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Nostocyclopeptide-M1: A Potent, Nontoxic Inhibitor of the Hepatocyte Drug Transporters OATP1B3 and OATP1B1

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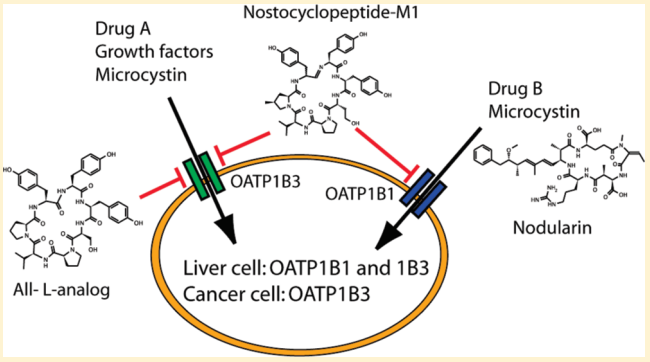
Supporting Information

ABSTRACT: We have isolated a novel cyanobacterial cyclic peptide (nostocyclopeptide M1; Ncp-M1) that blocks the hepatotoxic action of microcystin (MC) and nodularin (Nod). We show here that Ncp-M1 is nontoxic to primary hepatocytes in long-term culture. Ncp-M1 does not affect any known intracellular targets or pathways involved in MC action, like protein phosphatases, CaM-KII, or ROS-dependent cell death effectors. In support of this conclusion Ncp-M1 had no protective effect when microinjected into cells. Rather, the antitoxin effect was solely due to blocked hepatocyte uptake of MC and Nod. The hepatic uptake of MC and Nod is mainly via the closely related organic anion transporters OATP1B1 and OATP1B3, which also mediate hepatic transport of endogenous metabolites and hormones as well as drugs. OATP1B3 is also expressed in some aggressive cancers, where it confers apoptosis resistance. We show that Ncp-M1 inhibits transport through OATP1B3 and OATP1B1 expressed in HEK293 cells. The Ncp-M1 molecule has several nonproteinogenic amino acids and an imino bond, which hamper its synthesis. Moreover, a cyclic all L-amino acid heptapeptide analogue of Ncp-M1 also inhibits the OATP1B1/1B3 transporters, and with higher OATP1B3 preference than Ncp-M1 itself. The nontoxic Ncp-M1 and its synthetic cyclic peptide analogues thus provide new tools to probe the role of OATB1B1/1B3 mediated drug and metabolite transport in liver and cancer cells. They can also serve as scaffolds to design new, exopeptidase resistant OATP1B3-specific modulators.



KEYWORDS: OATP, drug transport, nodularin, microcystin, liver, cancer, cyanobacteria, apoptosis

INTRODUCTION

The organic anion transporter proteins (OATP) transport endogenous hormones and metabolites, as well as xenobiotics, including microbial toxins and drugs, into cells.[1] The OATP transporters are highly conserved in evolution.[1] This may explain why humans are susceptible to microbial toxins presumably designed to counteract primitive organisms. A striking example is microcystin (MC), which can cause acute, hemorrhagic liver damage whether ingested from contaminated water[2] or dialysis fluid.[3] Once MC has entered the hepatocyte it inhibits major Ser/Thr protein phosphatases.[4] This leads to activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII), protein hyperphosphorylation, and reactive oxygen species (ROS) formation.[5] The ensuing apoptosis is accompanied by polarized cell blebbing,[6−10] which may contribute to the disruption of the hepatic microvasculature and fatal bleeding.[2]

The major MC transporter in rodent liver is Oatp1b2,[11,12] whose deletion protects mice against MC-induced liver damage.[12] Oatp1b2 has undergone a recent gene duplication to produce two human orthologues, OATP1B1 and OATP1B3.[13] OATP1B1 and 1B3 are expressed in the sinusoidal parenchymal hepatocyte membrane (see refs 14 and 15 for reviews), where they transport endogenous compounds like bilirubin, as well as xenobiotics, including important drugs.[16,17] Their importance for drug transport is illustrated by the altered drug pharmacokinetics in patients with OATP1B1/1B3 mutations.[18,19] The expression of OATP1B3 is normally limited to the liver, but it has recently been

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found to be expressed in cancers, notably colon carcinomas, where it facilitates the uptake of growth-promoting factors and confers apoptosis resistance.[20−23] A nontoxic inhibitor of OATP1B3 is therefore of interest not only to delay the uptake of potentially hepatotoxic natural compounds or drugs but also to target OATP1B3 in some aggressive cancers.[24]

The present study is based on our recent discovery and structure elucidation of a novel cyanobacterial cyclic peptide, nostocyclopeptide-M1 (Ncp-M1), which prevents MC-induced hepatocyte death.[25] We wanted to elucidate the antitoxin mechanism of Ncp-M1, which could provide a novel inhibitor of one or more of the important mediators of MC-induced hepatocyte death.

We found that Ncp-M1 is nontoxic. It has no effect on any of the intracellular actions of either microcystin (MC) or the closely related and even more potent cyanobacterial toxin nodularin (Nod). In contrast, Ncp-M1 blocks completely the uptake of MC and Nod into isolated rat hepatocytes, as well as into OATP1B1 or OATP1B3 expressing HEK293 cells. Ncp-M1 has unusual amino acids and an imino bond[25] and is therefore not readily synthesized. Its action was mimicked by a synthetic cyclic L-amino acid peptide, which provides a nontoxic, easily available agent for the selective blocking of human OATP1B1/3 and rodent Oatp1b2. The novel cyclic peptides can replace toxic and less specific hepatic transport inhibitors. They provide also an exopeptidase-resistant scaffold for the synthesis of novel modulators of OATP1B1 and the potential drug target OATP1B3.

