

Molecular Characterization and Inhibition of Amanitin Uptake into Human Hepatocytes

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Amatoxins are the main poison of the green death cap (*Amanita phalloides*) and among the most dangerous natural toxins causing hepatic failure. A possible therapeutic approach is the inhibition of the transporting systems mediating the uptake of amatoxins into human hepatocytes, which, however, have yet to be identified. In the current study we tested whether members of the organic anion-transporting polypeptide (OATP) family, localized in the sinusoidal membranes of human hepatocytes, are involved in amatoxin uptake. For this, Madin Darby canine kidney strain II (MDCKII) cells stably expressing human OATP1B3, OATP2B1, or OATP1B1, were assayed for the uptake of ³H-labeled O-methyl-dehydroxymethyl- α -amanitin. Under our conditions, only OATP1B3 was able to transport amanitin with a K_m value of $3.7 \mu\text{M} \pm 0.6 \mu\text{M}$. Accordingly, toxin uptake was inhibited by OATP1B3 substrates and inhibitors (cyclosporin A, rifampicin, the quinoline derivatives MK571 ([3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)(3-dimethylamino-3-oxopropyl)thio)methyl]thiopropionic acid)) and montelukast, the cholecystokinin octapeptide (CCK-8), paclitaxel, and bromosulfophthalein, as well as by some antidotes used in the past for the treatment of human amatoxin poisoning (silibinin dihemisuccinate, penicillin G, prednisolone phosphate, and antamanide). These transport studies are in line with viability assays monitoring the toxic effect of amanitin on the transfected MDCKII cells. Further support for amatoxin transport was found in primary human hepatocytes, expressing OATP1B3, OATP2B1, and OATP1B1, where CCK-8, a substrate specific for OATP1B3, prevented the fragmentation of nucleoli, a lesion typical for amanitin action. In conclusion, we have identified OATP1B3 as the human hepatic uptake transporter for amatoxins; moreover, substrates and inhibitors of OATP1B3, among others rifampicin, may be useful for the treatment of human amatoxin poisoning.

Key Words: organic anion transporter 1B3; rifampicin; hepatocellular transport; amanitin transport; amanitin poisoning.

Fatal mushroom poisonings are predominantly caused by members of the *Amanita* family, including the genera *Amanita phalloides*, *Amanita virosa*, and *Amanita verna* (Wieland and Faulstich, 1978). While occurring only sporadically, intoxications with these mushrooms cause serious symptoms including hepatic failure, sometimes with the need for liver transplantation. Among the toxic cyclopeptides found in *Amanita* species are the amatoxins, the phallotoxins, and the virotoxins (Wieland and Faulstich, 1978). Only amatoxins are responsible for fatal mushroom poisoning.

The mechanism of hepatocellular uptake of the phallotoxin phalloidin, a bicyclic heptapeptide, has been elucidated recently (Fehrenbach *et al.*, 2003; Meier-Abt *et al.*, 2004). However, phallotoxins are not lethal to humans, probably because of insufficient intestinal absorption (Wieland and Faulstich, 1978, 1991). In laboratory animals phallotoxins cause hemorrhagic necrosis of the liver only when administered parenterally. The amatoxins, bicyclic octapeptides, act on hepatocytes and cause cell death by inhibition of mRNA synthesis in hepatocytes (Kedinger *et al.*, 1970; Stirpe and Fiume, 1967). These toxic effects are enhanced by the enterohepatic circulation of the amanitins (Wieland and Faulstich, 1991). The main toxin of the amatoxins, α -amanitin, is a common tool in molecular biology and in biological research, due to its high and very specific inhibitory action on eukaryotic RNA polymerase II (Cochet-Meilhac and Chambon, 1974; Kedinger *et al.*, 1970; Lindell *et al.*, 1970).

Several antidotes for α -amanitin poisoning have been described in the literature, among them are penicillin, silibinin dihemisuccinate, and prednisolone phosphate (Enjalbert *et al.*, 2002; Floersheim, 1978; Vogel *et al.*, 1975), which may decrease the hepatocellular uptake of α -amanitin (Faulstich *et al.*, 1980; Kröncke *et al.*, 1986). Earlier studies on rat liver and rat hepatocytes suggested that the uptake is sodium independent and facilitated by bile acid transporters (Kröncke *et al.*, 1986). Given the marked species-specific differences in hepatic uptake transporters of rat and man (Abe *et al.*, 1999; Hagenbuch and Meier, 2004), it appeared important to us to identify the transporter involved in the uptake of amatoxins into human hepatocytes and to study this transport process on the molecular level.

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One large family of uptake transporters is the organic anion-transporting polypeptide (OATP) family of solute carriers (Hagenbuch and Meier, 2004). In contrast to the sodium-dependent bile salt transporter, Na(+)/taurocholate transport protein, uptake by OATPs is sodium independent. OATPs mediate the uptake of a wide variety of organic compounds. Endogenous substances, such as bile acids, steroids and steroid conjugates, thyroid hormones, prostaglandins, and various peptides are substrates for members of the OATP family (Abe *et al.*, 1999; Hsiang *et al.*, 1999; König *et al.*, 2000a,b; Kullak-Ublick *et al.*, 2001). Three OATP proteins have been localized to the basolateral membrane of human hepatocytes: OATP1B1 (encoded by *SLCO1B1*; formerly termed OATP2 or OATP-C), OATP1B3 (encoded by *SLCO1B3*; formerly termed OATP8), and OATP2B1 (encoded by *SLCO2B1*; formerly termed OATP-B) (König *et al.*, 2000a,b; Kullak-Ublick *et al.*, 2001; Tamai *et al.*, 2000). These uptake transporters were stably expressed in Madin Darby canine kidney strain II (MDCKII) cells, localizing OATP1B1, OATP1B3, or OATP2B1 to their basolateral membrane domain (König *et al.*, 2000a; Kopplow *et al.*, 2005; Letschert *et al.*, 2004). In our study we used a ^3H -labeled α -amanitin derivative to measure the uptake by recombinant human OATP transporters expressed in polarized cells.

