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Brief Article

Acyloxymethyl Esterification of Nodularin-R and Microcystin-LA Produces Inactive Protoxins that Become Reactivated and Produce Apoptosis inside Intact Cells

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We report the esterification of the carboxyl groups of the cyclic peptide toxins nodularin-R and microcystin-LA to produce stable diacetoxymethyl and dipropionyloxymethyl ester derivatives. The derivatives had no activity but were reactivated upon esterase treatment. When injected into cells, the acyloxymethyl moieties were cleaved off and apoptosis induced. Linking the acyloxymethyl-ester moiety of these potent toxins to carriers destined for endocytosis paves the way for selective apoptosis induction in target (e.g., cancer) cells.

Introduction

Acetoxymethyl (AM[a]) esterification has been used successfully to enhance the cell penetration of polar compounds, which become trapped intracellularly when the AM group is removed by ubiquitously expressed nonspecific cellular esterases. Well known examples are FURA-AM, used to determine the intracellular level of free calcium ion,[1] the Ca^{2+}-chelator BAPTA-AM,[2] AM-esters of cyclic nucleotides, used to modulate cAMP receptor actions,[3,4] and AM esters of inositol phosphates.[5]

The cyanobacterial toxins microcystins and nodularin are cyclic hepta- and penta-peptides (Figure 1), respectively. They inhibit major protein phosphatases[6] and are extremely potent inducers of cell death.[7] When ingested, microcystins and nodularin are hepatotoxic due to their specific uptake into parenchymal hepatocytes,[8] mediated through the liver-specific transport proteins OATP1B1 and 1B3.[9] When microinjected, however, the toxins induce apoptotic cell death within minutes in all cell types tested, including cancer cells.[7] The toxins are therefore excellent candidates to eradicate unwanted cells if they can be targeted to their interior. In fact, the PP2A-inhibitor fostriecin has entered phase II clinical studies as an anticancer drug.[10] Successful intracellular drug targeting of leukemia cells has been achieved using the toxin calicheamine conjugated to anti-CD33 antibody specifically recognizing acute myelogenic leukemia cells.[11] In the case of microcystin and nodularin, the conjugation should be reversible and allow the intracellular release of active toxin. Another avenue is to encapsulate toxins in the hydrophobic interior of nanoparticles equipped with surface ligands inducing receptor-mediated endocytosis.[12] For this, the hydrophilic cyclic peptide toxins must be rendered more lipophilic without interfering with the biological activity.

We reasoned that nodularin and microcystin could be attached to a carrier via an esterase-sensitive acyloxymethyl−ester linkage. We decided, therefore, to modify nodularin and microcystin through diacetoxymethyl (AM_2) and dipropyloxymethyl (PM_2) esterification. To produce a more hydrophobic, putatively active toxin, we introduced a hydrophobic phenylglyoxal (PGx) substituent onto nodularin. We achieved the synthesis of reversibly inactivated esters of both microcystin and nodularin, as well as the synthesis of active phenylglyoxal−nodularin, all of which were able to induce apoptotic cell death when injected into intact cells.

Results and Discussion

Synthesis of Microcystin-LA-AM_2, Nodularin-AM_2, Nodularin-PM_2, and Phenylglyoxal-Nodularin. The carboxyl groups of microcystin (MC) and nodularin-R (Nod) are predicted to be essential for high affinity binding to the toxin target protein phosphatases 1 (PP1) and 2A (PP2A).[13,14] Their reversible modification by an esterase-sensitive substitution would therefore produce a protoxin active only after exposure to esterases. We found that the conditions used to synthesize acetoxymethyl esters of cyclic nucleotides[3,4] were inefficient for peptide modification and reworked the conditions based on a number of experiments on the model peptide Z-Arg-Leu-Val-Gly-OH, which contains the free carboxylic and guanidine groups found within the nodularin structure (Figure 1). Successful and rapid (reaction completed in 1.5−2 min, yield 55−70% already after 30 s of incubation) esterification of model peptide as well as of MC-LA and Nod was achieved when the reaction was carried out in dimethylsulfoxide, and acetoxymethyl bromide was added

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[a]Abbreviations: AM, acetoxymethyl; PM, propionyloxymethyl; PGx, phenylglyoxal; PP2A, protein phosphatase 2A; Nod, nodularin-R; MC, microcystin-LR or microcystin-LA.

