



Kinetics of the bile acid transporter and hepatitis B virus receptor Na⁺/taurocholate cotransporting polypeptide (NTCP) in hepatocytes

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Background & Aims: The human liver bile acid transporter Na⁺/taurocholate cotransporting polypeptide (NTCP) has recently been identified as liver-specific receptor for infection of hepatitis B virus (HBV), which attaches via the myristoylated preS1 (myr-preS1) peptide domain of its large surface protein to NTCP. Since binding of the myr-preS1 peptide to NTCP is an initiating step of HBV infection, we investigated if this process interferes with the physiological bile acid transport function of NTCP.

Methods: HBV infection, myr-preS1 peptide binding, and bile acid transport assays were performed with primary *Tupaia belangeri* (PTH) and human (PHH) hepatocytes as well as NTCP-transfected human hepatoma HepG2 cells allowing regulated

NTCP expression, in the presence of various bile acids, ezetimibe, and myr-preS1 peptides.

Results: The myr-preS1 peptide of HBV inhibited bile acid transport in PTH and PHH as well as in NTCP-expressing HEK293 and HepG2 cells. Inversely, HBV infection of PTH, PHH, and NTCP-transfected HepG2 cells was inhibited in a concentration-dependent manner by taurine and glycine conjugates of cholic acid and ursodeoxycholic acid as well as by ezetimibe. In NTCP-HepG2 cells and PTH, NTCP expression, NTCP transport function, myr-preS1 peptide binding, and HBV infection followed comparable kinetics.

Conclusions: Myr-preS1 virus binding to NTCP, necessary for productive HBV infection, interferes with the physiological bile acid transport function of NTCP. Therefore, HBV infection via NTCP may be lockable by NTCP substrates and NTCP-inhibiting drugs. This opens a completely new way for an efficient management of HBV infection by the use of NTCP-directed drugs.

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Keywords: Hepatitis B virus; Receptor; Infection; NTCP; ASBT; SOAT; Therapy; Bile acids; Ezetimibe; Transport.

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Abbreviations: HBV, hepatitis B virus; WMHBV, woolly monkey hepatitis B virus; WHV, woodchuck hepatitis B virus; myr-preS1, N-terminal myristoylated and C-terminal strep-tagged preS1-lipoprotein from N-terminal amino acids 2–48 of the large surface protein of the respective HBV; myr-preS1-AX594, N-terminal myristoylated and C-terminal fluorescently labelled with dye Alexa594 from N-terminal amino acids 2–48 of the large surface protein of HBV; NTCP, Na⁺/taurocholate cotransporting polypeptide; SLC, solute carrier; ASBT, apical sodium-dependent bile acid transporter; SOAT, sodium-dependent organic anion transporter; DHEAS, dehydroepiandrosterone sulfate; TMD, transmembrane domain; TBHBV, tent-making bat HBV; PTH, primary *Tupaia belangeri* hepatocytes; PHH, primary human hepatocytes; HDV, hepatitis D virus; tNtcp, *Tupaia belangeri* Ntcp; HEK293, human embryonic kidney 293 cells; HepG2, human hepatocellular liver carcinoma cell line; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; TC, taurocholic acid; HGM, hepatocyte growth medium; HbeAg, hepatitis B e antigen; HbsAg, Hepatitis B surface antigen; GE, genome equivalents; ELISA, enzyme linked immunosorbent assay; PFA, paraformaldehyde; PBS, phosphate buffered saline; RT, room temperature; HbcAg, Hepatitis B core antigen; BSA, bovine serum albumin; PCC, Pearson's correlation coefficient; GC, glycocholic acid; UDC, ursodeoxycholic acid; TUDC, tauroursodeoxycholic acid; GUDC, glyoursodeoxycholic acid; DHC, dehydrocholic acid; EZ, ezetimibe; TLC, tauroithocholic acid; OATP, organic anion transporting polypeptide; PBC, primary biliary cirrhosis; pp, post preparation; DOX, Doxycycline.

Introduction

More than 2 billion people have been infected with hepatitis B virus (HBV), giving rise to 240 million chronic HBV carriers and ~620,000 HBV-associated deaths annually [1]. While there is an effective prophylactic vaccine, HBV therapy with interferon is often ineffective and inhibitors of HBV reverse transcription usually have to be given life-long. To maintain the high efficacy of the HBV vaccine and to improve the therapy options of hepatitis B, a better understanding of the early steps of infection would be useful.

HBV is a member of the still growing family of *hepadnaviridae*. Mammalian hepadnaviruses form the genus *orthohepadnavirus* also include woolly monkey hepatitis B virus (WMHBV), woodchuck hepatitis B virus (WHV), and three newly discovered bat hepatitis B virus species with a zoonotic potential [2]. The myristoylated preS1-lipoprotein comprising the N-terminal amino acids 2–48 (myr-preS1) of the large HBV surface protein is essential for the species- and differentiation-dependent infection of



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human hepatocytes, but the identity of the high-affinity HBV receptor(s) has been elusive for long [3]. Recently, the Na⁺/taurocholate cotransporting polypeptide (NTCP in human, Ntcp in other species) could be identified as the functional species-specific HBV receptor [4]. NTCP/Ntcp belongs to the solute carrier family 10 (SLC10), also referred to as the “family of sodium-dependent bile acid transporters” which comprises seven members (*SLC10A1-SLC10A7*) [5]. NTCP (*SLC10A1*) is specifically expressed at the basolateral membrane of hepatocytes and is essentially involved in the reuptake of bile acids from the portal blood into hepatocytes for their resecretion into bile [6]. From the gut, bile acids are efficiently recovered by the apical sodium-dependent bile acid transporter ASBT (*SLC10A2*), which is highly expressed at the apical brush-border membrane of enterocytes in the terminal ileum [7]. A further transporter closely related to NTCP and ASBT is represented by the sodium-dependent organic anion transporter SOAT (*SLC10A6*), which is mainly expressed in reproductive organs and has a dominant transport function for sulfated steroids, such as dehydroepiandrosterone sulfate (DHEAS) [8]. NTCP, ASBT, and SOAT share similar gene structure, high sequence similarity of >60%, and a common nine transmembrane domain (TMD) topology with N_{exo}/C_{cyt} orientation of the N- and C-terminal domains based on recent crystal structure analysis of a bacterial ASBT homologue [7].

