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Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin

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Abstract

Microcystins are toxins produced by freshwater cyanobacteria. They are cyclic heptapeptides that exhibit hepato- and neurotoxicity. However, the transport systems that mediate uptake of microcystins into hepatocytes and across the blood–brain barrier have not yet been identified. Using the *Xenopus laevis* oocyte expression system we tested whether members of the organic anion transporting polypeptide superfamily (rodent: Oatps; human: OATPs) are involved in transport of the most common microcystin variant microcystin-LR by measuring uptake of a radiolabeled derivative dihydromicrocystin-LR. Among the tested Oatps/OATPs, rat Oatp1b2, human OATP1B1, human OATP1B3, and human OATP1A2 transported microcystin-LR 2- to 5-fold above water-injected control oocytes. This microcystin-LR transport was inhibited by co-incubation with the known Oatp/OATP substrates taurocholate (TC) and bromosulfophthalein (BSP). Microcystin-LR transport mediated by the human OATPs was further characterized and showed saturability with increasing microcystin-LR concentrations. The apparent K_m values amounted to 7 ± 3 μM for OATP1B1, 9 ± 3 μM for OATP1B3, and 20 ± 8 μM for OATP1A2. No microcystin-LR transport was observed in oocytes expressing Oatp1a1, Oatp1a4, and OATP2B1. These results may explain some of the observed organ-specific toxicity of microcystin-LR. Oatp1b2, OATP1B1, and OATP1B3 are responsible for microcystin transport into hepatocytes, whereas OATP1A2 mediates microcystin-LR transport across the blood–brain barrier.

Keywords: Environmental toxins; Natural toxins; Microcystin; Cyanobacteria; Blood–brain barrier; Hepatocyte; Organic anion transport; Oatp; OATP

Introduction

Microcystins are naturally occurring toxins produced by freshwater cyanobacteria and can be found in lakes, ponds, and rivers that are often used for recreational activities (Chorus et al., 2000; Watanabe et al., 1996). Thus, besides menacing terrestrial and aquatic wildlife and livestock (Carmichael, 1994; Fischer and Dietrich, 2000; Schwimmer and Schwimmer, 1968), cyanobacteria also represent a threat to human health (Jochimsen et al., 1998; Teixeira et al., 1993). In 1996, a hemodialysis unit in Caruaru, Brazil, used water for dialysis from a reservoir, which was contaminated with blue-green algae and contained microcystins. Sixty of the 126 intoxicated patients died of severe microcystin-induced neurotoxicity or toxic liver failure (Jochimsen et al., 1998; Pouria et al., 1998). Microcystins act by inhibiting Ser/Thr protein phosphatase-1 and -2A (Mackintosh et al., 1990), which leads to disruption of the cytoskeleton and subsequent cell death (Eriksson et al., 1989).

Target organs of microcystin are mainly the liver and the brain. This requires uptake of microcystin across the sinusoidal (basolateral) plasma membrane of hepatocytes and its transport across the blood–brain barrier. In rat hepatocytes, basolateral uptake of microcystin was inhibited by cholate, taurocholate (TC), and bromosulfophthalein (BSP) (Eriksson et al., 1990; Runnegar et al., 1995). All

Abbreviations: OATP, human organic anion transporting polypeptide; Oatp, rodent organic anion transporting polypeptide; BSP, bromosulfophthalein; TC, taurocholate.

