

Title page

Hepatic transport mechanisms of Cholyl-L-Lysyl-Fluorescein.

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Running Title page

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Abbreviations:

CLF, cholyl-L-lysyl-fluorescein; CHO, Chinese-hamster-ovary; NTCP, Na⁺-dependent-taurocholic-cotransporting-polypeptide; OATP, organic-anion-transporting-polypeptide; ABC, ATP-binding-cassette-transporter; TR⁻, transport deficient; TC, taurocholate; E₂17βG, estradiol-17β-D-glucuronide; DNP-SG, 2,4-dinitrophenyl-S-glutathione; ASBT, apical-sodium-dependent-bile-acid-transporter; PBC, primary biliary cirrhosis

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Abstract

Cholyl-L-lysyl-fluorescein (CLF) is a fluorescent bile salt derivative that is being developed as an agent to determine *in vivo* liver function. However, the mechanisms of uptake and excretion by hepatocytes have not been rigorously studied. We have directly assessed the transport capacity of various hepatobiliary transporters for CLF. Uptake experiments were performed in CHO cells transfected with human *NTCP*, *OATP1B1*, *OATP1B3* and *OATP2B1*. Conversely, excretory systems were tested with plasma membrane vesicles from *Sf21* insect cells expressing human *ABCB11*, *ABCC2*, *ABCC3* and *ABCG2*. In addition, plasma clearance and biliary excretion of CLF was examined in wild type, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice. Human *NTCP* and *ABCB11* were incapable of transporting CLF. In contrast, high affinity transport of CLF was observed for *OATP1B3*, *ABCC2* and *ABCC3* with K_m values of 4.6 ± 2.7 , 3.3 ± 2.0 and 3.7 ± 1.0 $\mu\text{mol/L}$, respectively. In *Abcc2*^{-/-} mice biliary excretion of CLF was strongly reduced compared to wild type mice. This resulted in a much higher hepatic retention of CLF in *Abcc2*^{-/-} vs wild type mice: 64% vs 1% of the administered dose (2 h after administration). In mice intestinal uptake of CLF was negligible compared to that of taurocholate. Conclusion: Human *NTCP* and *ABCB11* are incapable of transporting CLF, whereas *OATP1B3* and *ABCC2/Abcc2* most likely mediate hepatic uptake and biliary excretion, respectively of CLF. CLF can be transported back into the blood by *ABCC3*. Enterohepatic circulation of CLF is minimal. This renders CLF suitable as an agent to assess *in vivo* liver function.

Bile formation is a vital function of the liver and involves sodium-dependent and sodium-independent uptake of bile salts from the portal blood as well as ATP-dependent canalicular excretion (Meier and Stieger, 2002). Uptake into human hepatocytes involves Na⁺-dependent taurocholate cotransporting polypeptide, NTCP (*SLC10A1*) (Hagenbuch and Dawson, 2004; Dawson, et al., 2009), and organic anion transporting polypeptides, OATP1B1 (*SLCO1B1*) and OATP1B3 (*SLCO1B3*) (Hagenbuch and Meier, 2004). The bile salt export pump, BSEP (*ABCB11*) mediates biliary excretion of bile salts (Stieger, et al., 2007), the functional absence of which leads to severe liver disease. In addition, multidrug resistance associated protein 2, MRP2/Mrp2 (*ABCC2/Abcc2*) can transport bile salt metabolites (Akita, et al., 2001). In various forms of intrahepatic cholestasis the biliary excretion capacity of ABCB11 and ABCC2 are decreased (Paumgartner, 2006). As a consequence, hepatic bile salt levels rise intracellularly and subsequently, the uptake transporters NTCP, OATP1B1 and OATP1B3 are down regulated (Keitel, et al., 2005). In primary biliary cirrhosis expression of bile salt uptake systems is reduced, while the canalicular export systems BSEP and MRP2 are minimally affected and the basolateral salvage system is induced (Zollner, et al., 2003; Zollner, et al., 2007). Hence, determination of the clearance of substrates of hepatocellular transport systems could be used as a measure for *in vivo* liver function. In the past, sulfobromophthalein and indocyanine green were used to measure liver function as a test for measuring transport activities, but the former was abandoned because of the possibility of severe systemic reactions and the latter test is of concern in patients with iodine allergy (Sakka, 2007).

Cholyl-L-lysyl-fluorescein (CLF) is a fluorescent bile salt and has been introduced as a potential agent to assess *in vivo* liver function (Milkiewicz, et al., 2000). However, the transport systems involved in hepatic handling of CLF have not been investigated in molecular detail so far. Indications of the involvement of bile salt transporters in hepatic handling of CLF were found by several investigators: Mills *et al.* showed that the kinetics of CLF excretion into bile of rats is similar to that of glycocholate (Mills, et al., 1991). In another study of Baxter *et al.* it was shown that CLF could induce phospholipid and

cholesterol output similar to what was found for glycocholate (Baxter, et al., 1995). Since ABCC2/Abcc2 is also capable of mediating the transport of (modified) bile salts this transporter should also be considered as a potential candidate for biliary CLF excretion. However, Mills *et al.* concluded from a study with normal and TR⁻ (Abcc2 deficient) Wistar rats that Abcc2 was hardly involved in the biliary excretion of CLF, based on the observation of similar biliary excretion in both strains (Mills, et al., 1999). Carboxyfluorescein is an anion and a substrate for ABCC2/Abcc2 (Lee and Piquette-Miller, 2001). In this respect the fluorescein moiety of CLF could render CLF to be an ABCC2/Abcc2 substrate. An indirect indication that CLF could be a substrate of ABCC2 was seen by Maglova *et al.* (Maglova, et al., 1995). The excretion from couplets of cholyglycylamidofluorescein, which resembles CLF, could partially be inhibited by dinitrophenyl-glutathione, a model substrate for ABCC2 (Evers, et al., 1998). CLF is going to be introduced as a novel agent for determination of *in vivo* liver function in a clinical setting. It is therefore crucial to define the mechanisms of uptake and excretion by the hepatocyte. Altered plasma clearance in patients with liver disease will reflect a change in expression and/or function of the transporters involved in uptake and biliary excretion of CLF. Since different transporters are regulated via different mechanisms, plasma clearance studies in patients can only be properly interpreted if it is known which transporters are involved. We come to the surprising finding that the established human bile salt transporters NTCP and ABCB11 (BSEP) are not at all capable of transporting CLF. Instead transport is mediated via OATP1B3 and ABCC2.

Materials and methods

Materials

CLF was a generous gift from Norgine (Harefield, UK). [³H]GSH (52 Ci/mmol) and [³H]TC (1.19 Ci/mmol) were obtained from Perkin-Elmer (Boston, USA). [³H]E₂17BG (40.5 Ci/mmol) was purchased from NEN (Boston, USA). [³H]DNP-SG synthesis was performed as described before (de Waart, et al., 2006). Cellulose-acetate membrane filters were bought from Schleicher & Schuell (Dassel, Germany). Creatine-phosphate was purchased from Boehringer-Mannheim (Almere, The Netherlands). Creatine-kinase was bought from Roche-Diagnostics (Mannheim, Germany). All other chemicals and reagents were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Animals

Abcc2^{-/-} mice were bred at the National Cancer Institute (Amsterdam, The Netherlands). The production and characterization of *Abcc2*^{-/-} and *Abcc3*^{-/-} was described by Vlaming *et al.* (Vlaming, et al., 2006) and Zelcer *et al.* (Zelcer, et al., 2006), respectively. All mice were bred against a FVB background.

Generation of CHO cells stably expressing NTCP and Cell Lines

cDNA for human NTCP (Hagenbuch and Meier, 1994) was cut with *EcoRV* and *HindI* and the coding region ligated into pcDNA5/FRT (Invitrogen, Life-Technologies, Carlsbad, CA). CHO FlpIn cells (Invitrogen, Life-Technologies) were transfected with the resulting construct using Lipofectamine[®] 2000 (Invitrogen, Life-Technologies). Stably transfected cells were selected with 550 µg Hygromycin-B in HAM-F12 medium (Gibco, Invitrogen, Life-Technologies). Transfected cells were cloned with the limiting dilution method. Clones expressing functional NTCP were identified by transport assays with radioactively labelled taurocholate (see below). To further characterize the cloned cells, immunofluorescence localization of NTCP using a polyclonal antibody against NTCP (Kullak-Ublick, et al., 1997) was performed as described (Huber, et al., 2007). CHO cells expressing OATP1B1, OATP1B3 and OATP2B1 were described previously (Gui, et al., 2008; Treiber, et al., 2007).

Transport experiments with stably transfected cell lines

For all transport experiments, cells grown on 3 cm culture dishes were cultured for 24 h in media supplemented with 5 mmol/L sodium-butyrate to increase expression levels of transfected transporters (Palermo, et al., 1991). Functionality of NTCP expressing CHO cells was assessed by transport studies with radioactively labelled taurocholate as described in (Huber et al., 2007). Transport experiments with CLF were performed in the same buffers as for the radioactive substrates above. For visualization of CLF uptake, cells were immediately after the uptake experiment inspected with a Leica-DM-IRBE-inverted-microscope (Leica-Microsystems, Wetzlar, Germany) equipped with a Hamamatsu-ORKA-ER-camera (Hamamatsu-Photonics, Japan). To determine uptake of CLF, cells were solubilized by the addition of 2 ml 1% (w/v) Triton-X-100. After complete solubilization, 1.5 ml was used to measure fluorescence in a Perkin-Elmer LS-5 luminescence-spectrometer set at λ_{exc} 486 nm (slit 10 nm) and λ_{em} 520 nm (slit 5 nm). Protein was determined with the bicinchoninic acid method using a kit from Interchim (Montfouçon, France). Transport data from OATP expressing cell lines were corrected for binding by subtracting 0 min time points (blank values) and except for figs. 2B and 2C were normalized per minute. Kinetic analysis was performed with nonlinear regression of the data to the Michaelis-Menten equation using GraphPad PRISM-V-4.00 (GraphPad-Software-Inc., San Diego, CA)

Preparation of membrane vesicles

ABCC3 and *ABCG2*-recombinant baculovirus were a kind gift from Prof. P. Borst (Breedveld, et al., 2004;Zelcer, et al., 2001). *ABCB11*-recombinant baculovirus was a kind gift from Dr. R. Thompson (Byrne, et al., 2002). The production of *ABCB11*-, *ABCC2*-, *ABCC3*- and *ABCG2*-recombinant baculovirus was as earlier described (Byrne, et al., 2002;de Waart, et al., 2006;de Waart, et al., 2009). *Sf21* cells grown at 27°C were infected with *ABCB11*, *ABCC2*, *ABCC3* and *ABCG2*-cDNA containing baculovirus. Cells were harvested at 2-4 days after infection. Membrane vesicle preparation was as described before (de Waart, et al., 2009).

Western blotting and protein analysis

Membrane vesicles were fractionated by 6% SDS-PAGE, blotted on nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) which were blocked in phosphate-

buffered saline (PBS)/5% milk powder/0.05% Tween-20. The following antibodies were used: anti-his probe; sc-803 (Santa Cruz, USA), anti-ABCG2; BXP-21 (Maliepaard, et al., 2001), anti-ABCC2; M₂III6 (Scheffer, et al., 2000) and anti-ABCC3; M₃III21 (Scheffer, et al., 2000). Immune complexes were visualized with horseradish-peroxidase-conjugated immunoglobulins and detected using chemiluminescence (Amersham, UK).

Transport assays with plasma membrane vesicles

Transport studies with membrane vesicles were performed using the rapid filtration technique as described (de Waart, et al., 2009). Radiolabel was measured with a scintillation counter. When CLF was used as a probe, filters were placed in a glass tube, 0.1% Triton-X-100 was added and the tubes vortexed. Samples were pipetted in 96-wells plates (Kartell, Noviglio, Italy) and the amount CLF quantitated by measurement of fluorescence at λ_{exc} 485 nm and λ_{em} 520 nm using a NovoStar (BMG-labtech, Offenburg, Germany).