The initial attachment of HBV to hepatocytes occurs at the basolateral membrane and is mediated mainly by low-affinity binding between the antigenic loop of the small hepatitis B surface protein domain and heparan-sulfate proteoglycans [9,10]. Thereafter, high-affinity interaction between HBV myr-preS1 and NTCP is regarded as initial step for HBV uptake and infection. Amino acids 157-165 of NTCP located at the N-terminal end of TMD5 as well as amino acids 84-87 in the extracellular loop upstream of TMD3 were shown to be involved in this interaction [4,11–13]. The interaction between NTCP and myr-preS1 determines the host range and liver tropism of primate HBVs and amazingly also those of the related TBHBV species of the tent-making bats. Primary hepatocytes of the Asian treeshrew *Tupaia belangeri* (PTH) also support infection with the primate viruses HBV and WMHBV [14,15] and with hepatitis D virus (HDV) pseudotyped with surface proteins of TBHBV [2]. This susceptibility seems to be caused by a conserved domain of the human NTCP and *Tupaia belangeri* Ntcp (tNtcp) at the site of virus binding [4]. However, PTH are unable to support infection of rodent HBV, e.g. WHV [15].

The well-established HBV susceptible models of PTH and PHH as well as human NTCP-transfected HEK293 and HepG2 cell cultures allowed us to study if the physiological bile acid transport function of NTCP may interfere with myr-preS1 peptide binding to NTCP and HBV infection of NTCP/tNtcp expressing cells. Interestingly, we found both processes to be highly connected and thereby provide evidence that HBV infection via NTCP may be lockable by NTCP substrates and NTCP-inhibiting drugs.

Materials and methods

Transporter cDNA constructs, stably transfected cell lines, and transport assays

Transporter cDNA constructs were generated containing the full open reading frames of human NTCP, ASBT, and SOAT in different expression vectors (for more details see [Supplementary materials and methods](#)), allowing expression of the respective FLAG-tagged or GFP-tagged fusion proteins after transfection in human embryonic kidney HEK293 or HepG2 hepatoma cell lines. Flp-In T-REx-293 cells

(Invitrogen) were used to generate stably transfected tetracycline inducible NTCP-HEK293, ASBT-HEK293, and SOAT-HEK293 cell lines as described in detail in the [Supplementary materials and methods](#). ASBT/NTCP and SOAT/NTCP chimeric constructs were generated by replacing amino acids 91-94 and 163-171 in ASBT and SOAT by the corresponding amino acids 84-87 and 157-165 of NTCP using site-directed mutagenesis.

Human HepG2 hepatoma cells were cultivated in Dulbecco's modified Eagle medium (DMEM) (PAA, Cölbe, Germany) supplemented with 5% fetal calf serum (FCS) (PAN, Aidenbach, Germany) and penicillin/streptomycin (PAA) at 37 °C, 5% CO₂ and 95% humidity. Generation of the stably transfected NTCP-HepG2 cell line, which is variably inducible by doxycycline (DOX) treatment is described in detail in the [Supplementary materials and methods](#).

All stably transfected cell lines as well as PTH and PHH were used for transport assays with [³H]taurocholic acid ([³H]TC) or [³H]dehydroepiandrosterone sulfate ([³H]DHEAS) (Perkin Elmer, Rodgau, Germany) in Na⁺-containing or Na⁺-free transport buffers as described [8]. Transport inhibition experiments were started by incubation with the respective inhibitory compounds or myr-preS1 peptides dissolved in transport buffer for 30 min at 37 °C, before the transport assay was started by adding [³H]TC or [³H]DHEAS to the cells. Uptake was terminated after 10 min and the cell-associated radioactivity was determined by liquid scintillation counting.

Primary tupaia and human hepatocytes

Primary hepatocytes from *Tupaia belangeri* (PTH) were isolated by a modified two-step collagenase method and cultivated in modified hepatocyte growth medium (HGM) as described [13]. PTH were used for infection and transport experiments at day three after preparation unless otherwise noted. PHH were obtained from Primacyt Cell Culture Technology GmbH (Schwerin, Germany). More detailed specifications about the PHH are provided in the [Supplementary materials and methods](#).

Isolation and purification of HBV virions from patient serum

HBV particles for infection experiments were purified from plasma, donated by three different hepatitis B e antigen (HBeAg) positive chronic HBV carriers (for more details see [Supplementary materials and methods](#)) using rate zonal ultracentrifugation through a sucrose density gradient (15 to 60% [wt/wt]) for 15 h at 25,000 rpm as described [13]. Fractions containing high amounts of HBV DNA were quantified by real-time PCR.

HBV infection experiments

For HBV infection 24-well plates with 1 × 10⁶ NTCP-HepG2 cells, PTH, or PHH were pre-incubated for 30 min with myr-preS1 peptides or inhibitors/substrates of NTCP in HGM at 37 °C and were infected with 100 genome equivalents (GE) of HBV per hepatocyte. After 16 h, the inoculum was collected and cells were washed twice with HGM. Infected hepatocytes were cultured until day 11 post infection and supernatants were harvested every 2–3 days and analysed for secretion of newly synthesized Hepatitis B surface Antigen (HBsAg, PTH) or HBeAg (HepG2). For quantitative determination of HBsAg, a mouse monoclonal antibody mAb (C20/02) against a conformational epitope within the antigenic loop of HBsAg was used in an in house ELISA system as described [13]. HBeAg was determined using an automated microparticle enzyme immunoassay (Architect, Abbott, Wiesbaden, Germany). For immunostaining of newly synthesized viral proteins in PTH, cells were plated on glass cover slips. 11 days after HBV infection cells were washed and fixed with 3% paraformaldehyde (PFA) (Sigma-Aldrich, Taufkirchen, Germany) at 4 °C. After permeabilization with 0.2% Triton X-100 in PBS for 30 min at room temperature (RT) unspecific binding epitopes were blocked by an incubation with 10% FCS/DMEM for 1 h at RT. Expression of HBV core (HBc) particles were detected by immunostaining for 2 h at 37 °C using a 1:200 dilution of a polyclonal rabbit anti-HBcAg antiserum (Dako, Hamburg, Germany). After three times washing with PBS, cells were incubated with anti-rabbit Alexa Fluor 594 (Invitrogen) diluted 1:200 in 0.1% BSA/DMEM for 2 h at 37 °C. After extensive washing with PBS, nuclei were stained with DAPI, again washed, mounted on glass slides using Mowiol (Sigma-Aldrich) and visualized by confocal microscopy.

