Comparative effects of nodularin and microcystin-LR in zebrafish:

1. Uptake by organic anion transporting polypeptide Oatp1d1 (Slco1d1)

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Abstract

Microcystin-LR (MC-LR) and nodularin are hepatotoxins produced by several cyanobacterial species. Their toxicity is based on active cellular uptake and subsequent inhibition of protein phosphatases PP1/2A, leading to hyperphosphorylation and cell death. To date, uptake of MC-LR and nodularin in fish is poorly understood. Here, we investigated the role of the organic anion transporting polypeptide Oatp1d1 in zebrafish (drOatp1d1, Slco1d1) in cellular uptake in zebrafish. We stably transfected CHO and HEK293 cell lines expressing drOatp1d1. In both transfectants, uptake of MC-LR and nodularin was demonstrated by competitive inhibition of uptake with the fluorescent substrate lucifer yellow. Direct uptake of MC-LR and nodularin was demonstrated by immunostaining, and indirectly by the high cytotoxicity in stable transfectants. By means of a synthesized fluorescent labeled MC-LR derivate, direct uptake was further confirmed in HEK293 cells expressing drOatp1d1. Additionally, uptake and toxicity was investigated in the permanent zebrafish liver cell line ZFL. These cells had only a low relative abundance of *drOatp1d1*, *drOatp2b1* and *drOatp1f* transcripts, which correlated with the lack of MC-LR induced cytotoxicity and transcriptional changes of genes indicative of endoplasmic reticulum stress, a known effect of this toxin. Our study demonstrates that drOatp1d1 functions as an uptake transporter for both MC-LR and nodularin in zebrafish.

Key Words: Cyanobacterial toxin; microcystin; nodularin; organic anion uptake transporting polypeptide; Oatp1d1; uptake transporters; cellular effects; zebrafish

1. Introduction

Many cyanobacteria produce toxic secondary metabolites that are released into the surrounding water during a cyanobacterial bloom. This may pose a hazard to fish populations (Wiegand and Pflugmacher, 2005), livestock (Naegeli et al., 1997) and humans (Azevedo et al., 2002; Ueno et al., 1996). Only about 40 of 2000 cyanobacterial species were identified as being toxic (Hitzfeld et al., 2000), but some of the toxins belong to the most harmful natural products. For example, microcystin-LR (MC-LR) is among the most toxic ones, and it is one of the 91 substances for which a provisional guideline in drinking water has been set (WHO, 2011).

Microcystins (MCs) are the most abundant and most studied cyanotoxins (Sivonen and Jones, 1999). All MCs are cyclic heptapeptides containing the non-proteinogenic (D-amino-acids), unusual amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) (Neilan et al., 1999). Two variable amino acids result in a number of different congeners of varying toxicities. So far, more than 80 different MCs were described. MC-LR containing leucine and arginine as variable amino acids was shown to be the most toxic one (Monks et al., 2007).

Like MCs, nodularins are cyclic cyanotoxins containing Adda, but the ring is built up by 5 amino acids with only one variable amino acid. Both nodularins and microcystins have the following amino acids in common, Adda, D-Glu, D-MeAsp and L-Arg. The toxicity of nodularins appears to depend on a cyclic structure. Nine naturally occurring isoforms of nodularin-R have been reported to date (Chen et al., 2013).

The toxicity of MCs and nodularin is based on the inhibition of serine/threonine phosphatases of the PP1 and PP2 families (MacKintosh et al., 1990). MCs were shown to have tumor promoting activity and higher rates of liver cancer in China could be due to drinking water contaminated by these toxins (Svircev et al., 2010; Ueno et al., 1996). Tumor initiating activity was also reported for nodularins (Chen et al., 2013). Recently, molecular effects of MC-LR including endoplasmic reticulum stress (ER-stress) and inflammatory effects were identified in human hepatoma cells (Christen et al., 2013).

