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Inhibition of transport across the hepatocyte canalicular membrane by the antibiotic fusidate

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Abstract

Hyperbilirubinemia is a frequent side effect induced by long-term therapy with the antibiotic fusidate. The aim of this study was to elucidate the molecular mechanisms of fusidate-induced hyperbilirubinemia by investigating its influence on hepatic transport systems in the canalicular membrane. Using canalicular membrane vesicles from rat liver, we determined the effect of fusidate on the adenosine 5′-triphosphate (ATP)-dependent transport of substrates of the apical conjugate export pump, multi-drug resistance protein 2 (Mrp2, symbol Abcc2) and the bile salt export pump (Bsep, symbol Abcb11). Fusidate inhibited the ATP-dependent transport of the Mrp2 substrates 17β-glucuronosyl estradiol and leukotriene C_4, and the transport of cholyltaurine by Bsep with K_i values of 2.2 ± 0.3, 7.6 ± 1.3, and 5.5 ± 0.8 μM, respectively. To elucidate the *in vivo* implication of these findings, the effect of fusidate treatment on the elimination of intravenously administered tracer doses of 17β-glucuronosyl estradiol and cholyltaurine into bile was studied in rats. Treatment with fusidate (100 μmol/kg body weight) reduced the biliary excretion rate of 17β-glucuronosyl [^{3}H]estradiol and [^{3}H]cholyltaurine by 75 and 80%, respectively. Extended treatment of rats with fusidate (100 μmol/kg body weight, three times daily i.p. for 3 days) reduced hepatic Mrp2 protein levels by 61% ($P < 0.001$). Our data suggest that there are at least two different mechanisms involved in the impairment of transport processes and hepatobiliary elimination by fusidate, direct inhibition of transport of Mrp2 and Bsep substrates by competitive interaction and impairment by a decreased level of hepatic Mrp2.

Keywords: Fusidate; Multi-drug resistance protein; Bile salt transport; Hyperbilirubinemia; Cholestasis; ATP-dependent transport

1. Introduction

Fusidate is a mono-anionic steroidal antibiotic widely used for the treatment of infectious diseases with methicillin-resistant or, more recently, multi-resistant *Staphylococcus aureus* strains [1–3]. One of the major side effects of fusidate treatment is the induction of conjugated hyperbilirubinemia which may occur as early as 2 days after starting the treatment [4,5]. The incidence of fusidate-induced hyperbilirubinemia ranged from 17 to 48% when given intravenously [6,7]. When treatment is discontinued, elevated serum bilirubin levels may return to normal within 4–6 days [7,8]. So far the molecular mechanisms responsible for fusidate-induced hyperbilirubinemia have not been elucidated.

Cholestasis may be a side effect of drug treatment provoked by a variety of substances such as macrolides [9], penicillins [9], ethinylestradiol [10] and cyclosporin A [11]. Interference with the ATP-dependent transport of amphiphilic anions into bile is one of the mechanisms considered to play a key role [12,13]. Membrane proteins mediating the ATP-dependent transport of amphiphilic anions across the canalicular membrane include the apical multi-drug resistance protein 2 (MRP2) [14,15] and the bile salt export pump (BSEP), a member of the ABCB subfamily of the ATP-binding cassette transporters [16].

MRP2 mediates the ATP-dependent transport of conjugated bilirubin from the hepatocyte into bile with high affinity [17]. The absence of functional MRP2 in the human hepatocyte canalicular membrane is the molecular basis of the Dubin–Johnson syndrome [18], which is

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Abbreviations: ATP, adenosine 5′-triphosphate; 5′-AMP, adenosine 5′-monophosphate; MDR, multi-drug resistance; PBS, phosphate-buffered saline; MRP (symbol ABCC), human multi-drug resistance protein; BSEP (symbol ABCB11), human bile salt export pump; Mrp (symbol Abcc), rat multi-drug resistance protein; Bsep (symbol Abcb11), rat bile salt export pump; LTC_4, leukotriene C_4; K_i, inhibition constant; K_m, Michaelis–Menten constant; i.p., intraperitoneal; CM, canalicular membrane-enriched fraction; TTBS, Tris-buffered saline containing 0.05% Tween 20.

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characterized by conjugated hyperbilirubinemia. Inhibition of MRP2 has so far been demonstrated for cyclosporin A [17,19] and 17β-D-glucuronosyl estradiol [13].