Binding and colocalization analysis of viral myr-preS1 peptides

N-terminal myristoylated preS1 (amino acids 2–48) lipopeptides (further referred to as myr-preS1 peptides) of HBV, WMHBV and WHV as well as a myr-preS1 AX594-labelled peptide (Biosynthesis, Lewisville, Texas, USA) were incubated

on carrier-expressing cells at the indicated concentrations below 10 °C to prevent internalization. For interaction studies, carrier-GFP transfected HepG2 cells were pre-incubated for 30 min in HGM with bile acids at 37 °C followed by incubation with 1 μM of the HBV myr-preS1 peptide for 30 min at <10 °C. When using the Alexa594-tagged myr-preS1 peptide, cells were washed several times with DMEM after incubation with myr-preS1-AX594. Binding was immediately detected by live cell confocal imaging. The myr-preS1-AX594 quantification and degree of colocalization was determined by using the LAS AF software (Leica, Wetzlar, Germany). The estimated Pearson's correlation coefficient (PCC) represents the degree of overlap between fluorescence signals obtained by the two channels. The values of the PCC range from 0 (completely separated structures) to +1 (complete colocalization).

Immunofluorescence of Ntcp in PHH and Ntcp-HepG2 cells

Primary human hepatocytes and Ntcp-HepG2 cells were cultivated on glass coverslips and fixed with 3% paraformaldehyde for 45 min at 4 °C at the indicated time points post plating. For the determination of Ntcp expression in Ntcp-HepG2 cells, a 1:5000 dilution of the mouse M2 mAb against the FLAG epitope (Sigma) were used. Ntcp expression in PHH was stained by using a 1:100 dilution of a rabbit polyclonal anti-human Ntcp-antibody (Prestige, Sigma Aldrich).

Primary antibody binding was detected by using 1:500 dilutions of Alexa488 anti-rabbit or anti-mouse secondary antibodies (Invitrogen).

Real-time PCR detection of Ntcp/tNtcp expression

Total RNA of PTH and DOX induced Ntcp-HepG2 cells was isolated using TriReagent (Sigma) according to the manufacturers recommendation. After DNase I digestion cDNA was synthesized using Superscript III (Invitrogen). Specific Tupaia Ntcp and beta-actin TaqMan probes covering exon-exon boundaries were selected and showed efficiencies of 99.7% and 107%, respectively. For human Ntcp detection commercially available Ntcp and GAPDH TaqMan probes were used. 50 ng cDNA per well were used for TaqMan analysis. Expression data were calculated by the 2^{-ΔΔCT} method.

WB detection of Ntcp expression in Ntcp-HepG2 cells

Proteins were isolated from Ntcp-HepG2 cells with RIPA buffer (Sigma), containing protease inhibitors. 50 μg protein were separated on polyacrylamide gel (8%) and transferred to nitrocellulose membrane. Ntcp detection was performed with mouse anti-FLAG M2 antibody (Sigma). The mouse anti-E-Cadherin antibody (Sigma) was used for control.

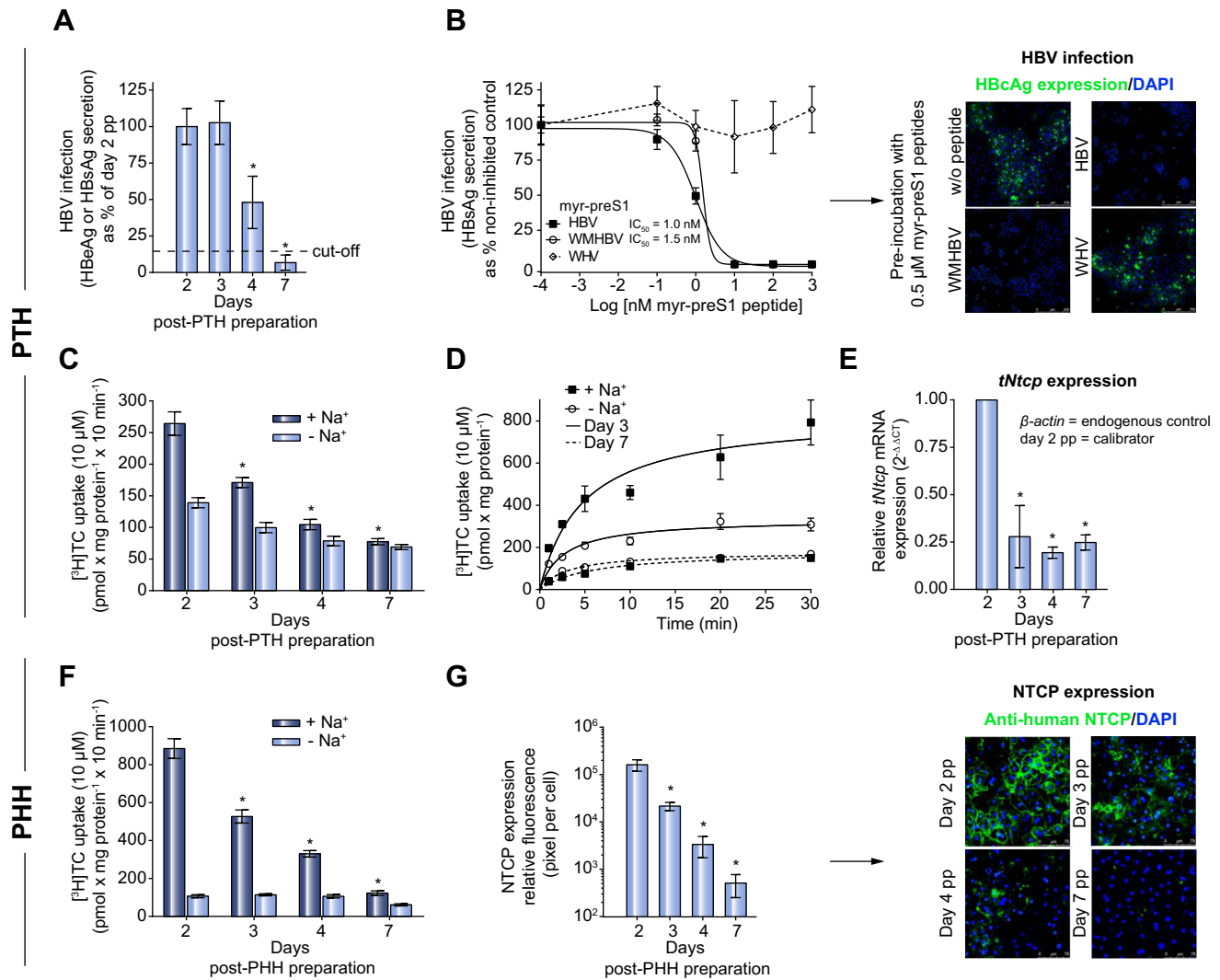


Fig. 1. HBV infection, Ntcp/tNtcp expression, and Na⁺-dependent [³H]TC uptake of PTH and PHH. PTH were infected with HBV at different days pp without (A) or (B) with the HBV, WMHBV, or WHV myr-preS1 peptides pre-incubated at increasing concentrations. Transport of [³H]TC in PTH (C) and PHH (F) at different days pp. (D) Time- and Na⁺-dependent [³H]TC uptake in PTH. (E) Time course of tNtcp mRNA and (G) Ntcp protein expression in PTH and PHH, respectively, at different days pp. *Significantly different from day 2 pp (1way ANOVA, p <0.05).

