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Selectivity and Potency of Microcystin Congeners against OATP1B1 and OATP1B3 Expressing Cancer Cells

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Abstract

Microcystins are potent phosphatase inhibitors and cellular toxins. They require active transport by OATP1B1 and OATP1B3 transporters for uptake into human cells, and the high expression of these transporters in the liver accounts for their selective hepatic toxicity. Several human tumors have been shown to have high levels of expression of OATP1B3 but not OATP1B1, the main transporter in liver cells. We hypothesized that microcystin variants could be isolated that are transported preferentially by OATP1B3 relative to OATP1B1 to advance as anticancer agents with clinically tolerable hepatic toxicity. Microcystin variants have been isolated and tested for cytoxicity in cancer cells stably transfected with OATP1B1 and OATP1B3 transporters. Microcystin variants with cytotoxic OATP1B1/OATP1B3 IC50 ratios that ranged between 0.2 and 32 were found, representing a 150-fold range in transporter selectivity. As microcystin structure has a significant impact on transporter selectivity, it is potentially possible to develop analogs with even more pronounced OATP1B3 selectivity and thus enable their development as anticancer drugs.

Introduction

Microcystins (MCs) are cyclic heptapeptides produced by several cyanobacterial genera such as Microcystis, Oscillatoria, Planktothrix, Nostoc, and Anabaena. They can be considered to be among the best studied cyanobacterial secondary metabolites [1–4]. The common structural feature of microcystins is a polypeptide synthase derived amino acid with the acronym “Adda”, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid, which is also one of the two mandatory substructures for the potent protein serine/threonine phosphatase (PP) 1 and PP2A inhibition observed for the microcystins [5–9]. Not only is the biosynthesis of these compounds by polypeptide synthases and non-ribosomal peptide synthetases remarkably well understood [10–12], but also more than 90 individual members of this chemically highly diverse compound family have been described in the scientific literature to date (Fig. 1) [13–56]. Especially prone to variation are the L-amino acids situated at positions 2 and 4 of the MC backbone. Related compounds are described in the scientific literature to date (Fig. 1) [13–56].

Being potent protein serine/threonine phosphatase inhibitors, microcystins and nodularins have a profound effect on cell signaling and cytoskeleton maintenance, leading to the death of affected cells [61,62]. However, the relatively large and amphiphilic MCs are unable to cross cell membranes by passive diffusion. Instead, they rely on active uptake by cells. Three members of the organic anion transporting polypeptides (OATP) family are able to mediate this uptake of MCs, namely OATP1B1, 1B3 and 1A2 [63,64]. OATP1B1 and OATP1B3 are the most efficient microcystin transporters, and as in healthy humans both transporters are exclusively found to be expressed in liver tissue [64,65], microcystins and nodularins are known to cause extensive liver damage [66–68]. Thus microcystins became infamous as hepatotoxins causing harm to humans and cattle when these compounds accumulated in sources of drinking water during algal water bloom times [69,70]. Inhibitors of these OATP transporters ameliorate the hepatotoxicity of microcystins and nodularins [68,71,72]. In contrast to OATP1B1, which is expressed in hepatocytes throughout the liver lobe, OATP1B3 localization is restricted around the central vein [64].

OATPs are currently in discussion as targets for cancer therapy [73–76]. Most interestingly, OATP1B3, but not OATP1B1, has been found to be functionally expressed in a number of cancer tissues, especially colon tumors, but also breast tumors, lung tumors, pancreatic and hepatocellular tumors [75,77–79]. As differential toxicity of natural microcystin variants on cell lines expressing either OATP1B1 or OATP1B3 has been observed [78,80], these findings raised the question whether microcystins might be suitable as leads for drug substances against these cancer types, and if there are microcystins among the more than 90 known variants that are selectively transported by OATP1B3 relative to OATP1B1. Selectivity that favors OATP1B3 over...
OATP1B1 should lead to a decreased hepatic clearance and increased uptake of MCs in OATP1B3-expressing tumors, creating a therapeutic window of the respective compound by decreasing the hepatic clearance rate and toxicity. MCs are interesting as novel lead structures because they have a mode of action not yet used but currently discussed for cancer therapy (phosphatase inhibition) [81–83], and in contrast to the majority of currently available anticancer drugs, they need active transport into cells and thus spare all tissues not expressing the mentioned OATPs.