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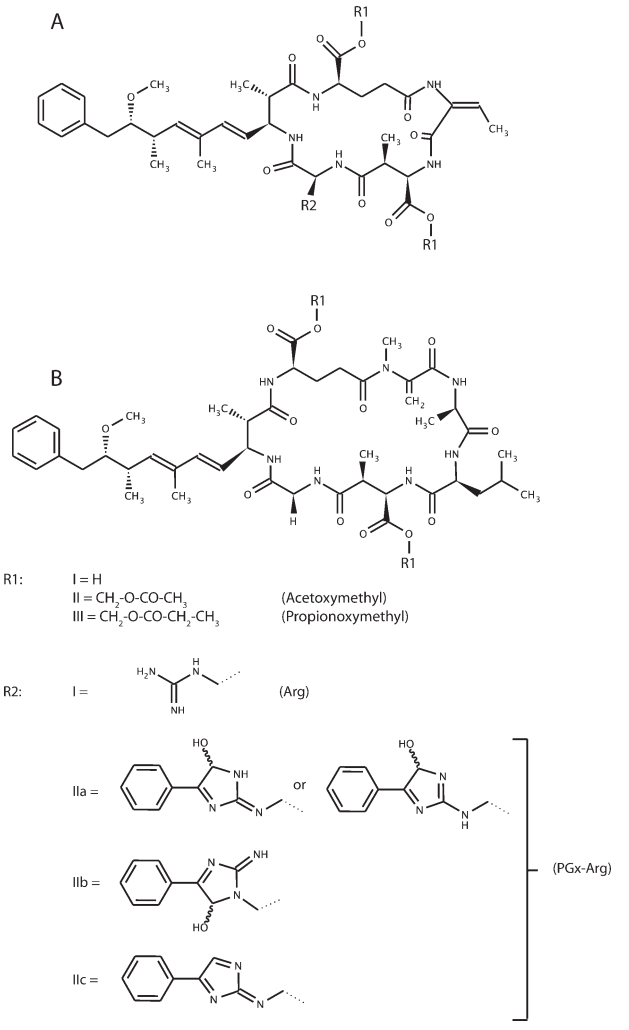

Figure 1. Nonionized structures of nodularin-R (A), microcystin-LA (B), and various carboxyl (R1) and arginine (R2) substituents used in the present study. The structures were drawn in Marvin version 5.0.1 for apple 2008, ChemAxon, www.chemaxon.com.

with diisopropylethylamine. AM_2 (R1-II) esters of Nod and MC-LA and PM_2 (R1-III) esters of Nod (I) (Figure 1) were obtained with purity >95% by RP-HPLC with acetonitrile/water/TFA as mobile phase. As expected from other AM-esters,[15] the acyloxymethyl-esters of Nod and MC-LA were stable in physiological buffers. Thus, less than 0.1% of the nodularin of PM_2-Nod was released during more than 1 h incubation with PP2A (Figure 2; data not shown). We conclude that the two carboxyl groups common to MC-LA and Nod can be substituted with acyloxymethyl to produce stable, potentially esterase cleavable toxin analogues.

The arginine of Nod is not predicted to be essential for high-affinity binding to PP2A.[14] We introduced therefore the charge neutralizing substituent phenylglyoxal in the arginine guanidine of Nod to obtain an active compound with improved hydrophobicity (Figure 1 and Table 1). In introductory experiments with the model peptide Z-Arg-Leu-Val-Gly-OH, we obtained consistently an increase of peptide mass by 99 or 117, corresponding to the incorporation of one molecule of phenylglyoxal minus one or two water molecules. This is in contrast to an early study on peptide arginine modification, which reported a 2:1 ratio,[16] and 2D NMR studies were undertaken showing the presence

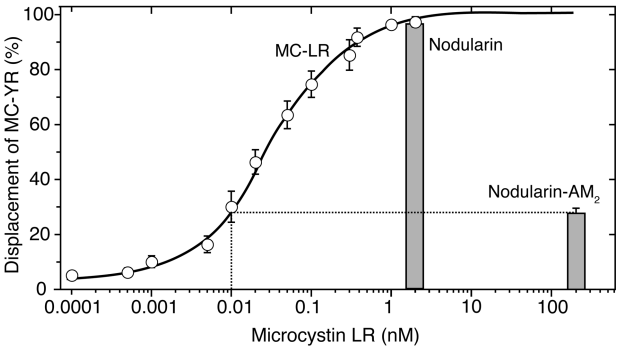

Figure 2. Nod-AM_2 has a low binding affinity to PP2A. Nod-AM_2, Nod, or MC-LR were mixed with radiolabeled MC-YR and incubated with PP2A. After separation of PP2A-bound from free MC-YR, the amount of protein-bound radioactivity was measured and the data plotted as percent displacement of MC-YR. The data are average and SEM from 3 to 8 independent experiments.