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latter compounds are substrates of the organic anion transporting polypeptide (rodent: Oatps; human: OATPs) superfamily of membrane transporters (Hagenbuch and Meier, 2003, 2004) that are classified within the *SLCO* family of solute carriers (Human Gene Nomenclature Committee Database) (Hagenbuch and Meier, 2004). Oatps/OATPs mediate Na^+-independent uptake of a wide variety of amphipathic organic compounds including bile salts, organic anionic dyes, steroids and steroid conjugates, drugs, various peptides, and toxins (Hagenbuch and Meier, 2003). Oatps/OATPs that are expressed in the brain (blood–brain barrier, choroid plexus) as well as in the liver include rat Oatp1a1 (Jacquemin et al., 1994), rat Oatp1a4 (Abe et al., 1998; Noé et al., 1997), human OATP1A2 (Kullak-Ublick et al., 1995), and human OATP2B1 (Kullak-Ublick et al., 2001). Rat Oatp1b2 (Cattori et al., 2000), human OATP1B1 (Abe et al., 1999; Hsiang et al., 1999; König et al., 2000b), and human OATP1B3 (König et al., 2000a) are primarily expressed in the liver. In the present study, we used the radiolabeled dihydromicrocystin-LR epimer [L-MeAla^7]microcystin-LR (Meriluoto et al., 1990), which was previously shown to have similar characteristics as native microcystins (Meriluoto et al., 1990). The study served to investigate whether the hepato- and neurotoxicity observed after microcystin intoxication (Jochimsen et al., 1998; Pouria et al., 1998) could be explained by microcystin transport mediated by members of the Oatp/OATP superfamily that are expressed in hepatocytes and in the blood–brain barrier.

Materials and methods

Animals. Female *Xenopus laevis* were purchased from the African Xenopus facility c.c, Noordhoek, Rep. South Africa.

Chemicals. [^{3}H]-dihydromicrocystin-LR (0.5 mCi/mmol) was prepared as described (Meriluoto et al., 1990). [^{3}H]Estrone-3-sulfate (53 Ci/mmol) was obtained from NEN Life Science Products (Boston, MA), [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham (Buckinghamshire, UK).

Uptake studies in X. laevis oocytes. Complementary RNA was transcribed in vitro from linearized cDNA using the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX). *X. laevis* oocytes were prepared as described (Hagenbuch et al., 1996) and injected either with 50 nl of water or with 5 ng of cRNA in 50 nl of water. After 3 days in culture at 18 °C with a daily change of Barth's solution, uptake of radiolabeled substrate was measured at 25 °C in 100 µl of uptake solution containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES adjusted to pH 7.5 with Tris as described (Reichel et al., 1999).

Protein phosphatase-1 and -2A inhibition assay. Preparation of [γ-^{32}P]phosphorylase-a and the protein phosphatase-1 and -2A inhibition assay was carried out as described (Cohen et al., 1988; MacKintosh, 1993). Assays were performed in 60-µl reactions consisting of 20 µl *X. laevis* oocyte-extract, 20 µl assay buffer, and 20 µl [γ-^{32}P]phos-32P]phosphorylase-a substrate for 10 min at 30 °C. The reaction was stopped by addition of 180 µl of 20% (w/v) trichloro-acetic acid, kept on ice for 10 min, and centrifuged at 17500 × *g* for 5 min. For acid molybdate extraction of inorganic [^{32}P]phosphate, 200 µl of the supernatant was mixed with 200 µl 1.25 mM KH_2PO_4 in 0.5 M H_2SO_4, 500 µl isobutanol/heptane (1:2), and 100 µl ammonium molybdate (5% w/v). After separation of the organic and aqueous phase, 300 µl of the organic phase was added to 3 ml of scintillation fluid (Ready Safe, Beckman, USA) and subjected to β-scintillation spectrometry.

Statistical analysis. Data are expressed as mean ± SE. To compare uptake into water injected with cRNA-injected oocytes, the unpaired Student's *t* test was used. For comparison of protein phosphatase-1 and -2A inhibition, data (mean ± SD) were subjected to ANOVA followed by a Tukey's test, significance levels were determined at $P < 0.05$.

