Liver and Biliary Tract Physiology/Pathophysiology

Long-term trans-inhibition of the hepatitis B and D virus receptor NTCP by taurolithocholic acid

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Abstract

Human hepatic bile acid transporter Na+/taurocholate cotransporting polypeptide (NTCP) represents the liver-specific entry receptor for the hepatitis B and D viruses (HBV/HDV). Chronic hepatitis B and D affect several million people worldwide, but treatment options are limited. Recently, HBV/HDV entry inhibitors targeting NTCP have emerged as promising novel drug candidates. Nevertheless, the exact molecular mechanism that NTCP uses to mediate virus binding and entry into hepatocytes is still not completely understood. It is already known that human NTCP mRNA expression is downregulated under cholestasis. Furthermore, incubation of rat hepatocytes with the secondary bile acid taurolithocholic acid (TLC) triggers internalization of the rat Ntcp protein from the plasma membrane. In the present study, the long-term inhibitory effect of TLC on transport function, HBV/HDV receptor function, and membrane expression of human NTCP were analyzed in HepG2 and human embryonic kidney (HEK293) cells stably overexpressing NTCP. Even after short-pulse preincubation, TLC had a significant long-lasting inhibitory effect on the transport function of NTCP, but the NTCP protein was still present at the plasma membrane. Furthermore, binding of the HBV/HDV myr-preS1 peptide and susceptibility for in vitro HDV infection were significantly reduced by TLC preincubation. We hypothesize that TLC rapidly accumulates in hepatocytes and mediates long-lasting trans-inhibition of the transport and receptor function of NTCP via a particular TLC-binding site at an intracellularly accessible domain of NTCP. Physiologically, this trans-inhibition might protect hepatocytes from toxic overload of bile acids. Pharmacologically, it provides an interesting novel NTCP target site for potential long-acting HBV/HDV entry inhibitors.

NEW & NOTEWORTHY The hepatic bile acid transporter NTCP is a high-affinity receptor for hepatitis B and D viruses. This study shows that TLC rapidly accumulates in NTCP-expressing hepatoma cells and mediates long-lasting trans-inhibition of NTCP’s transporter and receptor function via an intracellularly accessible domain, without substantially affecting its membrane expression. This domain is a promising novel NTCP target site for pharmacological long-acting HBV/HDV entry inhibitors.

entry inhibitor; HBV; NTCP; taurolithocholic acid; trans-inhibition

INTRODUCTION

Although an effective vaccine is available, hepatitis B virus (HBV) and hepatitis D virus (HDV) infections remain a major global health problem. More than 250 million people worldwide are chronically infected with HBV, and thousands of them suffer from hepatocellular carcinoma (HCC) or develop terminal liver cirrhosis. About 5% of chronic HBV carriers are coinfected with HDV, a situation often associated with disease progression and increased mortality rates (1). The hepatitis D virus is an HBV satellite virus, and both share identical envelope proteins (2). Current standard therapies include nucleoside reverse transcriptase inhibitors and interferon. However, both of these are not curative in the majority of cases (3–5).

In 2012, Yan et al. (6) identified the liver bile acid transporter Na+/taurocholate cotransporting polypeptide (NTCP, gene symbol SLC10A1) as the high-affinity hepatic entry receptor for HBV and HDV. NTCP belongs phylogenetically to the solute carrier family SLC10 and is typically expressed at the basolateral membrane of hepatocytes (7, 8), explaining the clear hepatotropism of HBV and HDV. NTCP, together with other bile acid carriers (including ASBT, OSTα/β, and BSEP), is essential for the maintenance of the enterohepatic circulation of bile acids between liver and gut (9). In detail, NTCP mediates a sodium-dependent reuptake of mostly
conjugated bile acids from portal blood back into hepatocytes (7).

It is already known that HBV and HDV attach to NTCP via their myristoylated preS1 domain comprising the NH2-terminal amino acids 2–48 of the large HBV surface protein, briefly called myr-preS1 lipopeptide (10, 11). To date, it is generally accepted that myr-preS1-mediated HBV/HDV binding to NTCP represents the mandatory high-affinity attachment step of viral entry into hepatocytes (12, 13). Previously, we showed that HBV/HDV myr-preS1-mediated viral binding to NTCP directly interferes with the physiological bile acid transport function of NTCP (14). Briefly, the myr-preS1 peptide blocked the bile acid transport in primary human hepatocytes and NTCP-overexpressing HepG2 hepatoma cells in a concentration-dependent manner. Vice versa, bile acids significantly inhibited myr-preS1 binding to NTCP as well as in vitro HBV infection (14). This effect most likely occurs via cis-inhibition at overlapping binding sites for bile acids and the myr-preS1 peptide at respective extracellular accessible domains of NTCP. In support of this assumption, we have recently shown that mutation of amino acid G158 at the second extracellular loop of human NTCP completely abolished myr-preS1 peptide binding to NTCP without affecting the bile acid transport function of NTCP (15). Other amino acids of NTCP, such as S267, which is located within transmembrane domain 8 (TMD8) in proximity to the extracellular loop connecting TMD8 and TMD9, proved to be essential for the binding of both bile acids and myr-preS1 peptide (16, 17). Interestingly, the naturally occurring NTCP variant S267F showed reduced transport activity for taurocholic acid (TC) but maintained transport of other substrates such as rosu-vastatin and estrone-3-sulfate (16, 18). Patients bearing this S267F variant were less susceptible to chronic HBV infection and showed decreased risk for HCC and liver cirrhosis under chronic HBV infection (19–23). Overall, these data indicate that the extracellular NTCP-binding sites for its transport substrates and for the myr-preS1 peptide overlap, but both can be separated by mutation (14, 15, 17).

In contrast to this cis-inhibitory effect, pulse preincubation with the secondary bile acid tauroliholocholic acid (TLC) followed by intensive washout had a long-lasting inhibitory effect on the transport function of NTCP, which cannot be explained by cis-inhibition (14, 24). Interestingly, this effect was not observed after preincubation with other bile acids such as TC, glycocholic acid (GC), or tauroursodeoxycholic acid (TUDC) (14). It was shown that NTCP mRNA expression is downregulated in patients with cholestasis, probably due to signals induced by bile acid overload (25). While long-term incubation with TLC in rat hepatocytes significantly decreased the plasma membrane expression of rat Ntcp, plasma membrane expression of human NTCP remained unaffected under comparable conditions (24). However, it is currently unclear how TLC mediates this long-term inhibitory effect on human NTCP without affecting substantially its membrane expression. Furthermore, it is of interest if and how this effect influences the susceptibility of hepatocytes for HBV/HDV infection. To address these questions, we analyzed the effect of pulse preincubation of TLC on the transport function, membrane expression, myr-preS1 peptide binding, and in vitro HDV infection in NTCP-overexpressing HepG2 and HEK293 cells. We found a long-lasting inhibitory effect of TLC on the NTCP transporter and receptor function as well as on in vitro HDV infection that might be explained by trans-inhibition of NTCP via a particular TLC-binding site at an intracellularly accessible domain of NTCP.