Table 1. In Silico Estimates of Isoelectric Point, Lipophilicity, and Net Charge Distribution at Physiological Conditions for Microcystin-LR, -LA, Nodularin-R, and Their Analogues[a]

		LogD	logD at	net charge distribution at pH 7.4			
compd	pI	at pI	pH 7.4	−2	−1	0	1
microcystin-LR	5.7	−7.4	−8.5	0	92	8	0
microcystin-LA	1.3	0.3	−6.8	99	1	0	0
MC-LA-AM_2	5.3	1.2	1.2	0	0	100	0
nodularin-R	3.2	−3.1	−8.5	0	99	0	0
PGx-Nod[b]	4.4	−1.4	−4.8	99	1	0	0
Nod-AM_2	12.4	0.4	−2.0	0	0	0	100
Nod-PM_2	12.4	1.6	−0.7	0	0	0	100

[a] MC-LA-AM_2: di-acetoxymethyl-microcystin-LA; PGx-Nod: phenylglyoxal-nodularin-R; Nod-AM_2: di-acetoxymethyl-nodularin-R; Nod-PM_2: di-propionyloxymethyl-nodularin-R. [b] Based on structure IIa of the R2 substituent of Nod in Figure 1.

of only one additional phenyl ring (not shown). For Nod, we obtained several adducts of phenylglyoxal (R2, IIa-c in Figure 1), all with adduct stoichiometry of 1:1 (PGx: arginine guanidyl). LC-MS analysis of the reaction mixture showed again that the MW of the precursor peptide had increased by no more than 99 or 117 (data not shown), indicating a 1:1 ratio PGx: arginine guanidyl. For the biological studies, we used PGx-Nod with the R2 substituent IIa (Figure 1A), which was separated from the other reaction products by HPLC and isolated at >95% purity. We conclude that the arginine residue of Nod could be substituted to produce a stable, lipophilic analogue.

The estimated isoelectric pH, lipophilicity, and charge distributions of the original and modified toxins are listed in Table 1. In agreement with the in silico predictions of hydrophobicity, all modified toxins were better retained on C18 columns than the corresponding nonmodified toxin (not shown).

The AM_2 and PM_2 Esters of Nod and MC-LA Failed to Inhibit Protein Phosphatases, But Were Reactivated by Esterases, Also in Intact Cells. The displacement of labeled MC-YR from the major microcystin target PP2A is the most sensitive assay to detect protein phosphatase inhibitors of the microcystin, nodularin class.[17] We found that Nod-AM_2 had about 4 orders of magnitude lower affinity for PP2A than the original Nod molecule (Figure 2). This shows that Nod-AM_2 (a) must contain at most 0.01% of free Nod even after incubation for more than one hour, and (b) had very low affinity for PP2A. The low affinity is explained by consideration of the