EXPERIMENTAL SECTION

Materials. Nostocyclopeptide-M1 (Ncp-M1) was purified to homogeneity from a culture of the *Nostoc* cyanobacteria strain XSPORK 13A, as described previously.[25] Ncp-M1 was kept at −80 °C until dissolved in DMSO before use.

The synthetic all L-amino acid cyclic and linear peptide analogues of Ncp-M1 (Tyr-Tyr-Ser-Pro-Val-Pro-Tyr) were from Peptides International (Louisville, KY). Microcystin YR (MC-YR) was labeled with [^{125}I] as described in ref 26 and purified by reversed phase HPLC.[27] Nod was labeled with ^{3}H by reduction with sodium boro[^{3}H]hydride as described in ref 28. ^{14}C-Glycocholic acid was from Amersham Biosciences (Little Chalfont, U.K.), and bromosulfophthalein (BSP) and cholic acid were from Sigma-Aldrich (St. Louis, MO).

Cell Experiments. Rat hepatocytes were isolated by *in vitro* collagenase perfusion.[29,30] Experimental conditions were as described in refs 25 and 31, except for assay of NaN_3 and general cytotoxicity, where the hepatocytes were seeded in collagen-coated 24-well culture dishes.[29] Microinjection of hepatocytes was as described previously.[32] The hepatocyte death (by apoptosis or necrosis) was evaluated by microscopy.[8] Long-term hepatocyte viability was assessed also by the WST-1 colorimetric assay of mitochondrial dehydrogenase activity (Roche Applied Sciences, Mannheim, Germany).

Human embryonic kidney cells (HEK 293T) were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum in 6-well cell culture plates. Enforced expression of green fluorescent protein (GFP) alone or with OATP1B3, OATP1B1 or OATP2B1 was by calcium phosphate transfection of 70% confluent HEK 293T cells for 6 h. After 20 h the cells were detached by gentle trypsin treatment and seeded in 48-well tissue culture plates (30 000 cells/well). After 24 h 80−90% of the cells were GFP positive by fluorescence microscopy, and they were exposed to MC or Nod with or without antitoxin. The effect of MC and Nod was assessed by microscopy of the GFP expressing cells. Vectors for OATP1B1,[33] 1B3[34] and 2B1[35] were a gift from Dietrich Keppler, German Cancer Research Center, Division of Tumor Biochemistry, Heidelberg, Germany.

Microinjection into normal rat kidney fibroblasts (NRK, ATCC no.: CRL-6509) and judgment of their apoptotic response were as described.[36] In brief, the apoptotis induced by MC or Nod in HEK 293T and NRK cells was characterized by cell retraction resulting in a rounded morphology, in contrast to the broadly attached, flattened morphology of the viable cells. The cell rounding was accompanied by polarized bud formation (blebbing). Necrosis occurred in less than 1.5% of the cells and was identified by gross swelling of the cell and rupture of the cellular membrane leading to influx of propidium iodide or trypan blue. The apoptotic cells remained attached to the substrate during the period of observation reported.

Determination of Hepatocyte Uptake of Radiolabeled MC-YR, Nodularin and Glycocholic Acid. Freshly prepared primary rat hepatocytes in suspension (800 000 cells/mL) were incubated in buffer[8] with Ncp-M1, BSP or vehicle for 15 min (37 °C in humidified atmosphere with 6% CO_2) under gyratory shaking. Radiolabeled [^{125}I]MC-YR or [^{3}H]Nod was added and the cells were incubated for another 5 min before rapid separation from the medium by centrifugation through a layer of a mixture of dibutyl and dinonyl phthalate.[37] The cell pellet was lysed in 1 mL of 2% sodium dodecyl sulfate (SDS) for 1 h and the radioactivity determined by scintillation counting (TriCarb 3100TR, Packard Bioscienses, CA). The accumulation of [^{14}C]-glycodeoxycholate was determined similarly, except that the cells were separated from the medium by gentle filtration (0.45 μm HA filters, Millipore Billerica, MA). The cell pellet was washed twice with 2 mL of incubation medium (37 °C), and the filters were processed for scintillation counting. We found that concentrations of BSP above 40−50 μM did not increase inhibitory effect further (not shown), and used 50 μM to obtain complete inhibition of Nod or MC uptake.

Determination of CaM-KII Activity and Protein Phosphorylation. The Ca^{2+}/calmodulin dependent protein kinase II (CaM-KII) in vitro activity was assayed at 30 °C in the absence and presence of 20 μM Ncp-M1 or 10 μM CaM-KII inhibitor KN-93 using syntide-2 (PLARTLSVAGLPGKK) as substrate. The basic incubation mixture contained 15 mM Hepes pH 7.40 with 10 mM $MgCl_2$, 0.5 mM [^{32}P]-γ-ATP (0.1 μCi/50 μL), 1 mM Ca^{2+}, 10 μg mL^{-1} calmodulin, and 1.5 μg mL^{-1} CaM-KII.