Animal experiments

Male mice were housed in a pathogen-free animal facility on a 12 h light-dark cycle. All animal experiments were approved by the institutional animal care and use committee of the Academic Medical Center. Mice were anesthetized with a combination of Hypnorm (VetaPharma, UK; 11.8 mg/kg fluanisone and 0.37 mg/kg fentanyl-citrate) and Diazepam (Centrafarm, Etten-Leur, The Netherlands; 5.9 mg/kg valium). Body temperature was maintained at $36 \pm 1^\circ\text{C}$ on thermostatted heating pads. Fore clearance studies, mice were infused with CLF by injecting 100 μL (2 mmol/L) CLF in the tail vein. Subsequently, blood was drawn from the carotis at indicated time points. Blood samples were deproteinized by addition of two volumes methanol and the amount CLF in the supernatant was quantitated by measurement of fluorescence as described above.

For biliary secretion studies, gallbladders were cannulated with PE10 polyethylene tubing and 100 μL (1 mmol/L) CLF was injected in the tail vein. Bile was collected in 10 min fractions; liver and blood were harvested at the end of the experiment. Homogenized livers were deproteinized by adding 2 volumes of MeOH and bile and blood samples were diluted with

0.1% Triton-X-100. The amount CLF was quantitated by measurement of fluorescence as described above.

For intestinal uptake studies of TC and CLF, mice were anesthetized and the gallbladder cannulated as described above. Mice received both TC and CLF by injecting 100 μ L (2 mmol/L) CLF into the ileum. Bile was collected every 15 min. Radioactivity was measured in a scintillation counter and CLF quantitated as described above.

Results

First, the role of different hepatocellular bile salt transporters in the hepatic uptake of CLF was investigated. For this purpose CHO cells stably expressing NTCP were generated (CHO-NTCP) and characterized. Figure 1A shows no expression of NTCP in wild type CHO cells but clear plasma membrane expression of NTCP in stably transfected cells. Furthermore, uptake of taurocholate (TC) into these cells was strictly sodium dependent and was saturable with increasing taurocholate concentrations (Fig 1B). The K_m and the V_{max} values were 16.0 $\mu\text{mol/L}$ and 6738 pmol TC/mg protein/min, respectively, for sodium-dependent transport of TC (Fig 1B). On the contrary, no sodium-dependent uptake of CLF mediated by NTCP could be observed by these cells as detected by fluorescence microscopy (Fig. 2A) and by quantitative fluorimetry of CLF (Fig. 2B). Uptake of CLF into NTCP-expressing cells was negligible, sodium-independent and comparable to transport in wild type cells. (Fig 2B). Next, uptake of CLF mediated by OATP1B1, OATP1B3 and OATP2B1 was examined in wild type and stably transfected CHO cells. High, time dependent transport rates of CLF were only seen in OATP1B3 expressing CHO cells (Fig. 2A and 2C, respectively). We consistently observed low, but significant uptake of CLF by OATP1B1 cells, but not by OATP2B1 cells (Fig 2A and 2C), indicating that CLF is not a substrate for OATP2B1. OATP1B3 mediated CLF transport was concentration-dependent (Fig 2D) with K_m and the V_{max} values of 4.6 ± 2.7 $\mu\text{mol/L}$ and 213 ± 42 pmol CLF/mg protein/min, respectively (mean of three independent determinations). In the case of OATP1B1, uptake experiments with increasing CLF concentrations also showed evidence for saturability (not shown). However, due to a low signal to noise ratio, in particular at higher CLF concentrations, no determination of the kinetic parameters was possible. Furthermore, we investigated if transport of model compounds mediated by NTCP, OATP1B1, OATP1B3 and OATP2B1 could be inhibited by CLF despite CLF being a substrate for OATP1B3 only. Strikingly, CLF very efficiently inhibited the transport of TC via NTCP (Fig 3A). Transport of TC via OATP1B3 could also be inhibited by CLF in a dose-dependent manner (Fig 3C) as expected. Finally, transport of the model substrate estrone-3-sulfate mediated by OATP1B1 and OATP2B1 was inhibited by

CLF, albeit much less efficiently (Fig 3B and 3D respectively), corroborating the uptake experiments with CLF. Of note, addition of CLF to wild type cells had no influence on substrate uptake indicating that it does not interfere with the structural integrity of the plasma membrane (Fig. 3).

In order to test whether the canalicular ATP-dependent transporters ABCB11, ABCC2 and ABCG2, as well as the basolateral transporter ABCC3 mediate transport of CLF, we expressed these human proteins in *Sf21* insect cells (Fig 4A). Figure 4B shows that slight ATP-dependent uptake occurred in control vesicles, indicating that an endogenous transporter is able to take up CLF in an ATP-dependent fashion. ATP-dependent transport of CLF in ABCB11 and ABCG2 protein containing membrane vesicles was not higher than in control vesicles (Fig 4C and D, respectively). However, ABCC2 and ABCC3 containing membrane vesicles showed much higher CLF transport rates compared to control wild type *Sf21* membrane vesicles (Fig 4E and F, respectively). ABCC2 and ABCC3 mediated transport of CLF was concentration-dependent (Fig 4G and H, respectively). The K_m values were $3.3 \pm 2.0 \mu\text{mol/L}$ and $3.7 \pm 1.0 \mu\text{mol/L}$ for ABCC2 and ABCC3, respectively, and V_{max} values were $436 \pm 215 \text{ pmol CLF/mg protein/min}$ and $188 \pm 55 \text{ pmol CLF/mg protein/min}$, respectively (mean of three independent determinations). Another model substrate for ABCC2 is dinitrophenyl-glutathione (DNP-SG) and CLF should inhibit transport of this compound. Half maximal inhibition of DNP-SG transport with CLF was achieved at approximately $1 \mu\text{mol/L}$ (Fig. 5A). Although we did not find any indication that CLF is a substrate for ABCB11, it contains the cholyl moiety and therefore, it might be able to inhibit bile salt transport via ABCB11. Indeed, TC transport could be inhibited by CLF in a dose dependent fashion and the concentration at which half maximal transport was observed was approximately $10 \mu\text{mol/L}$ (Fig 5B).

To investigate the role of *Abcc2* and *Abcc3* in plasma clearance of CLF, we examined CLF levels in plasma of wild type, *Abcc2*^{-/-} and *Abcc3*^{-/-} mice after a single injection of CLF in the tail vein. Clearance of CLF was strongly impaired in *Abcc2*^{-/-} mice in comparison with wild type mice but not affected in *Abcc3*^{-/-} mice (Fig 6). To examine the role of *Abcc2* in biliary

output of CLF, infusion experiments were performed with wild type and *Abcc2* deficient mice. Biliary excretion of CLF was very much delayed in the *Abcc2*^{-/-} mice (Fig 7A). As a consequence almost 70% of the CLF dose was excreted into bile in wild type mice within 20 min, whereas in the same time span less than 2% was excreted into bile in *Abcc2*^{-/-} mice. At 120 min after administration (Fig. 7B), the cumulative biliary excretion of CLF was still significantly higher in wild type mice than in *Abcc2*^{-/-} mice (85% vs 32% of the administered dose) which resulted in a significantly higher hepatic retention of CLF in *Abcc2*^{-/-} vs wt mice (64% vs 1% of the administered dose) and in higher blood levels in *Abcc2*^{-/-} vs wt mice (1306 ± 749 nmol/L vs 83 ± 21 nmol/L). All these data indicate that in mice *Abcc2* is the main transporter responsible for the biliary excretion of CLF. In line with the observations from Fig. 7A we observed that biliary output of CLF was not affected in *Abcc3*^{-/-} mice in comparison with wild type mice and we also found no differences in plasma and liver CLF contents (Fig 7B).

After excretion into bile and delivery into the duodenum, bile salts are taken up in the ileum via the apical sodium dependent bile acid transporter (ASBT) (Wong, et al., 1995). To investigate if CLF is taken up in the intestine, equimolar amounts of CLF and [³H]TC were injected into ileum of wild type FVB mice. Thereafter, bile was collected and the amounts of [³H]TC and CLF excreted in bile were quantified. At 4.5 h after ileal injection of both compounds only 2% of the CLF was recovered in bile whereas this was 68% for [³H]TC (Fig. 8). Hence, uptake of CLF in the terminal ileum is minimal. Apparently, CLF is not a good substrate for the intestinal uptake system *Asbt*.

Discussion

In this paper we show that uptake of CLF into hepatocytes is not likely to be mediated by NTCP, since we did not find any uptake of CLF into CHO cells expressing human NTCP. This was not due to a non-functional protein, since these cells were fully capable of mediating the uptake of the natural occurring bile salt, TC. The ileal counterpart of hepatic NTCP is ASBT and it mediates transport of conjugated and unconjugated bile salts (Dawson, et al., 2009). We obtained indirect evidence to suggest that CLF is not transported via (murine) Asbt: whereas TC injected in the ileal lumen of wild type mice was very efficiently recovered in bile, we found almost no CLF. These data suggest that both NTCP and Asbt, which are homologous sodium-dependent bile salt transporters, are unable to transport CLF. This result compares favourable with studies of bile salts conjugated via the side chain to fluorescein. These bile salts are very poorly absorbed in the small intestine of rats (Holzinger, et al., 1997). In contrast to CLF, another bile salt conjugate, taurocholy-chlorambucil, was found to be a substrate for human NTCP (Kullak-Ublick, et al., 1997). The latter compound is a conjugate at the 3-OH group of the bile salt whereas CLF is conjugated at the side chain. In this context it is interesting that Baringhaus *et al.* determined the pharmacophore of both NTCP and ASBT and found that the 3-OH group is not essential for transport whereas the acidic side chain is (Baringhaus, et al., 1999). Our new data are completely in line with this model.

The less specific bile salt transporters, OATP1B3 and, to a less extent, OATP1B1, turn out to be more likely candidates for uptake into the hepatocyte; which fits with the broad substrate specificity of these transporters (Hagenbuch and Meier, 2004).

Our data demonstrate, quite surprisingly but conclusively, that ABCC2/Abcc2 is the most prominent transporter responsible for biliary excretion of CLF and not ABCB11. In mice, the large majority of CLF in plasma is excreted into bile via Abcc2. It may be argued that the substrate specificity of human ABCC2 can be different from that in mice. However, in plasma membrane vesicle assays we demonstrated that transport of CLF via human ABCB11 is insignificant compared to that via human ABCC2. Therefore, in vitro studies with CLF

support the notion that the side chain of bile salts is essential for the substrate recognition by the bile salt transporters ASBT, NTCP and ABCB11. It has been demonstrated for rat *Abcb11* that unconjugated cholate is practically not transported (Gerloff, et al., 1998). This is supported by the observation that in patients with a defect in bile acid conjugation, practically no unconjugated bile acids are found in bile (Carlton, et al., 2003). Early reports involving CLF suggested that ABCB11 was mainly involved in this process. Firstly, Mills *et al.* showed that in rats the biliary excretion rate of CLF after jugular vein injection has similar kinetics as the excretion of glycocholate, and different from that of fluorescein which was slower. Therefore, the conclusion was drawn that CLF is also a substrate for ABCB11 like glycocholate. However, this experimental approach did not rule out the possibility that CLF is a substrate for ABCC2 but that the transport characteristics resemble those of glycocholate via ABCB11. Other indirect evidence that CLF is transported via ABCB11 was provided by Baxter *et al.* who administered glycocholate and CLF to isolated perfused rat livers under recycling conditions, and observed that CLF was capable of increasing the phospholipid and cholesterol output in a similar way as glycocholate. Again, this does not rule out the possibility that CLF is excreted via *Abcc2* and, once in the canalicular lumen, is subsequently able on the basis of its detergent properties to accept phospholipids and cholesterol from the membrane. It was shown in the same study that rat liver has a much greater capacity to transfer glycocholate from perfusate to bile than CLF and concomitantly, the increase in phospholipid and cholesterol output was less with CLF in comparison with glycocholate (Baxter, et al., 1995). All in all, no direct evidence has ever been provided to support the contention that CLF is (mainly) taken up by the hepatocyte via NTCP/Ntcp and/or excreted into bile via ABCB11/*Abcb11*. Our present data show decisively that CLF is not transported via these bile salt transporters. Opposite to this finding is that cholyglycylamidofluorescein (CGamF) incontrovertibly is an ABCB11 substrate. Because CLF and CGamF resemble each other, this difference is a remarkable finding (fig 9). What can be the explanation? The substrates CGamF and CLF contain both a cholyl and fluorescein moiety, but the bridge between the two moieties differs. First, the bridge in CLF is increased in comparison with