We have isolated various microcystin congeners from cyanobacteria of the genera *Microcystis*, *Planktothrix*, and *Nodularia* to test the hypothesis whether different transporter selectivities might be attainable. As tools for selectivity testing, cervical cancer HeLa cells and colon cancer RKO cells stably transfected with expression vectors for OATP1B1 and 1B3 have been used. In the present manuscript, the results of the testing of isolated MCs on these cell lines are described. The determined IC50 values as presented in Table 2. The determined IC50 values as presented in Table 2.

**Results and Discussion**

The amino acid (AA) compositions of the tested MCs are summarized in Table 1. The IC50 values of all isolated MCs against OATP1B1- and OATP1B3-selective adda (ADDA) are summarized in order of count, in position 4 Arg, Tyr, Trp, Hty, and Har are found. In addition to 22 MC variants, nodularin (23) as a cyclic Adda containing pentapeptide as well as okadaic acid (24) as a structurally not MC-related PP inhibitor have been tested.

The results are shown in Table 2.

Interestingly, marked differences for both potency and selectivity of the individual MC congeners could be observed.

**Selectivity**

While substitution of d-iso-β-MeAsp3 for d-iso-Asp3 (e.g., compounds 1/2 and 13/14) seems not to have a significant influence on neither potency nor selectivity, the influence of the presence of either Mdha7 or (E)-Dhb7 (e.g., 3/4, 5/6, 8/9, and 14/15) is ambiguous: While in the case of 3/4 and 5/6 substitution of Mdha7 for (E)-Dhb7 lead to a profound increase in selectivity for OATP1B3 over OATP1B1, this effect was not observed for 8/9 and 14/15. As the six least OATP1B3 selective compounds (1, 2, 3, 5, 7, 23) all feature Mdha7 or Mdhb7 while (E)-Dhb7 is found in 3 of the 6 most OATP1B3 selective compounds (4, 10, 12, 15, 16, 18), it is likely that (E)-Dhb7 has an influence in selectivity but is not the only structural feature conferring selectivity.

Indeed, especially the amino acid residues at positions 2 and 4 of the MC core structure seem to be important for both potency and selectivity. While 80% of the most OATP1B1 selective compounds feature Leu at position 2, this amino acid is completely absent in the most OATP1B3 selective compounds. However, Leu is also found in position 2 of several congeners that are weakly OATP1B3 selective. Arg in position 2 seems to induce OATP1B3 selectivity, while Arg in position 4 does not have any influence on selectivity. This is obvious with compounds 13 and 16, where exchange of the amino acids in positions 2 and 4 has a huge impact on selectivity. Arg in combination with the aromatic amino acids Phe, Tyr, and Hty are prominent monomers found in OATP1B3 selective compounds.

**Potency**

Potency of MC induced cell death in the test system used for this study has two facets. On one hand, toxicity of MC congeners depends on the extent of their PP inhibition [85]. On the other hand, toxicity of MC congeners depends on the extent of their PP inhibition [85]. On the other hand, toxicity of MC congeners depends on the extent of their PP inhibition [85].
hand, potency is depending on transporting capacity of the respective OATP. These two effects have not been distinguished in the present study. While some previous studies suggest that in vitro and in vivo toxicity are related to enzyme inhibition rather than transport [78,85], other reports show comparable PP inhibition of different microcystin congeners, implying that differences in transport contribute to in vivo toxicity [80,86].

Not surprisingly, 22, featuring O-methylated β-iso-Glu⁶, is one of the least potent compounds. Modifications of the free carboxylic acid of Ghu are likely to lead to a loss of efficiency due to loss of interaction with a positively charged arginine in the active center of the protein phosphatases [7], and Ghu(OMe) containing congeners have been found to inhibit PP1 to a much lesser extent than analogous Ghu containing MCs [87] and have lower in vivo toxicity [26]. Interestingly, also 10, 11 and 21 displayed weaker toxicity than most other congeners. While 10 inhibits PP2A about twenty times weaker compared with 1 [78,80,85,86], its toxicity in the present assay is about 500 times lower. This indicates that in the case of 10 (and also its analog 11), it is probable that toxicity is not only a matter of PP inhibition efficiency, but also of OATP transporting capability, as MC-RR is a dication under physiological conditions and thus less likely to be transported across cell membranes by anion transporters. In general, cytotoxicity in our assay does not correlate with protein phosphatase inhibition potency as described in the literature for compounds 1, 2, 6, 7, 8, 9, 10, 13, 18, 19, 20, 23 [56,78,80,85,86]. Our data thus indicate that differing transport efficiencies have a higher impact on in vivo toxicity than differing protein phosphatase inhibition potency.