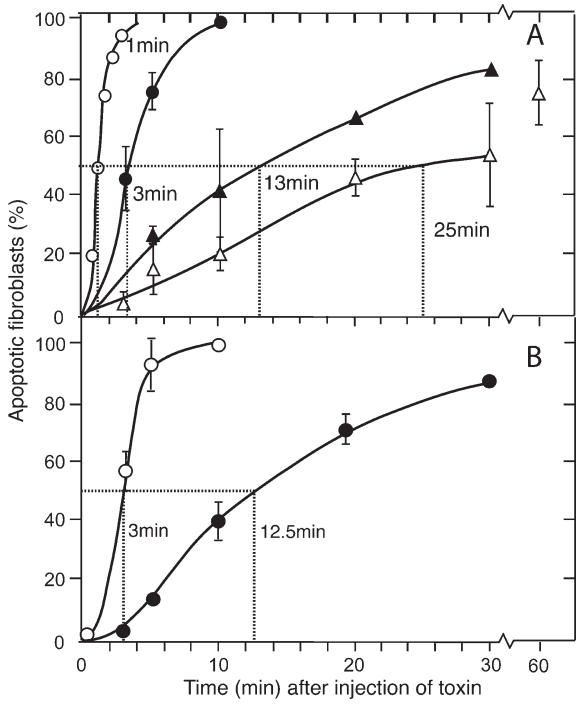

Figure 3. Acyloxymethyl-esters of Nod and MC-LR, and PGx-Nod induced apoptosis when microinjected. Swiss 3T3 fibroblasts were injected with 100 μM of Nod, MC-LR, or their analogues in vehicle with TRITC-dextran, and the percentage of the injected (fluorescent) cells with apoptotic morphology determined as a function of time after injection. (A) Apoptosis after injection with Nod (○), PGx-Nod (●), Nod-AM_2 (△), or Nod-PM_2 (▲). (B) Apoptosis after injection of MC-LR (open circles) or MC-LA-AM_2 (filled circles). The data represent average of three experiments and SEM.

Nod-PP2A complex in which the two carboxyl groups of either Nod or MC form tight hydrogen bonds with hydrophilic moieties in the PP2A inhibitor binding site.[13,14] Thus, the introduction of the acyloxymethyl moieties will not only disrupt the hydrogen bonds but also introduce unfavorable bulk and hydrophobicity in the tight, hydrophilic binding region.

Because the diacyloxymethyl-esters had severely decreased ability to interact with phosphatase and thus were nontoxic, they had to be de-esterified to restore the toxin activity. The ability of Nod-AM_2 and -PM_2 to be enzymatically processed to Nod was confirmed by HPLC analysis after in vitro incubation with pig liver esterase (not shown). More importantly, Nod-AM_2, Nod-PM_2 (Figure 3A), and MC-LA-AM_2 (Figure 3B) all induced apoptosis when injected into intact cells, albeit more slowly than Nod (Figure 3A) and MC-LR themselves (Figure 3B). This is expected if the compounds had to be converted by esterases before becoming biologically active. We conclude that the acyloxymethyl-esters of MC-LA and Nod could be activated by esterases and therefore become functional when introduced into intact cells. The PM_2 analogue of Nod was at least as efficient as the AM_2 analogue in inducing apoptosis (Figure 3A). This indicated that the extra bulk of the PM moiety did not interfere with esterase cleavage, indicating that esterase-sensitive attachment of Nod or MC to cell-targeting macromolecules destined for endocytosis could be achieved via longer acylesters than AM.

The phenylglyoxal-substituted Nod is biologically active, inducing apoptosis rapidly after microinjection into cells (Figure 3A). This fact and its improved lipohilicity relative to Nod (Table 1) renders it potentially useful for incorporation into the lipophilic center of nanoparticles destined for cell internalization.

The Nodularin-R and Microcystin-LA Analogues Had Decreased, But Not Abolished, Hepatocyte Toxicity. The intrinsically inactive and stable diacyloxymethyl esters of Nod and MC-LA could be activated only when exposed to esterases (Figure 3). Because primary hepatocytes can be studied at high dilution in synthetic medium with negligible esterase activity, their eventual death-inducing activity could be explained only by cellular uptake with subsequent toxin activation by intracellular esterases. Primary hepatocytes have efficient transporters, Oatp1b2 in rodents and OATP1B1/1B3 in humans,[9] for cyclic peptides like MC and Nod. We found that the esterified toxins had at least 1−2 orders of magnitude lower potency than the unmodified toxins as inducers of apoptotic hepatocyte death. Fifty percent death (LC_{50}) was obtained with 0.09 μM Nod (Figure 4A) or 0.27 μM MC-LR (Figure 4B), while Nod-AM_2 and -PM_2 had $LC_{50} = 14$ μM, and MC-LA-AM_2 had $LC_{50} = 8$ μM (Figure 4). PGx-Nod was more potent than the acyloxy-esters, with $LC_{50} = 1.4$ μM (Figure 4). All of the toxin analogues induced hepatocyte death with similar morphology, as did Nod and MC-LR (Figure 4). The morphology is characteristic with marked polarized cell blebbing (Figure 4; see also ref 18). We conclude that the esterified toxins were considerably less well transported into hepatocytes than the original toxins but otherwise acted as the unmodified toxins.