CGamF, 131 Da and therefore, the molecular weight of the molecule is larger. This could mean that substrates for ABCB11 have a size limit. The bigger the substrates become the lower the affinity for ABCB11. Furthermore, ABCC2 can possibly accommodate larger molecules than ABCB11. Second, in general ABCC2 mediates transport of anions with one or, preferably, two negative charges. Since CLF contains two COOH groups in comparison with CGamF, which contains one, therefore, CLF might be changed into a good ABCC2 substrate. Another way to identify the relevant transporters is the use of knockout animals. Mills *et al.* used *Abcc2* deficient, TR⁻ rats and measured CLF in bile after injection of CLF in the jugular vein. The cumulative amount of CLF in bile was similar in TR⁻ and normal Wistar rats after 30 min. They concluded from these data that *Abcc2* is not the main transporter for CLF. However, we now directly show that *Abcc2* is the main canalicular transporter for CLF in mice. We performed a similar but more extensive study using wild type and *Abcc2*^{-/-} mice. In mice deficient in *Abcc2*, biliary excretion of CLF was strongly impaired and retained in the liver, which suggests that *Abcc2* and not *Abcb11* is the major transporter responsible for biliary excretion of CLF. As there is still residual biliary transport of CLF in *Abcc2*^{-/-} mice a contribution of *Abcb11* and/or *Abcg2*, albeit small, cannot be excluded. Furthermore, reinterpretation of the data of the Mills *et al.* paper (1999) in the figure showing the biliary excretion (see supplemental data 1, the lower right panel represents our recalculation of the excretion based on the cumulative data given in the other panels) you get a similar picture as in mice: In the paper of Mills *et al.* the cumulative excretion is shown. If you change it to excretion per minute you get a peak for excretion of CLF in the Wistar rat and this is not seen for the TR⁻ rat. The decrease in excretion of CLF in the *Abcc2*^{-/-} mice is very similar as in the TR⁻ rat.

As CLF is being developed as an agent to assess liver function in patients it is crucial to know the exact mechanism of uptake into the hepatocyte and excretion into bile. Therefore, we examined human ABCB11 and ABCC2 mediated transport of CLF. No ABCB11 mediated transport was seen while ABCC2 mediated high affinity transport of CLF. This transport was time dependent and saturable with a K_m value of 3.3 $\mu\text{mol/L}$ and a V_{max} of 436 pmol CLF/mg

protein/min. In the light of a liver function test these observations are crucial, because *ABCB11* and *ABCC2* gene expression are differently regulated which leads to a different sensitivity of their expression towards various pathological conditions. For example, while *ABCB11* as well as *ABCC2* protein levels and localization were found to be maintained in PBC stages I and II (Zollner, et al., 2003;Zollner, et al., 2007), reduced protein levels and a disruption of canalicular localization have been reported for *ABCC2* in advanced PBC (stages III and IV) (Kojima, et al., 2003;Kullak-Ublick, et al., 2002). In a pilot study CLF elimination was analysed in liver cirrhosis patients and seemed to be impaired in comparison to healthy volunteers (Milkiewicz, et al., 2000). These cirrhotic patients suffered from high serum bilirubin and bile salt level, indicative of a cholestatic situation. In such conditions *ABCC2* function may not only be impaired but *ABCC3* expression may also be up regulated (Zollner, et al., 2003). To investigate if patients have a cirrhotic liver a biopsy can proof this. However, a less invasive test would be more favourable. CLF could be used as an agent to assess in vivo liver function because in our study not only *ABCC2* but also *ABCC3* mediate the transport of CLF, cirrhotic patients may have a lowered biliary excretion rate of CLF, but also a higher basolateral excretion due to a higher expression of *ABCC3*. Importantly, analysis of plasma CLF clearance will not be compromised by enterohepatic circulation, because ileal uptake was low in our animal study. In conclusion, our data are of importance for the interpretation of plasma CLF clearance in patients with liver disease.

Acknowledgments

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Footnotes

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Legends

Figure 1:

Characterization of CHO-NTCP cells.

(A) Expression of NTCP in stably transfected and wild type CHO cells. NTCP was visualized as described in materials and methods. (B) Kinetic characterization of CHO-NTCP. Cells were incubated with increasing TC concentrations for 45 sec in the presence of sodium (■) or potassium (▲) Sodium-dependent uptake rates (▼) were used to determine the kinetic parameters. Data represent means \pm SD of triplicates. Where error bars are absent, they were smaller than the symbol.

Figure 2:

Uptake studies into CHO cells expressing hepatocellular uptake transporters

(A) Fluorescence pictures after incubation of CHO transporter cells with 1 μ mol/L CLF during 5 min. Fluorescent pictures (right panels) are complemented with phase contrast pictures (left panels). Representative pictures are shown of 12 different experiments. (B) Transport of CLF mediated by NTCP in the presence (black bars) and absence of sodium (white bars) is given for 1 and 5 min. Shown is one out of two experiments performed. Each time point n=4. Significance was tested using two-sided Student's *t*-test: No significant differences were seen at 1 and 5 min for sodium versus choline buffer, respectively. (C) wild type cells and cells expressing OATP1B1, OATP1B3 and OATP2B1 were incubated with 1 μ mol/L CLF during 1 (white bar) or 5 min (black bars). Shown is one out of two experiments performed. Each time point n=4. Significance was tested using two-sided Student's *t*-test: *P<0.05 for OATP expressing CHO cells versus wild type CHO cells. (D) Kinetics of OATP1B3 mediated CLF transport. Cells were incubated with increasing concentrations of CLF for 45 sec. Shown are uptake rates corrected for 0 sec binding. Data of B through D represent means \pm SD. K_m and V_{max} values are the mean of three independent determinations.

Figure 3:

Inhibition of transport activity of hepatocellular uptake transporters by CLF.

CHO cells expressing NTCP, OATP1B1, OATP1B3 and OATP2B1 were incubated with 0, 5 or 50 $\mu\text{mol/L}$ CLF and with the following substrates, 2.5 $\mu\text{mol/L}$ TC, 1.0 $\mu\text{mol/L}$ estrone-3-sulfate, 10 $\mu\text{mol/L}$ TC and 1.0 $\mu\text{mol/L}$ estrone-3-sulfate, respectively. Data represent means \pm SD of triplicate determinations.

Figure 4:

Vesicular transport assays.

(A) Western blot of membrane vesicles of *Sf21* insect cells either or not infected with *ABCB11*, *ABCC2*, *ABCC3* and *ABCG2* cDNA containing baculovirus.

Time-dependent transport of CLF into plasma membrane vesicle from wild type *Sf21* cells (B), or *Sf21* cells expressing *ABCB11* (C), *ABCG2* (D), *ABCC2* (E) or *ABCC3* (F). The membrane vesicles (10 μg of total protein) were incubated with 4 $\mu\text{mol/L}$ CLF in the presence (closed symbols) or absence (open symbols) of 4 mmol/L ATP at 37°C. Data represent the average \pm SD. CLF uptake of a representative experiment with triplicate incubations is shown here. Transport of CLF is expressed as pmol/mg protein.

Concentration-dependent uptake of CLF into *Sf21* membrane vesicles containing *ABCC2* (G) or *ABCC3* (H). The membrane vesicles (10 μg and 30 μg of total protein for *ABCC2* and *ABCC3*, respectively) were incubated with different concentrations of CLF in the presence or absence of 4 mmol/L ATP. Fluorescence in the absence of ATP was subtracted from that in the presence of ATP and the average ATP-dependent CLF uptake \pm SD of one representative experiment with triplicate incubations is displayed.

Figure 5:

Inhibition of vesicular transport.

(A) CLF inhibition of DNP-SG transport into *ABCC2* containing *Sf21* membrane vesicles. (B) CLF inhibition of TC transport into *ABCB11* containing *Sf21* membrane vesicles. The values are expressed as a percentage of maximal ATP-dependent transport. Shown is the average \pm SD of a representative experiment with triplicate incubations. Significance was

tested using two-sided Student's *t*-test: * $P < 0.05$ for transport in the presence vs. absence of inhibitor.

Figure 6

Plasma clearance of CLF in mice.

Wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice received 100 μ L of 2 mmol/L CLF by i.v. injection.

After the indicated time points blood was drawn. Data are expressed as the mean percentage of the initial CLF level at 2 min. Significance was tested using two-sided Student's *t*-test:

* $P < 0.05$ for *Abcc2*^{-/-} vs wild type mice.

Figure 7

Biliary excretion of CLF in mice.

(A) Time dependent appearance of CLF in bile of wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice. (B)

CLF levels in blood, urine, liver and bile of wild type, *Abcc3*^{-/-} and *Abcc2*^{-/-} mice after 2 h.

Mice received 100 μ L of 1 mmol/L CLF by injection in the tail vein. Data represent means \pm SD. Plasma levels are expressed in nmol/L and levels in urine, liver and bile as percentage of administered dose. Significance was tested using two-sided Student's *t*-test: * $P < 0.05$ for *Abcc2*^{-/-} vs wild type mice.

Figure 8

Intestinal uptake of TC and CLF.

Appearance of TC and CLF in bile after ileal administration of 100 μ L mixture of 2 mmol/L TC and CLF in wild type mice. Bile was collected after indicated time points. Data represent means \pm SD. Cumulative biliary levels are expressed as percentage of applied dose.

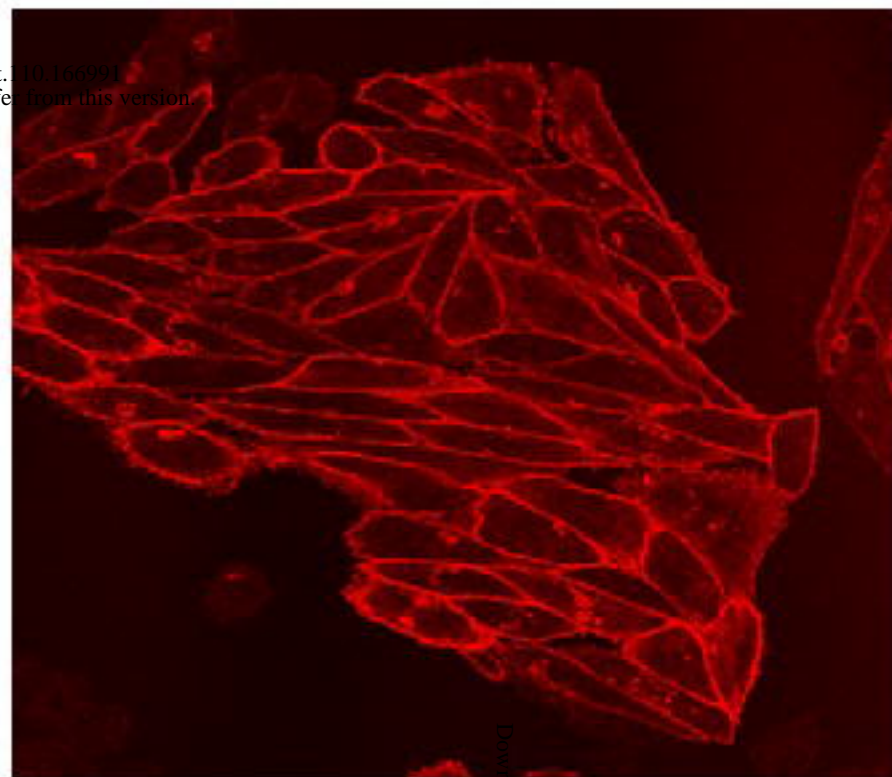
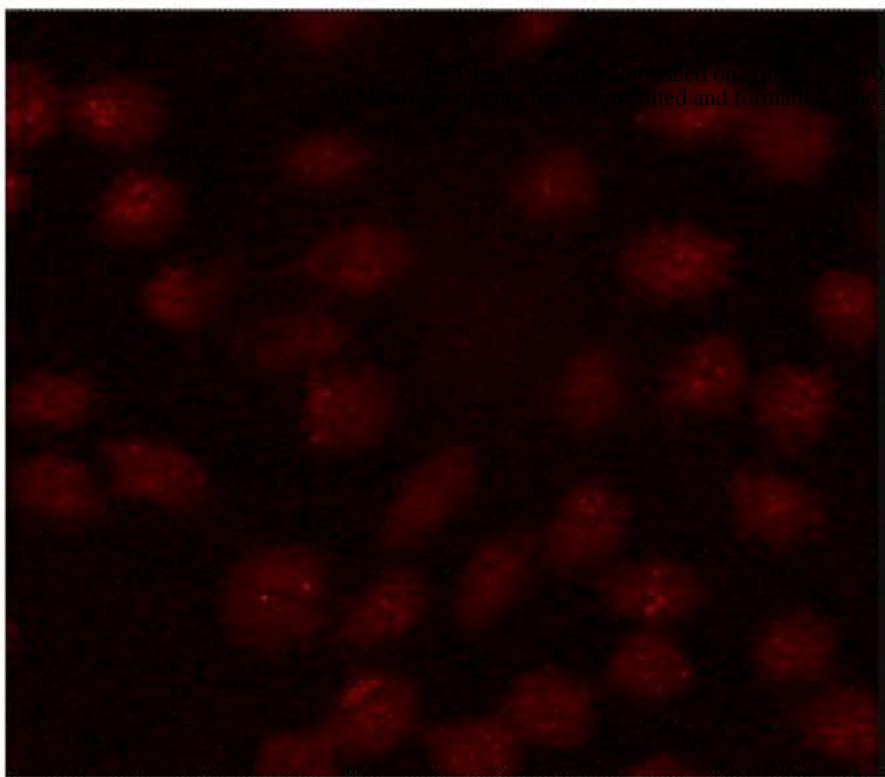
Significance was tested using two-sided Student's *t*-test: * $P < 0.05$ for TC uptake vs CLF uptake.