MATERIALS AND METHODS

Antibodies and Chemicals. The polyclonal antibodies SKT (König *et al.*, 2000b), SPA (Kopplow *et al.*, 2005), and ESL (König *et al.*, 2000a) were raised in rabbits against human OATP1B3, OATP2B1, and OATP1B1, respectively. Alexa Fluor 488-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Molecular Probes (Eugene, OR), and the anti-nucleolin antibody was obtained from Upstate (Lake Placid, NY). For the uptake experiments, ^3H -O-methyl-dehydroxymethyl- α -amanitin (^3H -amanitin; 0.65 Ci/mmol) was used, and for the viability studies and nucleolar fragmentation, O-methyl- α -amanitin was used (Fig. 1; Faulstich *et al.*, 1985; Jahn *et al.*, 1980; Wieland and Fahrmeir, 1970). Silibinin dihemisuccinate was kindly supplied by Madaus GmbH (Köln, Germany) and recrystallized in the laboratory of H.F. ^3H -bromosulphophthalein (^3H -BSP; 17 Ci/mmol) and ^3H -paclitaxel (4.8 Ci/mmol) were obtained from Hartmann Analytic (Braunschweig, Germany), and ^{14}C -penicillin G (benzyl- ^{14}C -penicillin; 0.06 Ci/mmol) was from Amersham (Little Chalfont, Buckinghamshire, United Kingdom). Other chemicals were commercially available and were obtained at the highest degree of purity.

Cell Culture and Transfection. MDCKII cells were cultured in minimum essential medium (Sigma, Taufkirchen, Germany), containing 10% fetal calf serum (Biowest, Nuaille, France), 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin, at 37°C and 5% CO_2 . MDCKII cells were transfected with pcDNA3.1(+) plasmids (Invitrogen, Groningen, Netherlands) containing the respective OATP cDNA using Metafectene (Biontex, Munich, Germany) according to the manufacturer's instructions. Since the most frequent single-nucleotide polymorphism, resulting in the amino acid exchange S112A, exhibits no functional differences compared to the so-called reference sequence (NM_019844) (Letschert *et al.*, 2004), we used OATP1B3-expressing cells with this polymorphism in the present study. MDCKII cells were grown on cell culture inserts to confluence for 3 days and induced with 10mM sodium butyrate for 24 h prior to analysis to obtain higher levels of the recombinant proteins (Cui *et al.*, 1999). The polyethylene terephthalate cell culture inserts (ThinCert, 24-mm diameter, pore size 0.4 μm , 1×10^8 pores/ cm^2) (Letschert *et al.*, 2005)

and the tissue culture multiwell plates (Cellstar) were obtained from Greiner Bio-One (Frickenhhausen, Germany). MDCKII cells transfected with the empty pcDNA3.1(+) vector served as a negative control in all experiments.

Transport Assays. MDCKII cells were grown and induced with butyrate as described above. For transport measurements, the cells were washed with uptake buffer (142mM NaCl, 5mM KCl, 1mM K_2HPO_4 , 1.2mM MgSO_4 , 1.5mM CaCl_2 , 5mM glucose, and 12.5mM HEPES, pH 7.3) for 10 min. Subsequently, 1 ml of uptake buffer was added to the apical compartment, and 1.5 ml of the uptake buffer containing the ^3H -labeled substrate was added to the basolateral compartment. For transport studies using BSP as substrate, cells were seeded on 12-well plates. After the respective time periods, the cells were washed three times with cold uptake buffer and solubilized with 2 ml 0.2% sodium dodecylsulfate (SDS) in water. In case of BSP as substrate, cells were washed two times with cold uptake buffer containing 0.5% bovine serum albumin and three times with cold uptake buffer without albumin prior to cell lysis. The radioactivity in the lysate was determined by liquid scintillation counting, and the appropriate protein concentration was determined by bicinchonic acid assay.

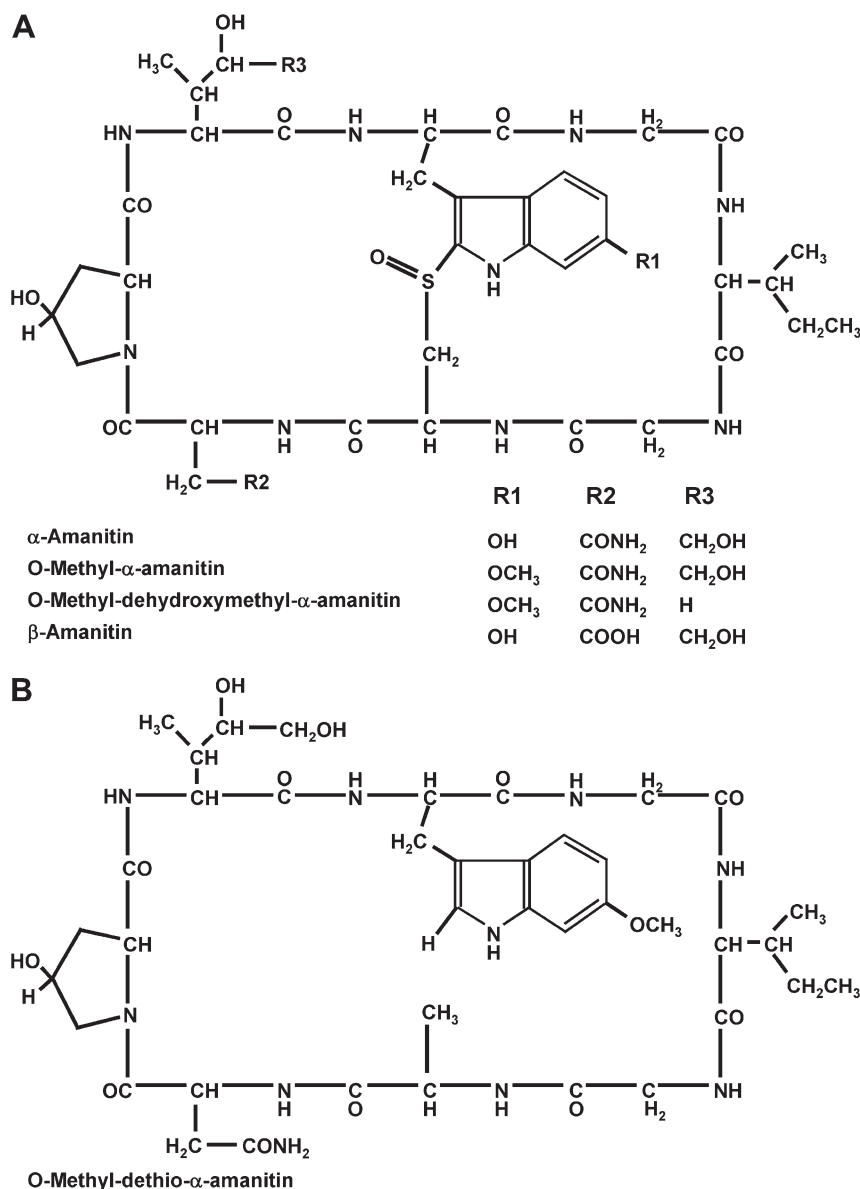
Cell Viability Assays. The viability of MDCKII cells was determined by AlamarBlue assays (Biosource, Camarillo, CA), a commercially available resazurin reduction assay (O'Brien *et al.*, 2000). Cells were grown in 96-well plates (50,000 cells per well) for 3 days and induced as described above. After a 24-h induction, cell culture medium in the absence or presence of O-methyl- α -amanitin was added to the cells. After the time points indicated, the cells were incubated with 10 μl fresh medium, diluted 1:10 with the AlamarBlue dye. After a 4-h incubation at 37°C under 5% CO_2 in a humidified atmosphere, the absorbance difference between 570 and 595 nm was determined (AlamarBlue reduction). Cell viability of the amanitin-treated cells was expressed as percentage of AlamarBlue reduction of the respective MDCKII cells cultivated for the respective period in the absence of amanitin.