Interestingly, the most potent MCs, with IC₅₀ values in the sub-nanomolar range (5, 6, 7, 8, 17, 18, 19), all feature combinations of either Leu² and Tyr/Phe/Trp⁴ or Tyr/Hty² and Tyr/Hty⁴. Arg is absent from the most potent congeners. The least potent congeners with IC₅₀ values > 100 nM (10, 11, 21, 22) all feature either Tyr² and Arg/Har⁴ or Arg² and Arg⁴. Presence of Arg in general lowers the potency, again supporting the hypothesis that the positively charged Arg residues hamper transport efficiency in addition to reducing PP2A inhibition, and thus lower in vivo toxicity.

**Summary and Conclusions**

In our screening of 23 normal MC variants, we have found several MCs with an IC₅₀ in the low nanomolar range and with transporter selectivity that favors OATP1B3 over OATP1B1 by a factor of up to thirty. While the presence of Arg in general lowers the potency of MC cytotoxicity, its presence in position 2 of the MC core structure also seems to be important for OATP1B3 selectivity, implying that this transporter might be more tolerant to the cationic nature of Arg under physiological conditions than OATP1B1. Furthermore, the presence of the slightly acidic aromatic amino acids Tyr or Hty in position 4 in addition to Arg in position 2 seems beneficial for OATP1B3 selectivity.

---

**Table 1. Structures of the tested MC congeners originating from Microcystis aeruginosa, Planktothrix rubescens, and Nodularia sp.**

<table>
<thead>
<tr>
<th>MC Congener</th>
<th>AA 1</th>
<th>AA 2</th>
<th>AA 3</th>
<th>AA 4</th>
<th>AA 5</th>
<th>AA 6</th>
<th>AA 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ᵃ MC-LR</td>
<td>Ala</td>
<td>Leu</td>
<td>MeAsp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>2ᵃ [D-Asp³]MC-LR</td>
<td>Ala</td>
<td>Leu</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>3ᵃ [D-Asp³]MC-HiIRR</td>
<td>Ala</td>
<td>Hil</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>4ᵇ [D-Asp³,β-Dhb⁷]MC-HiIRR</td>
<td>Ala</td>
<td>Hil</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>5ᵇ MC-LY</td>
<td>Ala</td>
<td>Leu</td>
<td>MeAsp</td>
<td>Tyr</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>6ᵇ [D-Asp³,β-Dhb⁷]MC-LY</td>
<td>Ala</td>
<td>Leu</td>
<td>Asp</td>
<td>Tyr</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>7ᵇ LF</td>
<td>Ala</td>
<td>Leu</td>
<td>MeAsp</td>
<td>Phe</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>8ᵇ LW</td>
<td>Ala</td>
<td>Leu</td>
<td>MeAsp</td>
<td>Trp</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>9ᵇ [D-Asp³,β-Dhb⁷]MC-LW</td>
<td>Ala</td>
<td>Leu</td>
<td>Asp</td>
<td>Trp</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>10ᵇ RR</td>
<td>Ala</td>
<td>Arg</td>
<td>MeAsp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>11ᵇ [D-Asp³,β-Dhb⁷]MC-RR</td>
<td>Ala</td>
<td>Arg</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>12ᵇ MC-RF</td>
<td>Ala</td>
<td>Arg</td>
<td>MeAsp</td>
<td>Phe</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>13ᵇ YR</td>
<td>Ala</td>
<td>Tyr</td>
<td>MeAsp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>14ᵇ [D-Asp³]MC-YR</td>
<td>Ala</td>
<td>Tyr</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>15ᵇ [D-Asp³,β-Dhb⁷]MC-YR</td>
<td>Ala</td>
<td>Tyr</td>
<td>Asp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>16ᵇ MC-RY</td>
<td>Ala</td>
<td>Arg</td>
<td>MeAsp</td>
<td>Tyr</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>17ᵇ MC-YY</td>
<td>Ala</td>
<td>Tyr</td>
<td>MeAsp</td>
<td>Tyr</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
</tr>
<tr>
<td>18ᵇ [D-Asp³,β-Dhb⁷]MC-HtyY</td>
<td>Ala</td>
<td>Hty</td>
<td>Asp</td>
<td>Tyr</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>19ᵇ [D-Asp³,β-Dhb⁷]MC-HtyHty</td>
<td>Ala</td>
<td>Hty</td>
<td>Asp</td>
<td>Hty</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>20ᵇ [D-Asp³,β-Dhb⁷]MC-HtyW</td>
<td>Ala</td>
<td>Hty</td>
<td>Asp</td>
<td>Trp</td>
<td>Adda</td>
<td>Glu</td>
<td>Dhb</td>
</tr>
<tr>
<td>21ᵇ [D-Asp³,β-Mser⁷]MC-YHar</td>
<td>Ala</td>
<td>Tyr</td>
<td>Asp</td>
<td>Har</td>
<td>Adda</td>
<td>Glu</td>
<td>Mser</td>
</tr>
<tr>
<td>22ᵇ [D-Glu⁶OMe]MC-YR</td>
<td>Ala</td>
<td>Tyr</td>
<td>MeAsp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu(OMe)</td>
<td>Mdha</td>
</tr>
<tr>
<td>23ᵇ Nodularin</td>
<td>Ala</td>
<td>MeAsp</td>
<td>Arg</td>
<td>Adda</td>
<td>Glu</td>
<td>Mdha</td>
<td></td>
</tr>
</tbody>
</table>