BSEP is predominantly expressed in the liver and localized to the canalicular membrane [16,20]. In contrast to MRP2 with its broad substrate specificity, BSEP has been defined as the specific bile salt transporter with high affinity for cholyltaurine [13,16]. Progressive familial intrahepatic cholestasis type 2, an inherited liver disease of childhood characterized by cholestasis and elevated serum γ-glutamyltransferase activity, was shown to be associated with mutations in the *BSEP* gene [20]. Cyclosporin A, rifamycin SV, rifampicin, and glibenclamide were found to inhibit BSEP transport function [12,13,19]. It is of interest to note that fusidate [3] is both, a structural analog of the prototypic Bsep substrate cholyltaurine [16] and of the prototypic Mrp2 substrate 17β-D-glucuronosyl estradiol [15].

The aim of the present study was to investigate whether fusidate affects the transport across the hepatocyte canalicular membrane. Although fusidate-induced hyperbilirubinemia was described in humans, we chose the rat model for our investigations as the human and the rat orthologs of MRP2 exhibit very similar transport characteristics for conjugated bilirubin [17]. Further, the rat represents a suitable model for *in vivo* experiments on hepatobiliary elimination of many substances. Evidence derived from both, *in vitro* and *in vivo* experiments supports the assumption that fusidate acts as an inhibitor of the ATP-dependent transport by Mrp2 and Bsep into bile. Furthermore, fusidate administration to rats leads to a decrease in Mrp2 protein levels upon long-term treatment.

2. Experimental procedures

2.1. Materials

[14,15,19,20-^{3}H]LTC_4 (6.1 TBq/mmol), [^{3}H]cholyltaurine (0.77 TBq/mmol), and 17β-D-glucuronosyl [6,7-^{3}H]-estradiol (2 TBq/mmol) were obtained from Du-Pont/New England Nuclear. Unlabeled LTC_4 was from Cascade Biochem Ltd., unlabeled 17β-D-glucuronosyl estradiol, cholyltaurine, ATP, adenosine 5′-monophosphate (AMP), creatine phosphate, and fusidate were obtained from Sigma. Nitrocellulose filters (pore size 0.2 μM) were purchased from Schleicher & Schüll. Cell culture media and supplements were obtained from Sigma–Aldrich Chemie. G418 (Geneticin) was purchased from Calbiochem. Sodium butyrate was from Merck Schuchardt.

2.2. Antibodies

The rabbit polyclonal antibody EAG15 was raised against a 12 amino acid peptide at the carboxyl terminus of rat Mrp2 [14]. A mouse monoclonal antibody C219 against multi-drug resistance (MDR) P-glycoproteins, including Mdr1a, Mdr1b, Mdr2, and the bile salt export pump Bsep was purchased from Centocor. The polyclonal antibody anti-SPGP directed against the carboxyl terminus of human and rodent Bsep was purchased from Kamiya Biomedical Company.

2.3. Animals

Male Wistar rats weighing 180–220 g were obtained from Charles River Wiga. The animals were kept in our department for laboratory animals and fed a stock diet. Animals were kept according to the Guide for the Care and Use of Laboratory Animals (NIH publication 86–23, revised 1985).

2.4. Cell culture

A *Mrp*2-transfected human embryonic kidney cell line 293 (HEK-293) was generated in our laboratory [15] using the cDNA encoding rat *Mrp2* as defined by EMBL/GenBank accession number X96393 [14]. HEK-293 cells transfected with the control vector served as control. HEK-293 cells were grown in minimum essential medium (Sigma) with 10% fetal calf serum (v/v) and were selected with G418 (Geneticin). Cells were kept in a humidified incubator (air/CO_2, 19/1, at 37°). Protein concentrations of cell homogenates were determined using the Lowry method.

2.5. Membrane preparations

Rat liver plasma membrane vesicles enriched in canalicular membranes were prepared as described by Böhme *et al*. [12] using a modified procedure of the one described by Meier and Boyer [21]. In the first low speed centrifugation step nuclear fragments were separated from the liver homogenate. A canalicular membrane-enriched fraction (CM) and a sinusoidal membrane-enriched fraction were isolated from the homogenate by subsequent differential and isopycnic zone centrifugation steps with sucrose and Percoll density gradients. Plasma membrane vesicles from HEK- 293 cells stably transfected with rat *Mrp2* cDNA or control vector were prepared as described previously [15]. Membrane vesicles were frozen and stored in liquid nitrogen.

2.6. Preparation of crude membrane fractions from rat liver

Liver tissue (2 g) was homogenized and connective tissue was removed by pressing the liver through a metal sieve plate (pore width 1 mm). The resulting homogenate was diluted with 10 mL hypotonic buffer (1 mM EDTA, 5 mM sodium phosphate; pH 7.0) and homogenized with 20 passes in a tight-fitting Dounce B homogenizer.