### MATERIALS AND METHODS

#### Cell Lines and Induction

Human embryonic kidney (HEK293; Thermo Fisher Scientific, Waltham, MA) cells, stably expressing the human NTCP protein, NH2-terminally tagged with the HA-epitope and COOH-terminally with FLAG-tag were maintained at 37°C, 5% CO2, and 95% humidity in Dulbecco’s modified Eagle medium (DMEM)/Ham’s F12 medium (Thermo Fisher Scientific) supplemented with 10% fetal calf serum (Sigma-Aldrich, Taufkirchen, Germany), 4 mM l-glutamine (PAA, Cölbe, Germany), and penicillin/streptomycin (PAA). Human hepatoma HepG2 cells, stably transfected with NTCP-FLAG [see more detailed description in (14)], and HepG2-tet-on cells (BD Clontech, Heidelberg, Germany) were cultured under the same conditions in DMEM with all supplements listed above, except for l-glutamine. For induction of the transgene, the medium was supplemented with 1 μg/mL tetracycline (Roht, Karlsruhe, Germany) for HA-NTCP-FLAG-HEK293 cells (further referred to as NTCP-HEK293 cells) or 2 μg/mL doxycycline (Sigma-Aldrich) for NTCP-FLAG-HEK293 cells (further referred to as NTCP-HepG2 cells). NTCP-HepG2 cells were used for all HDV infection experiments as reported before (14). HepG2-tet-on cells were transiently transfected using XtremeGENE 9 (Roche Diagnostics, Basel, Germany) with a pcDNA5-FRT-TO plasmid (Thermo Fisher Scientific) containing HA-NTCP-FLAG for plasma membrane expression studies. All cell lines were tested negatively for mycoplasma contamination according to the protocol of Uphoff and Drexler (26).

#### Synthesis of [3H]Taurolithocholic Acid

[3H]Taurine conjugation of lithocholic acid (LC) was done via a modified protocol of Kramer et al. (27), which represents an adapted Norman’s method (28). Briefly, 1 mg (2.7 μM) LC (Tokyo Chemical Industry, Tokyo, Japan) was dissolved in 80 μL of anhydrous dioxane. To this solution, 10 μL of triethylamine (Sigma-Aldrich) was added, followed by incubation for 10 min at 23°C. Then, 10 μL of 1% ethyl chloroformate (Thermo Fisher Scientific) solved in dioxane (Sigma-Aldrich) was added, and the mixture was incubated for additional 30 min at 23°C. A second cup, 200 μL of a 50 μM [3H]taurine hydrochloride solution (Bio Trend, Cologne, Germany; 20 Ci/mmol, 1 mCi/mL, 0.0225 fmol/dpm) was evaporated, and 40 μL of a 10 mM aqueous NaOH solution (Roth) and 40 μL of anhydrous dioxane were added. Finally, 15 μL of the LC-containing solution was added to the [3H]taurine solution and incubated for 24 h at 23°C. The resulting solution was cleaned up by thin-layer chromatography (Gina-Star, Raytest Isotopenmessgeräte GmbH, Straubenhardt, Germany) to a purity of 90%. Pre-coated plates with silica gel 60 F254 (Merck, Darmstadt, Germany) were used in the solvent system butanol-acetic acid-water 5:2.3 (vol/vol). [3H]Taurolithocholic acid ([3H] TLC) was eluted with methanol (Roth) from the silica gel.
Bile Acid Transport Assays

Qualitative and quantitative transport experiments were performed in NTCP-HEK293 cells with the fluorescent bile acid 4-nitrobenzo-2-oxa-1,3-diazole taurocholic acid (NB-DTC) in DMEM (29). Nuclei were stained with Hoechst33342 (Thermo Fisher Scientific), and qualitative transport was analyzed on a DM16600 B inverted fluorescent microscope (Leica, Wetzlar, Germany). Quantification of relative fluorescence was assessed by fluorescence reader GloMax (Promega, Madison, WI). Additional quantitative transport measurements were performed with [3H]taurocholic acid ([3H]TC, 20 Ci/mmol, 0.09 mCi/mL, PerkinElmer, Waltham, MA), [3H]TLC (see above), and [3H]dehydroepiandrosterone sulfate ([3H]DHEAS, 81.3 Ci/mmol, 1 mCi/mL, PerkinElmer) in NTCP-HEK293 cells by liquid scintillation counting on liquid scintillation analyzer Tri-Carb 2910 TR (PerkinElmer) as reported (30).

Determination of the Inhibitory Concentrations for [3H]TC Uptake

Bile acid transport measurements were performed in NTCP-HEK293 cells with [3H]TC as reported before (30). Briefly, cells were seeded onto polylisine-coated 96-well plates, induced with 1 μg/mL tetracycline, and grown to 100% confluence over 72 h at 37°C. After preincubation with TLC, cells were washed three times with tempered DMEM. Then, medium was replaced by 80 μL DMEM containing the respective inhibitor or solvent alone (positive control), and cells were further incubated for 5 min at 37°C. Bile acid transport experiments were started by adding 20 μL DMEM containing 25 μM [3H]TC (final concentration: 5 μM). Experiments were stopped after 10 min by washing twice with ice-cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, 7.3 mM Na2HPO4, pH 7.4). For negative control, the NTCP-HEK293 cells were not induced with tetracycline (-tet). Cell-associated radioactivity of [3H]TC was quantified by liquid scintillation counting in a Packard Microplate Scintillation Counter TopCount NXT (Packard Instrument Company, Meriden, CT). Transport rates were determined in disintegrations per minute (dpm). The mean of the negative control was subtracted, and the net transport was expressed as % of positive control. Irbesartan was purchased from Sigma-Aldrich. Inhibitory concentrations (IC50) were calculated using GraphPad Prism 6 (GraphPad, San Diego, CA).

Qualitative and Quantitative Myr-PreS1 Peptide Binding Assays

An NH2-terminally myristoylated and COOH-terminally AlexaFlour 568-coupled fluorescent myr-preSI2-48 peptide, consisting of amino acids 2-48 of the large HBV subgenotype D3 surface protein was purchased from Biosynthesis (Lewisville, TX) and will further be referred to as myr-preSIAX568 peptide. Furthermore, a tritium-labeled myr-preSI2-48 peptide (myr-GGQ-[4.5-3H-Leu]-STSNPLGGFPFDHQIDPAFRA-ANTANPDWFNPNKDTWPDAKVG) was purchased from Pharmaron (Cardiff, UK) and will further be referred to as [3H]myr-preSI1 peptide (120 Ci/mmol, 1 mCi/mL). Following bile acid preincubation, cells were washed three times with DMEM and incubated with 50 nM myr-preSI-AX568 peptide in DMEM at 37°C. After another step of washing, myr-preSI-AX568 peptide binding was assessed on Leica DM16600 B fluorescent microscope and quantified using ImageJ software (National Institutes of Health). In addition, quantitative binding measurements were performed with 5 nM [3H]myr-preSI1 peptide under the same experimental conditions, and detection was performed using liquid scintillation counting.