Figure 9

Chemical structures of the fluorescent bile salt derivatives, CLF and CGamF. In the cholyl-derivatives, R1 and R2 denote α -hydroxy groups.

Figure 1

A



Wild type CHO

CHO-NTCP

B

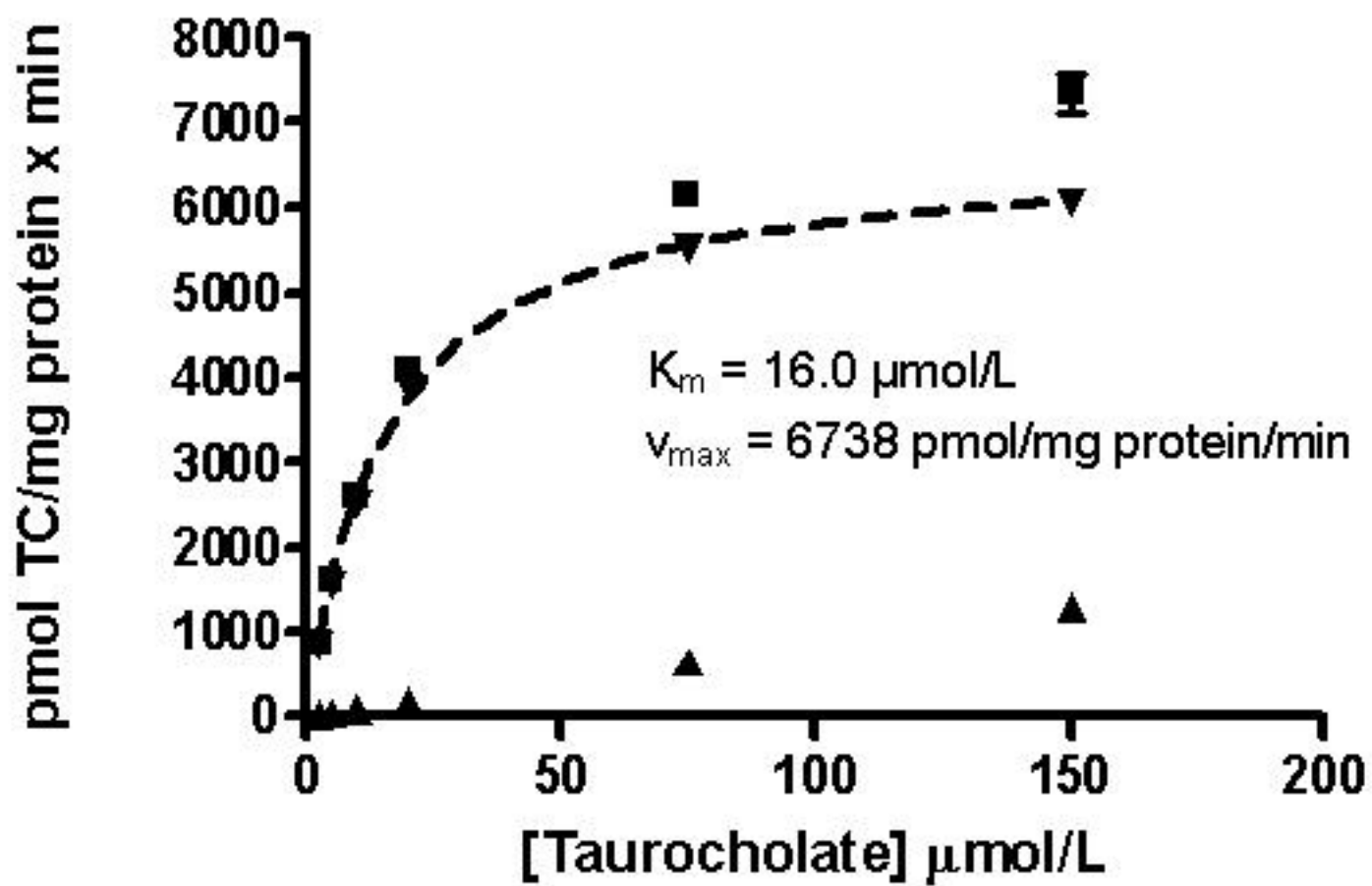
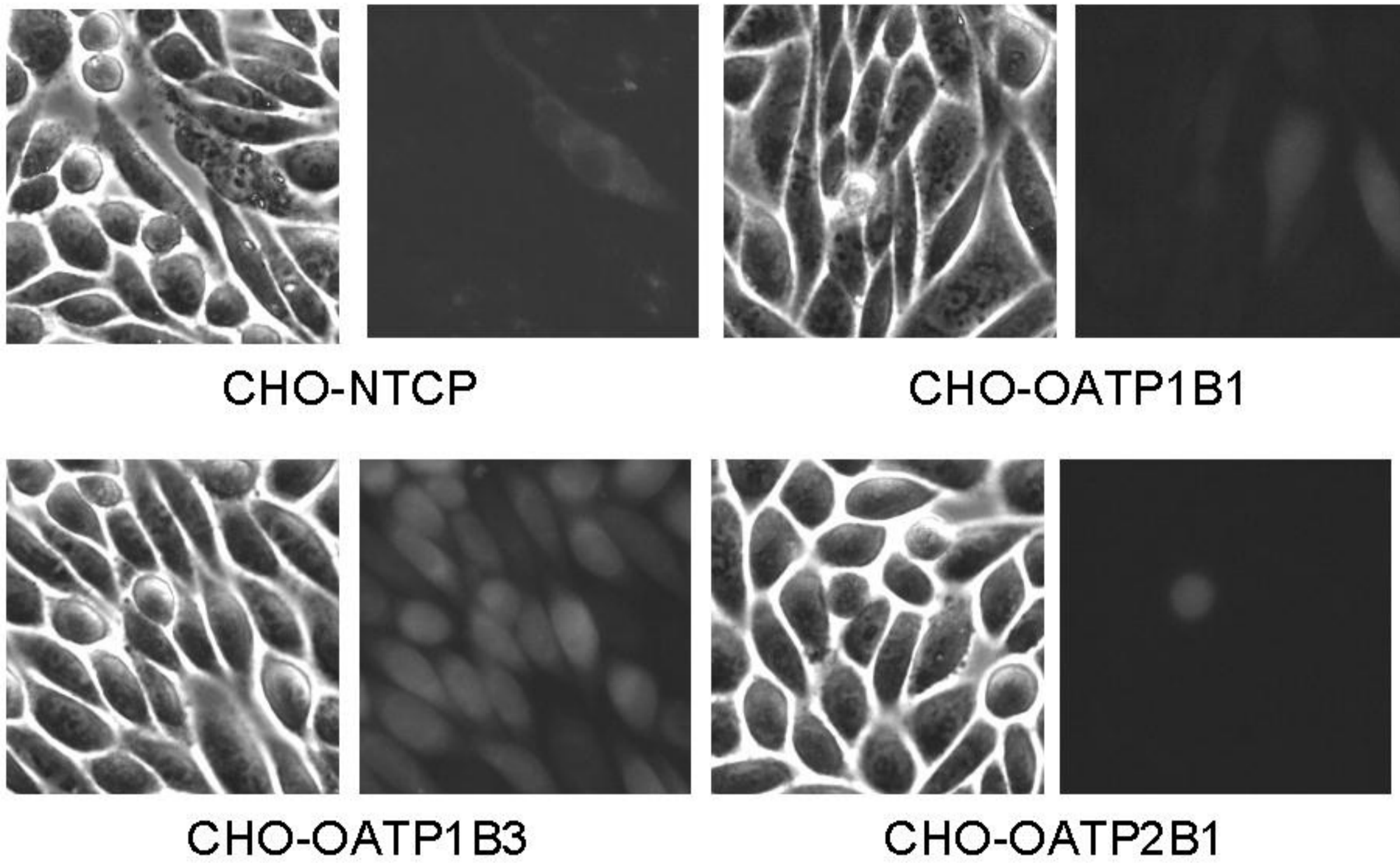