After centrifugation (100,000 *g*, 4°, 45 min) pellets were resuspended in Tris buffer (250 mM sucrose, 10 mM Tris–HCl; pH 7.4), frozen and stored in liquid nitrogen. All membranes were prepared in the presence of protease inhibitors (0.3 mM aprotinin, 1.0 mM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 mM pepstatin) at 4°.

2.7. Transport studies with membrane vesicles

The transport of labeled substrates into membrane vesicles was measured using a rapid filtration method [22]. Briefly, membrane vesicles (20 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM creatine phosphate, 100 μg/mL creatine kinase, and labeled substrate with and without fusidate in an incubation buffer (250 mM sucrose, 10 mM Tris–HCl; pH 7.4) at 37°. The final volume was 55 μL. Aliquots (20 μL) were removed at the indicated time points, diluted in 1 mL of ice-cold incubation buffer, and immediately filtered through a presoaked nitrocellulose membrane (0.2 μm pore size). Filters were rinsed eight times with 1 mL of incubation buffer, dissolved in scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5′-AMP. ATP-dependent transport was calculated by subtracting values obtained in the presence of 5′-AMP from those in the presence of ATP. For determination of kinetic constants, initial transport rates were measured at five different substrate concentrations (25–1000 nM for LTC_4 and 0.5–8 μM for 17β-glucuronosyl estradiol). The concentrations of the labeled substrate were kept constant and varying concentrations of unlabeled substrate were added. K_m values were determined as the substrate concentration at half-maximal velocity of transport under the experimental conditions described above using double-reciprocal plots according to Lineweaver and Burk (1934). Similar results were obtained by direct curve-fitting to the Michaelis–Menten equation using the computer program Sigma Plot.

2.8. Biliary excretion of 17β-D-glucuronosyl [^{3}H]estradiol and [^{3}H]cholyltaurine during fusidate infusion

Animals were anesthetized with xylazine (Bayer, 12 mg/kg body weight) and ketamine (Parke-Davis, 80 mg/kg body weight), and the common bile duct was cannulated with a polypropylene tube (outer diameter 0.6 mm). Bile was collected into preweighed tubes at 5 min intervals and bile flow was calculated based on the weight of the samples. After reaching constant bile flow, 3 mL fusidate solution (100 μmol/kg body weight in phosphate-buffered saline (PBS)) or PBS (controls) was infused into the femoral vein for 60 min. A single bolus of [^{3}H]cholyltaurine (1 μCi/kg body weight, corresponding to 31 pmol/kg, in 200 μL PBS) or 17β-D-glucuronosyl [^{3}H]estradiol (1 μCi/kg body weight, corresponding to 18 pmol/kg, in 200 μL PBS) was given intravenously 50 min after beginning of the fusidate infusion. Bile samples were collected for further 60 min after discontinuation of the fusidate infusion. Aliquots of bile samples were counted for radioactivity to determine the biliary excretion rate for the different tracers.

2.9. Biliary excretion of 17β-D-glucuronosyl [^{3}H]estradiol after fusidate treatment for 3 days

Male rats were treated three times daily intraperitoneally (i.p.) with fusidate (100 μmol/kg body weight) for 3 days. Animals were anesthetized, and bile was collected as described above. After reaching constant bile flow, a bolus of 17β-D-glucuronosyl [^{3}H]estradiol was given intravenously (1 μCi/kg body weight, corresponding to 18 pmol/kg, in 200 μL PBS). Bile samples were collected until 60 min after application of the tracer. Then the liver was perfused with 0.9% NaCl solution through the portal vein and harvested for immunoblot analysis. Aliquots of bile samples were counted for radioactivity to determine the biliary excretion rate of the tracer.

2.10. Immunoblotting and immunodetection

Samples of liver homogenates (20 μg protein) were mixed with sodium dodecyl sulfate-containing loading buffer, incubated at 37° for 30 min, and separated on 7.5% polyacrylamide gels in the presence of 3-mercaptoethanol. After transfer to nitrocellulose membranes, blots were blocked in 5% low-fat dried milk dissolved in Tris-buffered saline containing 0.05% Tween 20 (TTBS) for 2 hr. Incubation time of membranes with the primary and secondary antibody was for 1 hr each. The respective antibodies were diluted in 5% dried milk dissolved in TTBS. After each incubation, blots were washed three times with TTBS. Antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad) and the enhanced chemiluminescence technique (Amersham-Buchler). The following dilutions of antibodies were used: EAG15 at 1:10,000; C219 at 1:300; anti-SPGP at 1:2000 horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies at 1:3000. Immunoreactive bands on autoradiography films were scanned (HP ScanJet 5100C; Hewlett-Packard) and quantified using the Raytest Image Software.