HDV Infection Experiments

NTCP-HepG2 cells were inoculated with 700 genome equivalents of HDV particles per cell for 6 h. HDV was produced in vitro as reported before (31, 32). Genome equivalents were determined by RT-qPCR. Infection was performed in hepatocyte growth medium (HGM) consisting of William’s E medium (Thermo Fisher Scientific) supplemented with 2% bovine serum albumin (BSA; Roth), 2 mM L-glutamine (Thermo Fisher Scientific), 100 μg/mL gentamicin (Thermo Fisher Scientific), 10 nM dexamethasone (Sigma-Aldrich), 1 mM sodium pyruvate (Thermo Fisher Scientific), and 1× insulin-transferrin-selenium (Thermo Fisher Scientific). For the 6-h infection period, HGM was additionally supplemented with 2% DMSO (Merck), 4% polyethylene glycol 8000 (Sigma-Aldrich), and 2 μg/mL doxycycline (Sigma-Aldrich) as described (14). Subsequently, cells were washed with DMEM and further cultured in HGM supplemented with 2% DMSO and 2 μg/mL doxycycline. Medium was changed every 3–4 days until cells were fixed at days 9–11 post infection with 3% paraformaldehyde (Sigma-Aldrich) in PBS for 30 min at room temperature (RT). Cells were permeabilized with 0.2% Triton X 100 (Roth) in PBS for 30 min at RT, and blocked by incubation with 5% BSA in PBS for 30 min at RT. For HDV antigen (HDAg) detection, as a marker of HDV infection, cells were immunostained using purified human anti-HDV-positive serum at 37°C for 1 h (1:400 dilution) and goat anti-human IgG secondary antibody coupled to Alexa Fluor 568 (1:400 dilution, Thermo Fisher Scientific, Cat. No. A-21090, Lot No. 2126795) for 1 h at 37°C as described (31,32). Nuclei were stained with Hoechst33342 (1 μg/mL, Thermo Fisher Scientific).

Nonpermeabilized Immunofluorescence Assay

After bile acid incubation, NTCP-HEK293 as well as HepG2-tet-on cells, transiently expressing the HA-NTCP-FLAG protein, were fixed with 3% paraformaldehyde (Roth) and blocked with 5% BSA (Sigma-Aldrich) in PBS for 30 min at RT. Subsequently, monoclonal mouse anti-HA antibody (1:400 dilution for HEK293 and 1:800 for HepG2 cells, Thermo Fisher Scientific, Cat. No. 32–6700, Lot No. QL217972) was incubated for 1 h at 37°C, followed by goat anti-Mouse IgG Alexa Fluor 488 (1:800 dilution, Thermo Fisher Scientific, Cat. No. A-11001, Lot No. 1907294) and nuclear staining with Hoechst 33342 (1 μg/mL). NTCP-HEK293 cells were qualitatively analyzed on Leica DM6600 B inverted fluorescent microscope or quantitatively measured using multimode microplate reader Spark 10 M (Tecan, Männedorf, Switzerland) as reported before (33), with the following settings: fluorescence mode with bottom detection, 488±20 nm bandwidth monochromator for excitation, optimal gain, 4 × 4 reads per well, and 535±25 nm bandwidth.
emission filter. For quantification of relative fluorescence of HepG2-tet-on transiently expressing the HA-NTCP-FLAG protein, samples were assessed on Leica DMi6000 B fluorescent microscope and analyzed using LAS-X imaging software (Leica). Cell-based fluorescence was determined by defined regions of interest (ROI), and data are presented as the mean background-subtracted fluorescence intensity of nontransfected HepG2 cells.

**Lactate Dehydrogenase Release Cytotoxicity Assay**

Pierce lactate dehydrogenase release (LDH) cytotoxicity assay (Thermo Fisher Scientific, Cat. No. 88954) was performed according to the manufacturer’s protocol. Briefly, NTCP-HEK293 and NTCP-HepG2 cells were incubated with bile acids over 2 h or 6 h at 37°C. After 2 h, 50 μL of each sample and control medium was transferred to a new well plate, 50 μL of reaction mixture were added, and incubated for 30 min at RT. Finally, the reaction was stopped by adding 50 μM of stop solution and measured by ELISA reader (PHOmo, anthos Mikrosysteme GmbH, Krefeld, Germany). Lysis control (maximal LDH-control) was incubated 45 min with lysis buffer at 37°C. Cell-based infected HepG2 cells.

**MTS Cytotoxicity Assay**

The CellTiter 96 AQueous MTS colorimetric cell viability assay (Promega, Cat. No. G3581) was performed according to the manufacturer’s protocol. NTCP-HEK293 and NTCP-HepG2 cells were incubated 2 h with 100 μL DMEM without phenolred (Thermo Fisher Scientific) containing bile acids at 37°C and 5% CO₂ atmosphere. Thereafter, 20 μL of CellTiter 96 AQueous One Solution Reagent were added and incubated 1 h at 37°C and 5% CO₂. Using fluorescence reader GloMax (Promega) absorbance was recorded at 450 nm.

**Surface Biotinylation and Western Blotting**

NTCP-HepG2 cells were seeded onto six-well plates, were induced (NTCP-FLAG expression) or not induced (paternal control medium) and then two times with PBS. Cells of the respective wells were harvested in 1 mL lysis buffer (PBS containing L2% Triton X100, Sigma-Aldrich) and 10 μL of protease inhibitor cocktail (Thermo Fisher Scientific, Cat. No. 87785). The lysate was centrifuged at 10,000 g for 2 min to remove debris and DNA. Protein concentration of the supernatant was determined by bichinonic acid (BCA) assay following the manufacturer’s protocol (Novagen, Darmstadt, Germany, Cat. No. 71285-3) and was measured with NanoDrop One (Thermo Fisher Scientific) photometer in BCA assay application. Cell lysates were adjusted to 400 ng/mL by dilution with lysis buffer. For each cell lysate, 50 μL streptavidin magnetic beads (Pierce, Cat. No. 88817) were equilibrated with lysis buffer. For pull down, 50 μL equilibrated beads were incubated at 4°C overnight with 900 μL of lysate within 1.5 mL Eppendorf tubes on rotating stand. On the next day, beads were adsorbed to the tube wall by a magnetic stand, surplus buffer was withdrawn, 900 μL fresh buffer was added, and beads were resuspended and rotated for 10 min at 4°C. This washing process was repeated three times. To prepare for Western blotting, beads were adsorbed by a magnetic stand and the surplus was removed. Then, the beads were resuspended in 65 μL Laemmli buffer and incubated at 30°C for 10 min. After centrifugation, 20 μL of the obtained supernatant per well was separated on 10% SDS gel followed by Western blotting. The nitrocellulose membrane was blocked for 1h at RT in 5% milk powder (Sigma-Aldrich) in tris-buffered saline-Tween 20 [TBS-T; 137 mM NaCl, 10 mM Tris (Roth), pH 8.0, 0.05% Tween-20 (Roth)] and then washed with primary anti-FLAG antibody (anti-FLAG rabbit antibody, 1:2,000 dilution, Sigma-Aldrich, Cat. No. F7425, Lot No. 0000099715) overnight in 5% milk powder in TBS-T at 4°C. The membrane was washed three times in TBS-T and incubated at RT with secondary anti-rabbit-HRP antibody (anti-rabbit-HRP goat, 1:4,000 dilution, Thermo Fisher Scientific, Cat. No. 31460, Lot No. UK293475). Both antibodies were widely used before (34, 35). After final washing in TBS-T, the membrane was rinsed with Roti-Lumin substrate (Roth, Cat. No. P0781.1), transferred to an imager, and exposed for 20 min (Intas Science Imaging Instruments GmbH, Göttingen, Germany). Image analysis was done with Image Lab 6.1 (Bio-Rad Laboratories, CA).