Primary Human Hepatocytes. Primary human hepatocytes and their culture medium were obtained from Cytonet (Weinheim, Germany). The hepatocytes were freshly isolated and derived from a female living donor, 49 years of age, undergoing surgical removal of liver metastases. The hepatocytes were seeded on 8-well chamber slides (Nunc, Wiesbaden, Germany), coated with rattail collagen from Cytonet. Immunofluorescence studies, according to Chandra *et al.* (2005), and amanitin treatment were performed on the third day. Cells were fixed with acetone (-20°C) for 10 min on ice. After blocking with 2% fetal calf serum/1% bovine serum albumin in phosphate-buffered saline (PBS) for 45 min, primary antibodies were incubated for 1 h. After three washes with PBS, the respective secondary antibodies were incubated for 1 h. After three washes with PBS, cells were mounted with Moviol (Hoechst, Frankfurt, Germany). Confocal laser-scanning immunofluorescence microscopy was performed using an LSM-510 Meta apparatus from Carl Zeiss (Jena, Germany).

RESULTS

Uptake of ^3H -Amanitin by OATP1B3

The structures of α -amanitin and the different amanitin derivatives used in this study are shown in Figure 1. To see whether amanitin is a substrate for one of the OATP proteins expressed in human liver, uptake studies with stably transfected MDCKII cells were performed using 0.7 μM ^3H -amanitin. MDCKII-OATP2B1 and MDCKII-OATP1B1 cells did not show any significant changes compared to the vector-transfected MDCKII-Control cells, but MDCKII-OATP1B3 exhibited a significantly higher uptake ratio (Fig. 2). Data were verified by repeating this experiment with different ^3H -amanitin concentrations

FIG. 1. Chemical structures of α -amanitin and some of its derivatives.

and different cell batches with similar results. Kinetic analyses with OATP1B3-expressing MDCKII cells showed a K_m value of $3.7\mu\text{M} \pm 0.6\mu\text{M}$ ($n = 6$). A time course of the OATP1B3-mediated ^3H -amanitin uptake is shown in Figure 3. Whereas the background values of the MDCKII-Control cells remained in the same range, MDCKII-OATP1B3 cells showed a linear increase of ^3H -amanitin uptake within the studied time period. Table 1 shows the inhibitory action of the different amanitin derivatives on ^3H -amanitin uptake. All amanitins were able to inhibit the OATP1B3-mediated ^3H -amanitin uptake. β -Amanitin was the most potent inhibitor among the tested amanitins, followed by the nontoxic O-methyl-dethio- α -amanitin. The effect of α -amanitin was in the same range as O-methyl- α -amanitin.

Inhibition of ^3H -Amanitin Uptake by Different Organic Anions and Neutral Compounds

The uptake of ^3H -amanitin was inhibited by several known substrates and inhibitors of OATP1B3 as well as by reported antidotes for amanitin poisoning. The approximate concentration at 50% inhibition (IC_{50}) values for OATP1B3-mediated uptake of ^3H -amanitin are shown in Table 2. OATP1B3 inhibitors including cyclosporin A ($\text{IC}_{50} = 0.3\mu\text{M}$), silibinin dihemisuccinate ($\text{IC}_{50} = 0.4\mu\text{M}$), MK571 [(3-(3-(2-(7-chloro-2-quinolinyl)ethenyl)phenyl)((3-dimethylamino-3-oxopropyl)thio)methyl)thiopropionic acid)] ($\text{IC}_{50} = 0.5\mu\text{M}$), antamanide ($\text{IC}_{50} = 0.7\mu\text{M}$), and the antituberculosis antibiotic rifampicin ($\text{IC}_{50} = 0.8\mu\text{M}$) were the most potent inhibitors. Paclitaxel,

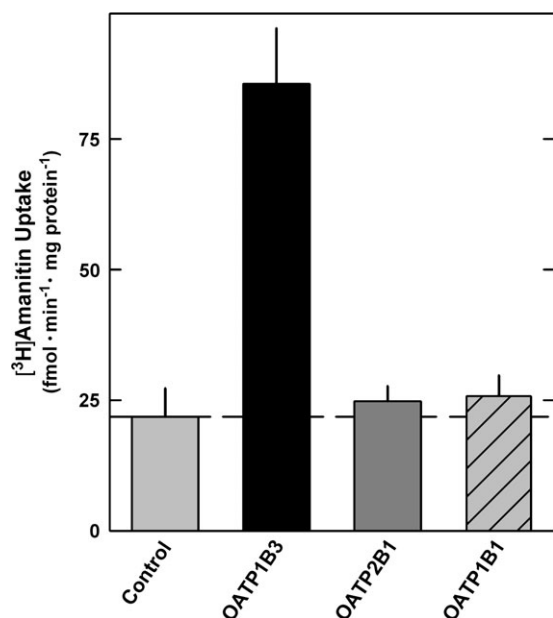


FIG. 2. Uptake of ³H-amanitin. MDCKII-Control cells and MDCKII cells expressing OATP1B3, OATP2B1, or OATP1B1 were grown on cell culture inserts (see Materials and Methods). ³H-amanitin (0.7 μM) was added to the basolateral compartment. After incubation for 30 min at 37°C, intracellular radioactivity was determined. Data represent means ± SDs from nine experiments, each determined in triplicate.