We considered the possibility that the effects on hepatocytes of the AM_2 and PM_2 esters (Figure 4A,B) could be due to transporter-independent membrane penetration in view of the enhanced lipophilicity of the modified toxins (Table 1). If so, the compounds should induce apoptosis also in fibroblasts and other cells lacking hepatocyte-specific transporters. Fibroblasts were, however, far less sensitive than the hepatocytes, only modest (less than 20%) apoptosis being noted after 3 h incubation with up to 120 μM of Nod-AM_2, Nod-PM_2, PGx-nodularin (Figure 4C), or MC-LA-AM_2 (Figure 4D). In fact, the modified toxins were not more potent than the unmodified toxins (Figure 4C,D). When microinjected with 100 μM toxin, an intracellular concentration of about 2 μM toxin is achieved based on the estimated 50-fold dilution of the injectate.[19] This is enough to induce rapid fibroblasts death (Figure 3). One can therefore assume that the intracellular concentration in fibroblasts exposed to 120 μM toxin in the medium must be far lower than 2 μM, and we conclude that the modified toxins had a very poor ability to enter cells lacking transport channels for cyclic peptides.

Concluding Remarks

In conclusion, we have demonstrated the inactivation of the apoptogenic toxins Nod and MC-LA through esterification of their essential carboxyl groups. The diacyloxymethyl-esters were readily reactivated upon exposure to esterases in vitro. When injected into cells, the ester linkage was cleaved and the liberated active toxin produced apoptosis (Figure 3). We believe therefore that cancer cells with specific receptors linked to endocytosis can be targeted by ester-linking Nod or MC to carriers, like cell-specific receptor ligands or antibodies destined for endocytosis.[11,20] We have also demonstrated that hydrophobic toxin derivatives, like phenylglyoxal-Nod (Table 1), can

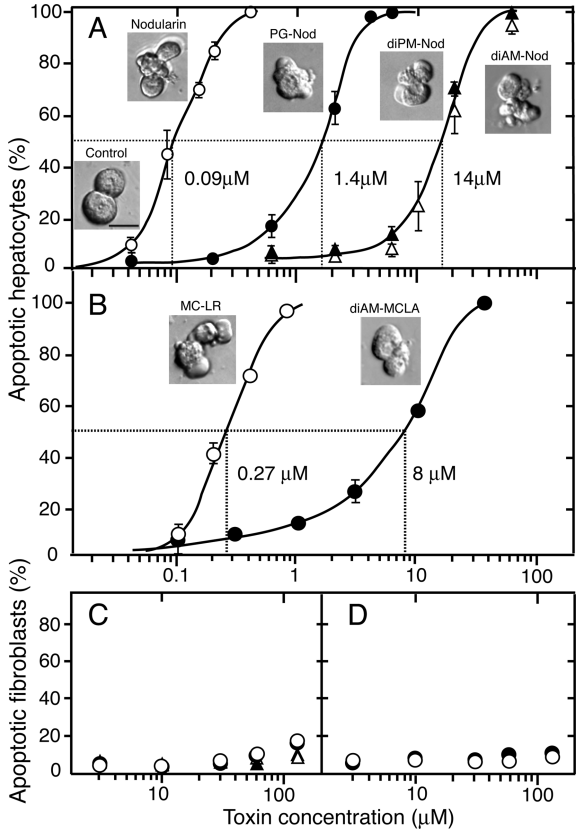

Figure 4. Acyloxymethyl esters of Nod and MC-LA, and PGx-Nod added to cell medium failed to induce fibroblast apoptosis but did induce hepatocyte apoptosis. (A,B) Freshly isolated rat hepatocytes were incubated in suspension culture for 90 min in the presence of various concentrations of the toxins. The percentage of apoptotic cells was determined by differential interference contrast microscopy. (A) Nod (○), PGx-Nod (●), Nod-AM_2 (△), and Nod-PM_2 (▲). (B) MC-LR (○), MC-LA-AM_2 (●). The insets of (A) show DIC-micrographs of cells treated with vehicle or with 80 nM Nod, 4 μM PGx-Nod, 60 μM Nod-AM_2, or 60 μM Nod-PM_2. (B) shows cells treated with 0.2 μM MC-LR or 35 μM MC-LA-AM_2. Bar is 15 μm. (C,D) Swiss 3T3 fibroblasts were incubated for 3 h in serum-free RPMI-medium in the presence of various concentrations of Nod, MC, or their protoxins. Apoptosis was assessed based on fluorescent microscopy of cells whose chromatin was stained (Hoechst 33342) and differential interference contrast microscopy. (C) Nod (○), PGx-Nod (●), Nod-AM_2 (△), and Nod-PM_2 (▲). (D) MC-LR (○) and MC-LA-AM_2 (●). The data represent the average and SEM from three experiments.

retain biological activity (Figure 3A). Such derivatives can allow more toxin to be loaded in the lipid core of nanoparticles, which emerge as a vehicle for the selective delivery of cytotoxic substances to cancer cells.[21]

Materials and Methods

Materials. DMSO and di-isopropylethylamine (DIEA) of highest purity available were stored over preactivated molecular sieves (3 Å). AM-Br was from Sigma-Aldrich (St. Louis, MO) and PM-Br a generous gift from Dr. C. Schultz (EMBL, Heidelberg, Germany).