The intact cell protein phosphorylation was determined in freshly isolated hepatocytes in suspension preincubated with $^{32}P_i$ and 15 μM Ncp-M1 or vehicle for 15 min before the addition of 200 nM Nod. After another 20 min incubation the cells were precipitated with 7% trichloroacetic acid (TCA), and the pellet was washed with 5% TCA followed by extraction with ether and solubilized in standard Tris buffer with SDS before separation by polyacrylamide gel electrophoresis in linear gradient polyacrylamide gel (7−15%). Phosphoproteins were visualized by exposing the dried gel to Kodak bio max film.

To estimate the effect of Ncp-M1 on in vitro phopshorylation of endogenous liver proteins, a cytosol fraction was prepared by ultracentrifugation (100000*g*, 1 h) of rat liver homogenized in 50 mM Hepes pH 7.2 containing 120 mM KCl, 5 mM EDTA, 3 mM EGTA, 2 mM dithioerythritol, 10 mM benzamidine, 50 μg mL^{-1} soya bean trypsin inhibitor, 50 μg mL^{-1} aprotinin, 7 μg mL^{-1} chymostatin, 6 μg mL^{-1} antipain, 48 μg mL^{-1} leupeptin,

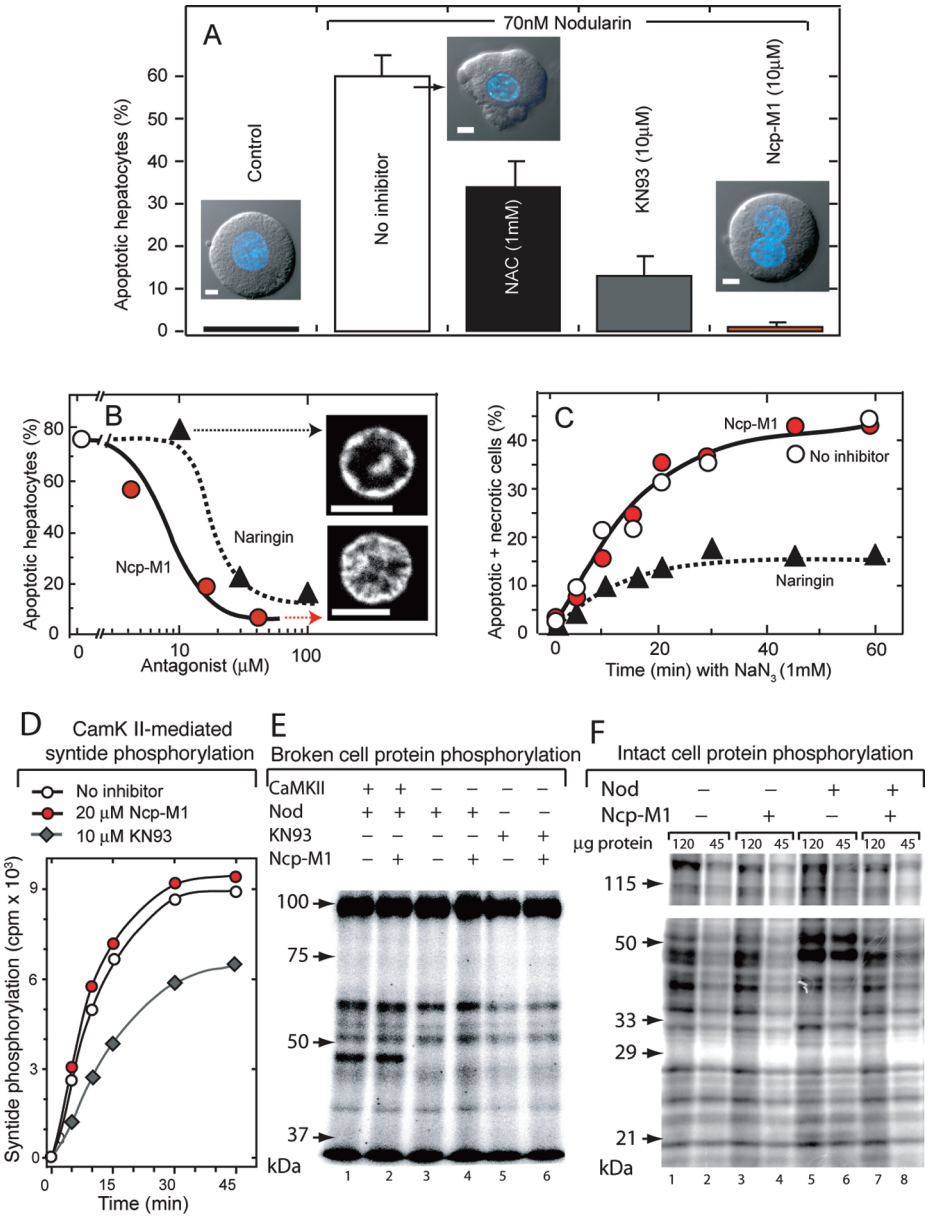


Figure 1. Ncp-M1 does not act on common microcystin (MC) or nodularin (Nod)-antagonist targets. (A) Freshly isolated primary rat hepatocytes in suspension were preincubated for 15 min with vehicle, 1 mM of the ROS scavenger NAC, 10 μM of the CaM-KII inhibitor KN93 or 10 μM Ncp-M1 and assessed for apoptosis after incubation for 1 h with 70 nM Nod. The insets show typical morphology of control hepatocyte, hepatocyte treated with 70 nM Nod alone and hepatocyte treated with 70 nM Nod and 10 μM Ncp-M1. Note the extensive surface budding and hypercondensation of nuclear chromatin in the Nod-treated hepatocyte compared to the control or that treated with Ncp-M1 in addition to Nod. (B) Hepatocytes in suspension were preincubated for 15 min with vehicle or various concentrations of Ncp-M1 or the antioxidant naringin before adding Nod to 70 nM. After coincubation for three hours, the cells were fixed and stained with Hoechst 33342, and the number of cells with hypercondensed chromatin determined. The insets show UV micrographs of hypercondensed, marginal chromatin distribution typical of Nod-induced apoptosis (upper) and normal chromatin distribution (lower). (C) Primary rat hepatocytes cultured for 18 h in monolayer culture were preincubated for 15 min with 40 μM Ncp-M1, 200 μM naringin, or vehicle and assessed for apoptosis after incubation for various periods of time with 1 mM NaN_3. (D) The phosphorylation of syntide-2 by CaM-KII in the presence or absence of 50 μM Ncp-M1 or 10 μM KN93. (E) Rat liver lysates were incubated with Nod, [^{32}P]-γ-ATP and Ca^{2+}/calmodulin alone or with added CaM-KII to study the effect of Ncp-M1 on endogenous and exogenous CaM-KII-dependent phosphorylation. Note the lack of effect of Ncp-M1 in either case. The extent of CaM-KII independent phosphorylation (in the presence of 10 μM KN-93) is shown for comparison. (F) Ncp-M1 blocks Nod-induced protein hyperphosphorylation in freshly isolated hepatocytes. Hepatocytes were preincubated with $^{32}P_i$ and Ncp-M1 or vehicle for 15 min before stimulation with Nod for another 20 min incubation, and the proteins were precipitated, separated by SDS−PAGE, and exposed to autoradiography to detect ^{32}P-labeled proteins. Each sample was loaded with 120 or 45 μg protein. Data in panel A are mean ± SEM of 3−5 experiments; panels B and C are typical of 3 separate experiments. The size bars represent 5 μm.