2.11. Statistical analysis

For biliary excretion experiments, data are expressed as the mean ± SD from six animals per group. For densitometry of immunoblots, 10 liver membrane preparations were analyzed from the control and the treatment group of the 72 hr experiment, and 4 liver membrane preparations from each group of the 24 hr experiment.

3. Results

3.1. Fusidate inhibits ATP-dependent transport in canalicular membrane vesicles

The inhibitory effect of fusidate on the ATP-dependent excretion of amphiphilic anions from the hepatocyte into the bile was studied with inside-out canalicular membrane vesicles from rat liver. As shown in Fig. 1A and Table 1 incubation of canalicular membrane vesicles with fusidate led to a competitive inhibition of the ATP-dependent transport of [^{3}H]cholyltaurine with a K_i of 2.2 ± 0.3 μM. This indicates that fusidate competes with the physiological substrate of the canalicular bile salt export pump Bsep.

Moreover, fusidate competitively inhibited ATP-dependent transport of [^{3}H]LTC$_4$ (Fig. 1B) and 17β-glucuronosyl [^{3}H]estradiol (Fig. 1C) with K_i values of 5.5 ± 0.8 and 7.6 ± 1.3 μM, respectively (Table 1). Since LTC$_4$ and 17β-glucuronosyl estradiol are high-affinity substrates for Mrp2, the data suggest that fusidate also leads to a strong inhibition of Mrp2-mediated transport.

3.2. Inhibition of ATP-dependent transport in membrane vesicles from rat Mrp2-transfected cells by fusidate

In order to confirm that Mrp2-mediated transport is inhibited by fusidate, transport studies were performed with membrane vesicles from HEK cells stably transfected with rat *Mrp2* cDNA (HEK–Mrp2). As depicted in Fig. 2 and Table 1 fusidate suppressed the ATP-dependent transport of [^{3}H]LTC$_4$ by recombinant rat Mrp2 with a K_i of 13 ± 2.1 μM.

3.3. Decrease of the biliary excretion of 17β-glucuronosyl [^{3}H]estradiol and [^{3}H] cholyltaurine in rats treated with fusidate

To elucidate the *in vivo* implication of the competitive inhibition of transport of Mrp2 and Bsep substrates by fusidate, we studied its effect on the recovery of intravenously administered tracer doses of 17β-glucuronosyl [^{3}H]estradiol and [^{3}H]cholyltaurine in bile. The tracers were administered 50 min after commencing the intravenous infusion of fusidate (100 μmol/kg body weight). Treatment with fusidate led to a strong decrease of the cumulative biliary excretion of both, 17β-glucuronosyl [^{3}H]estradiol (Fig. 3A) and [^{3}H]cholyltaurine (Fig. 4A). The maximal excretion rate of 17β-glucuronosyl [^{3}H]estradiol and [^{3}H]cholyltaurine decreased from $1.0\,\text{pmol} \times \text{min}^{-1} \times (\text{kg body weight})^{-1}$ to $0.26\,\text{pmol} \times \text{min}^{-1} \times (\text{kg body weight})^{-1}$ and from $4.2\,\text{pmol} \times \text{min}^{-1} \times (\text{kg body weight})^{-1}$ to $0.9\,\text{pmol} \times \text{min}^{-1} \times (\text{kg body weight})^{-1}$ (Figs. 3C and 4C), respectively. Total bile flow, as calculated under steady state conditions at 120 min, was reduced upon fusidate treatment (Figs. 3B and 4B) ($P < 0.001$).

