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Inhibition of hepatitis C virus RNA translation by antisense bile acid conjugated phosphorothioate modified oligodeoxynucleotides (ODN)

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ABSTRACT

Background: The 5′-noncoding region (5′NCR) of the HCV-genome comprises an internal ribosome entry site essential for HCV-translation/replication. Phosphorothioate oligodeoxynucleotides (tS-ODN) complementary to this region can inhibit HCV-translation *in vitro*. In this study, bile acid conjugated tS-ODN were generated to increase cell-selective inhibition of 5′NCR-dependent HCV-translation.
Methods: Different bile acid conjugated tS-ODN complementary to the HCV5′NCR were selected for their inhibitory potential in an *in vitro* transcription/translation assay. To analyze OATP (organic anion transporting polypeptides)-selective uptake of bile acid conjugated ODN, different hepatoma cells were stably transfected with the OATP1B1-transporter and primary human hepatocytes were used. An adenovirus encoding the HCV5′NCR fused to the luciferase gene (Ad-GFP-NCRluc) was generated to quantify 5′NCR-dependent HCV gene expression in OATP-overexpressing hepatoma cells and *in vivo*.
Results: A 17mer phosphorothioate modified ODN (tS-ODN4_13) complementary to HCV5′NCR was able to inhibit 5′NCR-dependent HCV-translation in an *in vitro* transcription/translation test system by more than 90% and it was also effective in Huh7-cells containing the HCV subgenomic replicon. Conjugation to taurocholate (tS-ODN4_13T) significantly increased selective ODN uptake by primary human hepatocytes and by OATP1B1-expressing HepG2-cells compared to parental HepG2-cells. Correspondingly, tS-ODN4_13T significantly inhibited HCV gene expression in liver-derived OATP1B1-expressing HepG2- or CCL13-cells up to 70% compared to unconjugated tS-ODN and compared to mismatch taurocholate coupled tS-ODN. *In vivo*, tS-ODN4_13T showed also a trend to block 5′NCR-dependent HCV gene expression.
Conclusions: The tested taurocholate conjugated 17mer antisense ODN complementary to HCV5′NCV showed an increased and selective uptake by hepatocytes and liver-derived cells through OATP-mediated transport resulting in enhanced specific inhibition of HCV gene expression *in vitro* and *in vivo*. Thus, this novel approach may represent a promising strategy to improve antisense approaches with ODN in the control of hepatitis C infection.

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1. Introduction

Hepatitis C virus (HCV) is a major cause of liver disease, affecting over 200 million patients worldwide. Despite progress in HCV treatment, the overall response of a therapy with ribavirin and pegylated interferon α (PEG-IFN) in patients with HCV genotype 1 or 4 remains unsatisfactory (McHutchison et al., 2002). Novel protease inhibitors targeting HCV-NS3 (boceprevir) or HCV-NS3/4A (telaprevir) have been recently developed and show high antiviral activity (Soriano et al., 2009). However, rapid HCV drug resistance to these agents has been shown to limit their efficacy, necessitating a combination with PEG-IFN and rivabirin, which may cause a wide range of serious

Abbreviations: Ad, adenovirus vector; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HCV, hepatitis C virus; MOI, multiplicity of infection; 5′NCR, 5′-noncoding region of HCV; IRES, internal ribosomal entry site; OATP, organic anion transporting polypeptide; ODN, oligodeoxynucleotide(s); RLU, relative light units; tS-ODN, phosphorothioate oligodeoxynucleotide; tS-ODN(T), taurocholate conjugated oligodeoxynucleotide.

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adverse reactions (Aman et al., 2012; Susser et al., 2009; Sarrazin et al., 2007; Mitchell and Rafael, 2012). Thus, development of novel, interferon-free therapeutic strategies without promoting HCV resistance is called for.

HCV is a positive-strand RNA virus of 9,600 nucleotides (nt) with a considerable sequence diversity (Choo et al., 1991; Simmonds, 1995). At the 5′-end, the HCV genome contains a noncoding region (5′NCR) of 341 nt, which is the most highly conserved region among all HCV strains (Bukh et al., 1992). It forms a stable secondary structure which contains an internal ribosome entry site (IRES) necessary for HCV translation/replication, representing an ideal target for antisense approaches.

In fact, we and others showed that phosphorothioate modified antisense oligodeoxynucleotides (ODN), RNA interference (siRNA), antisense RNA or hammerhead ribozymes complementary to this region can effectively inhibit HCV translation (Alt et al., 1995; Gonzalez-Carmona et al., 2006, 2011; Guerniou et al., 2007; Sen et al., 2003).