and 14 μg mL^{-1} pepstatin. The phosphorylation of cytosol proteins was studied in the presence or absence of the following: 1 μg mL^{-1} CaM-KII (Upstate, USA), 1 μM Nod, 20 μM Ncp-M1, or 10 μM KN-93. All samples were incubated at 25 °C in 50 mM Hepes pH 7.2 with 20 nM protein kinase A inhibitor, 5 mM $MnCl_2$, 50 μM [^{32}P]-γ-ATP (0.2 μCi/50 μL), 50 μg mL^{-1}

SBTI, 1 mM Ca^{2+}, 10 μg mL^{-1} calmodulin and 5 mg mL^{-1} cytosol protein. The phosphate incorporation increased rectilinearly with time for about 45 s, and the assay duration was 30 s.

Statistical Analysis and Estimation of Apparent K_m and K_i Values. The data for accumulated radiolabeled Nod and glycocholic acid into hepatocytes in the absence and presence of uptake inhibitor were analyzed for significance by Wilcoxon signed-rank and Student's *t* tests, using the SPSS statistical software (Chicago, IL). Competetiveness was confirmed by linear regression as previously described[38] (see Supporting Information). Estimations of the apparent K_m and K_i values for MC/Nod uptake as deduced from HEK 293 cell apoptosis were done using SigmaPlot software (Systat Software inc. San Jose, CA). A Hill equation (eq 1) was used to correct for sensitivity in the death process not accounted for in the Michaelis−Menten kinetic description of the drug transport. The apparent K_m and K_i values were determined by nonlinear regression analysis.

$$V = \frac{V_{max} S^h}{({}^{app}K_m^I)^h + S^h}: \quad (1)$$

V is velocity, i.e. percent apoptosis at a given time, S is transporter substrate concentration (MC or Nod), ${}^{app}K_m^I$ is the apparent K_m when an inhibitor is present, and h is the Hill constant.

RESULTS

The Ncp-M1 Antitoxin Did Not Antagonize Any of the Known Intracellular Microcystin Targets. The cyanobacterial hepatotoxins microcystin (MC) and nodularin (Nod) inhibit major protein phosphatases, leading to unchecked autophosporylation and activity of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase II (CaM-KII). This stimulates the production of reactive oxygen species (ROS) causing rapid, partially caspase dependent cell death.[4,5,7−9] The cyanobacterial cyclic peptide Ncp-M1 is an antitoxin against Nod and MC.[25] We show here that it protects primary hepatocytes against Nod more potently than the CaM-KII inhibitor KN-93 (Figure 1 A) and the antioxidants/free radical scavengers N-acetylcysteine (Figure 1 A) and naringin (Figure 1 B). Ncp-M1 does not displace labeled MC from its primary target protein phosphatase or modulate the phosphatase activity,[25] indicating that it must inhibit the MC/Nod action upstream and/or downstream of the phosphatase inhibition step.

We found that Ncp-M1, unlike naringin, was unable to protect against ROS forming death inducers, like H_2O_2 (not shown) or NaN_3 (Figure 1 C). Ncp-M1 was therefore unlikely to reduce oxidation in the cell compartment relevant for MC-induced apoptosis.