**Statistics**

Data are shown as means ± SD. Prism software (GraphPad Prism 6.0) was used for data presentation and statistical analysis. Statistical analysis was performed by one-way or two-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison post hoc test as indicated in the figure legends. For IC₅₀ calculations, statistical analysis was done by two-way ANOVA and Sidak’s multiple comparisons post hoc test.

**Graphical Abstract**

Chemical structures of bile acids and DHEAS were generated using PubChem (National Center for Biotechnology Information, Bethesda, MD).

**RESULTS**

**TLC-Induced Inhibition of NTCP’s Transporter and Receptor Function**

To analyze the effect of bile acid preincubation on the transporter and virus receptor function of NTCP, NTCP-HEK293 cells were used for transport experiments with the fluorescent bile acid NBD-TC and for binding experiments with the red fluorescent myr-preS1-AX568 peptide. In addition, anti-HA immunofluorescence was performed to detect the HA-NTCP-FLAG protein. Because the HA-coupled NH₂-terminus of NTCP is outward oriented and these experiments were performed under nonpermeabilized conditions, fluorescence signals indicate plasma membrane expression of NTCP under these experimental conditions. As expected, positive control cells without bile acid preincubation showed strong accumulation of NBD-TC and significant binding of
the myr-preS1-AX568 peptide to the surface of the cells (Fig. 1A). In addition, clear fluorescence staining of NTCP was detected at the plasma membrane of nearly all cells. This pattern was not much different, when NTCP-HEK293 cells were preincubated with 25 μM TC or TUDC for 30 min followed by thorough washout before the respective experiments. As both bile acids demonstrated a potential for NTCP cis-inhibition of bile acid transport and myr-preS1 peptide binding in our previous study (14), this indicates that the washout of the preincubated bile acids was sufficient to avoid such a cis-inhibition. In the case of taurochenodeoxycholic acid (TCDC), NBD-TC and myr-preS1-AX568 peptide showed a trend for lower relative fluorescence, but NTCP expression was still clearly detected at the plasma membrane. In contrast, preincubation of 25 μM TLC nearly abolished NBD-TC transport and myr-preS1-AX568 peptide
binding, but plasma membrane expression of NTCP was apparently not changed (Fig. 1A). The latter indicates that the drop in NTCP transport and receptor function was not caused by internalization or degradation of NTCP in these experiments. These qualitative data were verified by quantification of the relative fluorescence units for NBD-TC transport, myr-preS1-AX568 peptide binding, and anti-HA immunofluorescence (Fig. 1B). Most bile acids had no significant effect on the NBD-TC transport and myr-preS1 binding functions of NTCP. TCDC again showed a trend for lower NBD-TC transport and significantly reduced myr-preS1-AX568 peptide binding compared with the solvent-treated NTCP-HEK293 cells. Even more pronounced, 25 μM TLC and also lithocholic acid (LC) significantly reduced the NBD-TC transport via NTCP and the myr-preS1-AX568 peptide binding to levels of ~50% compared with the positive control. Of note, none of these bile acids induced any cytotoxic effect in the LDH cytotoxicity assay after preincubation even with 50 μM, indicating full cell viability in this experimental setup. NTCP cell surface expression detected by anti-HA immunofluorescence was also not significantly altered by preincubation with any of the bile acids used.

**TLC Preincubation Does Not Abolish Transport of [3H]TLC via NTCP**

During these experiments, the question arose whether the inhibitory effect of TLC on the transporter and receptor function of NTCP is limited only to the substrate NBD-TC. Because this is not a physiological bile acid, the effect of bile acid preincubation on the transporter and receptor function of NTCP was also investigated with the radiolabeled bile acid [3H]TC (Fig. 2A), as well as with [3H]DHEAS (Fig. 2C) and [3H]TLC itself (Fig. 2B) as additional substrates of NTCP. Bile acid preincubation was performed in sodium-containing buffer supporting active transport via NTCP (filled bars) or under sodium-free buffer conditions preventing bile acid transport (open bars). These assays were complemented by binding experiments with the [3H]myr-preS1 peptide lacking the covalently coupled fluorophore of the myr-preS1-AX568 peptide (Fig. 2D). TLC preincubation over 2 h induced a dramatic decline of the [3H]TC transport and [3H]myr-preS1 peptide binding (Fig. 2, A and D). In contrast, 2-h preincubation with GC had only moderate effects on both functions of NTCP. Interestingly, [3H]DHEAS behaved quite similarly to [3H]TC. Its transport was drastically blocked by TLC preincubation, while preincubation with GC only moderately, but significantly, reduced the [3H]DHEAS transport rate via NTCP (Fig. 2C). Of note, the inhibitory effect of TLC preincubation was only effective when the preincubation was performed in sodium-containing buffer, meaning that the sodium-dependent TLC uptake via NTCP during the preincubation phase is a prerequisite for this effect. Finally, the transport of [3H]TLC via NTCP was analyzed after 2-h preincubation with 25 μM TLC itself and with 25 μM GC (Fig. 2B). Remarkably, TLC preincubation did not alter its own ([3H]TLC) transport and basically behaved like GC preincubation only in the case of [3H]TLC as transport substrate. These data indicate that TLC preincubation retains not only plasma membrane expression of NTCP but also its principal transport function, at least for TLC. Therefore, it is unlikely that NTCP is just inactivated, for example, by phosphorylation, or dislocated from the plasma membrane by TLC preincubation. To verify that these effects are not just caused by cytotoxic side effects of the preincubated bile acids, additional MTS cytotoxicity assay was performed with TLC and GC under comparable experimental conditions (Fig. 2E). We found no cytotoxic effect for bile acid preincubation that could explain the specific long-term inhibitory effect of TLC.