Hil homoisoleucine, Hty homotyrosine, MeAsp β-methylaspartic acid, Har homoarginine, Mdha N-methyl dehydroalanine, Dhb dehydrobutyric acid, Mser N-methyl serine, Mdhb N-methyl dehydrobutyric acid.
doi:10.1371/journal.pone.0091476.t001
Further studies are needed to discriminate whether the observed differences in potency are due to differing PP inhibition or different transport capability of the OATP. However, earlier findings suggest that \textit{in vivo} toxicity is mainly depending on OATP transport kinetics than on differences in PP inhibition [80,86].

Although the deduced structure activity relationships are ambiguous in parts, the structural features observed for OATP1B3 selectivity can be condensed to the at present most likely selective general structure cyclo(- D-Ala$_{1}$-L-Arg$_{2}$-D-(Me)Asp$_{3}$-L-aromatic amino acid$_{4}$-Adda$_{5}$-D-Glu$_{6}$-\(E\)-Dhb$_{7}$). This general structure can be used as a starting point for generating novel compounds e.g. by precursor feeding or biocombinatorial strategies.

A challenge of using MCs as drug leads still remains: Even with selective MC variants, liver toxicity still might be a significant challenge, making it necessary to generate MC variants that could be metabolically detoxified by healthy liver cells and/or efficiently effluxed into the bile, and thus take advantage of hepatic detoxification and clearance mechanisms that would not be found in tumors.

Materials and Methods

\textbf{Cyanobacterial Material}

Several \textit{Microcystis aeruginosa} and \textit{Planktothrix rubescens} strains as well as a \textit{Nodularia} strain have been used to produce the studied microcystin congeners: 2 and 3, \textit{Microcystis aeruginosa} CBT 265; 4 and 15, \textit{Planktothrix rubescens} CBT 310; 5, 16, and 17, \textit{Microcystis aeruginosa} CBT 850, 6, 9, 18, 19, and 20, \textit{Planktothrix rubescens} CBT 862, 11, \textit{Planktothrix rubescens} CBT 329, 12, \textit{Microcystis aeruginosa} CBT 861, 14, 21, and 22, \textit{Microcystis aeruginosa} CBT 480, 23, \textit{Nodularia} sp. CBT 786. The strains were classified on the basis of PCR analysis and sequencing of various marker genes as well as their morphology, and have been deposited in the Cyano Biotech (CBT) culture collection under the accession numbers indicated above (Cyano Biotech, Berlin, Germany). The strains were cultivated in BG11 medium [88] at 20\textdegree C under continuous light (60-80 m$\text{mol m}^{-2}$ s$^{-1}$) in 20 L scale photobioreactors and harvested semi-continuously over a period of several weeks.