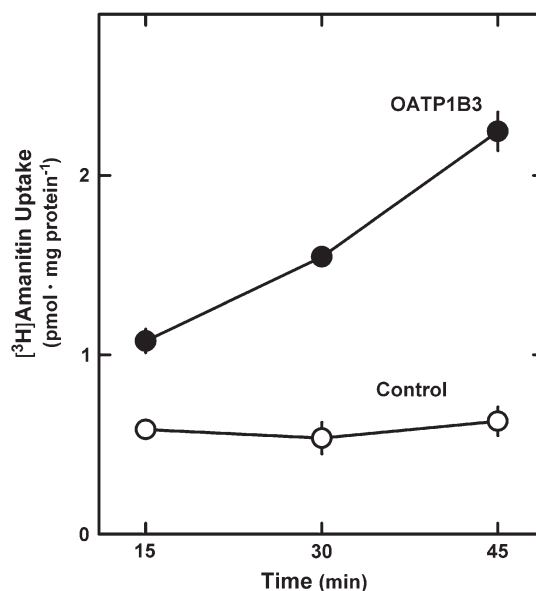


FIG. 3. Time dependence of ³H-amanitin transport in stably transfected cells. MDCKII-Control (○) and MDCKII-OATP1B3 cells (●) were grown on cell culture inserts as described under Materials and Methods. ³H-amanitin (0.7 μM) was given to the basolateral compartments. After 15, 30, and 45 min at 37°C, intracellular radioactivity was measured. Data represent means ± SDs from three experiments each determined in triplicate.

BSP, cholecystokinin octapeptide (CCK-8), montelukast, as well as previously used antidotes against amanitin poisoning, penicillin G, and prednisolone phosphate, also showed inhibition of ³H-amanitin uptake. Further kinetic analyses indicated that MK571 and antamanide were potent competitive inhibitors for OATP1B3-mediated amanitin uptake with K_i values of 0.2 and 0.7 μM, respectively. Silibinin dihemisuccinate inhibited the amanitin uptake in a noncompetitive manner ($K_i = 2.1$ μM).

Comparison of Inhibitory Effects on the Transport of the Common OATP Substrate BSP

To compare the sensitivity of the three human hepatocellular OATP proteins with the inhibitors of amanitin uptake, we tested the inhibition of the OATP-mediated uptake of ³H-BSP. The IC_{50} values are shown in Table 3. Cyclosporin A was the most potent inhibitor of OATP1B3-mediated BSP transport ($IC_{50} = 0.3$ μM). For OATP1B1 the IC_{50} was 3.5 μM, and for OATP2B1 it was 20 μM. MK571 inhibited OATP1B3 and OATP2B1 to a similar extent (IC_{50} values of 0.3 and 0.2 μM, respectively). The IC_{50} concentration for OATP1B1 was approximately 20 times higher. Comparing OATP1B3 with OATP1B1, similar results were shown for another quinoline derivative, montelukast (0.5 vs. 10 μM) and silibinin dihemisuccinate (1 vs. 10 μM). Rifampicin inhibited the OATP1B3-mediated BSP transport with an IC_{50} value of 1.5 μM. OATP2B1- and OATP1B1-mediated transport was inhibited only at higher concentrations

(IC_{50} value of 90 and 120 μM, respectively). Antamanide seemed to be a preferential inhibitor for OATP1B3-mediated transport at low concentrations ($IC_{50} = 15$ μM), and paclitaxel ($IC_{50} = 4$ μM) inhibited OATP2B1 and OATP1B1 only at cytotoxic concentrations (IC_{50} value of 25 and 50 μM, respectively; Table 3). Radiolabeled paclitaxel was shown to be transported by OATP1B3, but not by OATP1B1 and OATP2B1 (Table 4). Using radiolabeled penicillin G, uptake experiments indicated that this known antidote for α-amanitin poisoning is only a substrate for OATP1B3 (Table 4).

TABLE 1
Inhibition of OATP1B3-Mediated ³H-Amanitin Uptake by Different Amanitin Derivatives

	Inhibitor concentration (μM)	³ H-Amanitin transport (%)
O-Methyl-α-amanitin	130	83 ± 2
α-Amanitin	130	76 ± 8
O-Methyl-dethio-α-amanitin	110	33 ± 3
β-Amanitin	40	60 ± 5

Note. MDCKII-OATP1B3 cells were incubated with 0.7 μM ³H-amanitin for 30 min in the presence of several amanitin derivatives at the indicated concentrations. Rates of ³H-amanitin transport were calculated as percentage of transport in the absence of unlabeled amanitin derivative. Data represents mean values ± SDs from three determinations.

TABLE 2
Inhibition of OATP1B3-Mediated ^3H -Amanitin Uptake in OATP1B3-expressing MDCKII Cells

	IC ₅₀ (μM)
Prednisolone phosphate	75
Penicillin G	25
Montelukast	15
CCK-8	10
BSP	3
Paclitaxel	2
Rifampicin	0.8
Antamanide	0.7
MK571	0.5
Silibinin dihemisuccinate	0.4
Cyclosporin A	0.3

Note. Transport of 0.7 μM ^3H -amanitin was measured for 30 min in the presence or absence of the inhibitors. Data were obtained from at least three determinations each determined in triplicate.

Viability of Amanitin-Treated Cells

The uptake of amanitin by OATP1B3 was studied in addition in viability assays with nonlabeled amanitin. O-Methyl- α -amanitin was given to the MDCKII-Control, -OATP1B3, -OATP2B1, and -OATP1B1 cells growing on 96-well plates, and the viability was determined by the AlamarBlue assay. Figure 4 shows a time course with 0.1 μM amanitin from 24 to 72 h. Whereas MDCKII-Control, -OATP2B1, and -OATP1B1 cells remained unaffected, viability of MDCKII-OATP1B3 cells decreased depending on the duration of amanitin exposure. After 24 h there was already a significantly lower viability when compared to cells growing in medium without amanitin.