Results

Transport of microcystin by liver Oatps/OATPs

Several Oatps/OATPs are expressed in hepatocytes where they mediate uptake of numerous organic compounds from blood plasma. To determine whether one of these transporters could mediate uptake of microcystin-LR into hepatocytes, we expressed these transporters in the *X. laevis* oocyte expression system and measured uptake of radiolabeled microcystin-LR. As demonstrated in Fig. 1, rat Oatp1b2-expressing oocytes accumulated microcystin-LR about 2-fold. Furthermore, human OATP1B1 and OATP1B3 showed an approximately 3- to 4-fold higher uptake of microcystin-LR as compared to water-injected control oocytes. No significant microcystin-LR uptake was observed for rat Oatp1a1-, rat Oatp1a4-, and human OATP2B1-expressing oocytes although they were able to mediate uptake of the positive control estrone-3-sulfate (data not shown). To further characterize microcystin-LR transport mediated by the human OATPs, we performed inhibition experiments and saturation kinetics. Co-incubation of OATP1B1- and OATP1B3-expressing oocytes with microcystin-LR and two known OATP substrates, either 100 µM TC (Fig. 2, hatched bars) or 50 µM BSP (Fig. 2, open bars), reduced microcystin-LR uptake into the oocytes almost completely. Initial experiments demonstrated that microcystin-

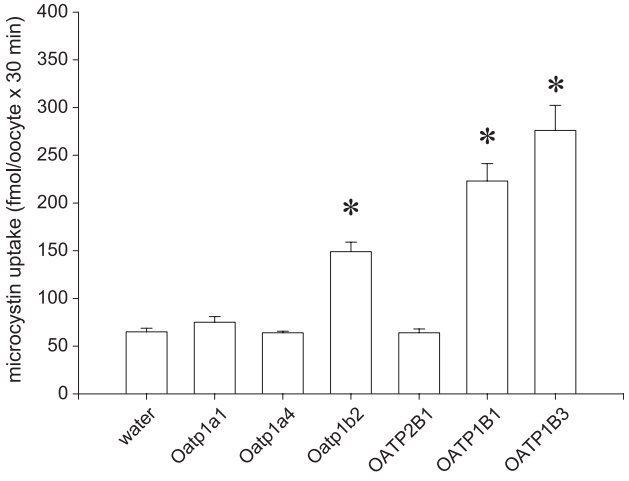

Fig. 1. Transport of microcystin-LR by liver Oatps/OATPs expressed in *X. laevis* oocytes. Oocytes were injected with 5 ng of the respective cRNAs or with 50 nl of water. After 3 days in culture, uptake of 4 μM microcystin-LR was measured over 30 min at 25 °C. Bars represent means ± SE of 8–10 determinations. * Denotes significantly different from water-injected oocytes ($P < 0.05$).

LR uptake was linear over at least 20 min (data not shown). Therefore, we measured uptake of microcystin-LR at increasing concentrations for 15 min to investigate saturability of microcystin-LR uptake with increasing concentrations. In fact, microcystin-LR uptake demonstrated saturability for both OATP1B1 (Fig. 3A) and OATP1B3 (Fig. 3B) with similar apparent K_m values of 7 ± 3 and 9 ± 3 μM, respectively. The two transporters differed, however, in their V_{max} values. OATP1B3 transported microcystin-LR with an about 4-fold higher capacity (V_{max} 168 ± 18 fmol/oocyte × 15 min) as compared to OATP1B1 (V_{max} 48 ± 8 fmol/oocyte × 15 min) (Fig. 3).

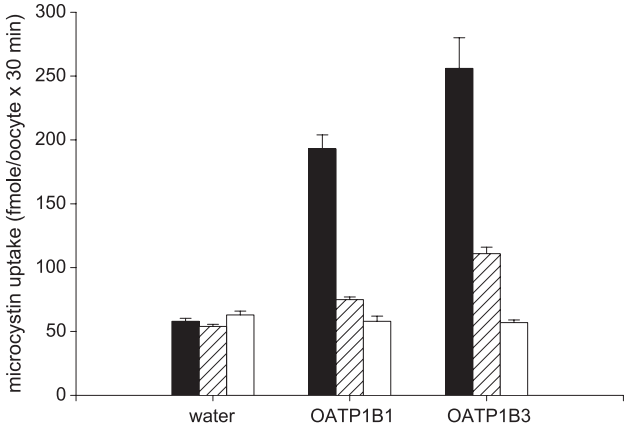

Fig. 2. Uptake of microcystin-LR into *X. laevis* oocytes expressing OATP1B1 and OATP1B3 and its inhibition by taurocholate and bromosulfophthalein. Oocytes were injected with 5 ng of the respective cRNAs or with 50 nl of water. After 3 days in culture, uptake of 4 μM microcystin-LR was measured over 30 min at 25 °C in the absence (filled bars) or presence of 100 μM taurocholate (hatched bars) or 50 μM bromosulfophthalein (open bars). The bars represent means ± SE of 9–10 determinations.