\textbf{Isolation of microcystins}

Cyanobacteria strains were screened by HPLC-DAD/IT-TOF-MS (Kinetex C$_{18}$, 2.6 $\mu$m, 100 x 3 mm column, phenomenex, Torrance, USA) using a linear scouting gradient of aqueous CH$_{3}$CN (5 to 80\% within 25 min at 0.6 ml/ min; 0.025\% v/v TFA) to confirm MC presence by their characteristic UV spectrum as well as the characteristic tandem MS fragment ion at m/z 375 [52,89]. Selected positive strains have been cultivated in BG11 medium [88] at 20\textdegree C under continuous light (60-80 $\mu$mol m$^{-2}$ s$^{-1}$) in 20 L scale photobioreactors. After biomass harvest and freeze-drying, the biomasses were resuspended in 50\% 

<table>
<thead>
<tr>
<th>IC$_{50}$ (nM)</th>
<th>Ratio IC$_{50}$ B1/B3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HeLa Control</td>
</tr>
<tr>
<td>1</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<tr>
<td>23</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>24</td>
<td>7.8</td>
</tr>
</tbody>
</table>

*Transiently transfected cell lines; results were reported previously [78].

doi:10.1371/journal.pone.0091476.t002

Table 2. IC$_{50}$ values of MC congeners (1-23) and okadaic acid (24) against stably transfected HeLa or RKO cell lines expressing either OATP1B1 or OATP1B3.
McOH (v/v), treated with an ultrasonication rod (Bandelin, Berlin, Germany) and extracted on a shaker for 30 min. After centrifugation the biomasses were subsequently extracted using 90% McOH (v/v). The 50% McOH and 80% McOH extracts of the respective biomasses were combined and dried in vacuo. The crude extracts were fractionated using water-methanol step gradients on C18 cartridges on a VersaFlash system (supelco, Bellefonte, USA), and the fractions containing MCs (monitored by HPLC) were dried in vacuo. After reconstitution, these fractions were subjected to semi-preparative HPLC. For a detailed description of the isolation procedures see File S1. Microcystins RR, LW, and LF and okadaic acid were obtained from Axxora, LLC (San Diego, CA), and microcystins LR and YR were obtained from Sigma-Aldrich (St. Louis, MO).

Structure elucidation

The structures of the isolated MCs have been determined by high-resolution tandem mass spectrometry [51,52,84], and confirmed by one- and two-dimensional NMR spectroscopy. Samples for NMR spectroscopy were dissolved in 600 μL d6-DMSO. NMR spectra were recorded at 600 MHz (1H frequency) on a Bruker AV-III spectrometer using cryogenically cooled 5 mm TCI-triple resonance probes equipped with one-axis self-shielded gradients. DQF-COSY spectra [90] were recorded using 2048×512 complex data points using 8 scans. Spectra were referenced indirectly to tetramethylsilane via the residual signals of 2048 gradients. DQF-COSY spectra [90] were recorded using TCI-triple resonance probes equipped with one-axis self-shielded gradients. DQF-COSY spectra [90] were recorded using 2048×512 complex data points using 8 scans. Spectra were referenced indirectly to tetramethylsilane via the residual signals of d6-DMSO (2.5 ppm for 1H). Tandem MS data have been acquired using an HPLC coupled to an IT-TOF mass spectrometer (Shimadzu Europe GmbH, Duisburg, Germany) with electrospray ionization in positive mode and were evaluated using the vendor’s software LCMSSolution version 3.60.361 with Formula Predictor version 1.13. For the calculation of sum formulae, the monoisotopic mass averaged from at least three scans has been used. The compounds were separated on a Kinetex C18 column (2.6 μm, 100×3 mm, phenomenex, Torrance, USA) using a gradient ranging from 5 to 80% CH3CN in water over 25 min (0.1% formic acid added as modifier). Precursor ions corresponding to [M+H]+ were isolated in the ion trap, fragmented by collision induced dissociation (CID) using argon as collision gas (collision energy set to 150%, collision gas to 100%, and q(Frequency) to 45.0 kHz), and separated in the TOF analyzer. MS/MS scans were averaged and converted to the mzXML format using the vendor’s software and evaluated using the software mMass (calculated fragment ions: M, a, b, c, and z; modifications: -H2O, -NH3, -CO, defined, combinations; matching with a tolerance of 0.01 Da) [91]. Annotated tandem HRMS and 1H-NMR spectra of all isolated compounds can be found in File S2.