TABLE 3
Comparison of Human Hepatocyte Uptake Transporters OATP1B3, OATP2B1, and OATP1B1: Inhibition of OATP-Mediated ^3H -BSP Uptake

	IC ₅₀ (μM)		
	OATP1B3	OATP2B1	OATP1B1
Antamanide	15	> 100	100
Paclitaxel	4	25	50
Rifampicin	1.5	90	120
Silibinin dihemisuccinate	1	1	10
Montelukast	0.5	1	10
MK571	0.3	0.2	5
Cyclosporin A	0.3	20	3.5

Note. Uptake of 0.5 μM ^3H -BSP was measured for 10 min in the presence or absence of the inhibitors. Data were obtained from at least three determinations each determined in triplicate.

TABLE 4
Comparison of Human Hepatocyte Uptake Transporters OATP1B3, OATP2B1, and OATP1B1: Transport of Radiolabeled Inhibitors of Amanitin Uptake

	Intracellular accumulation (pmol/min/mg protein)			
	Control	OATP1B3	OATP2B1	OATP1B1
^3H -Paclitaxel	0.96 ± 0.01	2.7 ± 0.3	1.01 ± 0.04	0.81 ± 0.06
^{14}C -Penicillin G	0.51 ± 0.12	1.7 ± 0.1	0.43 ± 0.08	0.60 ± 0.13

Note. Transport of 110 nM ^3H -paclitaxel or 1.3 μM ^{14}C -penicillin G was measured for 10 min. Data were obtained from three determinations, each determined in triplicate. Uptake values are given in bold and indicate statistically significant uptake in OATP-expressing compared to MDCKII-Control cells (Student's *t*-test, *p* < 0.01).

Concentration Dependency of Cytotoxicity Caused by Amanitin

The cytotoxic effect is shown in Fig. 5 in a concentration-dependent manner. Viability of MDCKII-OATP1B3 cells decreased depending on the amanitin concentration. The cells showed a strong decline of viability in the range of 0.1–10 μM O-methyl- α -amanitin after 24 h of exposure. For OATP1B3, the graph indicated an LD₅₀ value of approximately 0.3 μM. At a concentration of 10 μM there was also a slight cytotoxic effect on MDCKII-Control, -OATP2B1, and -OATP1B1 cells.

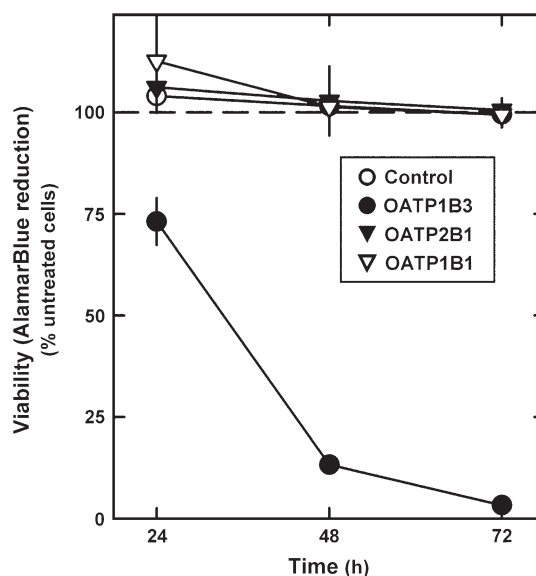


FIG. 4. Cell viability of stably transfected MDCKII cells after exposure to amanitin. MDCKII-Control (○), MDCKII-OATP1B3 (●), MDCKII-OATP2B1 (▼), and MDCKII-OATP1B1 (▽) were grown on 96-well plates. After induction for 24 h with butyrate (Cui *et al.*, 1999), the cells were incubated with O-methyl- α -amanitin (0.1 μM) for the time periods indicated. Cell viability at the time points indicated was determined by the AlamarBlue assay. Data represent the percentage of viable cells relative to untreated cells (*n* = 20).

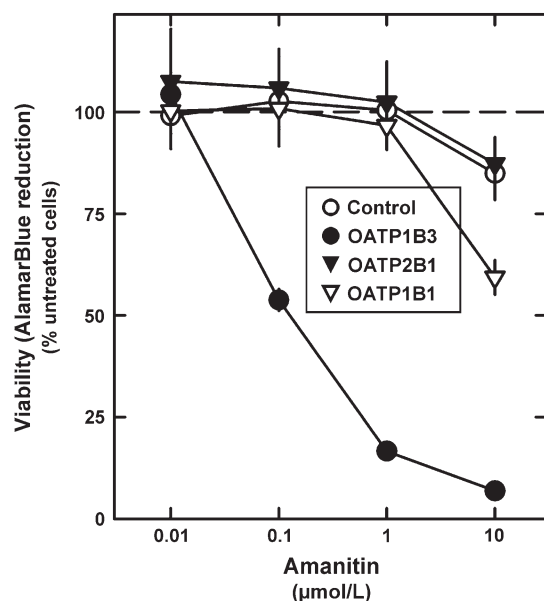


FIG. 5. Dependence of cell viability on amanitin concentration. MDCKII-Control (○), MDCKII-OATP1B3 (●), MDCKII-OATP2B1 (▼), and MDCKII-OATP1B1 cells (▽) were grown on 96-well plates. After induction for 24 h with butyrate (Cui *et al.*, 1999), the cells were incubated with different concentrations of O-methyl- α -amanitin (0.01, 0.1, 1, or 10 μ M) for 24 h, and the cell viability was determined by AlamarBlue assay. Data represents the percentage of viable cells relative to untreated cells ($n = 20$).