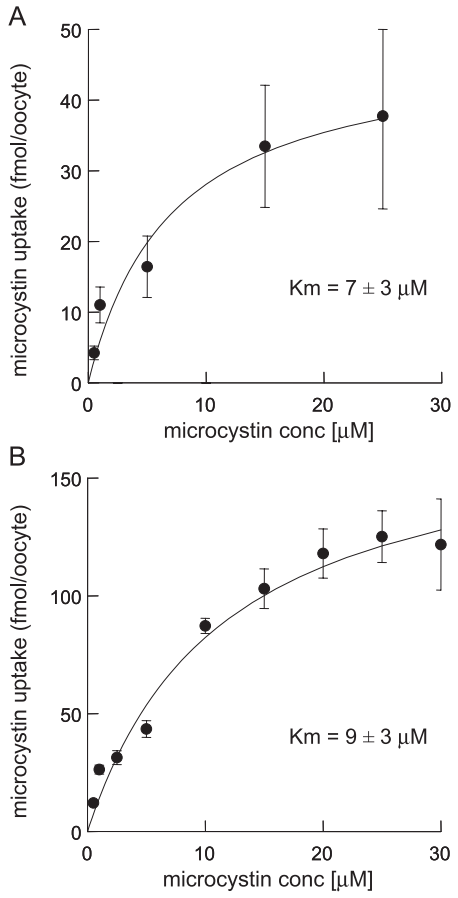

Fig. 3. Kinetics of microcystin-LR transport mediated by OATP1B1 (A) and OATP1B3 (B). Oocytes were injected with 5 ng of the respective cRNAs or with 50 nl of water. After 3 days in culture, initial (15 min) uptake of increasing concentrations of microcystin-LR was measured at 25 °C. Net microcystin-LR uptake was calculated by subtracting values obtained with water-injected oocytes from the values obtained with cRNA-injected oocytes. Data from 2–3 experiments were fitted by nonlinear regression analysis to the Michaelis–Menten equation (solid line) and apparent K_m values were calculated.

Transport of microcystin by brain Oatps/OATPs

Besides Oatp1a1, Oatp1a4, and OATP2B1 that are expressed in the brain as well as in the liver, OATP1A2 is strongly expressed in brain capillary endothelial cells (Gao et al., 2000). Because Oatp1a1, Oatp1a4, and OATP2B1 do not mediate transport of microcystin-LR (Fig. 1), we performed additional transport experiments with OATP1A2. As illustrated in Fig. 4 (control), OATP1A2-expressing oocytes were able to accumulate microcystin-LR approximately 4- to 5-fold above water-injected control oocytes. Co-incubation with 100 μM TC and 50 μM BSP resulted in a decrease of OATP1A2-mediated microcystin-LR uptake by 55% and 90%, respectively. OATP1A2-mediated microcystin-LR transport was saturable with an apparent K_m of 20 ± 8 μM and a V_{max} of 91 ± 21 fmol/oocyte × 15 min (Fig. 5), suggesting that OATP1A2 is involved in the transport of microcystin-