Purification of Nodularin-R and Microcystin-LA from Cyanobacteria. Nodularin-R was extracted from 130 g (dry weight) of *Nodularia spumigena* strain AV1 cells, first with a 1:1:1 mixture of methanol:butanol:water and next with 1 mM aq ammonia. After ion exchange chromatography (Macro-Prep Q system, Bio-Rad Laboratories, Hercules, CA) and preparative reversed phase chromatography, 220 mg of 94% pure Nod was obtained. Nod of >99.5% purity was obtained in >90% yield by semipreparative HPLC (stationary phase: Knauer Vertex column 250 mm × 16 mm packed with Kromasil 100, C-8, 5 μm; mobile phase: 18% CH_3CN, 0.1 M $NaClO_4$, 0.001 M phosphate, pH = 6.8). Desalting was on a Sep-Pak C-18 cartridge (Waters Corp. Milford, MA). After washing with water, Nod was eluted with 70% aq CH_3CN.

MC-LA was extracted from freeze-dried cells of the *Microcystis aeruginosa* strain IZANCYA1[22] using 1 mM aq triethylamine. The supernatant was applied on Sep-Pak C-18 cartridge and the activity eluted with acetonitrile before further purification by ion-exchange and reversed-phase chromatography. The final fractions were subjected to MS-analysis on a Bruker-Daltonic Esquire-LC-ESI-MS system.

Synthesis of AM- and PM-Esters of Nod and MC-LA. Nod or MC-LA (1.2 and 1.0 μmol, respectively) was dissolved in 7 μL of dry DMSO under argon protection. To the resulting solution DIEA (2.5 μmol) and AM-Br (2.4 μmol) were added and the mixture kept for 1.5−2 min at room temperature. The reaction was then stopped rapidly by freezing in liquid nitrogen. All volatile components were removed in vacuum. Purification of the AM-esters was performed on a semipreparative HPLC column (Knauer Kromasil RP-8, 5 μm, 250 mm × 10 mm) using water and acetonitrile (both added 0.05% TFA) as mobile phases. Purity of compounds was determined by analyses of the DAD-spectra of rechromatographed fractions. Synthesis of the PM-esters was performed similarly, but PM-Br was used for esterification.

The conditions for modification of the guanidine group of nodularin with phenylglyoxal were optimized using the model compound Z-Arg-Leu-Val-Gly-OH. Even under optimal conditions (aq THF pH 8.5−9), we achieved only a 1:1 stoichiometry of modification both for the model compound and for Nod itself. Synthesis of Nod modified at the guanidine group of arginine with phenylglyoxal (PGx-Nod; Figure 1A, R2, IIa-c) was performed in aq THF pH 8.8. Purification to homogeneity and analyses of reaction products were as for the ester analogues.

Cell Handling and Experimental Conditions. Hepatocytes were isolated from male Wistar rats (100−150 g) by in vitro collagenase perfusion[23] as previously described.[24] The hepatocytes were resuspended (8.0×10^5 cells/mL) in pregassed (5% CO_2/95% O_2) buffer (120 mM NaCl, 5.3 mM KCl, 0.01 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.0 mM $CaCl_2$, 10 mM Hepes (pH 7.4)) supplemented with lactate (5 mM) and pyruvate (5 mM). The cells were incubated with toxins in 48-well tissue culture plates in a 5% CO_2 atmosphere at 37 °C for 90 min, fixed in buffered (pH 7.4) formaldehyde to a final concentration of 2%, and percent apoptotic cells determined by differential interference contrast (DIC) microscopy.