We tested next if Ncp-M1 could inhibit CaM-KII. Ncp-M1did not affect the CaM-KII catalyzed phosphorylation of a standard peptide substrate (Figure 1D) or the CaM-KII mediated phosphorylation of endogenous proteins in a broken hepatocyte cytosol extract (Figure 1 E). Still, as shown in Figure 1 F, Ncp-M1 blocked completely the Nod-induced intact hepatocyte protein hyperphosphorylation (lanes 7 and 8 vs lanes 5 and 6) without affecting the basal protein phosphorylation pattern (lanes 3 and 4). We could thus not exclude that Ncp-M1 interfered with the CaM-KII activation through a mechanism not operating for purified CaM-KII or in broken cell preparations. In order to rule out this and other potential intracellular antitoxic effects of Ncp-M1, we studied whether plated primary

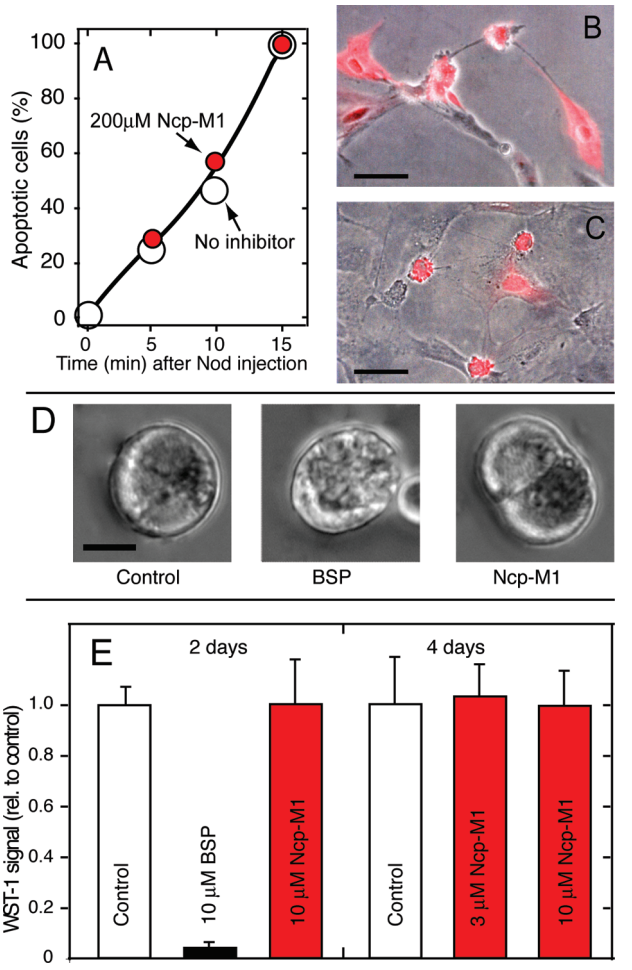


Figure 2. Ncp-M1 does not protect against nodularin (Nod) inside cells and is nontoxic. (A) NRK cells were microinjected with Nod (70 μM) alone (open circles) or with Ncp-M1 (300 μM, red filled circles), and the percentage of apoptotic cells was determined 5, 10, and 15 min after injection. Tetramethyl rhodamine isothiocyanate (TRITC)-conjugated dextran was coinjected to recognize the injected cells. (B, C) phase contrast and fluorescence composite micrographs of NRK-cells 10 min after microinjection with Nod (B) or a mixture of Nod and Ncp-M1 (C). Note the marked contraction and irregular outline of the Nod-injected cells, irrespective of the presence of Ncp-M1 in the injectate. The size bars are 20 μm. (D) Freshly isolated rat hepatocytes were incubated with 20 μM BSP for 1 h or 30 μM Ncp-M1 for three hours, and phase-contrast micrographs were recorded. Note the uneven appearance of the BSP-treated cells compared to the smooth surface of the control and Ncp-M1-treated cells. (E) Freshly prepared primary rat hepatocytes were treated with the indicated concentrations of Ncp-M1 and BSP, starting 3 h after seeding, and viability assessed by the WST-1 assay. All data are average with SEM of 3−6 separate experiments.

hepatocytes microinjected with Ncp-M1 still responded to extracellularly added Nod or MC, and if extracellular Ncp-M1 protected against microinjected Nod. We found no effect of Ncp-M1 in either case (not shown), suggesting that Ncp-M1 acted extracellularly. To probe this further Ncp-M1 and Nod were coinjected into NRK cells, which are highly sensitive to microinjected Nod.[5,31] Cells injected with Nod alone or together with excess Ncp-M1 had similar time course of death induction, and similar death-associated morphology, as judged by cell contraction and polarized membrane budding (Figure 2 A−C). We conclude that Ncp-M1 has no effect on ROS-mediated death,