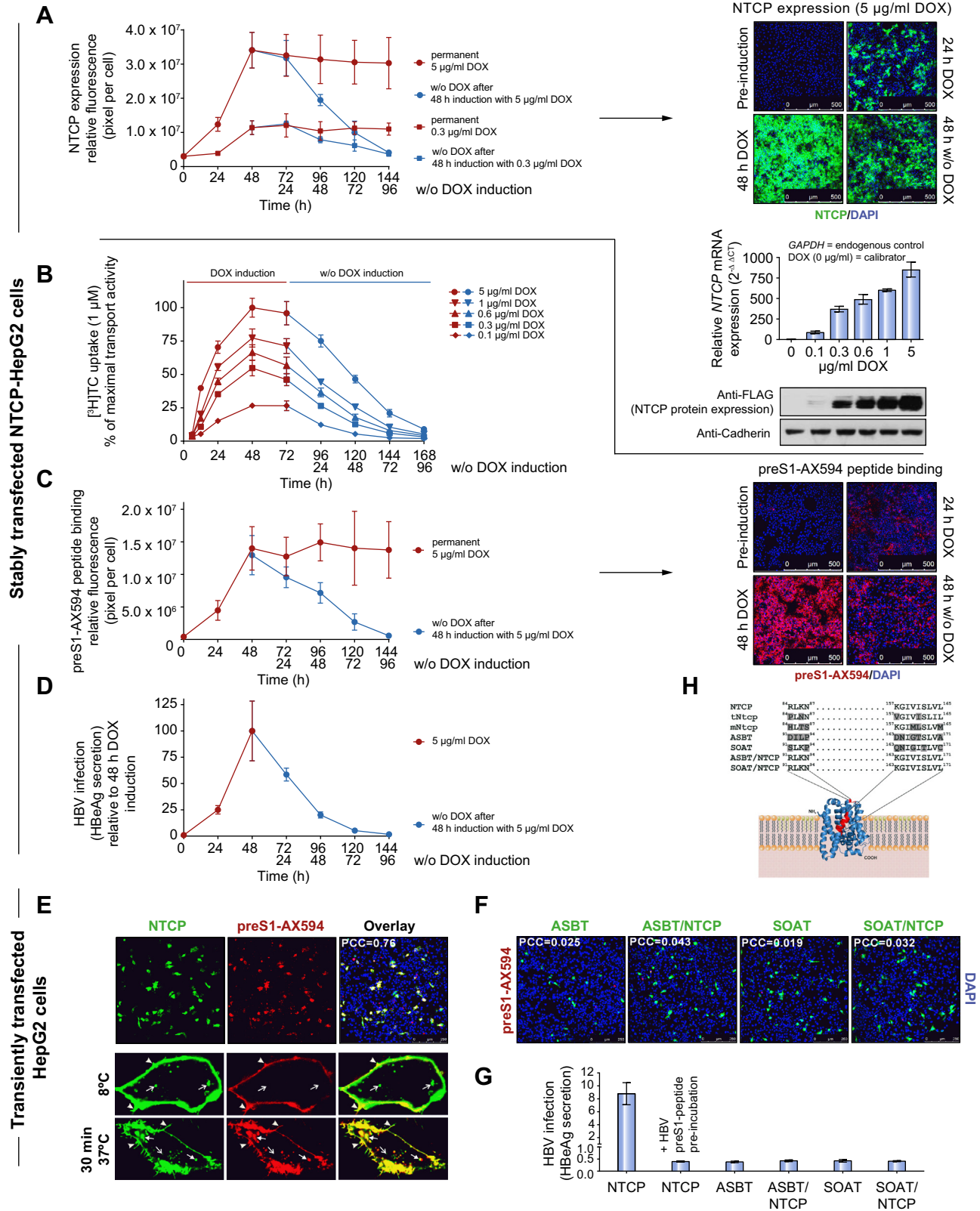


Fig. 2. NTCP expression, myr-preS1 peptide binding, HBV infection and ³H]TC transport in NTCP-HepG2 cells. (A) NTCP protein and mRNA expression, and (B) ³H]TC transport in NTCP-HepG2 cells could be variably induced by increasing DOX concentrations. (C) Time course of myr-preS1-AX594 peptide binding and (D) HBV infection of NTCP-HepG2 cells after induction with 5 µg/ml DOX. (E and F) Binding experiments with the HBV myr-preS1-AX594 peptide (red) and (G) HBV infection of transiently NTCP-, ASBT-, SOAT-, ASBT/NTCP-, and SOAT/NTCP-expressing HepG2 cells (green). (H) Localization and sequence alignment of the putative HBV binding domains in NTCP.