Cell lines and cytotoxicity assays

Primary hepatocytes are not suitable for studying microcystin toxicity and transport, as OATP transporters are downregulated within hours upon placing cells in culture [92]. Thus HeLa and RKO cell lines were obtained from ATCC and transfected with expression vectors for OATP1B1 and OATP1B3. Individual clones were isolated by a limiting dilution, and isolated clones showing high levels of expression by PCR were further characterized as previously described [72,93]. Individual HeLa and RKO clones were selected for further study by demonstration of increased uptake [1H]-BQ123, a substrate for both transporters. Further, the OATP1B3 clones demonstrated increased uptake of [1H]-CCK-8, a specific substrate for OATP1B3 but not OATP1B1, while the OATP1B1 clones did not show uptake of [1H]-CCK-8. In addition, RKO and HeLa cells stably transfected with an empty expression vector were used as controls. For cytotoxicity assays, the cells were plated in triplicate in 96-well microtiter plates in a medium containing 5% fetal bovine serum at densities of 1000 cells/well. After 24 hours, medium containing MC structural variants was added to the cells. After another 3 days, cell survival was determined with a sulforhodamine-based assay as we have previously described [93–95]. As the dose response curves for the apoptosis causing microcystins are steep and go down to 0 within 72 hours, the sulforhodamine assay is indicative of cell death [72,93]. The IC50 was calculated from the dose response curve as the concentration of drug that produced a 50% decrease in the mean absorbance compared to the untreated wells and reported as the average of at least three independent determinations performed in triplicate using Prism software.

Supporting information

File S1 Details on the Isolation of the Microcystin Congeners. (PDF)
File S2 Annotated tandem HRMS and 1H-NMR spectra of all isolated compounds. Raw NMR and MS data of these compounds are available free of charge via the Internet at http://dx.doi.org/10.6084/m9.figshare.380755. (PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: THJN JAM. Performed the experiments: THJN AD MSH. Analyzed the data: THJN AD JAM. Wrote the paper: THJN JAM.

References

4. Pearson L, Mihali T, Moffitt M, Kellmann R, Neilan B (2010) On the toxicity and transport, as OATP transporters are downregulated within hours upon placing cells in culture [92]. Thus HeLa and RKO cell lines were obtained from ATCC and transfected with expression vectors for OATP1B1 and OATP1B3. Individual clones were isolated by a limiting dilution, and isolated clones showing high levels of expression by PCR were further characterized as previously described [72,93]. Individual HeLa and RKO clones were selected for further study by demonstration of increased uptake [1H]-BQ123, a substrate for both transporters. Further, the OATP1B3 clones demonstrated increased uptake of [1H]-CCK-8, a specific substrate for OATP1B3 but not OATP1B1, while the OATP1B1 clones did not show uptake of [1H]-CCK-8. In addition, RKO and HeLa cells stably transfected with an empty expression vector were used as controls. For cytotoxicity assays, the cells were plated in triplicate in 96-well microtiter plates in a medium containing 5% fetal bovine serum at densities of 1000 cells/well. After 24 hours, medium containing MC structural variants was added to the cells. After another 3 days, cell survival was determined with a sulforhodamine-based assay as we have previously described [93–95]. As the dose response curves for the apoptosis causing microcystins are steep and go down to 0 within 72 hours, the sulforhodamine assay is indicative of cell death [72,93]. The IC50 was calculated from the dose response curve as the concentration of drug that produced a 50% decrease in the mean absorbance compared to the untreated wells and reported as the average of at least three independent determinations performed in triplicate using Prism software.

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References

phosphatases 1 and 2A from both mammals and higher plants. FEBS 364: 187–192.