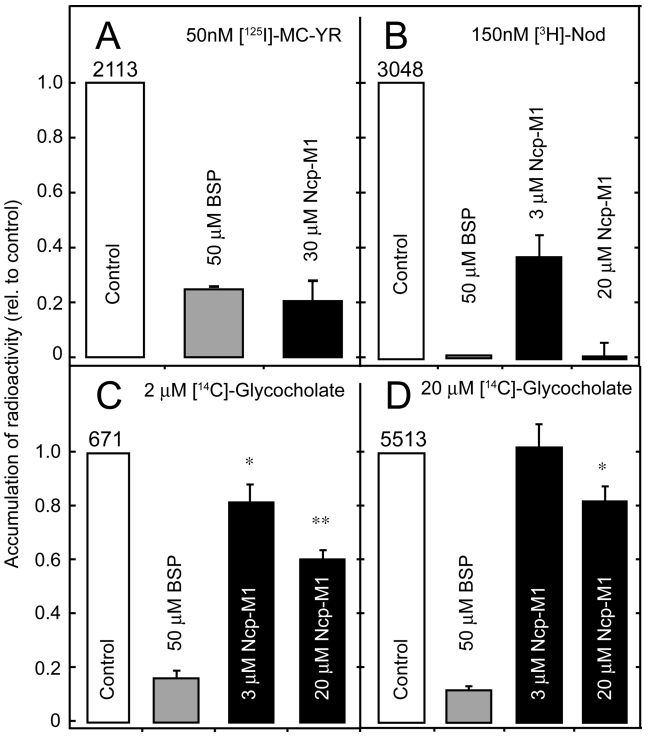


Figure 3. Ncp-M1 blocks the entry of nodularin (Nod) and microcystin (MC) into primary hepatocytes. Freshly isolated hepatocytes were preincubated with bromosulfophthalein (BSP), Ncp-M1 or vehicle for 15 min, incubated further with radiolabeled [^{125}I]-MC-YR (A), [^{3}H]-Nod (B) or [^{14}C]-glycocholic acid (C, D) for 5 min, and the accumulated intracellular radioactivity was determined (see Experimental Section for details). The total radioactivity counts for the control experiments are given. The asterisks (C, D) indicate significant difference from control at level $p < 0.05$ (*) and $p < 0.005$ (**).

gross basal protein phosphorylation, or CaM-KII activity, and antagonizes the MC/Nod toxicity through an extracellular action.

Ncp-M1 Is a Nontoxic Inhibitor of MC/Nod Transport into Hepatocytes. The complete lack of effect on major intracellular death-associated processes suggested that Ncp-M1 could be rather inert and with few side effects. This notion was supported by experiments on freshly isolated hepatocytes. In short-term experiments (up to 4 h) on hepatocytes in suspension no untoward effect of Ncp-M1 was noted up to the highest concentration (0.2 mM) tested (Figure 2 D; data not shown). This is in contrast to the widely used hepatocyte transporter blocker bromosulfophthalein (BSP), which already after 1 h incubation at 20 μM deformed the normally smooth surface membrane and caused nuclear condensation (Figure 2 D; data not shown).

For evaluation of longer-term toxicity the hepatocytes were plated in serum free medium and incubated with BSP or Ncp-M1. The cells were assayed for viability and morphological intactness[8] after 48 and 96 h of incubation. While cells incubated with 10 μM BSP were completely necrotic and had no discernible WST-1 signal after 48 h, the cells incubated with 10 μM Ncp-M1 were morphologically intact (not shown) and had full WST-1 signal (Figure 2 E).

That Ncp-M1 had to be present extracellularly to protect against MC and Nod suggested that it interfered with their uptake. Although Ncp-M1 was far less toxic than the hepatocyte MC transporter blocker BSP,[39] it was as efficient as BSP to inhibit

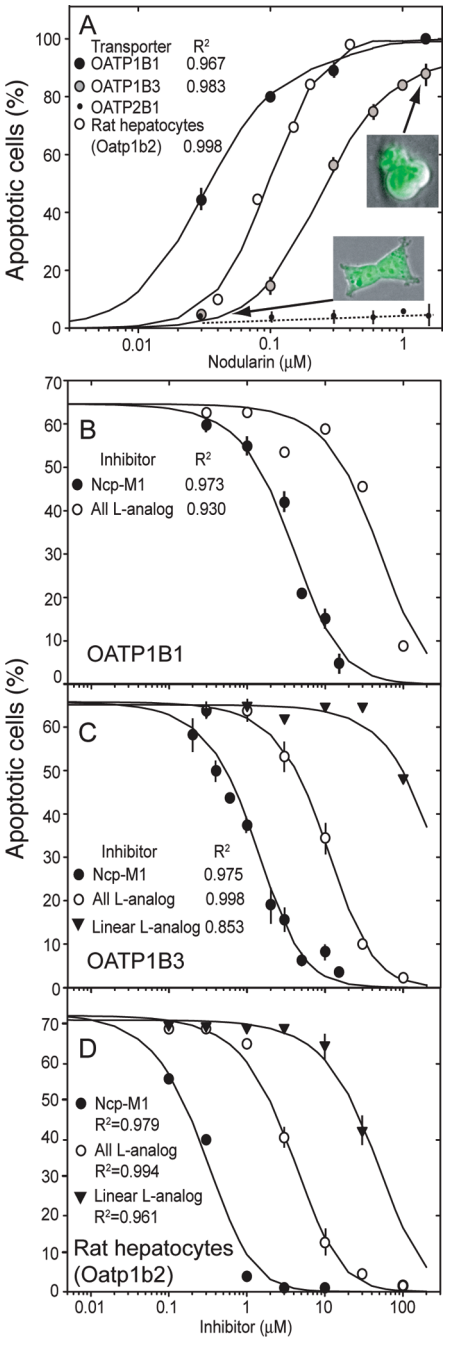
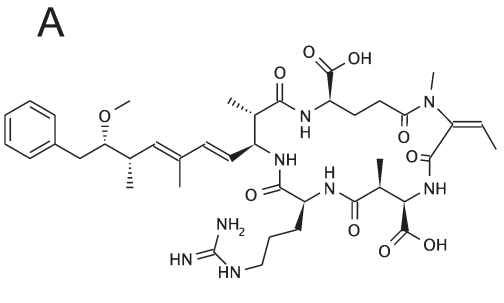
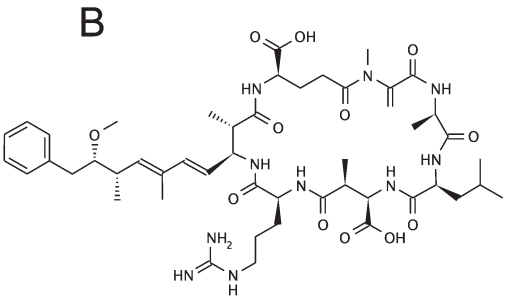