MCs and nodularins require active transport into human and animal cells. Organic anion-transporting polypeptides (OATPS/Oatps) were identified to mediate uptake into the cell (Boaru et al., 2006). Oatps belong to the superfamily of solute carriers (SLC) and these membrane-bound transporters

mediate the uptake of endogenous compounds, such as bile salts or hormones, but also a wide variety of xenobiotics (Popovic et al., 2013; Roth et al., 2012). The involvement of Oatps in uptake of MC-LR was shown in mammals (Feurstein et al., 2009; Fischer et al., 2005), in a lower vertebrate (Meier-Abt et al., 2007) and in rainbow trout (Steiner et al., 2014). However, not all Oatps are capable to transport MC-LR and affinities of a specific Oatp can vary for different MC variants (Fischer et al., 2005). The high expression of Oatps in liver seems to be correlated with the hepatotoxicity of MCs (Boaru et al., 2006). Knock-down mice lacking expression of hepatic mOatp1b2 were resistant to MC-LR induced hepatotoxicity (Lu et al., 2008). In humans, rats and mice 36 members of the OATP/Oatp family have been identified. Also in zebrafish 17 Oatp members have been detected in various tissues (Popovic et al., 2010). In the liver, Oatp1d1 and Oatp2b1 were the main expressed Oatps.

The aim of our current work was to evaluate in zebrafish, whether zebrafish Oatp1d1 (drOatp1d1) is involved in mediating the uptake of MC-LR and nodularin. To this end, we stably expressed drOatp1d in human embryonic kidney (HEK293) cells and Chinese hamster ovary cells (CHO). By different techniques, we demonstrated that drOatp1d1 transports MC-LR and nodularin, which resulted in cytotoxicity. In an associated paper, we assessed specific effects of MC-LR and nodularin to the liver and eleuthero-embryos of zebrafish (Faltermann et al. 2015b).

2. Material and Methods

2.1 Chemicals and reagents

Microcystin-LR (MC-LR) and nodularin were purchased from Enzo Life Science (Lausen, Switzerland). Lucifer yellow, probenecid, progesterone and MTT formazan powder were purchased from Sigma Aldrich (Buchs, Switzerland). All cell culture media were purchased from Invitrogen (LuBioscience, Luzern, Switzerland).

2.2 Cell culture

Zebrafish (*Danio rerio*) liver cells (ZFL cells) were maintained at 28 °C in a humidified incubator. Culture medium was prepared with 50 % Leibowitz-15, 35 % Dulbecco's modified Eagle's medium with 4.5 g/L glucose (LuBioScience) and 15 % Ham's F12 (LuBioScience). The medium was

supplemented with 1.78 mM NaHCO₃, 15 mM HEPES, 0.01 mg/ml insulin, and 50 ng/ml epidermal growth factor. Human embryonic kidney cells (HEK293) and Chinese hamster ovary cells (CHO cells) were cultured in DMEM/F12 Nutrient Mix supplemented with 10 % FBS, and 1 % penicillin-streptomycin (LuBioScience) and maintained in a humidified incubator (5 % CO₂, 37 °C).

2.3 Stable transfection of HEK293 and CHO cells with zebrafish drOatp1d1

To study cellular uptake of MC-LR, two different stable transfectants were developed. These included a Chinese hamster ovary (CHO) and a human embryonic kidney (HEK293) cell line, both stably expressing drOatp1d1. The drOatp1d1 expression construct was kindly provided by Marta Popovic (Rudjer Boskovic Institute, Croatia). The transfection of plasmids into CHO and HEK293 cells was performed using Lipofectamine2000 (LuBioScience) and 1.6 µg of DNA according to the manufacturer's protocol. The geneticin resistance gene on the plasmid allowed the selection of positive colonies by growing cells on culture medium containing 600 µg L⁻¹ geneticin for 3 weeks. Expression of the transporter in the colonies was determined by means of a fluorescent substrate, lucifer yellow. Colonies that showed fluorescence were picked with pads (Cloning discs, Sigma Aldrich), and after further culturing, cells were sorted by flow cytometry. Only cells with high intracellular fluorescence (due to high expression of drOatp1d1) were used for further experiments.

2.4 Uptake of microcystin-LR and nodularin (drOatp1d1 uptake assay)

The uptake assay is based on the uptake of fluorescent lucifer yellow, a substrate for drOatp1d1 (Popovic et al., 2013). The principle of this assay lies in the competitive transport of lucifer yellow and the compound in question (here MC-LR and nodularin) by drOatp1d1. Lucifer yellow uptake is lowered in the presence of a second substrate that is competing with transporter uptake, thus resulting in a decrease of intracellular fluorescence intensity. Both stable transfectants and wild-type cells (60,000 cells) were seeded on 96-well plates (black clear bottom, BD), containing culture media with 8 mM sodium butyrate to enhance Oatp1d1 expression (Eckhardt et al., 1999). After 24 h, the medium was replaced by 100 µL transport medium (HBSS buffer, Invitrogen) and cells were incubated at 37 °C for 15 min. Afterwards, buffer was replaced by HBSS supplemented with 50 µmol L⁻¹ lucifer yellow and different concentrations up to 500 µmol L⁻¹ of test substance (MC-LR, nodularin) for 5 min. The assay was established using 5