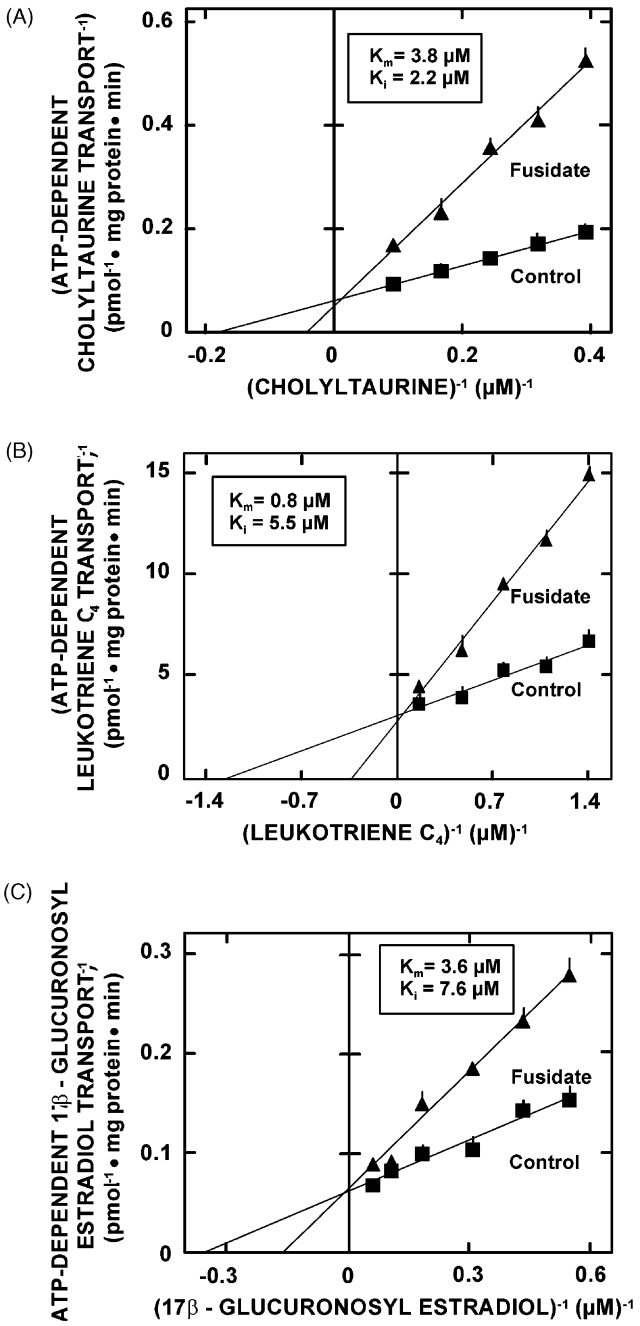

Fig. 1. ATP-dependent transport of [^{3}H]cholyltaurine (A), [^{3}H]leukotriene C$_4$ (B) and 17β-glucuronosyl [^{3}H]estradiol (C) into canalicular membrane vesicles from rat liver and their competitive inhibition by sodium fusidate. Double-reciprocal plots according to Lineweaver and Burk in the absence (control) or presence of 10 μM sodium fusidate with varying concentrations of substrate. The K_m values refer to the uninhibited transport of the respective substrates, whereas K_i values refer to fusidate. Mean ± SD of four determinations.

3.4. Influence of fusidate on the level of Mrp2 and Bsep protein in rat liver

As shown by immunoblot analysis, treatment of rats with fusidate (100 μmol/kg body weight i.p. three times daily) for 3 days reduced hepatic Mrp2 protein by 64%

Table 1
Kinetic constants for the inhibition of ATP-dependent transport into inside-out membrane vesicles by fusidate

Substrate	Membrane	K_m (μM)	K_i (μM)
17β-Glucuronosyl estradiol	CM	3.6 ± 1.0	7.6 ± 1.3
Cholyltaurine	CM	3.8 ± 1.1	2.2 ± 0.3
Leukotriene C_4	CM	0.8 ± 0.2	5.5 ± 0.8
Leukotriene C_4	HEK–Mrp2	0.7 ± 0.2	13 ± 2.2

ATP-dependent transport of [^{3}H]cholyltaurine, [^{3}H]leukotriene C_4, and 17β-glucuronosyl [^{3}H]estradiol into membrane vesicles and the competitive inhibition by fusidate were determined. The K_m values refer to the uninhibited transport of the respective substrates, and K_i values refer to fusidate. Mean ± SD from four measurements. CM, rat hepatocyte canalicular membranes; HEK–Mrp2, membrane vesicles containing recombinant rat Mrp2 [15].

of untreated controls (Fig. 5). Mrp2 protein levels were not significantly decreased when fusidate (100 μmol/kg body weight i.p.) was given twice over a time period of 24 hr (Fig. 5). The levels of hepatic P-glycoproteins and of Bsep were unchanged in both experiments (Fig. 5).

3.5. Influence of prolonged treatment with fusidate on biliary excretion of 17β-glucuronosyl [^{3}H]estradiol

To study the impact of the decrease of Mrp2 protein on biliary transport of Mrp2 substrates, we investigated the influence of prolonged treatment with fusidate on the biliary excretion of 17β-glucuronosyl [^{3}H]estradiol. Fusidate was applied i.p. three times daily at a dose of 100 μmol/kg body weight for 3 days. The final injection was given 14 hr before the injection of the radioactive tracer. Fusidate treatment decreased the maximal biliary excretion of 17β-glucuronosyl [^{3}H]estradiol from 1.3 ± 0.2 pmol × min^{-1}× (kg body weight)$^{-1}$ in the control group to 0.8 ± 0.2 pmol × min^{-1}× (kg body weight)$^{-1}$ in the treated group. The bile flow of the animals treated with fusidate remained unchanged (data not shown).

In animals undergoing prolonged administration of fusidate, blood levels of unconjugated and conjugated bilirubin remained unchanged when compared to controls (data not shown).