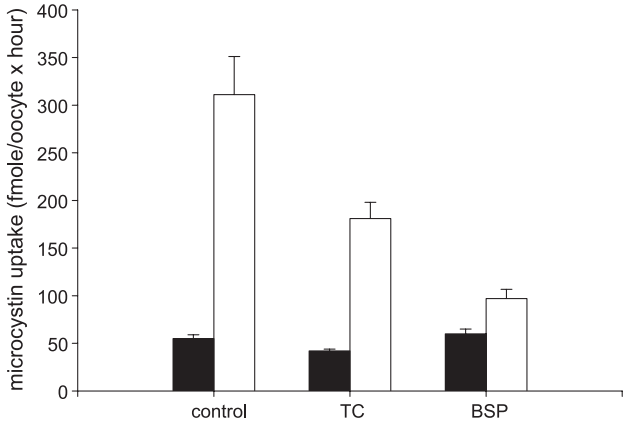

Fig. 4. Uptake of microcystin-LR into *X. laevis* oocytes expressing OATP1A2 and its inhibition by taurocholate and bromosulfophthalein. Oocytes were injected with 5 ng of OATP1A2-cRNA (filled bars) or with 50 nl of water (open bars). After 3 days in culture, uptake of 16 μM microcystin-LR was measured over 60 min at 25 °C in the absence (control) or presence of 100 μM taurocholate (TC) or 50 μM bromosulfophthalein (BSP). The values represent means ± SE of 12–14 uptake determinations.

LR across the blood–brain barrier (Gao et al., 2000). Unfortunately, we could not increase the microcystin-LR concentrations above 25 μM because the oocytes showed clear signs of microcystin-LR toxicity (Fig. 6) and disintegrated at higher concentrations of microcystin-LR.

Protection of microcystin-LR cytotoxicity in X. laevis oocytes

When water-injected control oocytes were incubated in the presence of 15 μM microcystin-LR over a time period of 15 min, no damage of oocytes was visible (Figs. 6A and B). As a consequence of microcystin-LR uptake, OATP1A2-expressing oocytes showed a temporal increase in toxic damage over the same time period (Fig. 6C). However, co-incubation of OATP1A2-expressing oocytes with 15 μM microcystin-LR and 50 μM BSP largely prevented the toxicity of microcystin-LR (Fig. 6D) because of competitive inhibition of microcystin-LR uptake by BSP. Similar findings were obtained when protein phosphatase-1 and -2A activities were measured (data not shown). In OATP1A2-expressing oocytes, protein phosphatase-1 and -2A activity was inhibited to 58 ± 2% of that of control oocytes after 15 min of incubation with 15 μM microcystin-LR. In the presence of 50 μM BSP, this inhibition was almost completely averted and protein phosphatase-1 and -2A activity remained at 87 ± 7% of control oocytes.

Discussion

The cyanobacterial toxin microcystin-LR, a cyclic heptapeptide, has been shown to exhibit hepato- and neurotoxicity in patients of a dialysis unit in Caruaru, Brazil, when the water was contaminated by blue-green algae (Pouria et al., 1998). In this study, we provide evidence that the organotropism of microcystin toxicity can be explained by microcystin transport mediated by OATPs that are expressed in the liver and at the blood–brain barrier.

Liver-specific rat Oatp1b2, human OATP1B1, and human OATP1B3 mediate uptake of microcystin-LR into hepatocytes. It has previously been shown that hepatocellular uptake of microcystins is inhibited by bile salts and BSP (Eriksson et al., 1990; Runnegar et al., 1995), suggesting that Oatps/OATPs could be involved in microcystin transport. Furthermore, several Oatps/OATPs that are expressed at the sinusoidal membrane of hepatocytes (Abe et al., 1999, 2001; Cattori et al., 2000; Hsiang et al., 1999; König et al., 2000a, 2000b) are able to transport cyclic peptides such as the endothelin antagonist BQ-123, the opioid peptide [D-penicillamin 2,5] enkephalin, and the mushroom toxin phalloidin (Meier-Abt et al., 2004). Therefore, we tested these hepatic Oatps/OATPs in the *X. laevis* oocyte expression system and could demonstrate that rat Oatp1b2, human OATP1B1, and human OATP1B3 indeed mediate uptake of radiolabeled microcystin-LR (Fig. 1). In addition, transport by human OATP1B1 and OATP1B3 was inhibited by co-incubation with the bile salt TC or with BSP (Fig. 2) and this OATP-mediated transport was saturable with apparent K_m values of 7 and 9 μM for OATP1B1 and OATP1B2, respectively (Fig. 3). These values are very similar to the apparent K_m value reported by Eriksson et al. (1990) for freshly isolated rat hepatocytes. Thus, these results clearly demonstrate that the OATP1B subfamily members are microcystin-LR transporters. Based on their