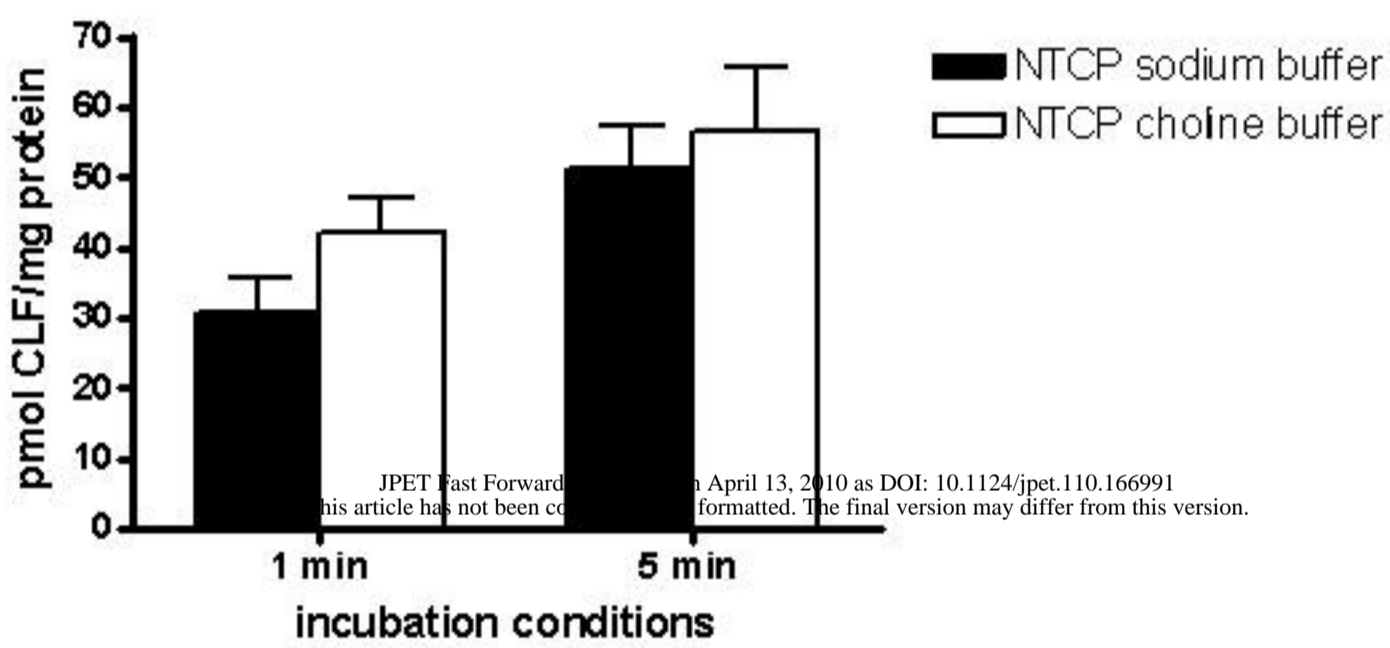
Figure 2

A

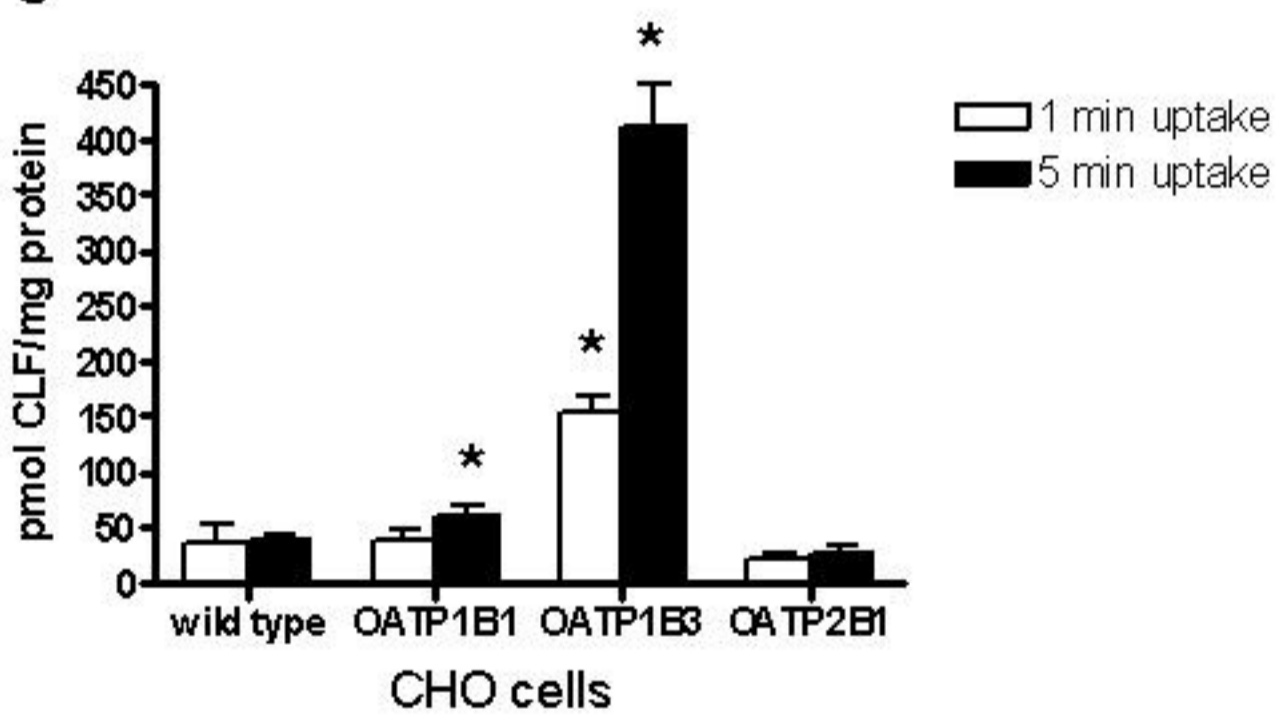


B

NTCP



C



D

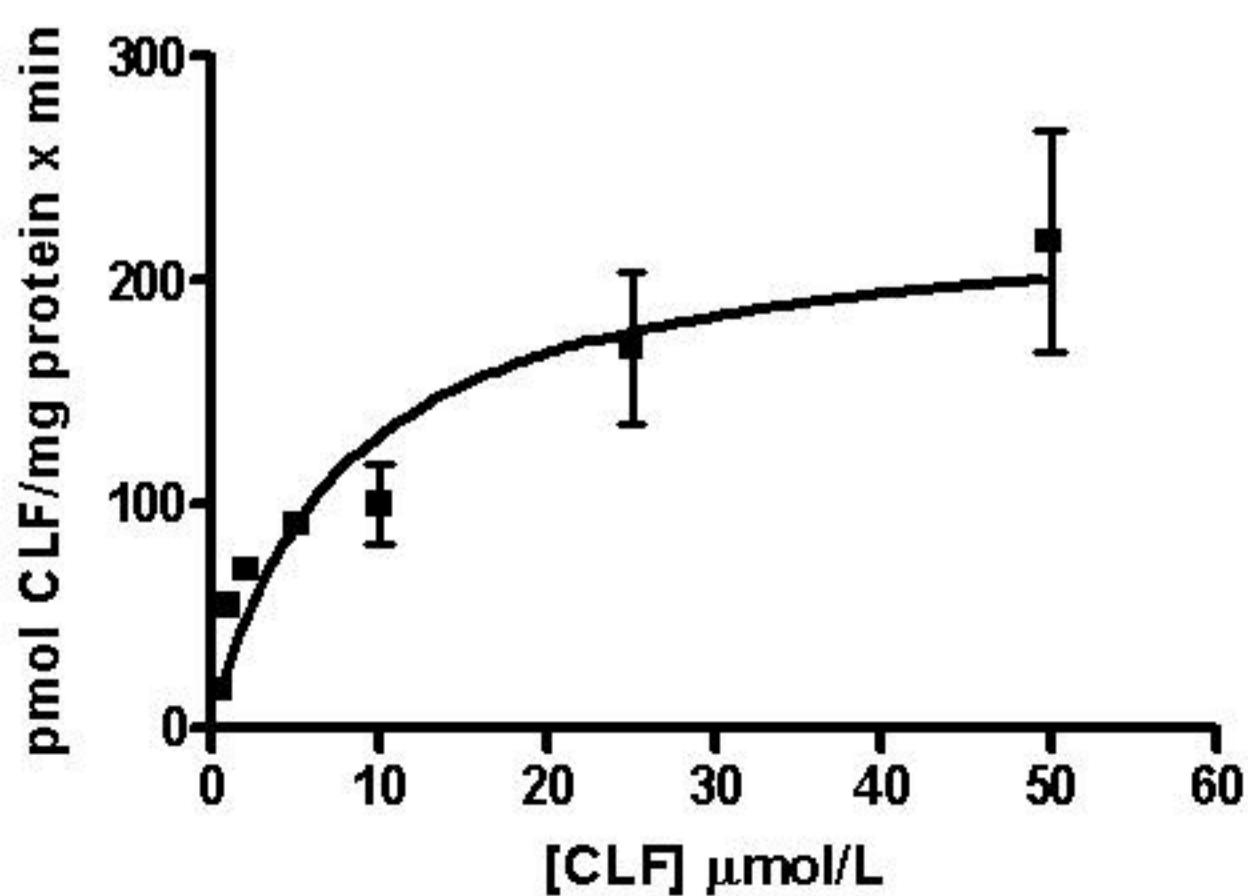


Figure 3

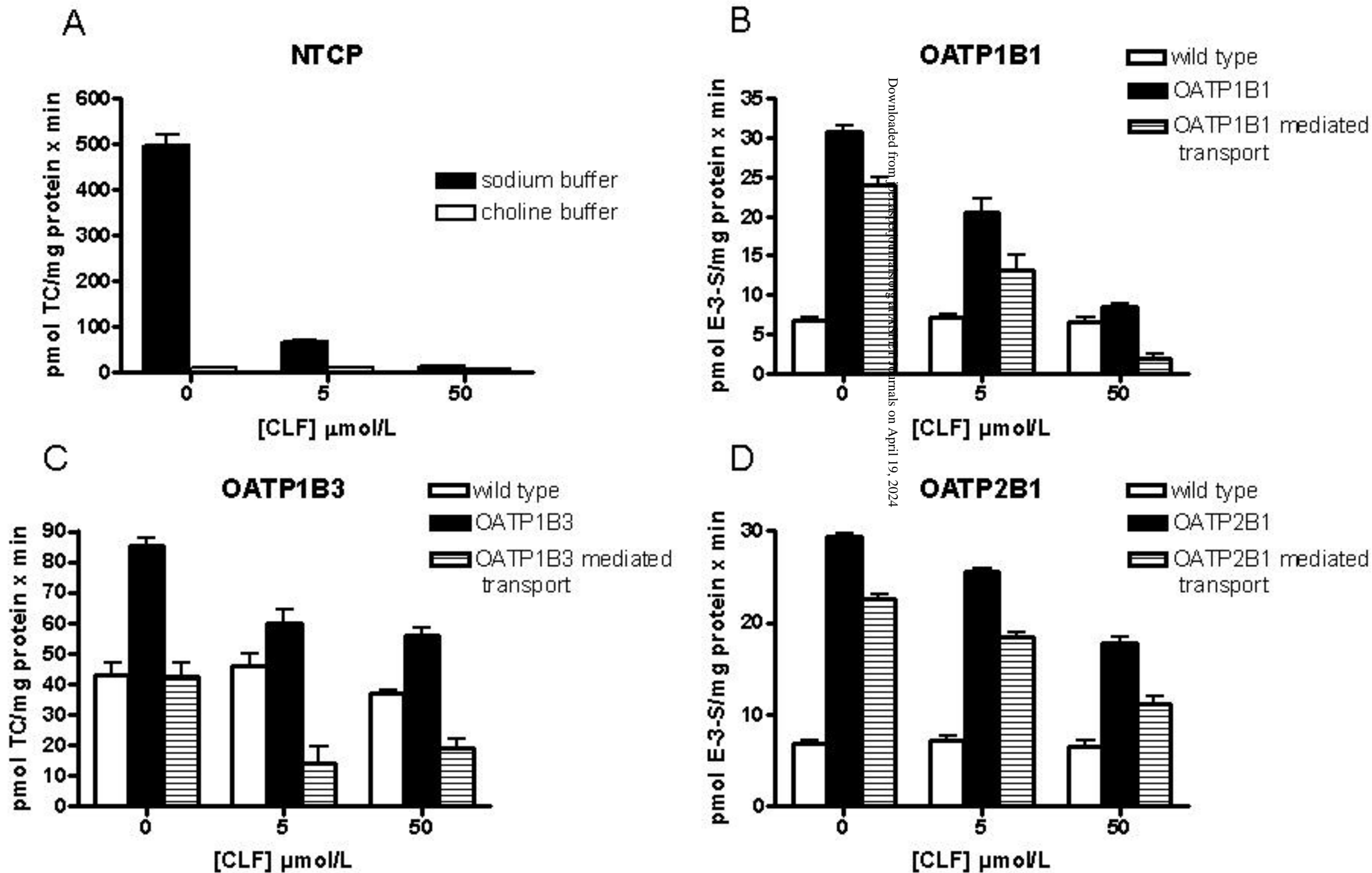
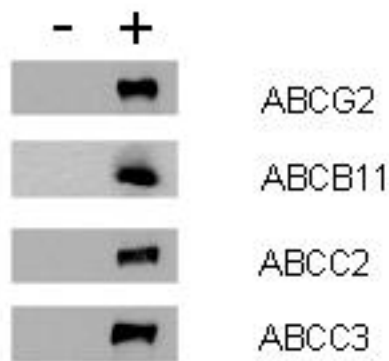