Swiss 3T3 mouse fibroblasts were cultured in RPMI-medium with 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere. For testing of toxin analogues in the medium, about 7000 cells were plated in 0.25 mL 48-well tissue culture plate wells and incubated for 3 h after changing to fresh medium without added serum. The incubation was stopped by adding 0.25 mL of 4% buffered formaldehyde solution (pH 7.4) containing the fluorescent DNA-stain Hoechst 33342. The percentage of apoptotic cells was determined as described previously.[7] For microinjection, the fibroblasts were plated in 3.5 cm tissue culture dishes at a density of 60000/dish. The microinjection was performed using an Eppendorf 5170 micromanipulator mounted on a Zeiss Axiovert 35 M inverted microscope. Microcapillaries (type BF100-10, outer and inner diameter 1.0 and 0.78 mm, respectively) and puller (model P-87) were from Sutter instruments Co (Novato, CA). For each toxin, between 50 and 60 cells were injected, and the percentage of apoptotic cells were determined by differential interference microscopy.[7] Each experiment was performed in triplicate, and the average and SEM was calculated.

Determination of ability of MC, Nod and their analogues to bind to the active site of protein phosphatase 2A was done with a MC-YR-[^{125}I] displacement assay as described.[17,25] Protein phosphatase 2A (PP2A) was obtained from rabbit muscle following the procedure of ref 26.

In Silico Predications of Physiochemical Properties. Estimations of logD, pI, and ionization conditions were done with the Calculator Plug-in in Marvin version 5.0.1 (2008) for Apple (ChemAxon Ltd., www.chemaxon.com). The molecules were drawn in 2D mode and converted to 3D structures with the fine build clean 3D mode in Marvin. Standard ionic condition (0.1 M Cl^- and 0.1 M Na^+/K^+) was used for estimation of logD at pI and physiological pH (7.4). pK_a was estimated in macro mode under the following conditions: min basic pK1, −10; max acidic pK_a, 20; temperature 310 K.