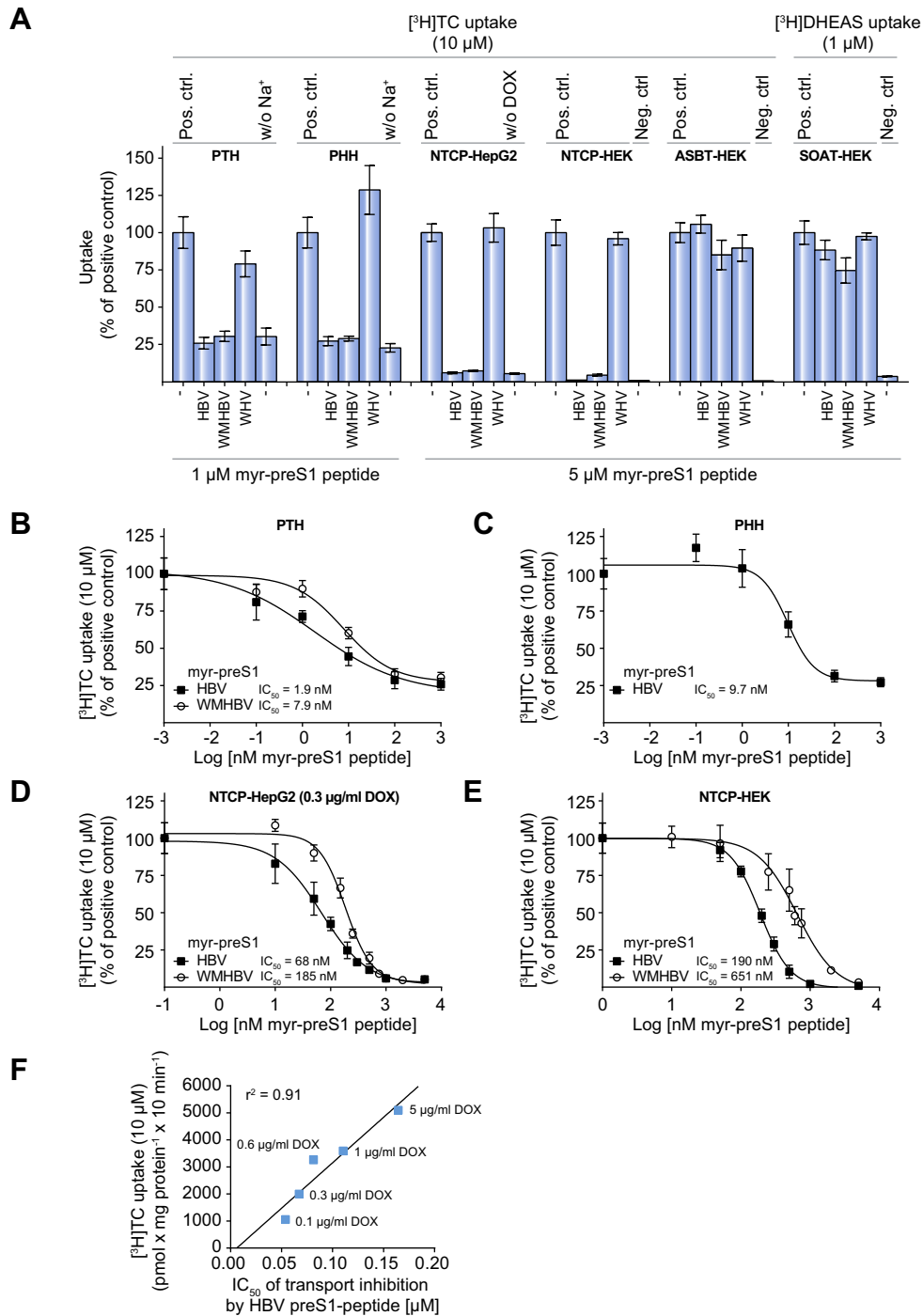


Fig. 3. HBV myr-preS1 peptides inhibit NTCP/tNtcp bile acid transport function. (A) PTH, PHH and stably transfected NTCP-, ASBT- or SOAT-HEK293 cells were pre-incubated with myr-preS1 peptides and uptake of $[^3\text{H}]\text{TC}$ or $[^3\text{H}]\text{DHEAS}$ was measured. Untransfected cells served as negative control. Inhibition of $[^3\text{H}]\text{TC}$ uptake in PTH (B), PHH (C), NTCP-HepG2 cells (D), or NTCP-HEK293 cells (E) by increasing concentrations of myr-preS1 peptides and calculated IC_{50} values. (F) Correlation between myr-preS1 IC_{50} values and the Na^+ -dependent uptake of $[^3\text{H}]\text{TC}$ via NTCP at increasing DOX concentrations. (This figure appears in colour on the web.)

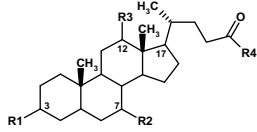
Results

Na⁺-dependent TC uptake of hepatocytes correlates with the NTCP/tNtcp expression and susceptibility for HBV infection