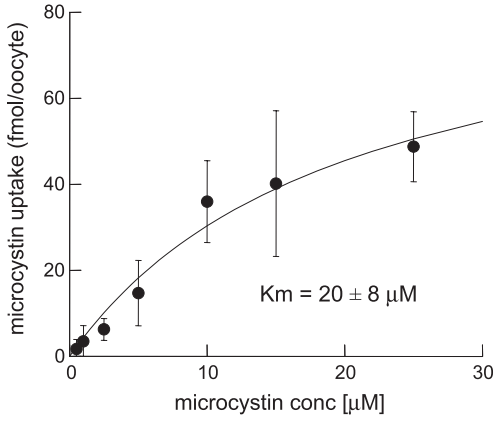

Fig. 5. Kinetics of OATP1A2-mediated microcystin-LR transport. Oocytes were injected with 5 ng of OATP1A2-cRNA or with 50 nl of water. After 3 days in culture, initial (15 min) uptake of increasing concentrations of microcystin-LR was measured at 25 °C. Net microcystin-LR uptake was calculated by subtracting values obtained with water-injected oocytes from the values obtained with cRNA-injected oocytes. Data from two independent experiments (24 to 28 individual uptake determinations per concentration) were pooled and fitted by nonlinear regression analysis to the Michaelis–Menten equation (solid line) and apparent K_m values were calculated.

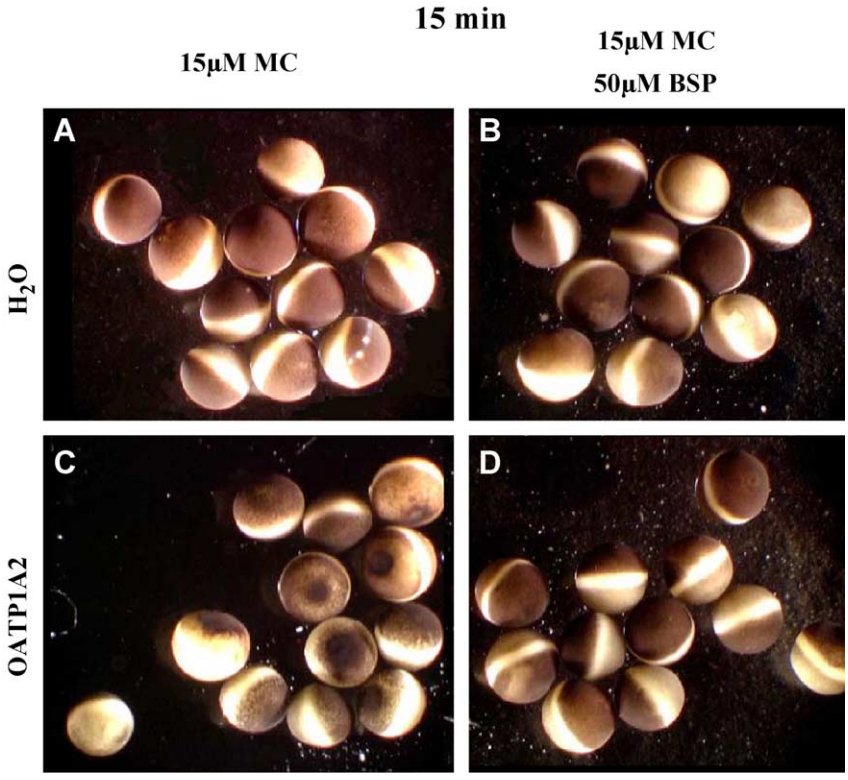

Fig. 6. Microcystin-LR-induced damage of OATP1A2-injected oocytes is prevented by bromosulfophthalein. Oocytes were injected with either 50 nl of water or 5 ng of OATP1A2 cRNA. After 3 days in culture, oocytes were incubated with 15 µM microcystin-LR (MC) for 15 min or 1 h in the presence or absence of 50 µM bromosulfophthalein (BSP) at 25 °C and pictures were taken. Healthy oocytes are characterized by a homogenous brownish coloration of the animal pole (A, B, and D). In contrast, damaged oocytes show a patchy discoloration and disintegration of the animal pole (C).