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References

(1) Hayashi, H.; Miyata, H. Fluorescence imaging of intracellular Ca^{2+}. *J. Pharmacol. Toxicol. Methods* **1994**, *31*, 1–10.
(2) Tsien, R. Y. A non-disruptive technique for loading calcium buffers and indicators into cells. *Nature* **1981**, *290*, 527–528.
(3) Bartsch, M.; Zorn-Kruppa, M.; Kuhl, N.; Genieser, H. G.; Schwede, F.; Jastorff, B. Bioactivatable, membrane-permeant analogs of cyclic nucleotides as biological tools for growth control of C6 glioma cells. *Biol. Chem.* **2003**, *384*, 1321–1326.
(4) Schwede, F.; Brustugun, O. T.; Zorn-Kruppa, M.; Døskeland, S. O.; Jastorff, B. Membrane-permeant, bioactivatable analogues of cGMP as inducers of cell death in IPC-81 leukemia cells. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 571–573.
(5) Schultz, C.; Rudolf, M. T.; Gillandt, H. H.; Traynor-Kaplan, A. E. Membrane-permeant, bioactivatable derivatives of inositol polyphosphates and phosphoinositides. In *Phosphoinositides: Chemistry, Biochemistry and Biomedical Applications*; Bruzik, K. S., Ed.; *Am. Chem. Soc. Symp. Ser.* **1999**; Vol. *718*, pp 232−243.
(6) Honkanen, R. E.; Codispoti, B. A.; Tse, K.; Boynton, A. L. Characterization of natural toxins with inhibitory activity against serine/threonine protein phosphatases. *Toxicon* **1994**, *32*, 339–350.
(7) Fladmark, K. E.; Brustugun, O. T.; Hovland, R.; Bøe, R.; Gjertsen, B. T.; Zhivotovsky, B.; Døskeland, S. O. Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. *Cell Death Differ.* **1999**, *6*, 1099–1108.
(8) Hooser, S. B. Fulminant hepatocyte apoptosis in vivo following microcystin-LR administration to rats. *Toxicol. Pathol.* **2000**, *28*, 726–733.
(9) Fischer, W. J.; Altheimer, S.; Cattori, V.; Meier, P. J.; Dietrich, D. R.; Hagenbuch, B. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. *Toxicol. Appl. Pharmacol.* **2005**, *203*, 257–263.
(10) Lê, L. H.; Erlichman, C.; Pillon, L.; Thiessen, J. J.; Day, A.; Wainman, N.; Eisenhauer, E. A.; Moore, M. J. Phase I and pharmacokinetic study of fostriecin given as an intravenous bolus daily for five consecutive days. *Invest. New Drugs* **2004**, *22*, 159–167.
(11) Singh, R.; Erickson, H. K. Antibody−cytotoxic agent conjugates: preparation and characterization. *Methods Mol. Biol.* **2009**, *525*, 1–23.
(12) Zheng, Y.; Cai, Z.; Song, X.; Chen, Q.; Bi, Y.; Li, Y.; Hou, S. Preparation and characterization of folate conjugated *N*-trimethyl chitosan nanoparticles as protein carrier targeting forlate receptor: In vitro studies. *J. Drug Target.* **2009**, DOI: 10.1080/10611860902737920.
(13) Xing, Y.; Xu, Y.; Chen, Y.; Jeffrey, P. D.; Chao, Y.; Lin, Z.; Li, Z.; Strack, S.; Stock, J. B.; Shi, Y. Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. *Cell* **2006**, *127*, 341–353.
(14) Kelker, M. S.; Page, R.; Peti, W. Crystal structures of protein phosphatase-1 bound to nodularin-R and tautomycin: a novel scaffold for structure-based drug design of serine/threonine phosphatase inhibitors. *J. Mol. Biol.* **2009**, *385*, 11–21.
(15) Vliem, M. J.; Ponsioen, B.; Schwede, F.; Pannekoek, W. J.; Riedl, J.; Kooistra, M. R.; Jalink, K.; Genieser, H. G.; Bos, J. L.; Rehmann, H. 8-pCPT-2′-O-Me-cAMP-AM: an improved Epac-selective cAMP analogue. *ChemBioChem* **2008**, *9*, 2052–2054.
(16) Takahashi, K. The reaction of phenylglyoxal with arginine residues in proteins. *J. Biol. Chem.* **1968**, *243*, 6171–6179.
(17) Serres, M. H.; Fladmark, K. E.; Døskeland, S. O. An ultrasensitive competitive binding assay for the detection of toxins affecting protein phosphatases. *Toxicon* **2000**, *38*, 347–360.
(18) Krakstad, C.; Herfindal, L.; Gjertsen, B. T.; Bøe, R.; Vintermyr, O. K.; Fladmark, K. E.; Døskeland, S. O. CaM-kinaseII dependent commitment to microcystin-induced apoptosis is coupled to cell budding, but not to shrinkage or chromatin hypercondensation. *Cell Death Differ.* **2006**, *13*, 1191–1202.
(19) Mellgren, G.; Vintermyr, O. K.; Boe, R.; Døskeland, S. O.; Hepatocyte, D. N. A. replication is abolished by inhibitors selecting protein phosphatase 2A rather than phosphatase 1. *Exp. Cell Res.* **1993**, *205*, 293–301.
(20) Singh, Y.; Palombo, M.; Sinko, P. J. Recent trends in targeted anticancer prodrug and conjugate design. *Curr. Med. Chem.* **2008**, *15*, 1802–1826.
(21) Stella, B.; Marsaud, V.; Arpicco, S.; Geraud, G.; Cattel, L.; Couvreur, P.; Renoir, J. M. Biological characterization of folic acid-conjugated poly(H2NPEGCA-co-HDCA) nanoparticles in cellular models. *J. Drug Target.* **2007**, *15*, 146–153.
(22) Vasconcelos, V. M.; Sivonen, K.; Evans, W. R.; Carmichael, W. W.; Namikoshi, M. Isolation and Characterization of Microcystins (Heptapeptide Hepatotoxins) from Portuguese Strains of *Microcystis aeruginosa* Kutz Emend Elekin. *Arch. Hydrobiol.* **1995**, *134*, 295–305.
(23) Seglen, P. O. Preparation of isolated rat liver cells. *Methods Cell. Biol.* **1976**, *13*, 29–83.
(24) Mellgren, G.; Vintermyr, O. K.; Døskeland, S. O. Okadaic acid, cAMP, and selected nutrients inhibit hepatocyte proliferation at different stages in G1: modulation of the cAMP effect by phosphatase inhibitors and nutrients. *J. Cell. Physiol.* **1995**, *163*, 232–240.
(25) Herfindal, L.; Oftedal, L.; Selheim, F.; Wahlsten, M.; Sivonen, K.; Døskeland, S. O. A high proportion of Baltic Sea benthic cyanobacterial isolates contain apoptogens able to induce rapid death of isolated rat hepatocytes. *Toxicon* **2005**, *46*, 252–260.
(26) Cohen, P.; Alemany, S.; Hemmings, B. A.; Resink, T. J.; Strålfors, P.; Tung, H. Y. Protein phosphatase-1 and protein phosphatase-2A from rabbit skeletal muscle. *Methods Enzymol.* **1988**, *159*, 390–408.