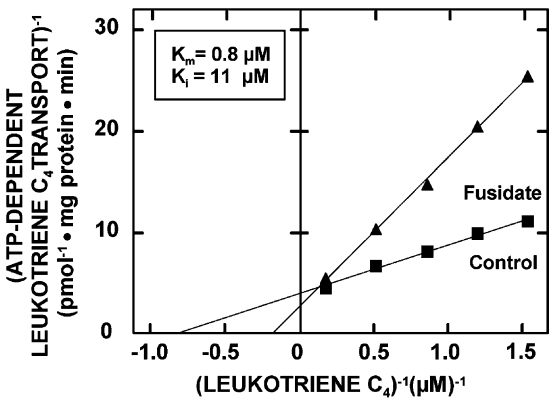

Fig. 2. ATP-dependent transport of [^{3}H]leukotriene C_4 into membrane vesicles from HEK cells stably expressing rat Mrp2 and its competitive inhibition by fusidate. Double-reciprocal plots according to Lineweaver and Burk in the absence (control) or presence of 30 μM fusidate with varying concentrations of substrate are shown. The K_m value refers to the uninhibited transport of [^{3}H]leukotriene C_4, the K_i value to fusidate. Mean ± SD of three determinations.

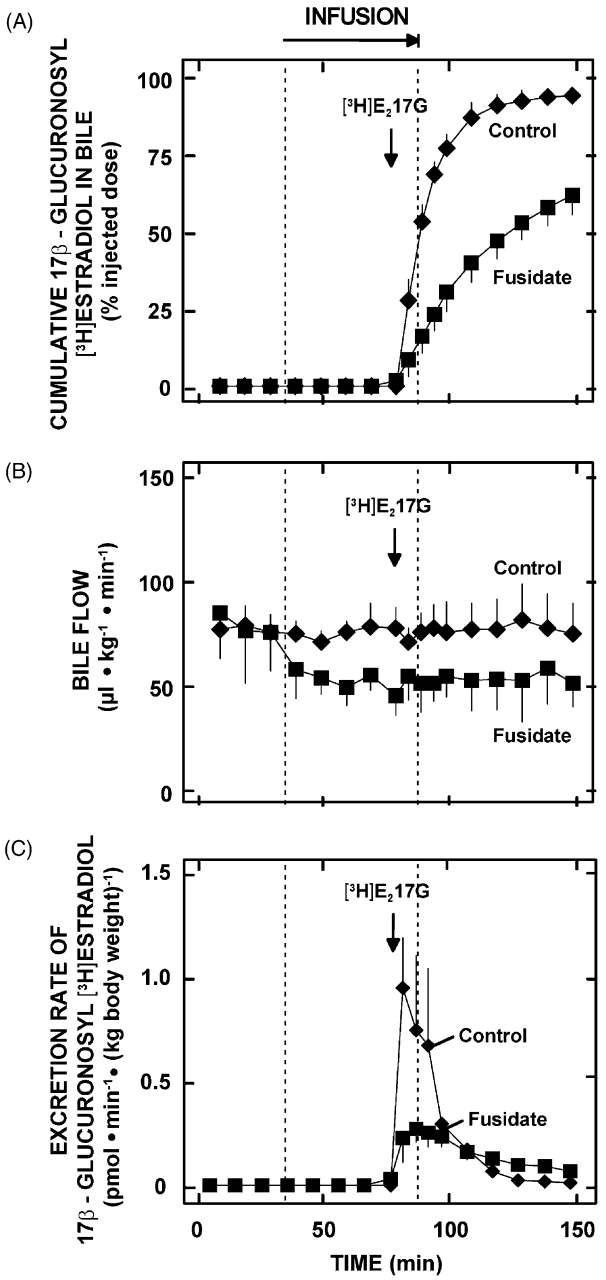

Fig. 3. Biliary excretion of 17β-glucuronosyl [^{3}H]estradiol ([^{3}H]$E_2$17G) and bile flow in rats treated with fusidate. Tracer doses of 17β-glucuronosyl [^{3}H]estradiol were administered intravenously after 50 min of intravenous infusion with fusidate (100 μmol/kg body weight) (■) or with PBS (◆). (A) Cumulative excretion of 17β-glucuronosyl [^{3}H]estradiol. (B) Bile flow. (C) Biliary excretion rate of 17β-glucuronosyl [^{3}H]estradiol. Results are mean ± SD from seven animals each in the control and in the treatment group.