Inhibition of Amanitin-Induced Cellular Damage

Viability assays were performed in order to test whether the decrease in cell viability could be inhibited by substrates and inhibitors of OATP-mediated transport (Table 5). The cells were exposed for 30 min to 1 μ M O-methyl- α -amanitin in the presence or absence of different substrates or inhibitors. The cells were washed with fresh medium and incubated overnight with culture medium. Cell viability was determined by AlamarBlue assay 24 h later. Exposure to 1 μ M α -amanitin had the same effect as the exposure to 1 μ M O-methyl- α -amanitin (viability of $18.5 \pm 1.7\%$ vs. $18.2 \pm 0.6\%$, $n = 20$). Incubation of OATP1B3-expressing MDCKII cells with 1 μ M amanitin for 30 min followed by washing and incubation for 24 h had the same effect as the incubation with 1 μ M amanitin for 24 h (viability of $18.2 \pm 0.6\%$ vs. $20.9 \pm 0.5\%$, $n = 20$). In contrast to the coincubation with amanitin and with inhibitors for only 30 min, a coincubation of the cells with amanitin and with inhibitors for 24 h had no protective effect (data not shown). This may be due to the clearance of the inhibitors after uptake into the cells and thus, exposure solely to amanitin after a while. The IC_{50} values of the different substances are shown in Table 5. Whereas the previously used antidotes for amanitin poisoning, prednisolone phosphate and penicillin G, showed some inhibitory action at higher concentrations (50 and 20 μ M, respectively), cyclosporin A, MK571, CCK-8, and rifampicin were able to prevent the cellular damage induced by amanitin

in concentrations of less than 1 μ M. MDCKII-Control, -OATP2B1, and -OATP1B1 cells did not change their viability significantly under any of these conditions. β -Amanitin also had a cytotoxic effect on MDCKII-OATP1B3 cells (data not shown).

Fragmentation of Nucleoli in Primary Human Hepatocytes Caused by Amanitin and Its Prevention by the OATP1B3-Specific Substrate CCK-8

Fig. 6 shows the localization of OATP1B3, OATP2B1, and OATP1B1 in primary human hepatocytes after 3 days of culture. All three OATPs were detected in the cultured primary human hepatocytes. Figure 7 shows nuclei and nucleoli of the human hepatocytes. The control cells, incubated with medium without amanitin for 8 h, showed intact nucleoli. Treatment with 1 μ M amanitin led to the fragmentation of the nucleoli. After counting of 56 nuclei, control cells showed nucleolar fragmentation in $14 \pm 4\%$ and in cells treated with 1 μ M amanitin $90 \pm 7\%$. The addition of 50 μ M CCK-8 to the amanitin decreased the number of damaged nucleoli to $26 \pm 8\%$ (Fig. 7).

DISCUSSION

This study describes the molecular basis of the uptake of toxic amanitin derivatives into human hepatocytes. We analyzed the transport of radioactively labeled O-methyl-dehydroxymethyl- α -amanitin (Fig. 1, 3H -amanitin) into transfected MDCKII cells stably expressing human OATP1B3, OATP2B1, or OATP1B1. Furthermore, we studied the toxic action of O-methyl- α -amanitin (Fig. 1) in the same cell system. Our results indicate that only OATP1B3-expressing MDCKII cells took up labeled amanitin while the MDCKII cells expressing OATP2B1 and OATP1B1 showed little, if any, transport activity under our conditions (Fig. 2). The radiolabeled amanitin was transported by OATP1B3 with a K_m value of $3.7 \pm 0.6 \mu$ M.

Several native or semisynthetic amanitin derivatives (Table 1) were able to inhibit the uptake of 3H -amanitin. This suggests that these amanitin derivatives are the likely substrates for the same transporter. The native β -amanitin, with its carboxyl group (Fig. 1), was a more potent inhibitor of the OATP1B3-mediated transport of 3H -amanitin than α -amanitin, probably due to its anionic character (Table 1). However, it remains to be elucidated whether β -amanitin is also the better substrate for OATP1B3. If so, the acidic amanitin may represent a component more dangerous for humans than the well-known α -amanitin. It should be noted that *A. phalloides* mushrooms contain α -amanitin and β -amanitin in nearly equal amounts (1.5–2.0 mg/g dry weight each; Wieland and Faulstich, 1983).

OATP1B1 and OATP2B1 (with amino acid identities relative to OATP1B3 of 80 and 35%, respectively; Hagenbuch and Meier, 2004; Kullak-Ublick *et al.*, 2001), did not transport the

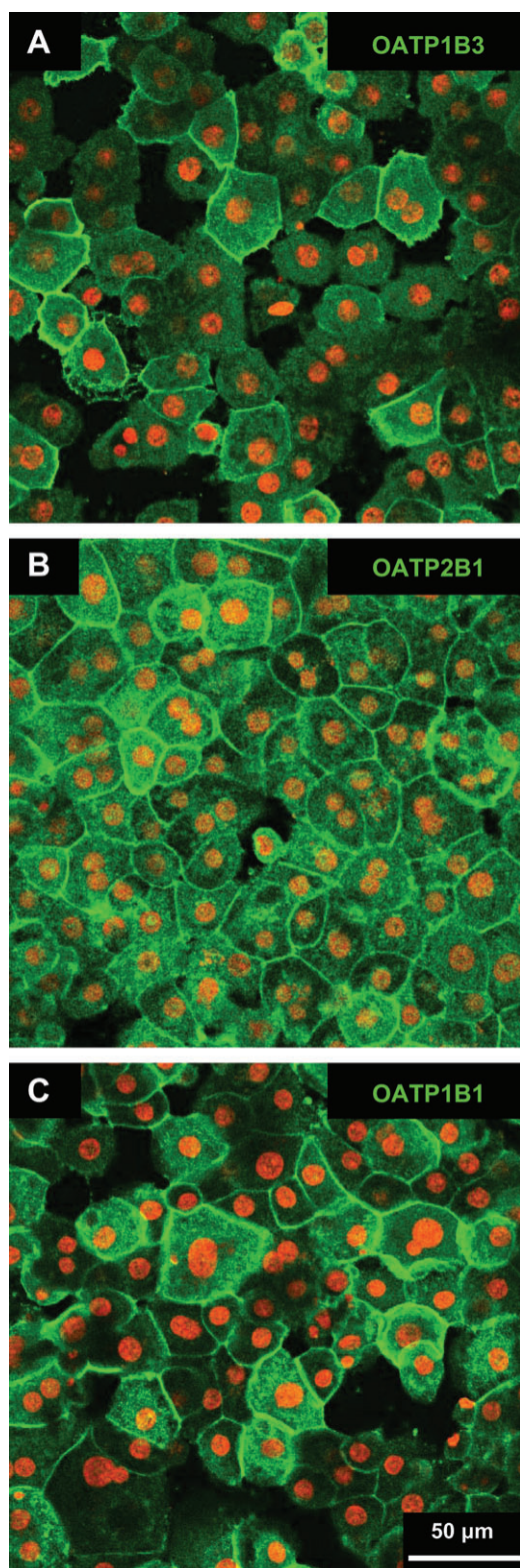


FIG. 6. Localization of OATP1B3, OATP2B1, and OATP1B1 in primary human hepatocytes. Immunofluorescence analyses of primary human hepatocytes were performed on day 3 of culture. The uptake transporters were detected with the respective antibodies described under Materials and Methods (green). The nuclei were stained with propidium iodide (red); bars, 50 μ m.