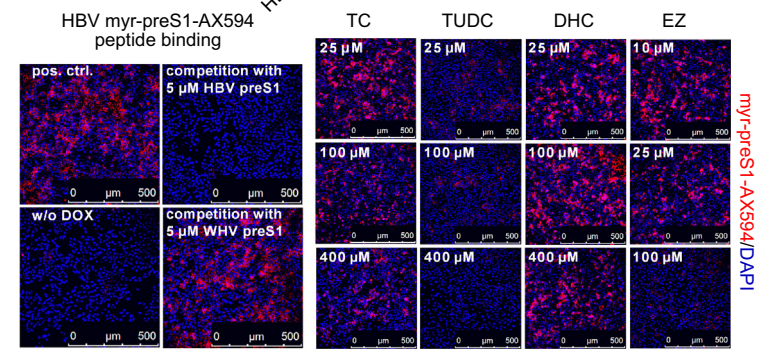
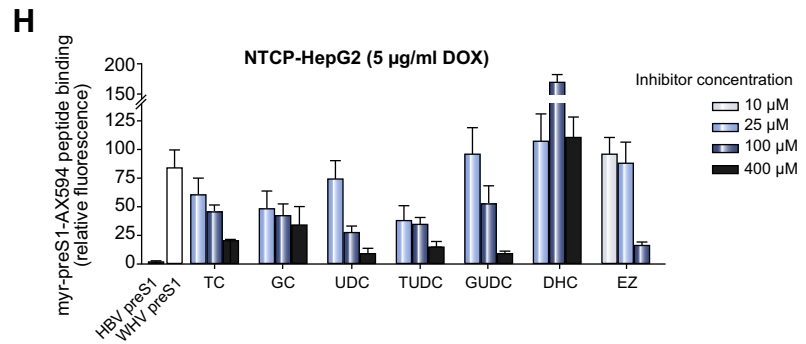
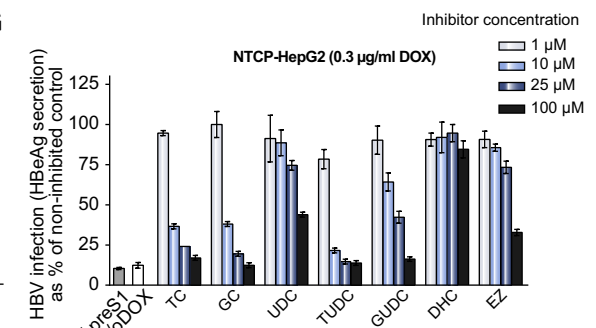
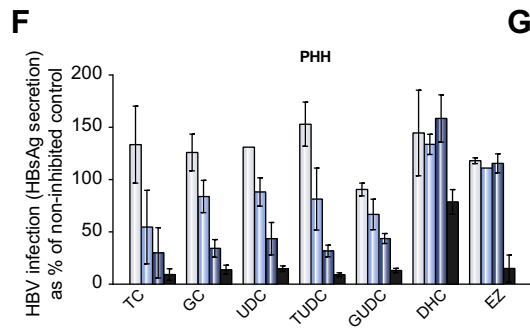
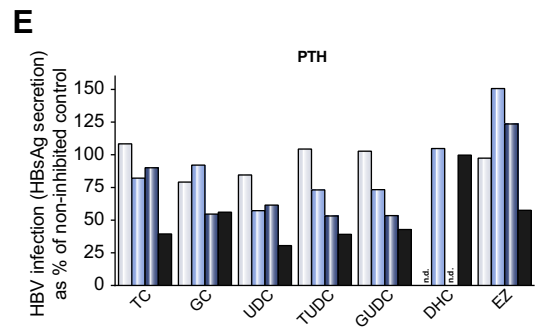
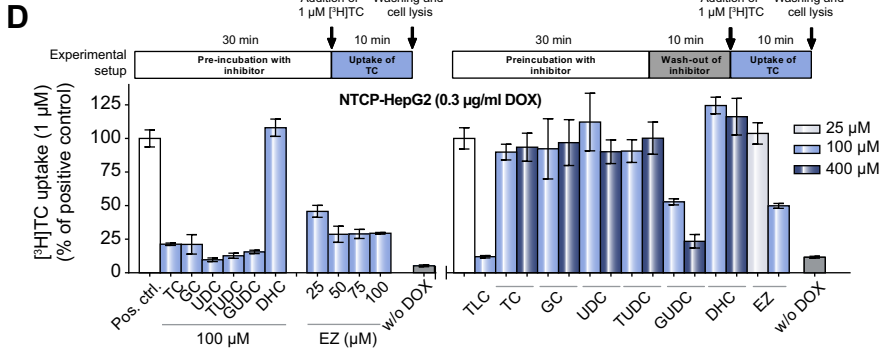
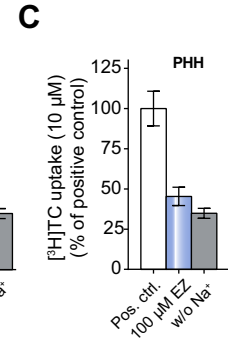
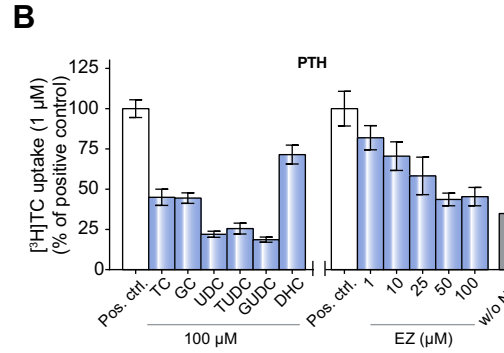
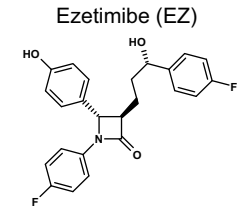
In PTH cells, TC uptake, HBV infection, and *tNtcp* mRNA expression were determined at different days post preparation (pp).

The susceptibility of PTH cells for HBV infection of purified viral inocula from different HBV donors significantly decreased from maximal susceptibility at day 2 after plating below the cut-off level for HBV infection at day 7 (Fig. 1A). HBV infection of PTH cells was a receptor-dependent process, because it could be efficiently inhibited by the myr-preS1 peptides of HBV and WMHBV with IC_{50} values of 1.0 nM and 1.5 nM, respectively, but not by a

A Basic structure of bile acids



Bile acid	R1	R2	R3	R4	
TC	Taurocholic acid	α-OH	α-OH	α-OH	Taurine
GC	Glycocholic acid	α-OH	α-OH	α-OH	Glycine
UDC	Ursodeoxycholic acid	α-OH	β-OH	H	OH
TUDC	Tauroursodeoxycholic acid	α-OH	β-OH	H	Taurine
GUUDC	Glyoursodeoxycholic acid	α-OH	β-OH	H	Glycine
TLC	Taurolithocholic acid	α-OH	H	H	Taurine
DHC	Dehydrocholic acid	O	O	O	OH



WHV myr-preS1 peptide (Fig. 1B). The Na⁺-dependent TC uptake in PTH cells showed a similar time-dependent decline as the HBV susceptibility and was nearly undetectable at day 7 pp (Fig. 1C). Overall, PTH cells showed Na⁺-dependent and Na⁺-independent transport of TC, which both significantly declined during cultivation (Fig. 1D). The *tNtcp* mRNA expression dropped already at day 3 pp to the level of day 7 pp (Fig. 1E). Similar to PTHs, Na⁺-dependent transport of TC in PHH cells significantly decreased within 7 days pp (Fig. 1F) and was in line with a decline of NTCP protein expression (Fig. 1G).

Establishment of a DOX inducible NTCP-HepG2 cell line and NTCP-specific HBV infection

By stable transfection we established a NTCP-HepG2 cell line in which NTCP expression was under control of a CMV-Tet-on expression system. By DOX treatment, NTCP expression could be induced in a concentration dependent manner (Fig. 2A and Supplementary Fig. 1A). In parallel with NTCP expression, Na⁺-dependent TC transport (Fig. 2B), myr-preS1 peptide binding (Fig. 2C and Supplementary Fig. 1B) and HBV infection (Fig. 2D) occurred in a time-dependent and DOX-dependent manner. Furthermore, we analysed, if the HBV myr-preS1 peptide not only interacts with NTCP, but also with its closely-related carriers ASBT and SOAT, or their functionally intact mutants ASBT/NTCP and SOAT/NTCP (see Supplementary Fig. 2), carrying putative HBV binding domains of NTCP [4,11,12] (Fig. 2H). NTCP-transfected HepG2 cells were labelled by the myr-preS1-AX594 peptide (Fig. 2E and F) and support HBV infection, shown by detection of newly produced HBeAg (Fig. 2G). At 37 °C a nearly complete co-localization of NTCP-GFP and myr-preS1-AX594 was detected at the plasma membrane (Fig. 2E, arrow heads) and in intracellular vesicles (Fig. 2E, open arrow, NTCP containing vesicle; closed arrow, NTCP and myr-preS1-AX594 containing vesicles), suggesting internalization of the peptide-NTCP complex, whereas at 8 °C only plasma membrane derived co-localization occurred (see also movie "Binding and uptake of HBV myr-preS1 peptide in NTCP expressing HepG2 cells" in Supplementary section). In contrast, no myr-preS1-AX594 peptide binding or HBV-infection of transiently SOAT-, ASBT-, SOAT/NTCP-, or ASBT/NTCP-GFP or -FLAG transfected HepG2, respectively, cells could be observed (Fig. 2F and G). The NTCP-directed HBV infection of HepG2 cells was completely abolished by the HBV myr-preS1 peptide (Fig. 2G).