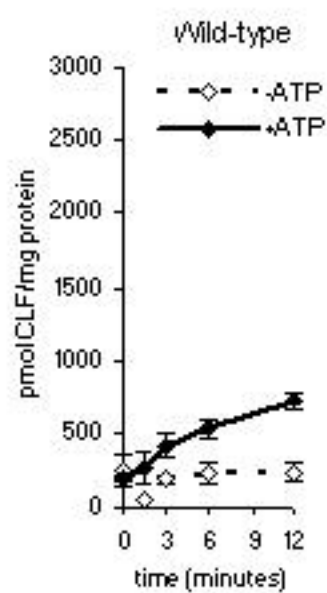
Figure 4

A

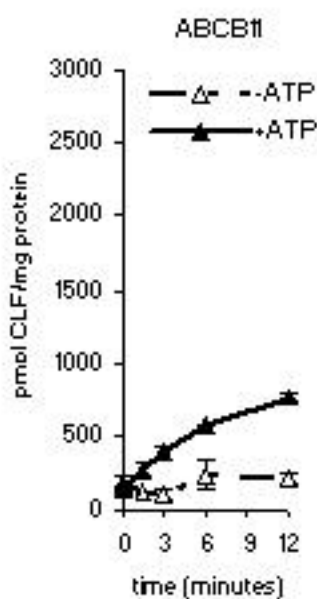


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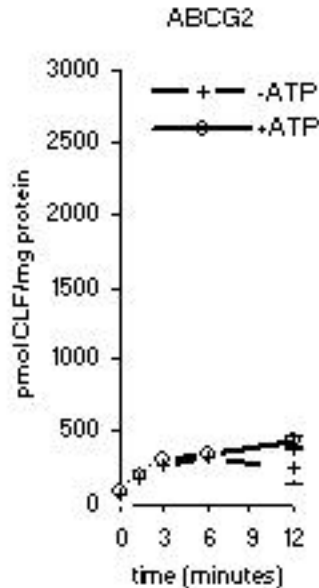
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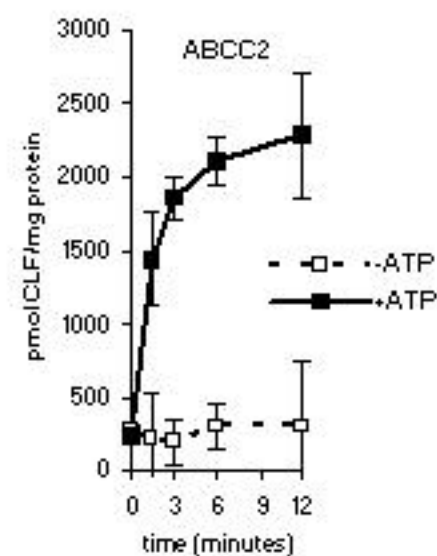
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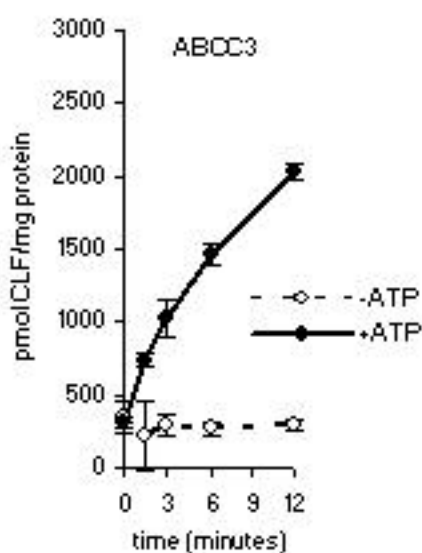
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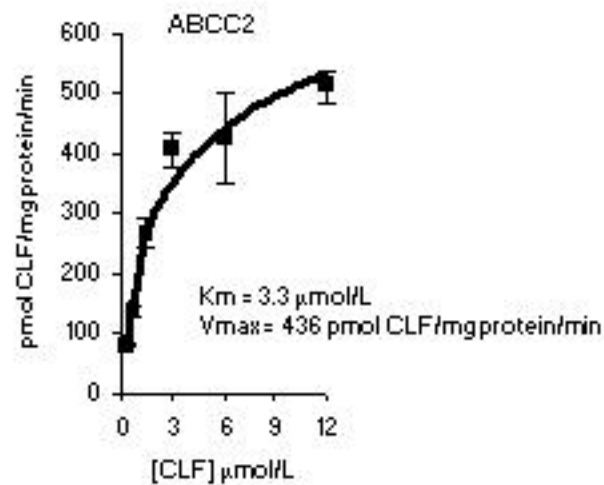
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F



G



H

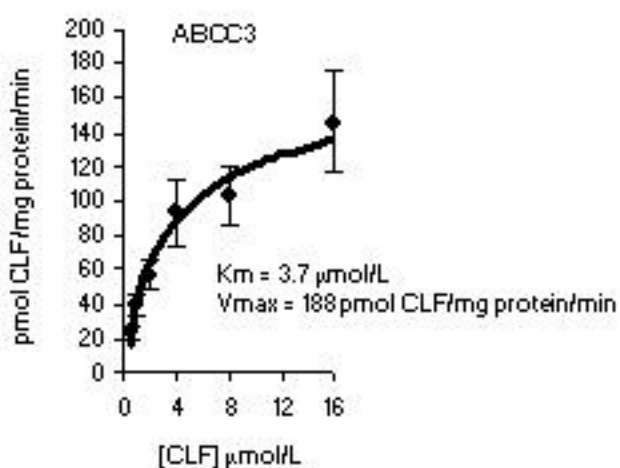
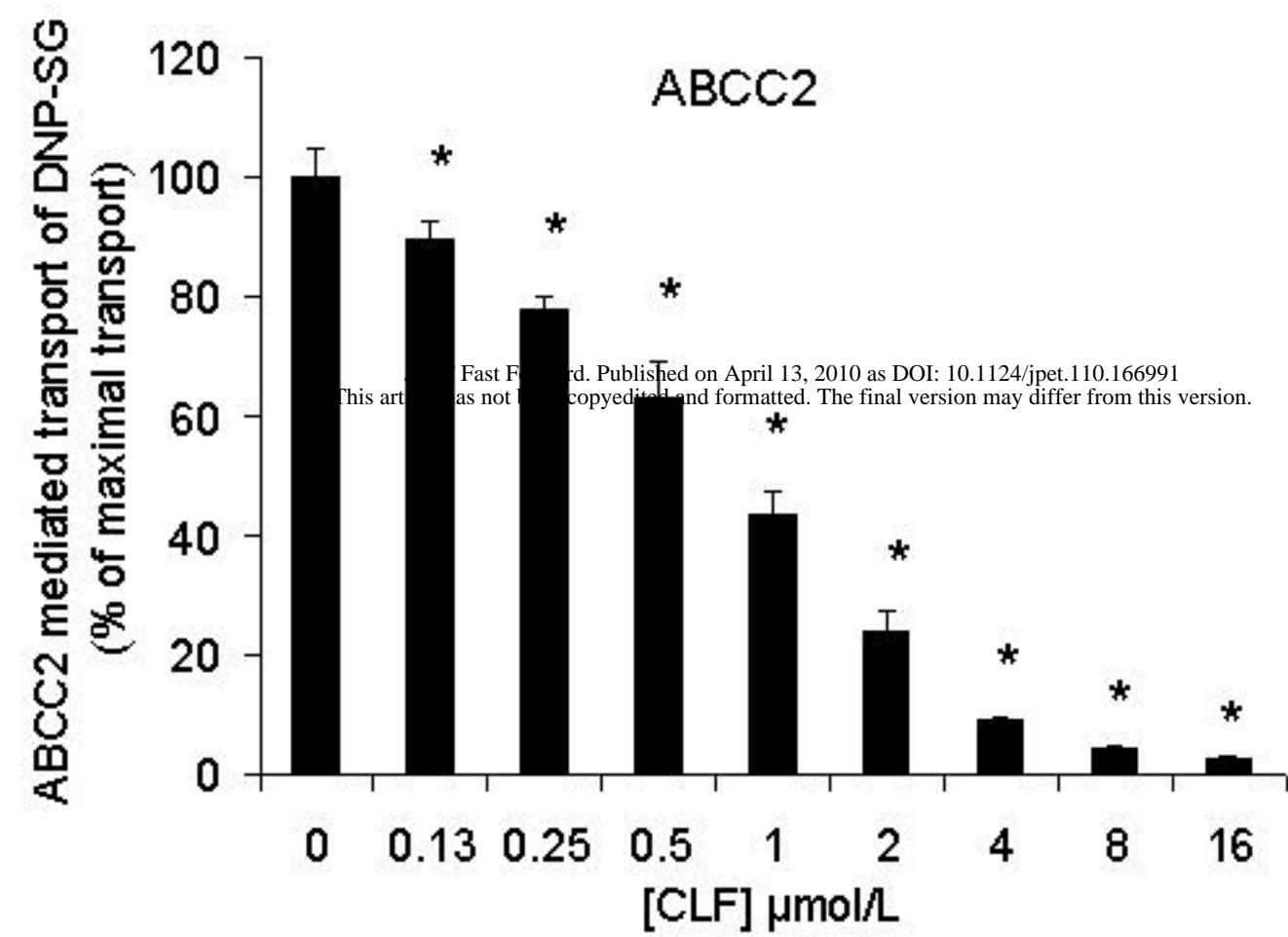