**Inhibitory Effect of TLC Depends on Its Intracellular Concentration**

Next, speed and duration of the inhibitory effect of TLC preincubation on the transport function of NTCP were analyzed. For these experiments, NTCP-HEK293 cells were preincubated with 25 μM TLC over time periods ranging from 10 s to 6 h (Fig. 3, A and C). In these experiments, the TC transport activity of NTCP started to decrease significantly after 2 min of TLC preincubation and reached the nadir already at 20 min (Fig. 3C). Longer preincubation times of up to 6 h did not further decrease the transport activity (Fig. 3A). Again, this effect was specific for [3H]TC as the tested transport substrate of NTCP, whereas TLC preincubation had no significant effect on its own ([3H]TLC) transport activity via NTCP even over longer periods of time (Fig. 3C, right). Furthermore, it was analyzed how long this inhibitory effect of TLC may last and if NTCP’s transport activity for TC would recover at any time. To address this question, NTCP-HEK293 cells were preincubated with 25 μM TLC over 2 h. After intensive washing, cells were further incubated in bile acid-free DMEM over time periods of 2 h up to 8 h at 37 C. As clearly

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**Figure 1.** Qualitative and quantitative effects of bile acid preincubation on NTCP’s transporter function, receptor function, and surface expression. A: HEK293 cells, stably expressing the HA-NTCP-FLAG protein were seeded and induced with 1 μg/mL tetracycline. Cells were incubated over 30 min with 25 μM TC, TLC, TCDC, TUDC, or solvent alone (positive control), respectively. After intensive washing, NBD-TC transport, myr-preS1-AX568 peptide binding, and anti-HA immunofluorescence under nonpermeabilized conditions were analyzed. NTCP transport was verified with 5 μM NBD-TC for 30 min at 37 C. Myr-preS1 peptide binding was analyzed with 50 nM of the myr-preS1-AX568 peptide for 20 min at 37 C. For detection of NTCP surface expression, nonpermeabilized cells were incubated with the anti-HA antibody for 1 h at 37 C. All assays were qualitatively assessed by fluorescence microscopy (green: NBD-TC and anti-HA fluorescence; red: myr-preS1-AX568 peptide fluorescence; blue: nuclei). Scale bars: 10 μm. B: for quantification of these effects, NTCP-HEK293 cells were induced with 1 μg/mL tetracycline (negative control; cells without tetracycline induction) and were preincubated with 25 μM of the indicated bile acids or solvent alone (positive control) for 2 h. After intensive washing with DMEM the following was analyzed: 5 μM NBD-TC uptake for 10 min at 37 C, 50 nM myr-preS1-AX568 peptide binding for 20 min at 37 C, and anti-HA immunofluorescence on nonpermeabilized cells. Cell viability was assessed under comparable experimental conditions with 50 μM of the indicated bile acids. Vehicle control represents cells incubated with solvent (DMSO) alone, and toxic control represents cells lysed with lysis buffer. RFU, relative fluorescent units. Data represent means ± SD of minimum triplicate determinations of representative experiments. **Significantly different from positive control, P < 0.05 (one-way ANOVA with Dunnett’s multiple comparison post hoc test).** DMEM, Dulbecco’s modified Eagle medium; GC, glycocholic acid; GUDC, glycursoxycholic acid; HA, HA tag; HEK293, human embryonic kidney cells; LC, lithocholic acid; NBD-TC, 4-nitrobenz-2-oxa-1,3-diazole taurocholic acid; neg.ctrl., negative control; NTCP, Na+/taurocholate cotransporting polypeptide; pos. ctrl., positive control; TC, taurocholic acid; TCDC, taurochenooxycholic acid; TLC, tauroxycholic acid; TUDC, taoursoxycholic acid.
NTCP INACTIVATION BY TAUROLITHOCHOLIC ACID

indicated in Fig. 3B, the inhibitory effect of TLC preincubation on the transport activity of NTCP remained relatively constant at its low level, even in the absence of TLC over 8 h post incubation. This indicates that TLC preincubation induced a long-lasting inhibitory effect on the TC transport activity of NTCP. Interestingly, the NTCP surface expression remained constant, even for very long periods of preincubation (up to 6 h) and postincubation (up to 8 h) (Fig. 3, A and B, bottom). Finally, parallel to the transport experiments with NBD-TC and [3H]TC after TLC preincubation, intracellularly accumulated [3H]TLC was quantified. Remarkably, [3H]TLC started to accumulate in the NTCP-HEK293 cells already at 10 s of incubation with 25 μM [3H]TLC. This accumulation then increased in a sigmoidal manner reaching a peak concentration at 60 min. These experiments clearly demonstrate that the inhibitory effect of TLC on the [3H]TC transport function of NTCP increased in the same manner as [3H]TLC accumulated inside the cells and, therefore, most likely occurred by trans-inhibition.

**TLC-Induced Inhibition of HDV Infection**

Next, it was analyzed if the inhibitory effect of TLC also affects the susceptibility of NTCP-HepG2 cells for in vitro HDV infection. Because HDV and HBV share identical surface proteins, HDV infection can be regarded as a surrogate of HBV infection. As in vitro infection experiments require incubation of the NTCP-HepG2 cells with HDV virus particles over several hours, it must be noted that only long-term effects can be monitored under these experimental conditions. NTCP-HepG2 cells were incubated with increasing concentrations of TLC or TC over 2 h, followed by intensive washing and incubation with bile acid-free HDV infection medium for 6 h (Fig. 4E). TLC significantly inhibited HDV infection in a concentration-dependent manner. With 25 μM TLC preincubation, the number of HDV-infected cells per well was reduced to ~50%. These data go in line with a strong inhibition of NBD-TC uptake that was also significantly inhibited over 6 h postincubation with 25 μM TLC (Fig. 3A). After 2-h preincubation with 250 μM TLC, the HDV infection rate even dropped to nearly zero infected cells per well. In contrast, 2-h preincubation with TC at 2.5 μM, 25 μM, and 250 μM concentrations reduced the HDV infection rate only slightly, but significantly, compared with the control (without bile acid preincubation). At the highest concentration of 250 μM TC, the infection rate still remained at 50% of the control (Fig. 4E). Of note, NTCP surface expression was mostly maintained at all bile acid preincubation conditions, with TC and TLC at least above 60% of the positive control in nonpermeabilized HepG2 cells expressing the HA-NTCP-FLAG protein (Fig. 4B, top). Additionally, cell surface biotinylated NTCP-HepG2 cells did not show any significant decrease in plasma membrane expression of NTCP after