Figure 4. Ncp-M1 and synthetic Ncp-M1 analogues as inhibitors of nodularin (Nod)-induced apoptosis in OATP1B1 and 1B3-expressing HEK293 cells and in primary hepatocytes. (A) HEK-293T cells co-transfected with GFP and OATP1B1, -1B3 or -2B1, and freshly isolated primary rat hepatocytes were assessed for apoptosis after 90 min incubation with various concentrations of Nod. The micrographs show OATP1B3-transfected HEK cells before (lower) and after (upper) exposure to Nod. (B) OATP1B1-transfected HEK cells were preincubated (15 min) with Ncp-M1 or a cyclic Ncp-M1 peptide mimic, and thereafter with 0.1 μM Nod for 90 min before assessment of apoptosis. (C) As for panel B except that the cells were transfected with OATP1B3, and the concentration of Nod was 0.6 μM. (D) Primary rat hepatocytes incubated with 70 nM Nod. The data are average values with SEM of 3−6 experiments. The curve estimations are calculated based on the model described in the Experimental Section. The R^2 values are given for the estimations.



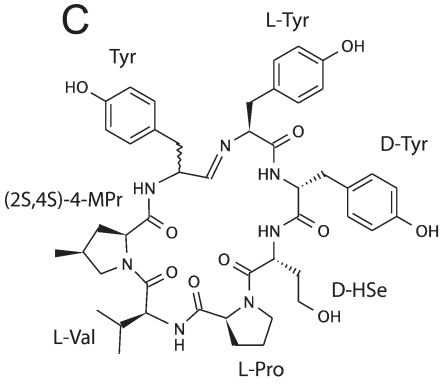
Nodularin

Transporter	Apparent Km (μM)
OATP1B1	0.032 ± 0.004
OATP1B3	0.23 ± 0.014
Rat hepatocytes (Oatp1b2)	0.098 ± 0.010



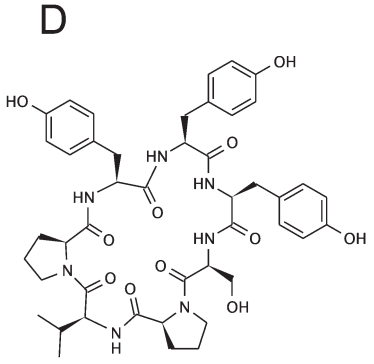
Microcystin LR

Transporter	Apparent Km (μM)
OATP1B1	0.97 ± 0.011
OATP1B3	1.03 ± 0.085
Rat hepatocytes (Oatp1b2)	0.27 ± 0.038



Ncp-M1

Transporter	Apparent Ki (μM)
OATP1B1	1.45 ± 0.21
OATP1B3	0.63 ± 0.08
Rat hepatocytes (Oatp1b2)	0.35 ± 0.09



All L-amino acid analog of Ncp-M1

Transporter	Apparent Ki (μM)
OATP1B1	17 ± 4.48
OATP1B3	5.0 ± 0.28
Rat hepatocytes (Oatp1b2)	4.6 ± 0.47

Figure 5. Structures of the cyclic peptides used, and their apparent K_m or K_i values with SEM for OATP1B1 and -1B3, and for rat hepatocytes (Oatb1b2). The values were determined by nonlinear regression from the data in Figure 4, as described in the Experimental Section.

the hepatocyte uptake of either [^{125}I]-MC-YR or [^{3}H]-Nod (Figure 3 A,B). The potency of Ncp-M1 as Nod uptake inhibitor (Figure 3 B) was sufficient to explain its inhibition of Nod-induced death (Figure 1A), supporting that the only antitoxic mechanism of Ncp-M1 was by antagonizing the MC/Nod uptake.

Several of the hepatocyte transporters carry bile acids, and they are all inhibited by BSP,[1] as demonstrated also in the present study, in which BSP (50 μM) counteracted completely the hepatocyte uptake of labeled glycocholate (Figure 3 C,D). In contrast, Ncp-M1 was only a partial antagonist of glycocholate uptake, suggesting specificity for only a subset of the bile acid transporters. In conclusion, Ncp-M1 is stable and nontoxic and inhibits the MC uptake with higher specificity than BSP.

Characterization of the Ncp-M1 Inhibition of the OATP1B/1B3 Transporters. The MC uptake in rodent hepatocytes is mainly mediated by Oatp1b2,[11] whose two human orthologues, OATP1B1 and OATP1B3,[13] allow MC entry when expressed in frog oocytes[11] or HEK 293 cells.[40] We found that HEK 293T cells with enforced expression of OATP1B1 or OATP1B3 became about as sensitive as rat hepatocytes toward Nod-induced apoptosis (Figure 4A). Cells expressing the BSP-sensitive hepatocyte sinusoidal membrane transporter OATP2B1 were, as expected,[15] insensitive (Figure 4A). The Nod-induced apoptosis in cells expressing OATP1B1 (Figure 4B) or OATP1B3 (Figure 4C) could be completely counteracted by Ncp-M1. This provides direct evidence that Ncp-M1 inhibits the two human MC-carrying transporters.