$\mu\text{mol L}^{-1}$ lucifer yellow and up to $50 \mu\text{mol L}^{-1}$ progesterone as known drOatp1d1 substrate. The incubation time was 15 minutes. The reaction was stopped by addition of $100 \mu\text{L}$ ice-cold HBSS containing 2.5 mmol L^{-1} of the Oatp inhibitor probenecid. Subsequently, cells were washed three times with the same solution (HBSS, 2.5 mmol L^{-1} probenecid). Before fluorescence was measured on a plate reader (FlexStation, Molecular Devices) the medium was replaced by HBSS/probenecid/brilliant black to quench extracellular fluorescence (Macioszek and Kononowicz, 2004). Wild-type cells were used as blanks for measurements. The uptake is presented in % fluorescence compared to control cells. Fluorescence (measured as relative fluorescence units) of control cells that were incubated with $50 \mu\text{mol L}^{-1}$ LY only was set to 100 %. Based on this, fluorescence of cells incubated with $50 \mu\text{M}$ LY and the test compounds was transformed into % fluorescent compared to control cells and this was given as % uptake compared to control cells.

2.5 Determination of uptake of microcystin-LR and nodularin by immunoblotting

For intracellular detection of MC-LR and nodularin, CHO wild-type cells, CHO cells overexpressing drOatp1d1, and ZFL cells were seeded on small Petri dishes 24 h before exposure. Exposure to $0.05 \mu\text{mol L}^{-1}$ MC-LR and nodularin and the solvent control (0.001 % DMSO) was performed in HBSS buffer for 24 h. Subsequently, cells were lysed in lysis buffer (1 mM PMSF, 50 mM , L^{-1} Tris, 150 mmol L^{-1} NaCl_2 , 15 mmol L^{-1} EDTA, 0.1 % Triton) and proteins separated by centrifugation ($10,000 \text{ g}$ for 5 min). Protein concentration in the supernatant was determined by a BCA assay kit (Thermo Scientific). $20 \mu\text{g}$ of protein of each sample was first separated by SDS polyacrylamide gel electrophoresis and then blotted onto a nitrocellulose membrane (Amersham Protran $0.45 \mu\text{mol L}^{-1}$ NC; GE Healthcare Life Science). After blocking with 1 % BSA for 1 h, the membrane was incubated with the primary antibody (anti-Adda, Enzo lifescience) in a dilution of 1:100 at 4°C overnight, and subsequently with a fluorescent secondary antibody (DyLight 488, horse anti mouse IgG, Axxora) in a dilution of 1:80 000 for 1 h at room temperature. Fluorescence of the antibody was visualized using a FLA9000 apparatus (FUJIFILM).

2.6 Uptake of fluorescent labeled MC-LR

A fluorescent derivate of MC-LR was synthesized by covalently binding the red fluorophor Texas-red to MC-LR (MC-LR-Texas-red) (Grundler et al., 2015). The uptake of MC-LR-Texas-red was determined using drOatp1d1 expressing HEK293 cells in comparison to HEK293 wild-type cells. The cells were exposed in 96-well plates (black, clear bottom) for 30 min to 2.5 $\mu\text{mol L}^{-1}$ MC-LR-Texas-red in the transport medium.

2.7 Effects of MC-LR and nodularin to cell morphology

Toxic actions of MC-LR and nodularin were determined by observing changes in the morphology and appearance of exposed cells in comparison to unexposed drOatp1d1-expressing cells, and wild-type cells that do not express uptake transporters. To determine effects on morphology, HEK293 and CHO cells stably expressing drOatp1d1 were exposed to 1 $\mu\text{mol L}^{-1}$ of MC-LR and 5 μM nodularin (both containing 0.5 % DMSO), respectively, for 30 min or 15 min in HBSS buffer. Images were taken using an Olympus CKX41 microscope.

2.8 Oatp expression in zebrafish liver cell line (ZFL) and detection of transcriptional changes

To demonstrate that cellular uptake of MC-LR and nodularin had taken place, we determined alterations in mRNA abundance induced by the cyanotoxins as a determinant of their effects. One of the mostly and commonly applied key markers for ER stress detection is the glucose regulated protein 78, also called bip, that dissociates from - and thereby activates - ER stress transducer molecules, when unfolded proteins accumulate in the ER. This results in transcriptional activation of various target genes, including *bip*. Its transcriptional activation is commonly used as a marker for ER stress and the onset of the unfolded protein response (Baumeister et al., 2005).