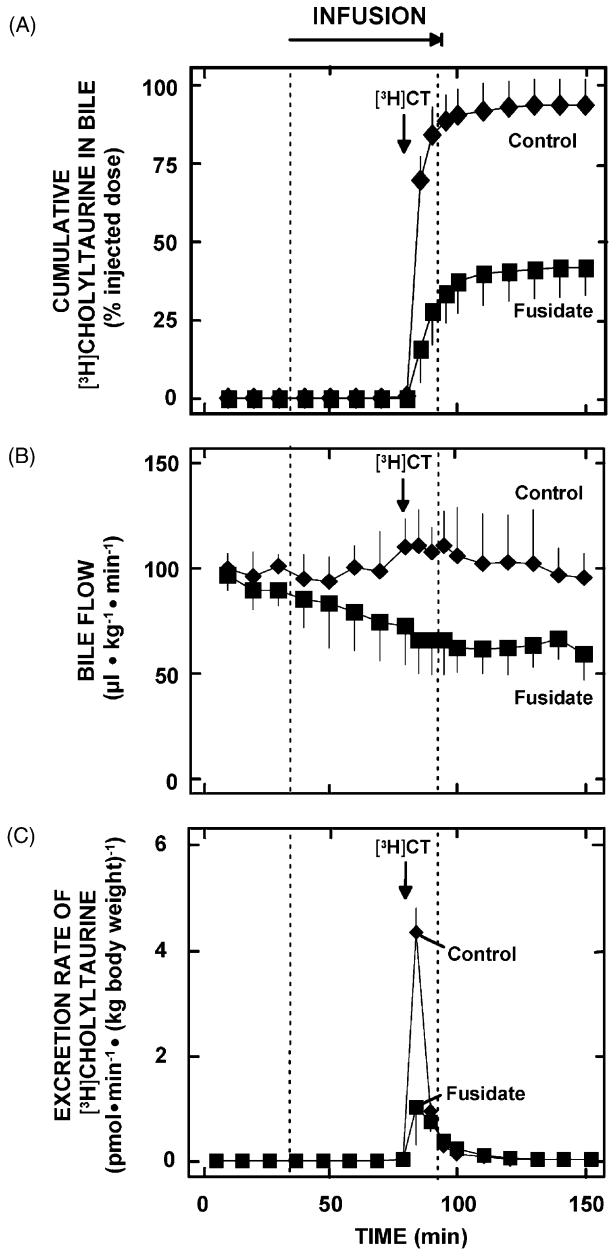

Fig. 4. Biliary excretion of [^{3}H]cholyltaurine (CT) and bile flow in rats treated with fusidate. Tracer doses of [^{3}H]cholyltaurine were administered intravenously after 50 min of intravenous infusion with fusidate (100 µmol/kg body weight) (■) or with PBS (◆). (A) Cumulative excretion [^{3}H]cholyltaurine. (B) Bile flow. (C) Biliary excretion rate of [^{3}H]cholyltaurine. Results are mean ± SD from seven animals each in the control and in the treatment group.

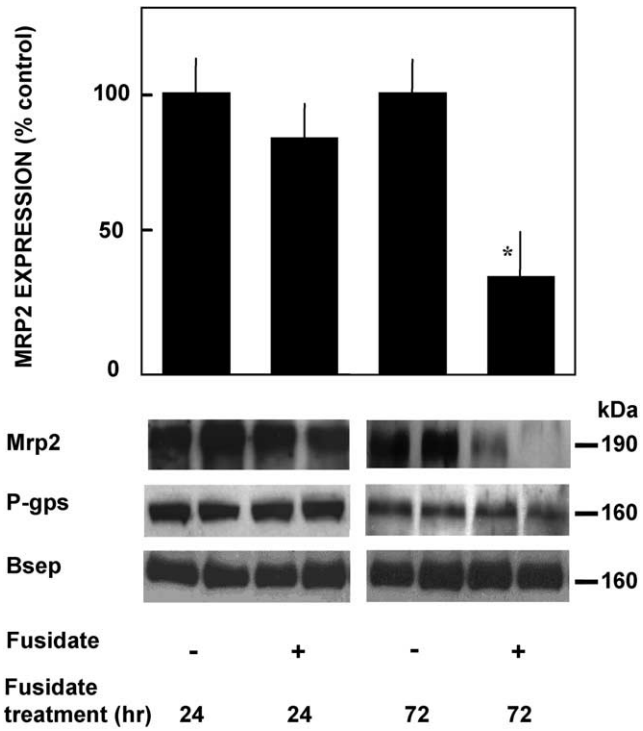

Fig. 5. Immunoblot analysis of Mrp2 in rat liver after treatment with fusidate for 24 and 72 hr. Homogenates of rat liver (30 µg protein per lane each) for Mrp2 and C219 and canalicular membrane of rat liver (10 µg protein per lane each) for Bsep were separated by SDS–PAGE, transferred to nitrocellulose membranes, and probed with the polyclonal antibody EAG15 [14] and anti-SPGP, and the monoclonal antibody C219. Protein levels of Mrp2 were determined densitometrically. A standard curve was prepared from the control homogenates. Results are mean ± SD of 4 (24 hr) or 10 (72 hr) animals in the control and in the treated group. $^*P < 0.001$.