Inhibition of NTCP-mediated TC transport by myr-preS1 peptides

Binding of the HBV myr-preS1 peptide to NTCP suggested that these peptides may interfere with the bile acid transport function of NTCP. Therefore, we performed transport experiments with TC on PTH, PHH, NTCP-HepG2 cells, and NTCP-HEK293 cells as well as for control on ASBT-HEK293 and with DHEAS on SOAT-HEK293 cells in the presence of the respective myr-preS1 peptides (Fig. 3A). HBV myr-preS1 peptides significantly inhibited the TC uptake of PTH (Fig. 3B) and PHH cells (Fig. 3C) down to ~25% of the control uptake with IC₅₀ values of 1.9 nM and 9.7 nM,

respectively. Furthermore, the TC uptake of NTCP-HepG2 (Fig. 3D) and NTCP-HEK293 cells (Fig. 3E) was completely blocked by the HBV myr-preS1 peptides with IC₅₀ values of 68 nM and 190 nM, respectively. In NTCP-HepG2 cells, IC₅₀ values for myr-preS1 mediated transport inhibition clearly correlated with the overall NTCP transport rate (Fig. 3F). The transport of ASBT and SOAT was not influenced by myr-preS1 peptides (Fig. 3A).

Competition between HBV attachment and NTCP ligand binding

To analyse if ligands of NTCP may interfere with the attachment and infectivity of HBV, several taurine and glycine conjugates of cholic acid (TC and GC) and ursodeoxycholic acid (UDC, TUDC, GUDC) as well as dehydrocholic acid (DHC), and ezetimibe (EZ) (Fig. 4A) were tested for their inhibitory potential on the TC uptake by PTH (Fig. 4B) and NTCP-HepG2 cells (Fig. 4D). Inhibition of TC uptake in PHH was only tested with EZ (Fig. 4C). All three cell systems were pre-incubated with up to 100 μM of the respective compounds for 30 min before the TC uptake was analysed over 10 min in the presence of inhibitors. In PTH, PHH, and NTCP-HepG2 cells, all tested compounds except of DHC strongly inhibited the TC uptake. After 30 min pre-incubation and subsequent wash-out, TC, GC, UDC, TUDC, and EZ (at 25 μM) did not affect the TC uptake over 10 min, whereas GUDC and EZ (at 100 μM) reduced the NTCP activity in NTCP-HepG2 cells by >50% (Fig. 4D, right panel). As a control, we used tauro lithocholic acid (TLC) that has been reported to result in a rapid loss of NTCP from the plasma membrane during pre-incubation of the cells [13,16]. In parallel we investigated, if these inhibitory effects interfere with the HBV susceptibility. Therefore, PTH (Fig. 4E), PHH (Fig. 4F) and NTCP-HepG2 cells (Fig. 4G) were incubated with increasing concentrations of the indicated compounds for 30 min, before HBV infection. Similar to the transport inhibition, all compounds (except of DHC), showed a concentration-dependent inhibition on the HBV infection. No cytotoxicity was observed at given concentrations of the inhibitory compounds (Supplementary Fig. 3). Apart from HBV infection, we also analysed the effect of bile acids and EZ on the myr-preS1 peptide binding to NTCP (Fig. 4H, Supplementary Fig. 4). Similar to HBV infection (Fig. 4G), inhibition of HBV myr-preS1-AX594 peptide binding was particularly effective with TUDC (Fig. 4H). EZ also showed strong inhibitory effects on HBV myr-preS1-AX594 peptide binding and HBV infection (Fig. 4E-H) at least at 100 μM.

Discussion

Recently, Yan *et al.* were the first to demonstrate that NTCP represents a functional high-specific receptor for HBV infection [4]. The data of the present study fully support this finding and show that myr-preS1 peptide binding and infectivity of HBV in PTH, PHH as well as in HepG2 cells clearly depend on functional NTCP/*tNtcp* expression. In PTH and PHH cells, HBV susceptibility and Na⁺-dependent bile acid transport simultaneously declined during few days pp and were nearly undetectable at day 7 pp. This decline was first observed at the mRNA level and was fol-

Fig. 4. Bile acids and ezetimibe block HBV infection. (A) Chemical structures of inhibitory compounds. Transport of [³H]TC in PTH (B), PHH (C), and NTCP-HepG2 cells (D) was inhibited by the indicated compounds at the given concentrations. HBV infection of PTH (E), PHH (F), and NTCP-HepG2 cells (G) was inhibited by bile acids and ezetimibe (EZ) at the indicated inhibitory concentrations. (H) Inhibition of HBV myr-preS1-AX594 peptide binding (red) to NTCP-HepG2 cells by bile acids and EZ.

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lowed by the functional protein expression (Fig. 1). Similar results were reported in freshly isolated rat hepatocytes, in which a time-dependent decline in the Na⁺-dependent TC uptake was associated with down-regulation of the rat Ntcp mRNA [17].

Although in primary hepatocyte cultures nearly every cell expresses NTCP shortly after plating, the number of HBV-infected cells in those cultures is usually around 20–25% as seen by us and others [4]. A similar magnitude of infection can also be seen with our NTCP-HepG2 cell line and was also reported for NTCP-transfected HepG2 and HuH7 cell lines [4]. The reason for this phenomenon is still unclear and might be due to restrictions of a still unknown HBV post-entry step.