Total RNA was extracted from exposed and control cells using TRIZOL reagent according to the manufacturer`s instruction. Purity and amount of RNA was measured spectrophotometrically, and RNA integrity was analysed by gel electrophoresis. Single strand cDNA synthesis and RT-qPCR was performed as described before (Faltermann et al., 2014). Primers for Oatps are listed in Popovic et al., (2010), all others are listed in the Table 1. Quantification of *drOatp1d1* transcripts was performed according to Simon (2003), using the relative quantification method:

$MNE = E_{ref} \Delta C_{tref} / \text{mean } E_{target} \Delta C_{target}; \text{mean}$

Expression was normalized to the housekeeping gene *RPL13a* expression. The expression of this gene was constant over the exposure period.

For transcription analysis of the ER-stress marker gene *bip* in response to MC-LR and nodularin exposure, CHO wild-type cells, CHO cells expressing drOatp1d1, and ZFL cells were seeded on small Petri dishes 24 h before exposure. CHO wild-type cells and drOatp1d1 expressing CHO cells were exposed to 0.05 $\mu\text{mol L}^{-1}$ MC-LR, and 0.05 $\mu\text{mol L}^{-1}$ nodularin. The solvent control received 0.001 % DMSO. Exposure was performed in HBSS buffer for 24 h. ZFL cells were exposed to 5 and 35 $\mu\text{mol L}^{-1}$ MC-LR for 48 h. A solvent control (0.001 % DMSO) was also included. Each treatment was performed in triplicate.

2.9 Determination of cytotoxicity by MTT assay in MC-LR treated ZFL cells

ZFL cells were seeded in a 96-well plate (100,000 cells per well). 24 h after seeding, cells were exposed to 3, 6, 12, 25, 50, and 100 $\mu\text{mol L}^{-1}$ MC-LR in ZFL media without FBS for 24 h or 48 h. Included were also solvent controls with 0.5 % DMSO. The experiments consisted of a total of 3 to 8 replicates per group. Following exposure, media were replaced by fresh ZFL media without FBS containing 0.5 mg mL^{-1} MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide in PBS). Incubation was stopped after 3 h and media were replaced by 200 μL DMSO. After 10 min on a shaker, 25 μL Soerensen buffer (50 mmol L^{-1} glycine, 50 mmol L^{-1} NaCl, pH 10.5) was added 5 min later, absorption was measured at 570 nm in a microtiter plate reader (Tecan, Stäfa, Switzerland).

2.10 Uptake of lucifer yellow into ZFL cells

Cells were incubated with lucifer yellow under the same conditions as in the MTT assay. Cells were seeded onto a 96-well plate with black, clear bottom (100,000 cells / well). 24 h after seeding, cells were incubated with 6.125, 12.5, 25, 50 and 100 $\mu\text{mol L}^{-1}$ lucifer yellow in ZFL medium without FBS for 48 h. Before fluorescence measurement, cells were washed three times with HBSS/probenecid and HBSS/probenecid/brilliant black was added.

2.11 Statistics

Uptake into drOatp1d1 expressing cells was statistically analysed using One-way ANOVA with Dunnett's post-hoc test. Significance level was set at $p \leq 0.05$.

3. Results

3.1 Uptake of microcystin-LR and nodularin by drOatp1d1

Uptake kinetics of lucifer yellow was investigated by measuring the intracellular fluorescence in CHO cells expressing drOatp1d1 (Figure 1a). The K_m value was 42.19 ± 6.07 , confirming previous data performed with transient transfections (Popovic et al., 2010). Due to competition for the binding site of the uptake transporter, co-incubation of lucifer yellow and progesterone, a known drOatp1d1 substrate (Popovic et al., 2010), resulted in a decreased uptake of the fluorophore (Figure 1b). Co-incubation of $50 \mu\text{mol L}^{-1}$ lucifer yellow and both, MC-LR and nodularin, led to concentration-dependent decrease of intracellular fluorescence in CHO cells, stably expressing drOatp1d1, as compared to control cells (Figure 2a). In case of MC-LR and nodularin, intracellular fluorescence decreased to 32 % and 58 %, respectively. Differences in fluorescence intensities between cells incubated with lucifer yellow only and co-exposed with MC-LR and nodularin, respectively, were also observed visually under the fluorescence microscope. Representative fluorescence images of the cells are shown in Figure 2b. When incubated with lucifer yellow only, fluorescence was much higher, and cells appeared much brighter compared to co-exposure with cyanobacterial toxins. These experiments indicate that drOatp1d1 is a transporter for both MC-LR and nodularin.