labeled amanitin at the concentrations studied. Such substrate selectivity is also known for the transport of other substrates by OATP1B3, as exemplified for ouabain, digoxin, the peptide hormone CCK-8, and fexofenadine (Ismair *et al.*, 2001; Kullak-Ublick *et al.*, 2001). More commonly, OATP1B3, OATP2B1, and OATP1B1 share a large number of substrates, as shown, e.g., for BSP, dehydroepiandrosterone 3-sulfate, and fluvastatin (Kopplow *et al.*, 2005; Kullak-Ublick *et al.*, 2001). There is also a broad overlap of substrate specificity between OATP1B3 and OATP1B1 for endogenous substrates such as 17β -glucuronosyl estradiol and bile acids like cholytaurine and cholyglycine (Hagenbuch and Meier, 2004; Ismair *et al.*, 2001; Shimizu *et al.*, 2005), as well as for peptide substrates such as the endothelin antagonist BQ-123, the opioid receptor agonist [D-Pen², D-Pen⁵]-enkephalin (Kullak-Ublick *et al.*, 2001), and the cyanobacterial toxin microcystin-LR, a cyclic heptapeptide (Fischer *et al.*, 2005). Notably, OATP1B1 is the main transporter of the heptapeptide phalloidin, the main toxin of the phalloxin family produced by the same mushroom (Fehrenbach *et al.*, 2003; Meier-Abt *et al.*, 2004).

In order to obtain more evidence for OATP1B3 as the main uptake transport protein for amatoxins, the viability of cells transfected with the various hepatic OATP proteins was studied in the presence of amanitin. The toxin in these experiments was O-methyl- α -amanitin, a toxin structurally related to the radiolabeled O-methyl-dehydroxymethyl- α -amanitin, with a toxicity similar to α -amanitin. We found that after 24 h the viability of OATP1B3-expressing cells was strongly inhibited at low toxin concentrations, whereas cells expressing OATP2B1 or OATP1B1 remained unaffected (Fig. 4); at higher toxin concentrations (10 μ M), viability also decreased in these latter cells (Fig. 5), suggesting that additional, yet unidentified pathways of amanitin uptake may exist. Here it is of interest to note that in many cell lines tested *in vitro*, for which OATP transporters were not reported, viability was inhibited by α -amanitin at concentrations above 1 μ M after 72 h. Obviously, the duration of toxin exposure of cells in culture is important, as indicated by MDCKII-OATP1B1 and MDCKII-OATP2B1 cells, which showed a significant loss of viability at low toxin concentrations, when incubated for 72 h (data not shown).

In the past, the uptake of amanitin into rat hepatocytes was studied in much detail (Faulstich *et al.*, 1974; Floersheim, 1971, 1978; Jahn *et al.*, 1980; Wieland and Faulstich, 1978). It was shown that this uptake can be inhibited by prednisolone phosphate, penicillin G, silibinin dihemisuccinate, and antamanide (Wieland and Faulstich, 1978). Subsequent studies with rat hepatocyte basolateral membrane vesicles suggested that the uptake of amanitin may occur both in a sodium-dependent and sodium-independent way (Kröncke *et al.*, 1986). Interestingly, some of the inhibitors that interfered with the uptake into rat hepatocytes, like antamanide and silibinin dihemisuccinate, also inhibited the transport of radiolabeled amanitin by the human uptake transporter OATP1B3 (Table 2), which markedly differs from rat hepatocyte OATP transporters