We tested next a synthetic cyclic Ncp-M1 mimetic in which the unusual Ncp-M1 amino acids (2*S*,4*S*)-4-methyl-proline, D-homoserine, and D-tyrosine[25] were replaced by L-proline, L-serine, and L-tyrosine, and the imino-bond replaced by an ordinary peptide bond (see Figure 5 for structures). It inhibited completely the uptake of Nod through OATP1B1/B3 and the rat hepatocyte transporter Oatp1b2, albeit with lower potency than Ncp-M1 (Figure 4 B−D). Like Ncp-M1, the analogue was nontoxic both for primary rat hepatocytes and for HEK 293T cells with and without OATP1B1/B3 overexpression (not shown). A linear version of the same peptide was considerably less potent (Figure 4 C,D), indicating that the cyclic structure is important for high-affinity transporter recognition.

The Nod and MC concentration-dependent HEK 293T cell death is a direct biophysical measure not of toxin uptake alone but also of alterations in intracellular signaling. Still, the death

response (Figure 4 and Figure S1 in the Supporting Information) obeyed kinetics that allowed reproducible and inherently coherent estimates of the apparent K_m for MC and Nod uptake, as well as the apparent K_i of Ncp-M1 and its synthetic cyclic peptide mimetic. The estimated values are listed in Figure 5. Ncp-M1 and MC-LR had similar affinity for OATP1B1 (apparent K_i = 1.5 μM, apparent K_m = 1 μM), OATP1B3 (apparent K_i = 0.63 μM, apparent K_m = 1 μM) and the rat hepatocyte (Oatp1b2) transporter (apparent K_i = 0.35 μM, apparent K_m = 0.27 μM). This does not mean that toxic cyclic peptides and Ncp-M1 cannot discriminate between the transporters, since Nod had much higher affinity for the OATP1B1 transporter (apparent K_m = 0.032 μM) than Ncp-M1 (apparent K_i = 1.5 μM).

We conclude that a stable, nontoxic, and inexpensive Ncp-M1 mimetic can inhibit transport through both OATP1B1/B3 and their rodent orthologue Oatp1b2 and therefore represents a novel useful tool to block transport through these transporters. It can also serve as a scaffold for the production of second-generation transporter modulators.

DISCUSSION

The newly discovered cyclic peptide antitoxin Ncp-M1[25] protects hepatocytes against the death-inducing effect of MC and Nod. We show here that Ncp-M1 blocks the hepatocyte uptake of MC and Nod as well as the uptake of Nod and MC into HEK 293 cells with enforced expression of either of the two human hepatocyte MC transporters OATP1B1 and OATP1B3. Ncp-M1 did not interfere with intracellular steps of MC action, such as protein phosphatase inhibition, CaM-KII activation, ROS production, or actin rearrangement.[8,10] The apparent intracellular inertness of Ncp-M1 distinguishes it from other OATP1B1/1B3 inhibitors. Common inhibitors are usually biologically active endogenous substances (e.g., steroid hormones, bilirubin or bile salts), toxins (e.g., phalloidin), biologically active xenobiotics (e.g., ursolic acid), or drugs (e.g., fluvastatin and methotrexate).[15,24]

Ncp-M1 showed no apparent inherent toxicity, as evidenced by the normal morphology, growth and mitochondrial dehydrogenase activity (Figure 2 D,E) of hepatocytes incubated with it for 96 h. Ncp-M1 has also other advantages like high solubility in physiological saline and exopeptidase resistance due to its cyclic peptide nature. The much used hepatocyte transporter blocker BSP[15] was under similar conditions highly toxic for hepatocytes (Figure 2 D,E). Ncp-M1 appears also to be a more selective than BSP, since it, unlike BSP, is unable to block all the bile acid transporters carrying glycocholic acid (Figure 3). This suggests that Ncp-M1, unlike BSP, is selective to a subset of transporters, namely, those able to transport cyclic peptides.

We propose therefore Ncp-M1 as a novel inhibitor of the human hepatocyte OATP1B1/1B3 and rodent hepatocyte Oatp1b2. Ncp-M1 is apparently as potent to inhibit OATP1B3 transport as the best inhibitors recently found by screening the 1100 drugs of the Prestwick library.[24] In addition it has preference for inhibition of OATP1B3. The synthetic all L-amino acid cyclic peptide Ncp-M1 analogue, which also inhibits OATP1B1/1B3, provides an inexpensive alternative to Ncp-M1. That it lacks the inherently unstable imino bond of Ncp-M1 is another advantage. The synthetic peptide can easily be further modified to further enhance its specificity and potency and provide even higher protease resistance. The synthetic all L-amino analogue has higher OATP1B3 preference than Ncp-M1 itself (Figure 5D), suggesting that it can be a useful scaffold for the synthesis of novel nontoxic inhibitors with further improved affinity for this transporter. A selective inhibitor is needed to discriminate between the transport through the related OATP1B1 and OATP1B3 in the liver itself. It has also the potential to spare the uptake of many endogenous compounds taken up redundantly by the liver through OATP1B1 and OATP1B3, while depriving aggressive tumors of the growth factors apparently obtained by ectopic expression of the OATP1B3 transporter.[23]

ASSOCIATED CONTENT

Supporting Information. Description of the competetive relationship between the toxins (MC or Nod) and the inhibitors (Ncp-M1 and its synthetic analogue). This material is available free of charge via the Internet at http://pubs.acs.org.

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