3.2 Demonstration of uptake by immunoblotting

We provided a direct demonstration of MC-LR uptake by Western blotting using an antibody directed to microcystin. No antibody staining is detectable in control cells, or CHO wild-type cells exposed to $0.05 \mu\text{mol L}^{-1}$ MC-LR. In contrast, MC-LR is clearly detectable in cells stably expressing drOatp1d1, as demonstrated by strong antibody staining (Figure 3).

3.3 Uptake of fluorescent labeled MC-LR

Direct uptake was furthermore demonstrated by means of fluorescent MC-LR-Texas-red. Intracellular fluorescence was clearly observed when HEK293 cells overexpressing drOatp1d1 were exposed for 30 min to 1 $\mu\text{mol L}^{-1}$ MC-LR-Texas-red. Uptake was observed directly under the fluorescence microscope (Figure 4). In wild-type cells lacking drOatp1d expression, no fluorescence was detected (data not shown).

3.4 Indirect demonstration of MC-LR and nodularin uptake by changes in cell morphology and gene transcripts

An indirect method to evaluate, whether drOatp1d1 mediates the uptake of MC-LR and nodularin, is the identification of effects in CHO and HEK293 cells expressing drOatp1d1. To this end, we compared the morphology of HEK293 and CHO cells expressing drOatp1d1 after exposure to MC-LR and nodularin in comparison to wild-type cells. Strong morphological changes occurred in stable HEK293 transfectants exposed to 1 $\mu\text{mol L}^{-1}$ MC-LR for 30 minutes. Furthermore, CHO cells stably expressing drOatp1d also showed strong morphological changes already after 15 minutes, when exposed to 5 $\mu\text{mol L}^{-1}$ nodularin (Figure 5a). In response to both treatments, cells became round in shape and detached very rapidly from the bottom of the plate, which represents a clear sign of cytotoxicity. In contrast, no changes in the morphology of wild-type HEK293 and CHO cells were visible when exposed to the same concentrations of MC-LR and nodularin as the transfectants. They looked identical as control cells exposed to medium only. Therefore, stable expression of drOatp1d1 in HEK293 and CHO cells resulted in high sensitivity towards toxic action of MC-LR and nodularin, which was lacking in wild-type cells.

Furthermore, we compared transcriptional changes that indicate ER-stress induced by MC-LR and nodularin in CHO wild-type cells and CHO cells expressing drOatp1d1. MC-LR induces ER-stress in human hepatoma cells (Christen et al., 2013), which results in transcriptional induction of key marker genes. One of these key markers is the *glucose regulated protein 78*, also called *bip* the product of which dissociates from - and thereby activates - ER stress transducer molecules, when unfolded proteins accumulate in the ER. This results in transcriptional activation of various target genes, including *bip*. Its transcriptional activation is commonly used as a marker for ER stress and the onset of the unfolded

protein response (Baumeister et al., 2005). In CHO cells expressing drOatp1d1, transcription of *bip* was significantly altered after exposure to MC-LR and nodularin compared to control, but not in CHO wild-type cells (Figure 5b). Significant induction occurred following exposure to 0.05 $\mu\text{mol L}^{-1}$ MC-LR and nodularin for 24 h.

3.5 Expression of drOatps and uptake of lucifer yellow in ZFL cell line

DrOatp1d1 is highly expressed in the liver of zebrafish (Popovic et al., 2010), but it is not known, whether it is expressed in a permanent fish liver cell line. Thus, we analysed the expression of three *Oatps* in ZFL cells. Transcripts of *drOatp1d1*, and particularly *drOatp2b1*, were detected and transcripts of *drOatp1f* showed only very low abundance (Figure 6a). This is similar as in adult zebrafish liver (Popovic et al., 2010). The functionality of the Oatp-mediated transport was assessed by incubation of ZFL cells with lucifer yellow, which resulted in a concentration-dependent uptake of the fluorophore after 48 h (Figure 6b). However, the measured fluorescence was extremely low, suggesting only minimal transport activity.