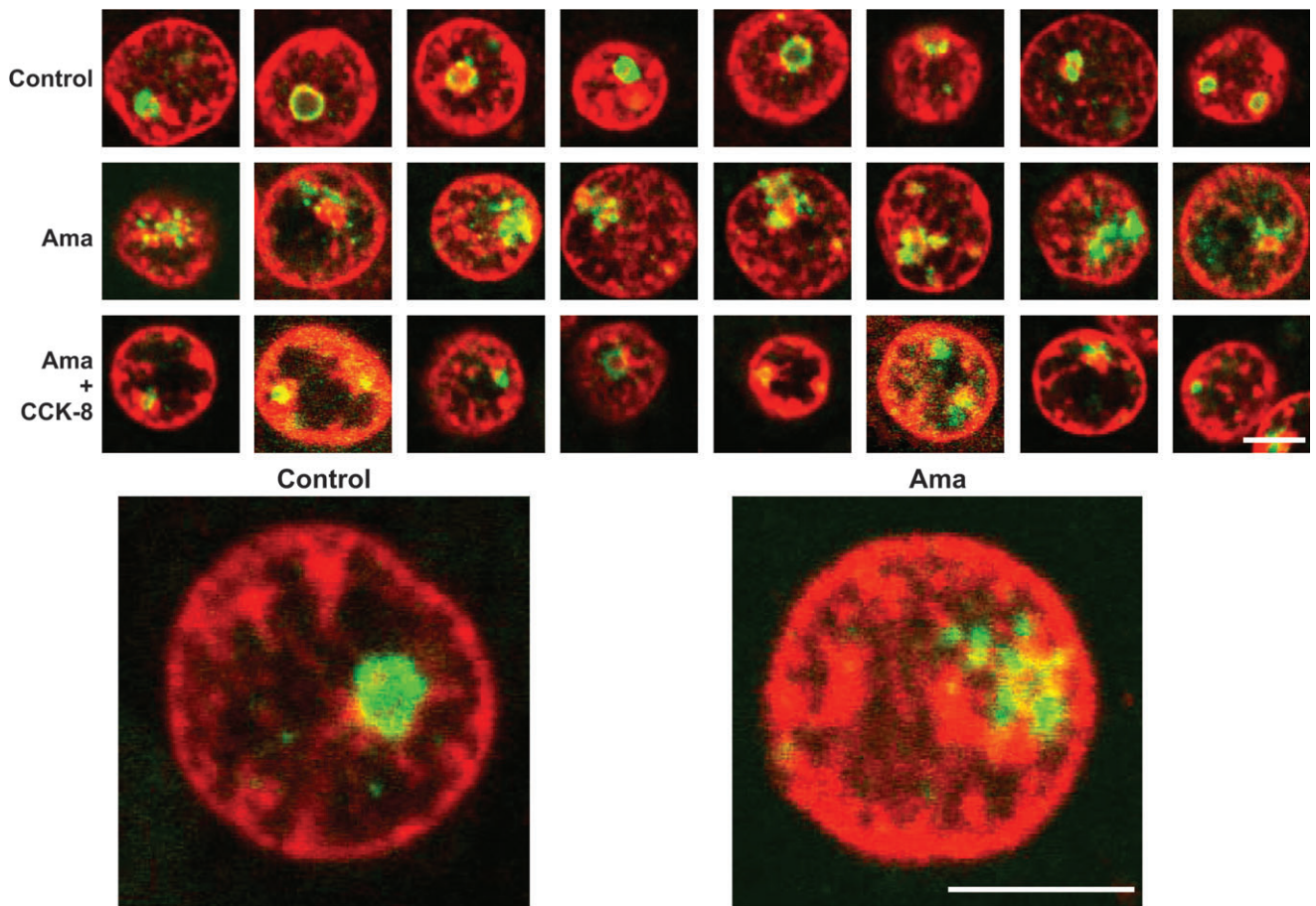


FIG. 7. Fragmentation of nucleoli of primary human hepatocytes by amanitin. Intoxication studies were performed on day 3 of culture. Cells were incubated with O-methyl- α -amanitin (1 μ M) in the absence (Ama) or presence of CCK-8 (50 μ M) (Ama + CCK-8) for 8 h. Hepatocytes cultured without amanitin served as controls. Nucleoli were stained with anti-nucleolin antibody (green), the nuclei with propidium iodide (red); bars, 5 μ m.

in its amino acid sequence (Hagenbuch and Meier, 2004). On the other hand, substances identified in our recent studies as inhibitors of human OATP1B3, like cyclosporin A (Letschert *et al.*, 2004), MK571 (Letschert *et al.*, 2005), and rifampicin (Cui *et al.*, 2001; Vavricka *et al.*, 2002) were now shown to be potent inhibitors of amatoxin uptake (Table 2). Paclitaxel (Smith *et al.*, 2005), BSP (König *et al.*, 2000b), CCK-8 (Ismair *et al.*, 2001), and montelukast also inhibited 3 H-amanitin uptake, as well as penicillin G and prednisolone phosphate, known as antidotes for human amanitin poisoning (Table 2). Some of the substances newly described in the present study as inhibitors of OATP1B3-mediated amatoxin transport represent potential new antidotes in human amanitin poisoning, provided their use is not restricted by additional intrinsic biological activities.

Measurement of cell viability after exposure to unlabeled amanitin in the absence or presence of potential antidotes seemed to be useful as a screening assay for potential inhibitors of OATP1B3 (Table 5). The reduced amanitin toxicity in MDCKII-OATP1B3 cells coincubated with substrates and

TABLE 5
Inhibition of Amanitin-Induced Cell Damage in
OATP1B3-Expressing Cells

	IC ₅₀ (μ M)
Prednisolone phosphate	125
Penicillin G	20
Montelukast	17
BSP	15
Antamanide	6
Paclitaxel	5
Silibinin dihemisuccinate	3
CCK-8	0.9
MK571	0.6
Rifampicin	0.6
Cyclosporin A	0.3

Note. MDCKII cells, cultured in 96-well plates were incubated with 1 μ M O-methyl- α -amanitin in the absence or presence of the inhibitors in culture medium. After 30 min the cells were washed and cultured for additional 24 h, and differences in viability was measured by AlamarBlue assays. Control cells and OATP2B1- and OATP1B1-expressing cells did not show a significant loss of viability under these conditions ($n = 20$).

inhibitors indicates that administration of high-affinity substrates of OATP1B3 could provide a therapeutic option to reduce liver damage in amanitin-intoxicated patients. As shown in Table 2, the OATP1B3-mediated transport of ^3H -amanitin was inhibited by several substances with IC_{50} values below $1\text{ }\mu\text{M}$. We therefore asked whether these inhibitors would affect the transport of other OATP substrates as well, e.g., of ^3H -BSP. We found that the uptake of BSP is reduced by these inhibitors in the same concentration range ($0.3\text{--}4\text{ }\mu\text{M}$), except for antamanide which required a concentration of about 20-fold higher (Tables 3 and 4). There was almost no inhibition of BSP transport by antamanide with the other two transporting proteins OATP2B1 and OATP1B1.

Our observations suggest that amanitin intoxication may be reduced by the administration of inhibitory or competitive OATP1B3 substrates, which may inhibit primary uptake and particularly secondary amanitin uptake during enterohepatic circulation and, by this, alleviate the cytotoxic effects on hepatocytes. This is in line with previous observations describing a protective effect against *Amanita* mushroom poisoning in humans by the OATP1B3 substrate penicillin G (Jander and Bischoff, 2000). Rifampicin was a potent inhibitor of OATP1B3-mediated BSP transport (Cui *et al.*, 2001) whereas OATP2B1- and OATP1B1-mediated uptake was inhibited only at higher concentrations (Table 3). Serum concentrations 4 h after oral administration of 1200 mg rifampicin may reach $36\text{ }\mu\text{M}$ (Acocella, 1983). Kinetic analyses after human amanitin intoxications showed that plasma concentrations of α -amanitin, approximately 36 h after ingestion, were in a range between 9 and 210 nM (Jaeger *et al.*, 1993). Thus, rifampicin treatment may have a therapeutic impact on amanitin intoxications superior to that of previously used antidotes.

Cytotoxicity was not only studied in stably transfected MDCKII cells but also in primary human hepatocytes. We demonstrated the prevention of nucleolar fragmentation in human hepatocytes by coinubation with the OATP1B3-specific substrate CCK-8 (Fig. 7). Since the fragmentation of nucleoli is a characteristic event in cells treated with α -amanitin (Brasch and Sinclair, 1978; Fiume, 1975; Kedinger and Simard, 1974), we used it as an indicator of damage in primary human hepatocytes. The uptake of amanitin into human hepatocytes was inhibited by CCK-8, as suggested by the reduced nucleolar fragmentation (Fig. 7). Thus, our identification of the uptake transporter for amanitin and its derivatives in human hepatocytes may contribute to improved therapeutic interventions in *Amanita* poisoning.

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