However, poor specific tissue delivery and transport through cell membranes are major obstacles in the therapeutic application of antisense nucleotides (Iversen et al., 1992). Various strategies using liposomes, nanoparticles, cholesterol conjugates or cell-penetrating peptides have been developed to enhance ODN or siRNA delivery. However, their tissue specificity and use *in vivo* remain limited (De Rosa and La Rotonda, 2009; Delogu et al., 2009; Oh and Park, 2009; Stewart et al., 2008).

Two different transport systems are involved in the uptake of bile acids of hepatocytes: the Na^+-taurocholate-cotransporting polypeptide (NTCP) and several multispecific organic anion transporting polypeptides (OATPs) (Hagenbuch and Meier, 1996; Kramer and Wess, 1996; Trauner and Boyer, 2003). Conjugation of therapeutic drugs to bile acids has been shown to increase uptake into hepatoma cells via these transport systems (Briz et al., 2002; Kullak-Ublick et al., 1997; Barbara et al., 2006). Thus, conjugation of ODN to bile acids may also increase a liver-specific and more effective inhibition of HCV translation of antisense ODN.

In this study, conjugation of phosphorothioate-modified antisense oligonucleotides to taurocholate allowed OATP-selective ODN uptake in liver-derived cells and primary human hepatocytes resulting in enhanced, 5′NCR-specific inhibition of HCV translation *in vitro* and *in vivo*, pointing to a promising new approach to improve antisense ODN for the control of HCV infection.

2. Materials and methods

2.1. Chemical synthesis of bile acid conjugated oligonucleotides (ODN)

Starting from the previously described phosphorothioate modified 23mer ODN complementary to 326–348 nt of HCV5′NCR (tS-ODN4), several shortened antisense ODN were generated for subsequent bile acid conjugation (Table 1) (Alt et al., 1995). The control tS-ODN4K, which was described previously, targets the same sequence of HCV5′NCR (nt 326–348) than tS-ODN4 but 4 nt have been mismatched (Alt et al., 1995). After testing their inhibitory capacity, tS-ODN4_13 was chosen for conjugation to cholate (C), taurocholate (T) or cholesterol (X) as described previously, (Fig. 1A) (Lehmann et al., 2001). Briefly, synthesis of cholic and taurocholic acid phosphoramidites started from commercially available cholic acid. In a sequence of seven reaction steps, 3α-(2-hydroxyethoxy)cholic acid could be obtained. The analogous taurocholic acid phosphoramidite starting from 3α-(2-hydroxyethoxy)cholic acid was achieved via an amidation reaction with 2-aminoethane-ethyl-sulfonate. ODN synthesis was carried out on a PerSeptive Biosystems DNA-synthesizer. To increase the nuclease stability of ODN, thioate modifications were introduced at three phosphodiesters at each end. In control ODN, fluorescein isothiocyanate (FITC) was covalently attached to the ODN at the 3′-position. ODN were purified by RP-HPLC. All compounds were characterized by analytical RP-HPLC, PAGE and electrospray ionization mass spectrometry (Lehmann and Engels, 2001).

Table 1
Shortened ODN derived of tS-ODN4 (23mer) complementary to HCVRNA326–348 nt.

tS-ODN4	5′-T*G*C*TCATGGTGCACGGTCTA*C*G*A-3′	23mer
tS-ODN4_11	5′-T*C*A*TGGTGCACGGTCTA*C*G*A-3′	20mer
tS-ODN4_13	5′-T*G*G*TGCACGGTCTA*C*G*A-3′	17mer
tS-ODN4_26	5′-T*C*A*TGGTGCAC*G*G*T-3′	14mer
tS-ODN4_27	5′-T*C*A*TGGTG*C*A*C-3′	11mer
tS-ODN4_28	5′-T*C*A*TG*G*T*G-3′	08mer
tS-ODN4_30	5′-C*T*C*AT*G*G*T-3′	03m er
tS-ODN4_31	5′-G*C*T*CA*T*G*G-3′	08mer
tS-ODN4_33	5′-T*G*C*TC*A*T*G-3′	08mer
tS-ODN4 34	5′-A*T*G*GT*G*C*A-3′	08mer

t: terminally modified; S: phosphorothioate. * = phosphorothioate.

2.2. Cell lines

911 cells are human embryonic retinoblasts used to propagate E1-deleted adenoviruses (Fallaux et al., 1996). The human hepatoma cells HepG2 (ATCC HB8065) and CCL13 (ATCC CCL-13) were stably transfected with the OATP1B1-transporter to study selective delivery of bile acid conjugated ODN. The previously described Huh7 cell clone Con1/Luc-ubi-neo/ET containing the HCV subgenomic replicon of genotype 1b was used for inhibition experiments (Lohmann et al., 1999). Kidney-derived cells HEK-293 (ATCC CRL-1573) and colorectal adenocarcinoma cells HT29 (ATCC HTB-38) served as control. Cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Life Technologies, Karlsruhe, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin (Sigma, Taufkirchen, Germany). Stable transfected cells and cell clones containing the HCV replicon were selected with neomycin (PAA, Cölbe, Germany).