**Figure 2.** Effect of TLC on NTCP’s transporter and receptor function. The effect of TLC on the transporter and receptor function of NTCP was analyzed in NTCP-HEK293 cells. NTCP expression was induced by tetracycline (except for the negative control) for 72 h. A–E: cells were preincubated with 25 μM TLC, 25 μM GC or solvent alone (positive control) solved in sodium-containing (black columns) or sodium-free (white columns) transport buffer over 2 h at 37°C. Then, cells were rinsed three times with sodium-free buffer. For uptake experiments, cells were incubated at 37°C for 10 min with 10 μM [3H]TC (A), 10 μM [3H]TLC (B), or 10 μM [3H]DHEAS (C), respectively, solved in sodium-containing transport buffer. D: binding of 5 nM [3H]myr-preS1 peptide was analyzed for 10 min at 37°C in DMEM after preincubation with the respective bile acid at 25 μM also solved in DMEM. To determine transport and myr-preS1 binding rates, cell-associated radioactivity was assessed using liquid scintillation counting. E: following 2-h preincubation with 25 μM bile acids, MTS cytotoxicity assay was performed. Data represent means ± SD of two independent experiments, each with quadruplicate determinations (n = 8). *Significantly different from positive control with P < 0.001 (two-way ANOVA with Dunnett’s multiple comparison post hoc test). DHEAS, dehydroepiandrosterone sulfate; DMEM, Dulbecco’s modified Eagle medium; GC, glycocorticoid; HEK293, human embryonic kidney cells; MTS, MTS tetrazolium; neg. ctrl., negative control; NTCP, Na+ taurocholate cotransporting polypeptide; pos. ctrl., positive control; TC, taurocholic acid; TLC, taurolithocholic acid.
preincubation with TLC or TC (Fig. 4B, bottom). Therefore, the decrease in HDV infection rates cannot be explained by a quantitative loss of NTCP plasma membrane expression. For comparison, 2.5 μM, 10 μM, 25 μM TLC or TC were used for cis-inhibition of HDV infection (Fig. 5C). In these experiments, the medium contained HDV virus particles and TLC/TC during the 6-h in vitro infection period. As expected, both bile acids had comparable potent inhibitory effects on the HDV in vitro infection rates, with only 8% infected cells per well in the presence of 25 μM TC, and hardly any infected cells per well in the presence of 250 μM TLC, compared with the control (without bile acid coincubation). Both bile acids fully maintained cell viability (measured by LDH cytotoxicity assay) after 6 h of bile acid coincubation and at 4 days postincubation even at the highest concentrations, so that toxic effects on the cells can be excluded (Fig. 5, A and B). This was basically confirmed by additional MTS cytotoxicity tests on NTCP-HepG2 cells that showed full cell viability after 2-h incubation with up to 250 μM TLC (Fig. 4C). These data clearly indicate that TC only had strong inhibitory effects on HDV infection during coincubation (cis-inhibition), whereas TLC showed comparable inhibitory effects after preincubation (trans-inhibition) and during coincubation (cis-inhibition).

To analyze if parts of the trans-inhibitory effect may be induced by bile acid reflux from the cells during the HDV infection, bile acid concentrations were additionally determined in the supernatant at the end of the HDV infection period. Only trace amounts of bile acids refluxed from the cells after preloading with 25 μM and 250 μM of TC or TLC in the preincubation medium (Fig. 4D vs. Fig. 4A). These were determined to 7 μM for TLC and 4 μM for TC after preincubation with 250 μM of the respective bile acid. Of note, both concentrations were not sufficient to cis-inhibit in vitro HDV infection (see Fig. 5C) and, therefore, bile acid reflux from the cells is not a relevant factor for interpretation of the in vitro HDV infection experiments after bile acid preincubation.

**TLC Trans-Inhibition Does Not Change the Cis-Binding Kinetics of NTCP**

To investigate if the trans-inhibitory effect of TLC only affects the transport rate and the myr-preS1 peptide binding capacity of NTCP, or if cis-binding kinetics are additionally affected, transport experiments with [3H]TC were performed...
**Figure 4.** Trans-inhibitory effect of TLC preincubation on in vitro HDV infection. NTCP-HepG2 cells were seeded and incubated with 2 μg/mL doxycycline to induce NTCP expression. A: after 3 days, cells were preincubated for 2 h at 37°C with 25 μM and 250 μM of [3H]TC or [3H]TLC, followed by intensive washing. B: at 6-h postincubation with DMEM (without any bile acid), the amount of radiolabeled bile acids was measured in the supernatant by liquid scintillation counting. B, top: HepG2 cells transiently transfected with the HA-NTCP-FLAG construct were incubated for 2 h with 25 μM and 250 μM of TC or TLC, followed by anti-HA immunofluorescence detection under nonpermeabilized conditions. Samples were analyzed by the LAS-X imaging software, and cell-based fluorescence of 60 individual cells was determined by defined ROI. Data present means ± SD background-subtracted relative fluorescence of two combined experiments (n=120). B, bottom: NTCP-HepG2 cells were incubated for 2 h with 25 μM and 250 μM of TC or TLC, or with solvent alone (positive control) and were subjected to surface biotinylation. The used biotinylation agent EZ-Link NHS-PEG12-Biotin is highly hydrophilic due to its polyethyleneglycol12 spacer arm and allows no membrane penetration. Data represent means ± SD of combined data from four independent experiments. *Significantly lower values compared with positive control with P < 0.001 (one-way ANOVA with Dunnett’s multiple comparison post hoc test). In addition, a representative Western blot is depicted. C: cell viability was assessed under comparable experimental conditions with 25 μM and 250 μM of TC or TLC, or with solvent alone (positive control). Data represent means ± SD of three independent experiments each with triplicate determinations (n=9). E: NTCP-HepG2 cells were preincubated with the indicated concentrations of TC or TLC at 37°C, followed by intensive washing. Then, cells were incubated with bile acid-free HDV infection medium, containing 700 HDV genome equivalents/cell for 6 h at 37°C. Medium was changed every 3–4 days. At days 9–11 post infection, cells were fixed and immunostained against the expression of HDV antigen (HDAg), as a marker of HDV infection. The numbers of infected cells per well were counted using fluorescence microscopy. Data represent means ± SD of three independent experiments each with triplicate determinations (n=9). *Significantly lower infection rates compared to positive control with P < 0.001 (two-way ANOVA with Dunnett’s multiple comparison post hoc test). DMEM, Dulbecco’s modified Eagle’s medium; HDV, hepatitis D virus; HepG2, HepG2 hepatoma cells; NTCP, Na+/taurocholate cotransporting polypeptide; ROI, region of interest; TC, taurocholic acid; TLC, taurolithocholic acid.
with and without TLC preincubation in the presence of increasing concentrations of different cis-acting ligands. Ursodeoxycholic acid (UDC) was used as a prototypic substrate of NTCP, irbesartan as a potent inhibitor of NTCP, and myr-preS1 as a surrogate parameter for HBV/HDV binding. As expected, TLC preincubation decreased the binding. As expected, TLC preincubation decreased the binding. As expected, TLC preincubation decreased the binding. As expected, TLC preincubation decreased the binding.