3.6 Cytotoxicity of MC-LR to ZFL cells and ER-stress induction

We further tested, whether the occurrence of *Oatp* transcripts was correlated with uptake of cyanobacterial toxins in ZFL cells and whether this would lead to cytotoxicity and induction of ER-stress. Exposure of ZFL cells to MC-LR at concentrations up to 100 $\mu\text{mol L}^{-1}$ did not result in cytotoxicity. Figure 7 shows that cell viability was not affected, in contrast to the positive control (10 % DMSO).

To analyse for ER-stress induction as a toxic reaction of the cell, we determined the transcript of *bip* upon exposure to MC-LR. There was no induction of the transcript at 5 and 35 $\mu\text{mol L}^{-1}$ MC-LR (Figure 8). In principle, ZFL cells are able to induce ER-stress, as shown by thapsigargin (Figure 8). Therefore, the lack of ER-stress induction by MC-LR was probably due to the lack of sufficient uptake.

4. Discussion

Due to their large size and structure, MC-LR and nodularin do not readily penetrate through the cell membrane by diffusion, but require multi-specific Oatps for uptake. In our study, we provide several direct and indirect lines of evidence that drOatp1d1 is able to mediate the uptake of MC-LR. We also show for the first time, that drOatp1d1 is an uptake transporter for nodularin. Despite the demonstration of the importance of drOatp1d1, this does not preclude that additional Oatps or other uptake transporters might also be involved in the uptake of these cyanobacterial toxins.

Oatps can serve as transporters for endogenous and exogenous compounds. Thus far, expression and function of Oatps in fish have only rarely been investigated. Several Oatps of zebrafish have been annotated in phylogenetic analyses (Popovic et al., 2010, 2013). One of the two major Oatps mostly expressed in the liver is drOap1d1 and its functional property has been characterized. Typical substrates are bile salts and steroid hormones, but also a wide range of pharmaceuticals and environmental pollutants (Popovic et al., 2013). Thus, this transporter plays a crucial role in the xenobiotic defense system, similar to OATP1A, OATP1B1 and OATP1B3 in humans (Roth et al., 2012). For this reason, our study was focused on drOatp1d1 for the uptake of cyanobacterial toxins.

drOatp1d1 uptake assay. Our first evidence for the uptake of MC-LR and nodularin by drOatp1d1 resulted from transport assays in CHO and HEK293 cells overexpressing drOatp1d1. Similar to Popovic et al., (2013), in which transient transfections were used, we employed stable transfectants to determine the competitive uptake of lucifer yellow and MC-LR and nodularin, respectively. Both toxins resulted in a concentration-dependent decrease in lucifer yellow uptake due to substrate competition (Figure 2a). Decreased uptake upon co-exposure was also observed under the fluorescence microscope (Figure 2b). This indicates that both MC-LR and nodularin are substrates of drOatp1d1. However, inhibition of uptake was lower than with progesterone, which suggests that the affinities of MC-LR and nodularin to drOatp1d1 were much lower. Data on MC-LR confirms previous experiments in little skate (Meier-Abt et al., 2007) and rainbow trout (Steiner et al., 2014), where the involvement of Oatp1d1 for uptake of MC-LR was shown. In addition, our experiments indicate that drOatp1d1 is also a transporter for nodularin. Collectively, this transporter has a wide variety of substrates, both endogenous and exogenous ones. This makes drOatp1d1 a very important and versatile uptake transporter in liver cells.

Direct evidence of uptake. Direct evidence for the uptake of MC-LR by transfectants was provided by Western blot analysis (Figure 3) and fluorescently Texas-red labeled MC-LR (Figure 4). Uptake of this fluorescent MC-LR derivative is clearly demonstrated in HEK293 cells expressing drOatp1d1 (Figure 4). In contrast, uptake did not occur in HEK293 wild-type cells (data not shown). The fluorescent labeling of MC-LR most likely did not alter transport characteristics: similar experiments with another labeled Oatp substrate, fluorescent chenodeoxycholic acid, showed that the labeling did not alter transport by human OATP1B1 (Yamaguchi et al., 2006). Thus, the demonstration of intracellular fluorescent MC-LR is an indication that drOatp1d1 mediated the uptake of MC-LR. This indication is verified by immuno-labeling of MC-LR, that was detectable in transfectants but not in wild-type cells. We hypothesize that drOatp1d1 similarly mediates the uptake of nodularin.