Figure 5

A



B

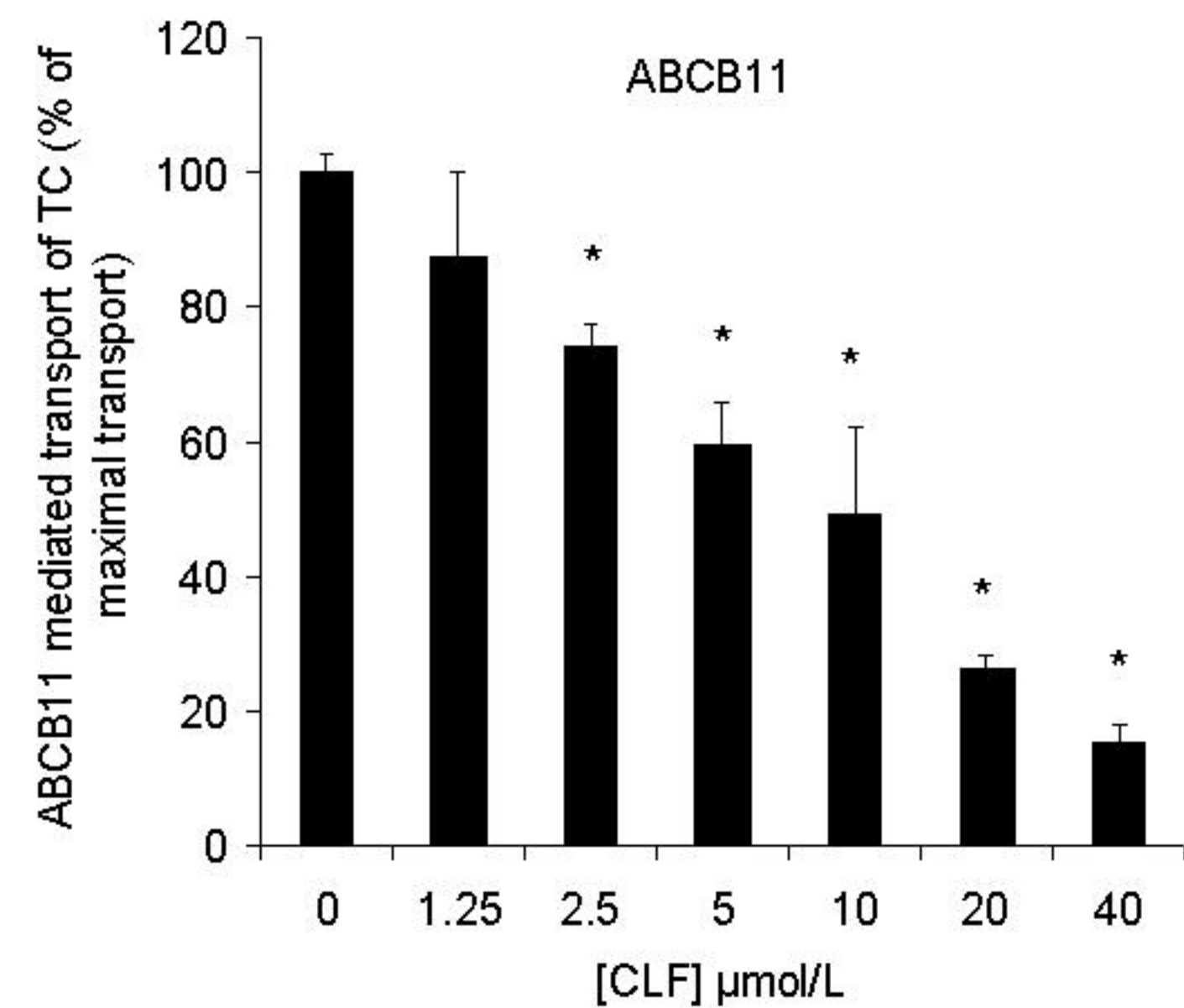
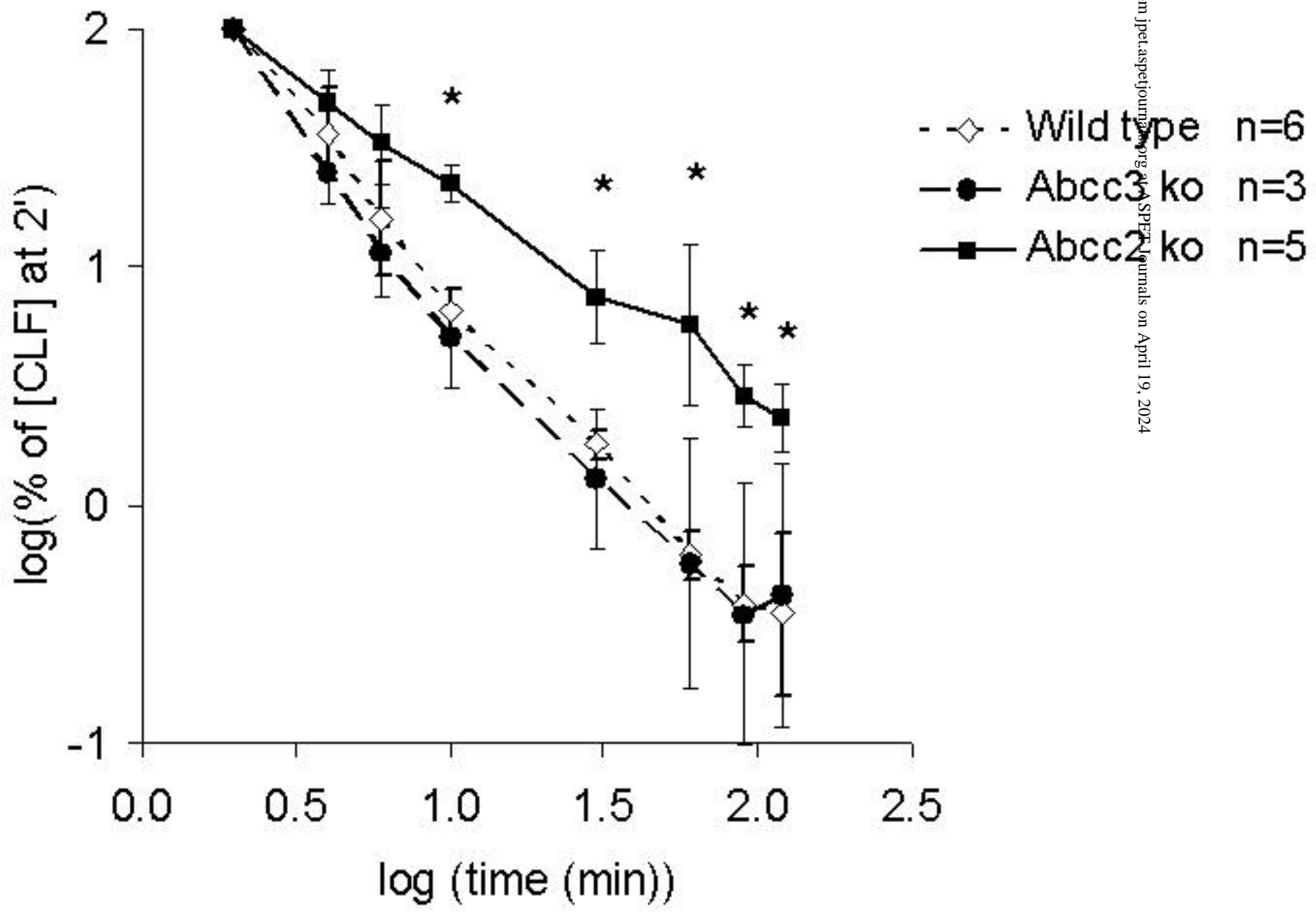