**DISCUSSION**

Under physiological conditions, bile acids undergo an efficient enterohepatic circulation. This process consists of their continuous hepatocellular canalicular efflux, intestinal reabsorption, and hepatic reuptake. The latter process is mediated by NTCP and members of the organic anion transporting polypeptide (OATP, gene symbol SLCO) family (7). During cholestasis, this circulation is disrupted and bile acids start to accumulate in the liver. Via the farnesoid X receptor (FXR, gene symbol NR1H4) and some other nuclear receptors, this intrahepatic bile acid increase leads to an adaptive transcriptional regulation of the hepatic bile acid transporters to prevent hepatocytes from bile acid-induced cellular toxicity (36). Consequently, NTCP is downregulated under cholestasis to reduce the hepatic bile acid uptake, and alternative basolateral efflux transporters such as OSTα/β, MRP3 (gene symbol ABCC3), or MRP4 (gene symbol ABCC4) are induced to facilitate the transport of bile acids from cholestasis hepatocytes back into the blood (7, 25, 37).

In addition, bile acid uptake via NTCP undergoes dynamic regulation at the protein level (38). In rat hepatocytes as well as in Huh7 hepatoma cells stably expressing rat Ntcp or human NTCP, the plasma membrane expression of the Ntcp/NTCP protein as well as its bile acid transport rates are stimulated by cAMP (24, 39–43). In contrast, bile acids such as TLC induced retrieval of rat Ntcp from the plasma membrane of cholestatic hepatocytes, this bile acid, overall, produces acute cholestasis by preventing biliary organic anion and bile acid secretion (46, 47). For rat Ntcp, this retrieval process, initiated by cholesstatic bile acids, is quite well understood (38) and involves clathrin-dependent endocytosis via the dileucine motif L222/L223 (48). In contrast, short-term regulation of human NTCP has only been
sparsely analyzed so far. Interestingly, human NTCP transport rates can also be inhibited by preincubation with TLC. However, TLC did not decrease the plasma membrane expression of human NTCP in HuH cells as in the rat (24). A previous study by Schonhoff et al. (24) analyzed more closely whether this TLC-induced transport inhibition of human NTCP may involve phosphoinositide-3-kinase (PI3K)-dependent activation of protein kinase PKCɛ. However, as they could not find a role of the PI3K/PKB pathway, the cellular mechanism that TLC uses to inhibit the transport function of human NTCP is still unclear.

Based on the data of the present study, we suppose that TLC-induced transport inhibition of NTCP involves TLC binding to NTCP from the cytoplasmic side and, thus, can be classified as a trans-inhibition mode that was already discussed in a previous publication by Schonhoff et al. (24). In addition to the bile acid transport function of NTCP, its HBV/HDV virus receptor function was analyzed as well, to figure out whether trans-inhibition of NTCP could contribute to a better understanding of NTCP-mediated HBV/HDV entry and its pharmacological inhibition. We confirmed that preincubation with particular bile acids significantly inhibited the transport function of NTCP. TLC and LC were equally potent in this regard, whereas TCDC showed only partial inhibition (Fig. 1). In contrast, other bile acids such as TC, GC, or TUDC showed no effect on the transport function of NTCP. This clearly demonstrates that this is a compound-specific effect and does not generally occur in the presence of bile acids. Based on the experimental setup used in the present study, it can be excluded that this inhibitory effect only occurs by competitive cis-inhibition at the outward-open bile acid-binding site of NTCP. Remarkably, apart from the transport function, the HBV/HDV receptor binding function of NTCP (measured by myr-preS1 peptide binding) was also significantly inhibited by TLC, LC, or TCDC preincubation. Of note, under these experimental conditions and in agreement with the previous study by Schonhoff et al. (24), NTCP was still detected at the plasma membrane (Fig. 1 and Fig. 4B). It, therefore, can be excluded that a retrieval of NTCP from the plasma membrane, which was shown for rat Ntcp under comparable experimental conditions (see earlier text in DISCUSSION), is responsible for this drop of NTCP’s transporter and receptor function. An alternative explanation for the long-lasting inhibitory effect of TLC could be that the intracellular accumulation of this bile acid would induce cell stress and so cells would require longer time to fully recover their transport function. However, as the MTS cytotoxicity assays showed full cell viability after 2-h preincubation with TLC (Fig. 2E) and NTCP-HEK293 cells still showed full transport activity at least for TLC (Fig. 2B), this possibility seems unlikely.

The inhibitory effect of TLC on the NTCP transporter function occurred within seconds and reached its maximum at 20 min. This maximum could not be further increased.

**Figure 6.** Cis-binding kinetics of NTCP at TLC trans-inhibition. NTCP-HEK293 cells were incubated with tetracycline to induce NTCP expression and were used for bile acid transport experiments with 5 μM [3H]TC over 10 min at 37°C. Cells without tetracycline treatment were used as negative control. Measurements were performed with solvent alone (positive control) and with increasing concentrations of ursodeoxycholic acid (UDC), irbesartan, and myr-preS1 peptide as cis-acting inhibitors of TC uptake in two experimental setups. For the first setup, cells were preincubated with 25 μM TLC over 1 h at 37°C in DMEM, then washed three times with DMEM before the [3H]TC uptake experiments were started (results shown in gray). For the second setup, cells were preincubated with pure DMEM over 1 h at 37°C before the same inhibition experiment was performed (results shown in black). The mean of the negative control was subtracted to calculate net [3H]TC transport rates expressed as dpm (A) or as % of control (B) at the y-axis. Half maximal inhibitory concentrations (IC50) were calculated from the data expressed as % of control by nonlinear regression analysis using the equation log(inhibitor) vs. response (GraphPad Prism 6). Absolute transport rates are expressed as dpm. Data represent means ± SD of two independent experiments, each with quadruplicate determinations (n = 8). *Significantly lower transport rates with P < 0.01 (two-way ANOVA with Sidak’s multiple comparisons post hoc test). DMEM, Dulbecco’s modified Eagle’s medium; HEK293, human embryonic kidney cells; NTCP, Na+/taurocholate cotransporting polypeptide; TC, taurocholic acid; TLC, taurolithocholic acid.
after prolonged preincubation up to 6 h (Fig. 3, A and C). Interestingly, TLC preincubation did not inhibit all functions of NTCP. Although the myr-preS1 binding competence as well as the transport activity for the NTCP substrates TC and DHEAS were significantly reduced, this was not the case for TLC itself (Fig. 2B). This indicates that the NTCP protein is not entirely inactivated, for example, by covalent protein modifications. NTCP is instead selectively inhibited but still active in transporting TLC as a substrate. We hypothesized that TLC does not share the typical binding site and transport route of the bile acid and steroid substrates of NTCP. Thus, TLC transport is still maintained even when TLC preincubation inhibits transport of TC and DHEAS. Another interesting observation was that the inhibitory effect of TLC on the transporter and receptor function of NTCP was long-lasting (Fig. 3B). Even under very long incubation of the NTCP-HEK293 cells in the absence of TLC for up to 8 h, the same inhibitory effect was observed as directly after preincubation and washing. Therefore, this long-lasting effect might indicate that TLC trans-binding to NTCP occurs with high affinity.