Indirect evidence: morphological changes and cytotoxicity. Further indirect evidence of MC-LR and nodularin uptake by drOatp1d1 comes from the observed morphological changes following cyanotoxin exposure of transfectants expressing drOatp1d1 (Figure 5a). Exposed cells started to rapidly detach from the plate, which is indicative for cytotoxicity. This confirms similar observations by Vesterkvist et al., (2012) in caco-cells exposed to microcystin. Similar morphological changes were also found in HeLA cells overexpressing human OATP1B1 and OATP1B3 after exposure to MC-LR (Monks et al., 2007).

In addition to morphological changes, we also observed changes on the molecular level. MC-LR is known to induce ER-stress in human hepatoma cells (Christen et al., 2013). Transcriptional induction of *bip* is often used as marker for ER stress, although it should be noted that this is on the mRNA level, and not always reflected on protein level. Transcriptional induction of the ER-stress marker *bip* occurred in Oatp1d1 expressing CHO cells after exposure to 0.05 $\mu\text{mol L}^{-1}$ MC-LR and 0.05 $\mu\text{mol L}^{-1}$ nodularin for 24 h (Figure 5b). However, no transcriptional alteration of *bip* was detected in CHO wild-type cells lacking the uptake transporter.

ZFL cell lines are not suitable for uptake and effects assessment of cyanotoxins. Cell lines are important tools in toxicology and often preferred over *in vivo* testing. This in mind, we assessed the

suitability of the permanent zebrafish liver cell line ZFL for research on cyanobacterial toxins and analyzed the expression of *drOatp1d1*, as well as uptake and effects of cyanotoxins. Transcription analysis included *drOatp1d1* and *drOatp2b1* that are expressed in the liver, and *drOatp1f* mainly expressed in the kidneys of adult fish (Popovic et al., 2010). In ZFL, transcripts of *drOatp1d1* and *drOatp2b1* were present, and, as expected, the abundance of *drOatp1f* was very low (Figure 6a). In contrast to the adult liver, transcripts of *drOatp2b1* were much more abundant than of *drOatp1d1* in ZFL cells. Functional analysis showed a concentration-dependent intracellular increase of lucifer yellow concentration (Figure 6b), thus suggesting that uptake transporters were active. However, low fluorescence levels suggested that the uptake was extremely low, probably due to only minimal expression of Oatps. This could explain the lack of cytotoxicity in ZFL cells, even at high MC-LR concentrations (up to 100 $\mu\text{mol L}^{-1}$). Furthermore, no indication for induction of ER-stress was observed (Figure 7) on the mRNA level. This is in contrast to effects in Huh7 cells (Christen et al., 2013) where induction of *Bip* was shown both on transcriptional and protein level, as well as in freshly isolated zebrafish liver organ cultures (Faltermann et al. 2015b), where transcription of *bip* was increased after exposure to the cyanotoxins. In both systems, expression of *Oatps* resulted in uptake of cyanobacterial toxins, and subsequent changes on the mRNA level that are indicative of ER-stress. This underlines that MC-LR and nodularin affect exclusively cells that express the uptake transporter. We should also emphasize here that changes on mRNA abundance may but not necessarily must translate into changes on the protein level.

While generally, permanent cell lines seem to be relative resistant to MC-LR induced toxicity (Chong et al., 2000), this was not the case for ocadaic acid-induced toxicity (Bøe et al., 1991). Like MC-LR, ocadaic acid inhibits serine/threonine phosphatases, but in contrast to MC-LR and nodularin, it can easily cross cell membranes by diffusion. Low expression of uptake transporters is indicative for the very low sensitivity of several other permanent cell lines, including HepG2 cells (Hilgendorf et al., 2007; Komatsu et al., 2007; Zegura et al., 2011). Furthermore, freshly isolated primary trout hepatocytes became resistant to MC-LR after only a short period of culturing (Boaru et al., 2006). Our data in ZFL cells lead to the conclusion that these cells lack the levels of Oatps that would be required for substantial uptake. Thus, they do not serve as appropriate model for analysing effects of cyanobacterial toxins.

In summary, our study demonstrated an important role of drOatp1d1 in the uptake of MC-LR and nodularin in zebrafish. This represents the first study identifying the transporters responsible for nodularin uptake. Functional expression of drOatp1d1 in stable CHO and HEK293 transfectants clearly demonstrated the uptake of MC-LR. The high phylogenetic relationship of drOatp1d1 with human OATP1B1 and OATP1B3 (Popovic et al. 2013) indicates its fundamental role in the uptake of cyanobacterial toxins. However, other uptake transporters may also play an additional role in this process. Further experiments should investigate the contributing role of Oatp2b1, since this transporter may also be involved in the uptake, as suggested by its high expression in the adult liver (Popovic et al. 2013).