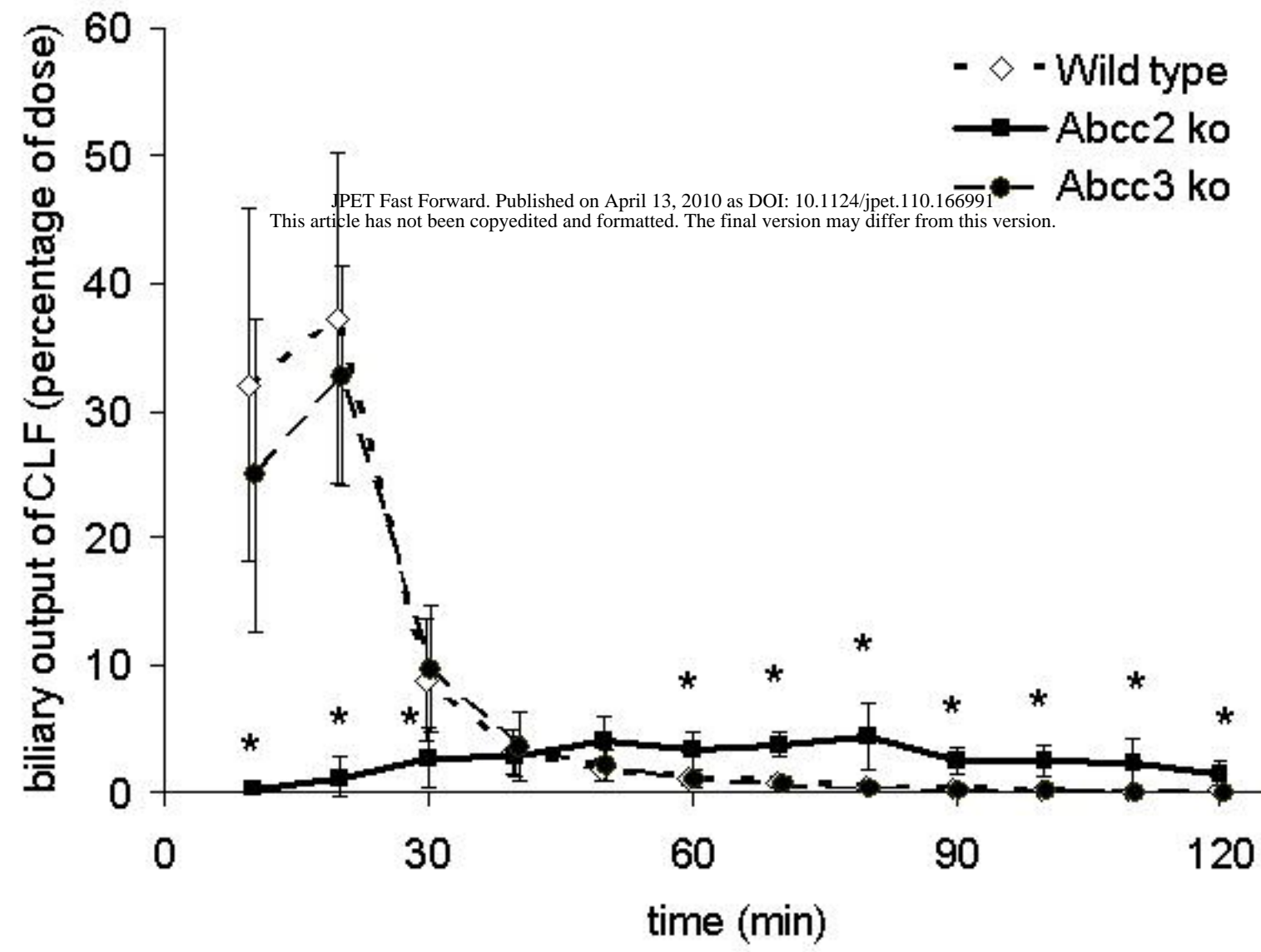
Figure 6



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Figure 7

A



B

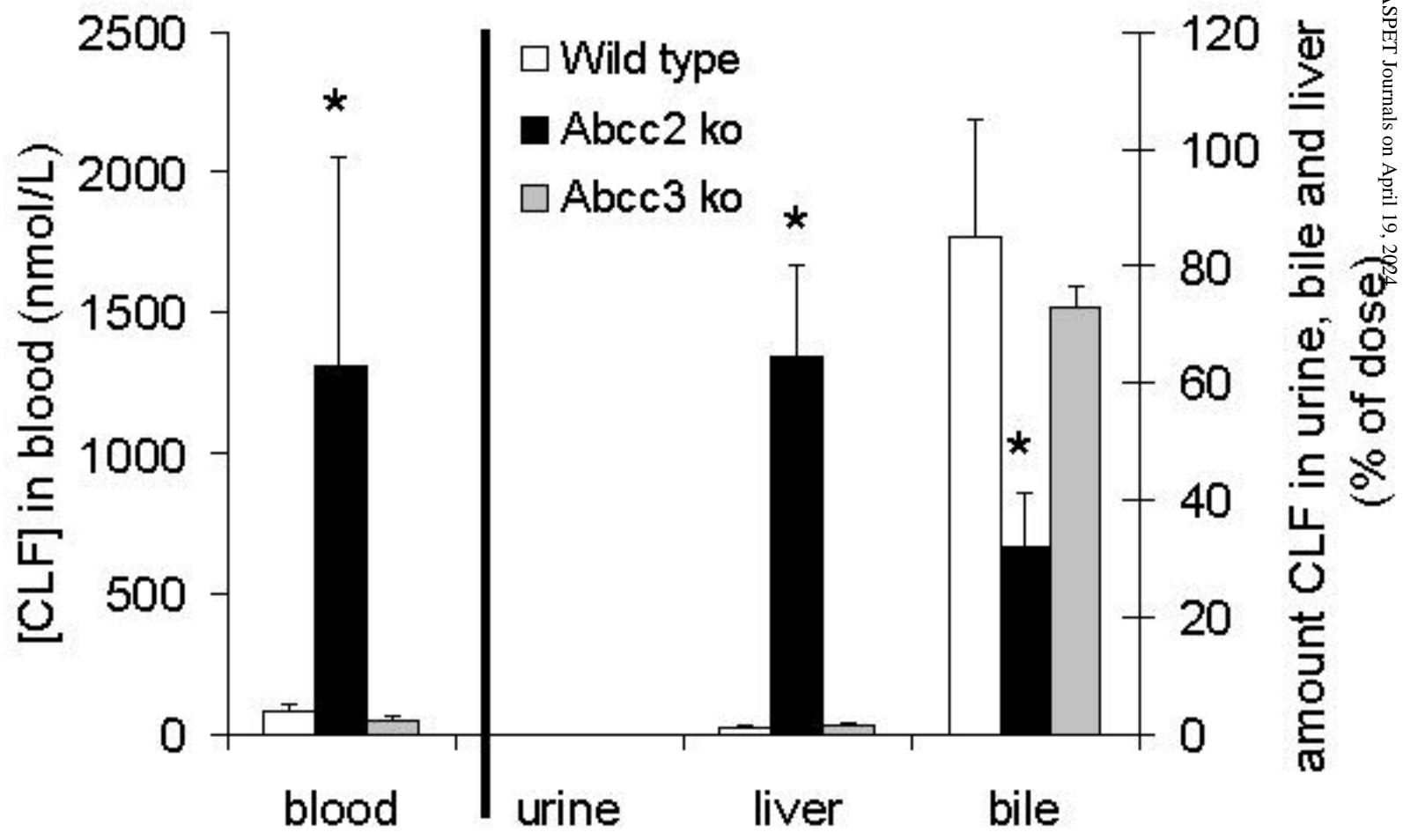


Figure 8

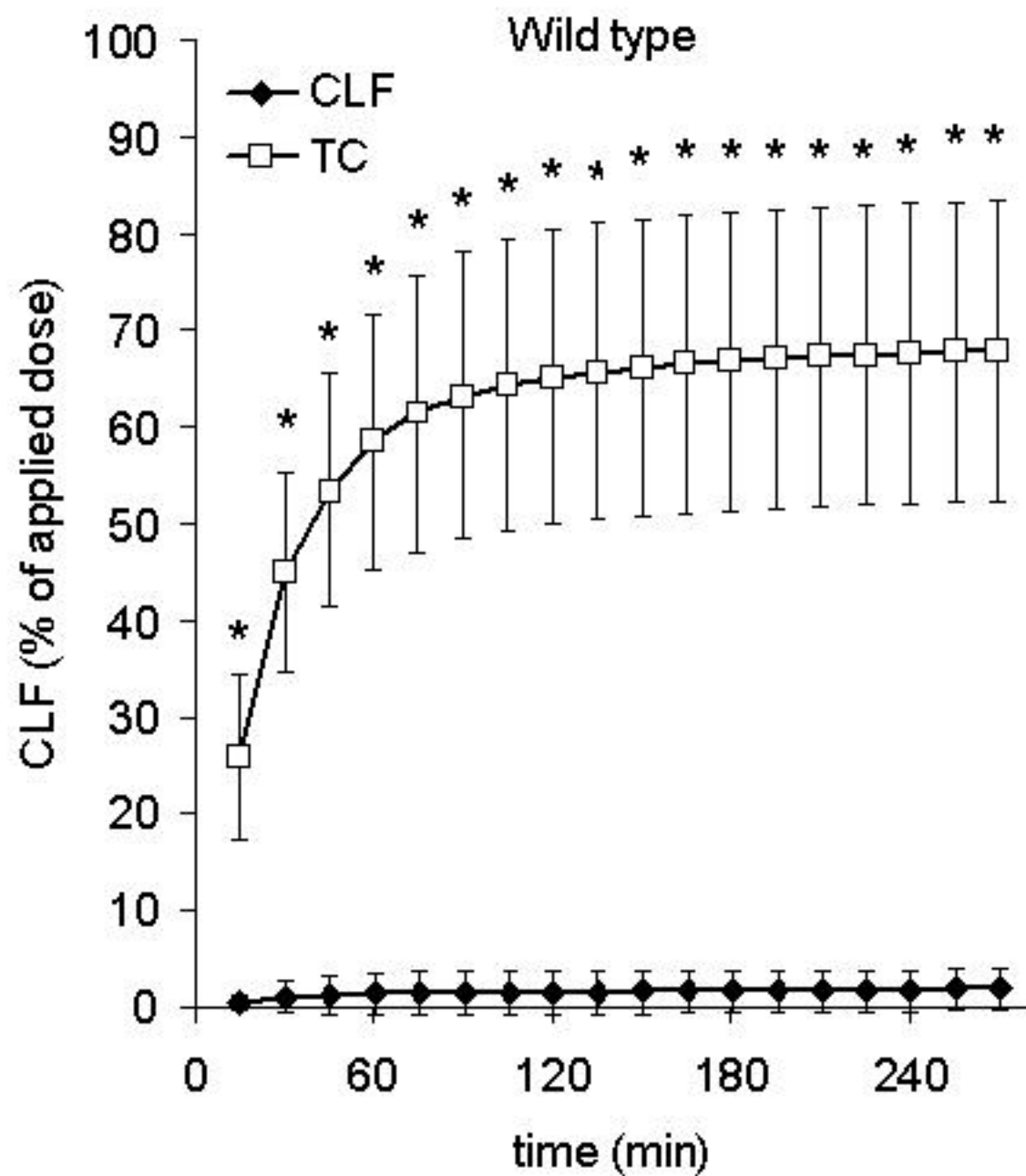
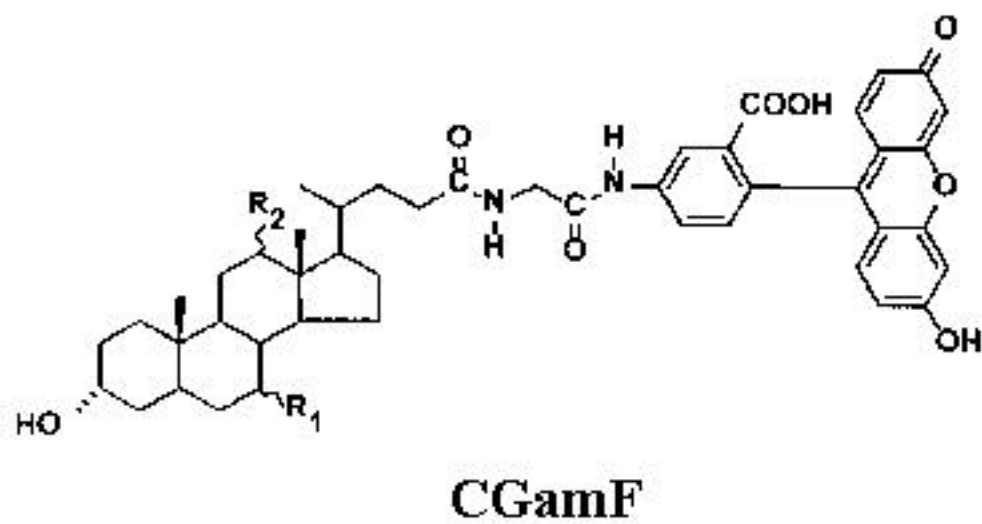
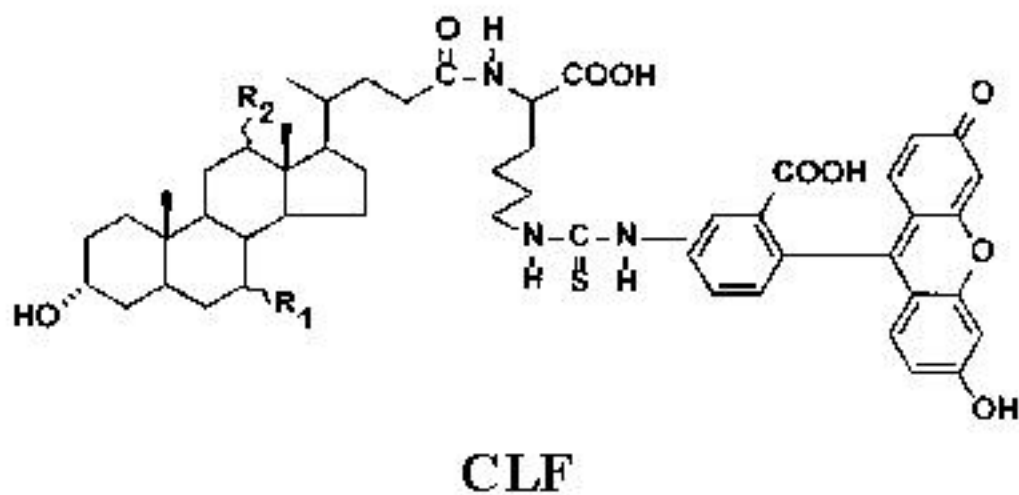


Figure 9



Supplemental data 1

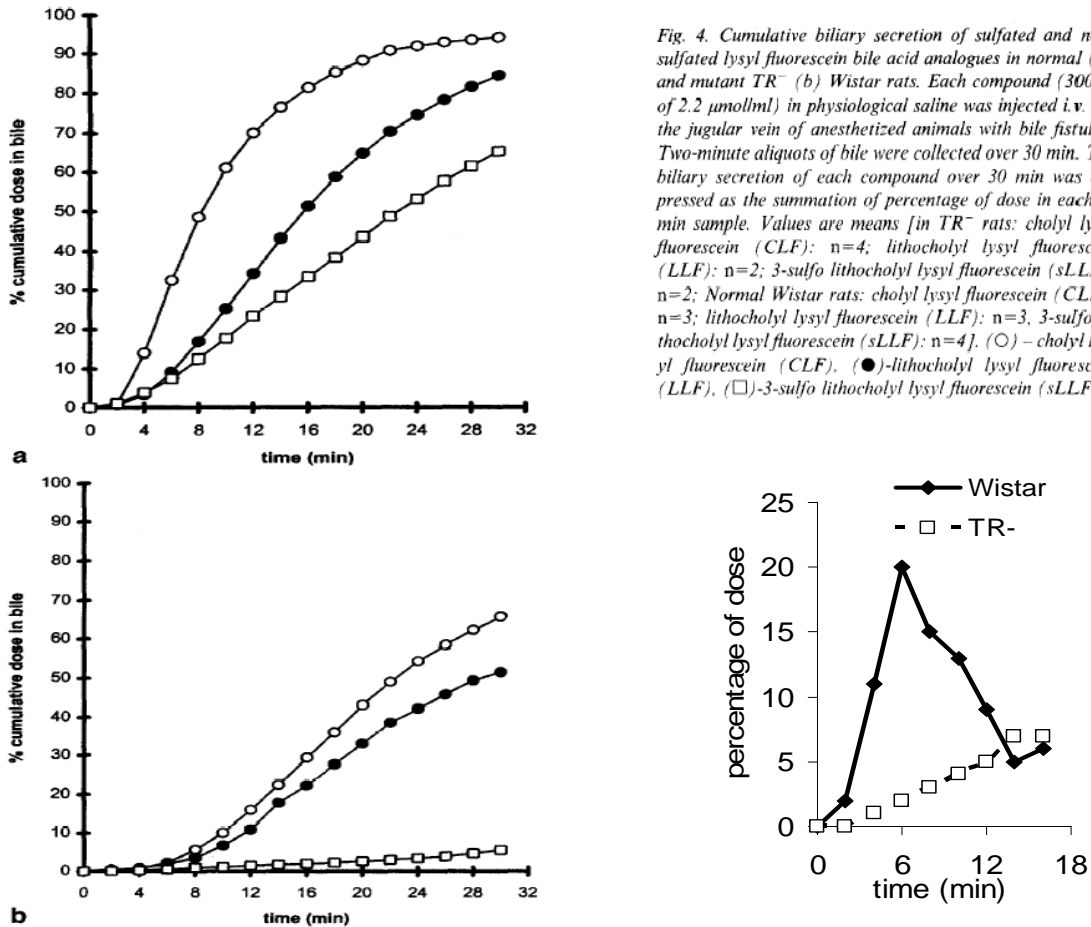


Fig. 4. Cumulative biliary secretion of sulfated and non-sulfated lysyl fluorescein bile acid analogues in normal (a) and mutant TR⁻ (b) Wistar rats. Each compound (300 μ l of 2.2 μ mol/ml) in physiological saline was injected i.v. via the jugular vein of anesthetized animals with bile fistulae. Two-minute aliquots of bile were collected over 30 min. The biliary secretion of each compound over 30 min was expressed as the summation of percentage of dose in each 2-min sample. Values are means [in TR⁻ rats: cholyl lysyl fluorescein (CLF): n=4; lithocholyl lysyl fluorescein (LLF): n=2; 3-sulfo lithocholyl lysyl fluorescein (sLLF): n=2; Normal Wistar rats: cholyl lysyl fluorescein (CLF): n=3; lithocholyl lysyl fluorescein (LLF): n=3; 3-sulfo lithocholyl lysyl fluorescein (sLLF): n=4]. (○) - cholyl lysyl fluorescein (CLF), (●) - lithocholyl lysyl fluorescein (LLF), (□) - 3-sulfo lithocholyl lysyl fluorescein (sLLF).

Figure 4 with legend of paper of Mills *et al.* The lower right panel represents our recalculation of the excretion based on the cumulative data given in the other panels.

Reprinted from Mills *et al.*: *J Hepatol* **31** Mills CO, Milkiewicz P, Muller M, Roma MG, Havinga R, Coleman R, Kuipers F, Jansen PL and Elias E: "Different pathways of canalicular secretion of sulfated and non-sulfated fluorescent bile acids: a study in isolated hepatocyte couplets and TR- rats" (1999), with permission from Elsevier. <http://www.jhep-elsevier.com/>