2.3. Plasmids

Previously described plasmids pCMVNCRluc and pT7NCRluc contain HCV5′NCR and the first 66 nt of the core gene fused in frame to the firefly luciferase sequence (Alt et al., 1995). The adenoviral backbone vector pAdEasy-1 and the transfer vector pAdTrackCMV, which possesses a GFP-gene were kindly provided by Dr. B. Vogelstein, University of Baltimore, USA. Full-length OATP1B1-cDNA was isolated from the previously described plasmid pCMV6-XL4 and cloned into pcDNA3.1/V5-His (Invitrogen, Karlsruhe, Germany) to generate pcDNA3.1/V5-His-OATP1B1 (Kullak-Ublick et al., 2001).

2.4. In vitro transcription/translation assay

The *in vitro* transcription/translation test system has been described previously (Alt et al., 1995). Briefly, 1 μg of linearized pT7NCRluc was incubated with T7RNA-Polymerase (Promega, Mannheim, Germany) at 37 °C for 1 h. Fifty nanograms of DNA-free RNA was used for translation into a commercially available rabbit reticulocyte lysate according to the manufacturer's instructions (Promega). ODN were added to the translation mixture and incubated at 75 °C for 10 min. Next, luciferase assay was performed.

2.5. Construction of adenoviral vectors

The HCV-luciferase sequence NCRluc was cut from pCMVNCRluc and cloned into pAdTrackCMV. The generated plasmid pAd-

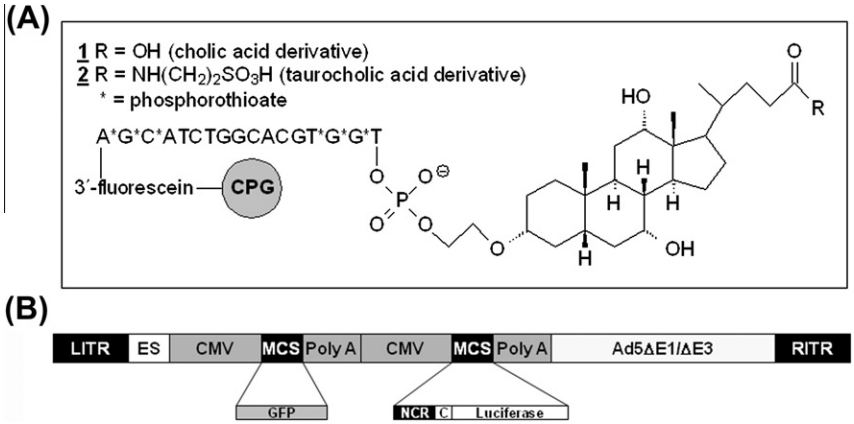

Fig. 1. (A) 5′-Bile acid and 3′-fluorescein modified antisense ODN (17mer) complementary to HCV5′NCR. (B) Map of Ad-GFP-NCRluc. Ad-GFP-NCRluc encodes the HCV5′NCR and 66 nt of core-sequence, fused to the firefly luciferase gene. LITR: left inverted terminal repeat, ES: encapsidation signal, CMV: cytomegalovirus immediate early promotor, MCS: multiple cloning site, Poly A: polyadenylation signal, Ad5E1/E3: human adenovirus type 5 with E1- and E3-deletions, RITR: right inverted terminal repeat.

TrackCMV-NCRluc was recombined with pAdEasy-1 according to the published protocol by He et al. (1998). Finally, recombinant plasmids were transfected into 911 cells to generate Ad-GFP-NCRluc viruses (Fig. 1B).

2.6. Generation of stably OATP1B1-transfected cells

HepG2- and CCL13-cells were transfected with 20 μg pcDNA3.1/V5-His-OATP1B1 using lipofectamine according to the manufacturer's instructions (Invitrogen). 72 h later cells were cultured under G418-selection (800 μg/ml).

2.7. Polymerase chain reaction (PCR)

To demonstrate 5′NCR transcription in HepG2-OATP1B1 and CCL13-OATP1B1 cells after transduction with Ad-GFP-NCRluc, a PCR of cellular RNA was performed using the following primers: forward, HCV5′NCR 5′-GCTCTAGACCCAAGCTTGCCAGCC-3′ and reverse, HCV5′NCR 5′-GGCCGGTACCGACGTCCTGTGGG-3′. To confirm OATP1B1 transcription in stable transfected HepG2-OATP1B1 and CCL13-OATP1B1 cultures, the following primers, corresponding to bp 1188–1211 and 1664–1688 of the OATP1B1 cDNA, were used for reverse transcription: forward, 5′-AGAGCAACAGTATGGTC AGCCTTC-3′ and reverse, 5′-AAGCATCATCTCTTGGGCATTCAC-3′.

