finney, D.J., 1963. Probit analysis (rev. ed.). Cambridge University Press, London.

Goldberg, J., Huang, H.-B., Kwon, Y.-G., Greengard, P., Nairn, A.C., Kuriyan, J., 1995. Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1. Nature 376, 745–753.

Harada, K.I., 1996. Chemistry and detection of microcystins. In: Watanabe, M.F., Harada, K., Carmichael, W.W., Fujiki, H. (Eds.), Toxic Microcystis. CRC Press, New York, pp. 103–148.

Honkanen, R.E., Zwiller, J., Moore, R.E., Daily, S.L., Khatra, B.S., Dukelow, M., Boynton, A.L., 1990. Characterization of microcystin-LR, a potent inhibitor of type 1 and type 2A protein phosphatases. J. Biol. Chem. 265, 19401–19404.

Huang, H.-B., Horiuchi, A., Goldberg, J., Greengard, P., Nairn, A.C., 1997. Site-directed mutagenesis of amino acid residues of protein phosphatase 1 involved in catalysis and inhibitor binding. Proc. Natl Acad. Sci. 94, 3530–3535.

Ito, E., Takai, A., Kondo, F., Masui, H., Imanishi, S., Harada, K.I., 2002. Comparison of protein phosphatase inhibitory activity and apparent toxicity of microcystins and related compound. Toxicon 40, 1017–1025.

Janssens, V., Goris, J., 2001. Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signaling. Biochem. J. 353, 417–439.

Kaya, K., 1996. Toxicology of microcystins. In: Watanabe, M.F., Harada, K., Carmichael, W.W., Fujiki, H. (Eds.), Toxic Microcystis. CRC Press, New York, pp. 175–202.

Lawton, L.A., Edwards, C., 2001. Purification of microcystins. J. Chromat. A 912, 191–209.

Lee, T.-H., Chou, H.-N., 2000. Isolation and identification of seven microcystins from a cultured M.TN-2 strain of *Microcystis aeruginosa*. Bot. Bull. Acad. Sin. 41, 197–202.

Lee, T.-H., Chen, Y.-M., Chou, H.-N., 1998. First report of microcystins in Taiwan. Toxicon 36, 247–255.

MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P., MacKintosh, C., 1995. The cyanobacterial toxin microcystin bind covalently to cysteine-273 on protein phosphatase 1. FEBS Lett. 371, 236–240.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., Fujiki, H., 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118, 420–424.

Runnegar, M., Berndt, N., Kong, S.-M., Lee, E.Y.C., Zhang, L., 1995. *In vivo* and *in vitro* binding of microcystin to protein phosphatases 1 and 2A. Biochem. Biophy. Res. Commun. 216, 162–169.

Theiss, W.C., Carmichael, W.W., Wyman, J., Bruner, R., 1988. Blood pressure and hepatocellular effects of the cyclic heptapeptide toxin produced by the freshwater cyanobacterium (blue-green alga) *Microcystis aeruginosa* strain PCC-7820. Toxicon 26, 603–613.

Toivola, D.M., Eriksson, J.E., 1999. Toxins affecting cell signaling and alternation of cytoskeletal structure. Toxicol. in Vitro 13, 521–530.

Wera, S., Hemmings, B.A., 1995. Serine/threonine protein phosphatases. Biochem. J. 311, 17–29.