Another question was if reflux of TLC after the preincubation phase may be responsible for the inhibitory effect and so would only mimic a cis-inhibition. However, in respective control experiments, only trace amounts of TLC refluxed from the cells after washout that would not be sufficient to explain the measured effects by cis-inhibition alone (Fig. 4, A and D). This was most pronounced in the HDV infection experiments, where TLC concentrations below 10 μM had only minimal effects in cis-inhibition experiments (Fig. 5C). These concentrations would be achieved at most in the extracellular medium after preincubation with 250 μM TLC. In contrast, preincubation with 250 μM TLC completely abolished HDV infection in the trans-inhibition approach (Fig. 4E). These findings go in line with the observation that TLC only gains its trans-inhibitory potential when the preincubation is performed in sodium-containing buffer, which is the prerequisite for NTCP-mediated TLC uptake and accumulation in the preincubation phase (Fig. 2). In contrast, under sodium-free preincubation conditions, TLC largely lost its inhibitory potential, because its uptake via NTCP is abolished in the absence of sodium.

Finally, the question was addressed if the trans-inhibition of NTCP after TLC preincubation only affects the transport rates of the extracellularly provided substrate or if, in addition, the binding kinetics of an extracellular ligand were altered. Bile acids typically show competitive inhibition at the same extracellular binding site after coincubation, that is, when both bile acids are provided from the extracellular site of the transporter (7, 24, 45). However, in the experimental setup of the present study, TLC was first accumulated in the cells and washed out from the extracellular compartment, so that TLC is supposed to bind to an intracellularly accessible domain of NTCP (trans), whereas the transporter substrates and the myr-preS1 peptide were provided from the extracellular site (cis). In addition to the extracellularly provided transporter substrate [3H]TC, different compounds were coincubated together with [3H]TC to verify if these still interfere competitively with the transporter function of NTCP, even under trans-inhibition with TLC. As shown in Fig. 6, UDC, irbesartan, and myr-preS1 all inhibited the [3H] TC transport rate with comparable inhibition kinetics, irrespective of TLC preincubation. Nevertheless, the absolute transport rate of [3H]TC was reduced significantly by TLC preincubation. These data clearly indicate that TLC represents a long-lasting trans-acting noncompetitive inhibitor of the transporter and HBV/HDV receptor function of NTCP. Furthermore, these data demonstrate that NTCP is still functionally active, so inactivation of NTCP by postranslational modifications or abrogation from the plasma membrane can be excluded.

**Trans**-inhibition of membrane transporters by their substrates has previously been reported for other carriers, including ion transporters (49), ATP-binding cassette (ABC) transporters like human TAPL (50), ModBC from Methanosarcina acetivorans (51), TmrAB from Thermus thermophilus (52), and the ABC importer MetNI from Escherichia coli (53). Furthermore, **trans**-inhibition has been described for various amino acid transporters (54–57). Also, bile acid transporters from the OATP family have been reported to be **trans**-inhibited, for example, by cyclosporine A (CsA) (58). Similar to the present study, CsA acted as cis-inhibitor during coincubation with the substrate as well as **trans**-inhibitor after preincubation and washing before the transport experiments. In the case of OATP1B1 and OATP1B3, CsA preincubation induced a concentration-dependent and long-lasting inhibitory effect on the transport function over at least 16 h (58, 59). From the group of ABC transporters, Stieger et al. showed **trans**-inhibition of rat Bsep by the cholestatic estrogen metabolite estradiol-17β-glucuronide (E217G) (60, 61). In addition, Akita et al. (62) observed that sulfated bile acids show **trans**-inhibition of rat Bsep. Most **trans**-inhibitors described in these publications showed similar inhibition patterns, including time- and dose dependency of the inhibitory effect, rapid and long-lasting inhibition of the respective transporter, as well as high-affinity binding of the inhibitor to the **trans**-inhibition site of the transporter. In the present study, all these characteristics were also observed for the NTCP **trans**-inhibitor TLC, which showed a time- and dose-dependent inhibition of the bile acid transport. Furthermore, **trans**-inhibition was maintained over at least 8 h after removing of TLC from the incubation medium and, therefore, was long-lasting (Fig. 3B).

Blocking of HBV/HDV infection of hepatocytes by preventing virus binding to NTCP or blocking of endocytosis of the virus/NTCP receptor complex is a promising novel treatment strategy for acute and chronic HBV/HDV infections. This might be achieved by downregulation of NTCP expression in the hepatocytes, for example, by tumor necrosis factor-α or interleukin-6 (63, 64). In addition, retrieval of NTCP from the plasma membrane by induction of clathrin- or caveolin-dependent endocytosis of NTCP might be a option (65, 66). However, most approaches focus on the cis-inhibition of the virus binding to NTCP, which can be achieved by peptide-based drugs such as Myrcludex B, mimicking the myr-preS1 peptide, or by small molecules that address the extracellular myr-preS1 and/or bile acids-binding site of NTCP (64, 67). Myrcludex B provided proof-of-concept that NTCP-directed inhibition can effectively suppress HBV and HDV infection and was very recently approved as the first drug treatment of HDV-infected patients in Europe and Russia
(68). Based on the data of the present study, we suggest a novel potential target site of trans-acting NTCP inhibitors that can block virus binding to NTCP and, thus, may prevent HBV/HDV infection. Although TLC as a cholestatic bile acid is not an appropriate candidate, other compounds could mimic this long-lasting, trans-inhibitory effect of TLC and could be even more effective then short-acting cis-inhibitors of NTCP. To define this potential trans-located binding site of NTCP more closely, 3-D structural information of the human NTCP protein would be needed and would provide the possibility to address this target site by docking strategies.

In conclusion, we found that even after short pulse preincubation, TLC had a potent and long-lasting inhibitory effect on the transporter function of NTCP, while NTCP was still present at the plasma membrane. Furthermore, binding of the HBV/HDV myr-preS1 peptide and susceptibility for infection studies; K.A.L., M.K., S.F.M., and J.G. drafted manuscript; K.A.L., M.K., and S.F.M. prepared interpretations of experiments; K.A.L., M.K., and S.F.M. prepared figures; N.G., F.L. and D.G. provided materials and laboratories for infection studies; K.A.L., M.K., S.F.M., and J.G. provided final version of manuscript.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A.L., M.K., S.F.M., and J.G. conceived and designed research; K.A.L., M.K., S.F.M. performed experiments; K.A.L., M.K., S.F.M., and J.G. analyzed data; K.A.L., M.K., S.F.M., and J.G. interpreted results of experiments; K.A.L., M.K., and S.F.M. prepared figures; N.G., F.L. and D.G. provided materials and laboratories for infection studies; K.A.L., M.K., S.F.M., and J.G. drafted manuscript; K.A.L., M.K., S.F.M., N.G., F.L., D.G., and J.G. edited and revised manuscript; K.A.L., M.K., S.F.M., N.G., F.L., D.G., and J.G. approved final version of manuscript.

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