4. Discussion

Intrahepatic cholestasis and conjugated hyperbilirubinemia observed in patients treated with fusidate raised the question whether this antibiotic influences canalicular transport processes and expression of hepatocellular Mrp2. In this study, we have shown that fusidate competitively inhibits ATP-dependent transport of the Mrp2 substrates 17β-glucuronosyl estradiol and LTC_4 and the Bsep substrate cholyltaurine into rat canalicular membrane vesicles. K_i values were well below plasma concentrations reached during regular administration of fusidate to rats [23] or man [24,25]. Using Mrp2-transfected cells we have demonstrated that the inhibition of the transport of Mrp2 substrates by fusidate is mediated by a direct inhibition of Mrp2. In *in vivo* experiments fusidate reduced bile flow and inhibited biliary excretion of cholyltaurine and of 17β-glucuronosyl estradiol (Figs. 3 and 4). Prolonged treatment with fusidate resulted in a marked decrease of hepatic Mrp2 protein (Fig. 5) and reduced biliary excretion rates of 17β-glucuronosyl estradiol, whereas short-term treatment had no significant influence on Mrp2 protein levels (Fig. 5).

From the transport experiments we conclude that fusidate acts as a competitive inhibitor of Mrp2 and Bsep. Because of the competitive type of the inhibition, fusidate will probably bind at the substrate binding site of both transporters. Since radioactively labeled fusidate was not available, we have not determined whether fusidate itself is a substrate of Mrp2 or Bsep.

Since it is known that fusidate is metabolized in the liver by acetylation or conjugation with glucuronate [26], it would be of interest whether the conjugated antibiotic also has an inhibitory effect on canalicular transport processes, particularly on Mrp2. In addition to the inhibition shown

here, such effects of the metabolites of fusidate could further contribute to the inhibition of biliary excretion of Mrp2 and Bsep substrates measured in the short-term experiments.

Although fusidate was shown to competitively inhibit Mrp2 function and prolonged treatment with fusidate decreased hepatic Mrp2 levels, we did not find changes in the plasma bilirubin concentration during fusidate treatment for 3 days in the rats. Since fusidate plasma levels rapidly decrease to 36% of peak levels 4 hr following a single injection [23], no direct inhibition of biliary excretion of Mrp2 substrates by fusidate would be expected when plasma bilirubin concentration was determined 14 hr after the last fusidate injection. The finding that plasma bilirubin levels were unchanged in rats inspite of decreased hepatic Mrp2 levels upon drug treatment is also known for ethinylestradiol. Ethinylestradiol treatment of rats for 5 days led to a decrease of hepatic Mrp2 levels to 41% of controls [27], whereas plasma bilirubin remained unchanged [28]. Thus, even under conditions of strongly reduced Mrp2 levels, biliary excretion of bilirubin glucuronide in rats is still sufficient to keep the plasma bilirubin concentration within the normal range. As a more sensitive parameter of Mrp2 function we determined the biliary excretion rate of 17β-glucuronosyl [^{3}H]estradiol. The biliary excretion rate of 17β-glucuronosyl [^{3}H]estradiol in fusidate-treated animals was reduced, demonstrating the physiological relevance of reduction in hepatic Mrp2 levels in rats.

For the *in vivo* study we used 17β-glucuronosyl estradiol as tracer substance to analyze the Mrp2 function [15]. Takikawa *et al*. [29] demonstrated that in Mrp2-deficient Eisai hyperbilirubinemic rats [30] there is very little biliary 17β-glucuronosyl estradiol excretion compared to normal rats. Therefore, it is appropriate to conclude that Mrp2 represents the main canalicular transporter for 17β-glucuronosyl estradiol, which makes this substrate a suitable tracer substance for *in vivo* analysis of Mrp2 function.

In our studies we focused on the influence of fusidate on canalicular transport processes since all known deficiencies of biliary excretion are due to defects in ATP-dependent canalicular transporters [12,19,20]. However, we cannot exclude that, in addition to the fusidate-induced inhibition of the excretion of endogenous substances from the hepatocyte into the bile, there is a partial interference of the hepatocellular uptake in the *in vivo* experiment. From the results of this study we conclude that at least two different mechanisms are involved in the reduction of canalicular transport by fusidate: the direct inhibition of Mrp2 and Bsep by competitive interaction and the down-regulation of the protein level of Mrp2.

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