selective expression at the sinusoidal membrane of hepatocytes, they are most likely responsible for the observed hepatotoxicity of microcystin intoxications in rats, mice, and humans (Hooser et al., 1989; Jochimsen et al., 1998; Meriluoto et al., 1989; Pouria et al., 1998). However, this suggestion could only be proven directly in knockout animals that no longer express the hepatic OATP1B subfamily members. Furthermore, the identification of the cyclic heptapeptide microcystin-LR as a new substrate for Oatp1b2, OATP1B1, and OATP1B3 supports recent observations that OATP1B subfamily members are particularly good transporters for cyclic peptides (Meier-Abt et al., 2004).

Human OATP1A2, which is expressed in endothelial cells of the blood–brain barrier (Gao et al., 2000), also mediates microcystin-LR transport. Several Oatps/OATPs such as rat Oatp1a4 (Gao et al., 2000), human OATP1A2 (Gao et al., 2000), and human OATP2B1 (Gao and Meier, unpublished) are expressed at the blood–brain barrier. Among these transporters, only OATP1A2 was able to transport microcystin-LR (Figs. 1 and 4) and, similar to the liver OATPs, OATP1A2-mediated microcystin-LR transport was inhibitable by TC and BSP (Fig. 4). Furthermore, we could determine saturation kinetics with an apparent K_m value of 20 µM (Fig. 5), which is only 2- to 3-fold above the values obtained for the OATP1B subfamily members. These results clearly demonstrate that besides the OATP1B subfamily members also OATP1A2 is able to transport microcystin-LR. Its high expression in brain (Kullak-Ublick et al., 1995) and its localization to the endothelial cells of the blood–brain barrier (Gao et al., 2000; Hagenbuch et al., 2002) suggest that human OATP1A2 could be involved in microcystin-LR transport into brain. However, we cannot exclude that additional transporters as, for example, OATP1C1 or its rodent homologues Oatp1c1, are also involved in microcystin-LR transport across the blood–brain barrier. Thus, our results suggest that OATP1A2-mediated microcystin transport across the blood–brain barrier is one of the prerequisites for the neurological symptoms of microcystin intoxication as reported by Pouria et al. (1998) and Jochimsen et al. (1998).

Microcystin-LR-induced toxicity can be prevented with known Oatp/OATP substrates. Microcystin-LR-induced damage of oocytes expressing OATP1A2 could be prevented by co-incubation with TC and BSP (Fig. 6). Similarly, we could demonstrate an alleviation of intracellular protein phosphatase-1 and -2A inhibition by the same substrates. These observations suggest that microcystin intoxication could be reduced or even prevented in the presence of Oatp/OATP substrates. They confirm previous studies that demonstrated complete protection of lethal intoxications in mice by the known OATP1B1 and

OATP1B3 substrate rifampin (Hermansky et al., 1991; Vavricka et al., 2002). Therefore, one can speculate that any drug with reasonable high affinity for OATP, for example, statins (Hsiang et al., 1999), could prevent low-dose microcystin intoxication, provided it is given simultaneously with or shortly after the intoxication.

In conclusion, we have identified the organic anion transporting polypeptides Oatp1b2, OATP1A2, OATP1B1, and OATP1B3 as microcystin-LR transporters in rat and man. The tissue distribution of the identified microcystin-LR transporting Oatps/OATPs explains well the preferential organ toxicity of microcystins in liver and brain. Thus, Oatp1b2, OATP1B1, and OATP1B3 are responsible for uptake of microcystin-LR into rat and human hepatocytes, respectively, while OATP1A2 mediates microcystin-LR transport across the blood–brain barrier. The reduced microcystin-LR toxicity in Oatp/OATP-expressing oocytes co-incubated with BSP and TC indicates that administration of high affinity co-substrates of OATP1B1, OATP1B3, and OATP1A2 could provide a therapeutic option to reduce liver damage or neurotoxicity in microcystin-intoxicated patients.

Acknowledgments

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