The establishment of a HepG2 cell line with inducible NTCP-expression allowed us to analyse NTCP-related HBV susceptibility, myr-preS1 peptide binding and bile acid uptake more in detail (Fig. 2). Differential induction of NTCP-expression by dose-dependent DOX-treatment correlated with NTCP mRNA and protein expression. Kinetics of NTCP-induction, TC uptake and myr-preS1 peptide binding reached its maximum after 48 h DOX treatment. After DOX withdrawal, NTCP expression, TC uptake, myr-preS1-peptide binding and HBV infection in NTCP-HepG2 cells followed similar kinetics as in primary hepatocytes. Thus, it can be concluded that the decline of HBV susceptibility and Na⁺-dependent bile acid transport observed in PTH and PHH cells within few days pp clearly depends on functional NTCP expression. At least in PTH also the Na⁺-independent part of the total TC uptake declined over time, which was most likely mediated by hepatic carriers of the organic anion transporting polypeptide (OATP) family [6,17]. Our NTCP-HepG2 cell line now allows to balance the NTCP expression at the physiological level, giving advantage over other maximally over-expressing systems [4,12,13,18,19].

Apart from NTCP, the SLC10 carrier family contains two further carriers, ASBT and SOAT, with high sequence and structural homology to NTCP [5]. However, whereas the ASBT and SOAT transport functions are quite specific for bile acids and sulfated steroids, respectively, NTCP has a broader substrate pattern including both kinds of substrates [5–8]. Therefore, we were interested if HBV may also bind to ASBT or SOAT via its myr-preS1 peptide. But we clearly show that both carriers do neither bind the myr-preS1 peptide nor support HBV infection. Even integrating the two putative HBV myr-preS1 binding domains of NTCP [4,11,12] into the respective sites of ASBT and SOAT did not support myr-preS1 binding or HBV infection (Fig. 2E–G). These data underline the liver-specific HBV susceptibility via NTCP binding, whereas the homologous carriers ASBT and SOAT, mainly expressed in gut and testis [5,8], respectively, are not targeted by HBV.

A further intriguing finding of the present study is that the myr-preS1 peptide-dependent attachment of HBV to NTCP and the bile acid transport function of NTCP clearly interfere with each other: The myr-preS1 peptides of HBV and WMHBV (but not of WHV) significantly inhibited the TC transport in PTH and PHH, and completely blocked the TC uptake in NTCP-HepG2 and NTCP-HEK293 cells (Fig. 3); *vice versa*, certain bile acids clearly inhibited HBV infection of PTH, PHH, and NTCP-HepG2 cells and dramatically blocked the myr-preS1-AX594 peptide binding to NTCP (Fig. 4E–H). However, it has to be noted that a 30 min incubation of NTCP-HepG2 cells with GUDC at 100 μM and 400 μM as well as EZ at 100 μM significantly reduced the NTCP transport activity, even after a 10 min wash-out phase

(Fig. 3D). This could be explained by NTCP retrieval from the plasma membrane, which was previously reported for taurolithocholic acid (TLC) and taurochenodeoxycholic acid [13,16]. Nevertheless it can be concluded that bile acid substrate binding and myr-preS1 peptide binding to NTCP directly interfere with each other, also supporting very recent findings from other groups [12,13]. Yan *et al.* also used PTH to show myr-preS1 peptide-induced inhibition of the Na⁺-dependent [³H]TC transport. However, in contrast to the present study, Yan *et al.* found stronger effect of TUDC on HBV infection in PTH [13].

In the present study we used UDC and TUDC as potential myr-preS1 peptide binding inhibitor (Fig. 4H). UDC is an approved drug for a variety of chronic liver diseases such as dissolution of cholesterol gallstones and the improvement and delay of progression of primary biliary cirrhosis (PBC), and lacks any cytotoxicity up to concentrations of 500 μM [20]. Standard treatment doses of UDC are at 12–15 mg/kg body weight and make UDC and UDC-conjugates to the predominant bile acids within the endogenous bile acid pool. Under treatment of PBC patients, UDC and UDC-conjugates reach plasma concentrations up to 17 μM [21]. However, this concentration seems to be too low for >50% inhibition of HBV infection at least in our *in vitro* assay (Fig. 4E–G). Clinical studies about the influence of bile acids on the course of hepatitis B came to inconclusive results [22], and currently there is no clear evidence for a protective role of UDC treatment in hepatitis B under standard treatment conditions.

Apart from bile acids, *in vivo* experiments already confirmed that hepadnaviral myr-preS1 peptides are potent inhibitors of HBV infection, even at low nanomolar concentrations, and the myr-preS1 peptide-derived drug Myrcludex B is currently under development as an elegant and promising approach for intervention of HBV infection [23]. However, it cannot be excluded that therapeutically used myr-preS1 peptide derivatives may hamper the enterohepatic circulation of bile acids by blocking the NTCP-mediated bile acid transport into hepatocytes. While there might be no serious side effects during short-term treatment, e.g. for HBV post exposure prophylaxis, effects on the bile acid homeostasis should be carefully monitored under long term treatment, e.g. in chronically HBV-infected patients.

In subsequent studies it will be interesting to investigate if already labelled drugs which have been shown to inhibit the transport function of NTCP, in the same way and with comparable inhibition kinetics could also block myr-preS1 peptide binding and HBV infection of NTCP-expressing liver cells. These drugs include cyclosporine A, propranolol, bumetanide, simvastatin, irbesartan, doxazosin, and many others [24,25]. Very recent studies already demonstrated that cyclosporine A efficiently can block myr-preS1 peptide binding to NTCP and HBV infection of HBV susceptible cells [18,19]. In the present study we closer analysed the lipid lowering drug ezetimibe, which is a specific inhibitor of the intestinal Niemann-Pick C1-like cholesterol transporter and has shown before a significant inhibition of NTCP with a K_i of 25 μM [25]. In our study, ezetimibe significantly inhibited the TC transport in PTH, PHH, and NTCP-HepG2 cells, and also inhibited the HBV infection of these cells (Fig. 4). However, ezetimibe at a concentration of 100 μM significantly reduced the TC transport activity of NTCP after 30 min pre-incubation and 10 min wash-out (Fig. 4D). Therefore, the inhibition of myr-preS1 peptide binding and HBV infection by 100 μM ezetimibe might be a mixed effect of NTCP inhibition and internalization. Furthermore, as ezetimibe plasma concentrations under standard

treatment are far below these values effective for HBV inhibition, i.e., in the range of about 15 nM [26], ezetimibe might not be the appropriate drug for treatment of chronic HepB patients. Nevertheless, our data indicate that HBV infection indeed may be treatable with a chemical compound of low molecular weight.

In conclusion: Myr-preS1 dependent viral binding to NTCP, necessary for productive HBV infection, interacts with the physiological bile acid transport function of NTCP. Therefore, HBV infection via NTCP may be lockable by NTCP substrates and NTCP-inhibiting drugs. This opens a completely new way for an efficient management of HBV infection by the use of NTCP-directed drugs.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2014.05.018>.

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