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Figure Legends.

Figure 1. a) Transport of different lucifer yellow concentrations ($25 \mu\text{mol L}^{-1}$ to $400 \mu\text{mol L}^{-1}$) into drOatp1d1 expressing CHO cells in the linear uptake range at 5 min incubation. Intracellular fluorescence is given in relative fluorescence units (RFU). **b)** Decrease of lucifer yellow uptake with increasing progesterone concentrations is due to competition of both substrates with drOatp1d1. Values are presented as mean $\pm$ SEM.

Figure 2. a). Inhibition of lucifer yellow (LY) uptake (concentration $50 \mu\text{mol L}^{-1}$) and resulting decrease in intracellular fluorescence with increasing MC-LR and nodularin concentrations in percent of control fluorescence. **b)** Corresponding phase contrast (A, B, E, F) and fluorescence (B, D, F, G) images of cells incubated for 5 min with $50 \mu\text{mol L}^{-1}$ lucifer yellow only (A, B, E, F), or in combination with $500 \mu\text{mol L}^{-1}$ MC-LR (C, D) or $500 \mu\text{mol L}^{-1}$ nodularin (F, G).

Figure 3. Immunoblot against MC-LR in CHO wild-type cells exposed to solvent control (WT/Ctr) or to $0.05 \mu\text{mol L}^{-1}$ MC-LR (WT/+MC) and in CHO cells expressing drOatp1d1 exposed to solvent control (CHO-Oatp/Ctr) and to $0.05 \mu\text{mol L}^{-1}$ MC-LR (CHO-Oatp/+MC).

Figure 4. Phase contrast (A) and fluorescence image (B) of HEK293 cells expressing drOatp1d1 after exposure for 30 min to $2.5 \mu\text{mol L}^{-1}$ of fluorescent MC-LR.

Figure 5 a) Microscope images of HEK293 cells stably expressing drOatp1d1 and HEK293 wild-type cells exposed to $1 \mu\text{mol L}^{-1}$ MC-LR for 30 min, and drOatp1d1 expressing CHO cells and CHO wild-type cells exposed to $5 \mu\text{mol L}^{-1}$ nodularin for 15 min. **b)** Transcriptional changes of *bip* in CHO wild-type cells and *drOatp1d1* transfected CHO cells compared to respective control cells, after exposure to $0.05 \mu\text{mol L}^{-1}$ MC-LR for 24 h. Values are presented as mean $\pm$ SEM. Statistical analysis were performed using an unpaired t-test and significant changes were marked by asterisks, with $*p < 0.05$.

Figure 6. **a)** Mean normalized transcripts of *drOatp1d1*, *drOatp1f* and *drOatp2b1* in ZFL cells cultivated at 100 % confluency. **b)** Uptake of different concentrations of lucifer yellow into ZFL cells given as relative fluorescence units (RFU) after incubation for 48 h.

Figure 7. Cytotoxicity of MC-LR in ZFL cells after 48 h exposure as determined by MTT assay. Data represent the results of two independent experiments. Shown is also the positive control (10 % DMSO).

Figure 8. Relative expression of *bip* after 48 h exposure to 5 and 35 $\mu\text{mol L}^{-1}$ MC-LR and the positive control thapsigargin (1 $\mu\text{mol L}^{-1}$) in ZFL cells (grown to 100 % cell density).

Tables

Table 1. Primer sequences used for Quantitative Real-Time PCR Analysis

Target gene	Primer	Sequence (5' - 3')
rpl13a ¹	Forward	AGC TCA AGA TGG CAA CAC AG
	Reverse	AAG TTC TTC TCG TCC TCC
bip ¹	Forward	CGA AGA AGC CAG ATA TCG ATG A
	Reverse	ACG GCT CTT TTC CGT TGA AC
GAPDH ²	Forward	TGT TGC GAG ACA AGA CGA
	Reverse	CCT GCA CCT GCT GCG GAC T
BIP ²	Forward	CCA TCA AGA CCA AAG GAG GA
	Reverse	TCC ATT TTG TGG AAG CAT GA

1: Primers for ZFL, 2: Primers for CHO

Figures.

Figure 1

a)

b)

Figure 2

Figure 3

Figure 4

Figure 5

a)

b)

Figure 6.

a)

b)

Figure 7.

Figure 8.