2.8. Luciferase assay

Luciferase activity from protein lysates was determined with the Luciferase Reporter System (Promega, Mannheim, Germany) according to the manufacturer's instructions. The relative light units (RLU) were measured during 30 s in a luminometer (Berthold, Pforzheim, Germany).

2.9. Western blot analysis of OATP1B1 expression in hepatoma cells

Expression of OATP1B1 was determined by Western blot using standard protocols. A primary monoclonal antibody mESL kindly provided by Prof. Dietrich Keppler, University of Heidelberg (Germany) and a horse peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (Sigma, Munich, Germany) were used (Cui et al., 2003). Visualization was achieved by enhanced chemoluminescence (SuperSignal Chemiluminescent-Substrate, Perbio-Science, Bonn, Germany).

2.10. Flow cytometry

Efficiency of cell transduction and GFP-expression in hepatoma cells after transduction with Ad-GFP-NCRluc were measured by flow cytometry (Coulter Epics XL-Cytometer, Immunotech, Krefeld, Germany). FITC-marked bile acid conjugated ODN served to analyze the cellular ODN uptake.

2.11. Adenoviral transduction of hepatoma cells and inhibition experiments

For experiments in HepG2-OATP1B1 and CCL13-OATP1B1, cells were seeded onto 24-well plates, grown for 48 h and transduced with Ad-GFP-NCRluc (MOI 50) in DMEM supplemented with 2% horse serum for 2 h. Six hours later, 2–6 μM ODN were added to the culture medium without any transfection reagent. For inhibition experiments in the Huh7-cells with the HCV subgenomic replicon, 4 μM ODN were added to the culture medium. Cell viability was assessed by trypan blue dye exclusion.

2.12. Primary human hepatocytes

Primary human hepatocytes from human liver resectates were purchased from Primacyt Cell Culture Technology (Schwerin, Germany). These hepatocyte cultures are free from non-parenchymatous liver cells. FITC-conjugated ODN with or without bile acid conjugation were added at 4 μM concentration to the culture medium of hepatocytes without any transfection reagent. To test the competitive effect of a bile acid on the uptake of ODN, taurocholate at 4 μM concentration was added in addition to ODN to the cell culture medium. The uptake of ODN was monitored using fluorescent microscope IX81 (Olympus, Hamburg, Germany) and Cell P Analysis Software (AnalySIS, Soft Imaging System GmbH, Münster, Germany). Cell viability was assessed by trypan blue dye exclusion.

2.13. Imaging and quantification of in vivo bioluminescence

Mice were injected intravenously (i.v). with the reporter vector Ad-GFP-NCRluc (5×10^7 pfu/mouse). Luciferase was measured *in vivo* by live imaging using a highly sensitive, cooled charge-couple device camera (IVIS 200 Xenogen, Alameda, CA, USA). For imaging, luciferin (Synchem, Alternburg, Germany) was injected intraperitoneally (150 mg/kg body weight). After 5 min, light emitted from the mice was imaged and electronically displayed using a Living Image Software (Xenogen, Ivis). For inhibition experiments, 20 mg ODN/kg

body weight were i.v.-injected in mice and luciferase activity was measured at different time points after the treatment. For toxicity, taurocholate-coupled ODN were injected to healthy mice i.v. and weight control or occurrence of gastrointestinal symptoms such as diarrhea were evaluated at different time points. For some mice, blood samples were collected 24 h after the treatment, and aminotransferases (ALT and AST) were measured in order to determine liver toxicity.

2.14. Statistical analysis

For descriptive statistics, means and standard errors were noted. Paired *t*-test was used for statistical significance. A *p*-value of less than 0.05 was considered significant. The IC_{90} (concentration of ODN, which inhibited the luciferase activity by 90%) was calculated for each tested ODN in the different *in vitro* transcription/translation assays.

3. Results

3.1. Inhibition of HCV translation by different shortened phosphorothioate ODN in vitro

The previously identified 23mer phosphorothioate modified oligonucleotide tS-ODN4 as potent inhibitor of HCV translation was chosen for bile acid modification (Alt et al., 1995). To increase cell uptake after bile acid conjugation, we shortened the tS-ODN4 sequence and generated several ODN (Table 1). The effect of ODN length on their inhibitory potential was evaluated in an *in vitro* transcription/translation assay. As shown in Fig. 2A, tS-ODN shortened to 20–17 bp could still inhibit translation of the fusion HCV-RNA by >95%. With regard to the number of nucleotides, the 17 bp ODN (tS-ODN4_13) was the shortest sequence with the best inhibitory effect on the 5′NCR-dependent luciferase expression (IC_{90} 2.8 μM). This effect was HCV5′NCR-sequence specific, as the 17 bp mismatch tS-ODN4_13(K) could not inhibit HCV-translation (Fig. 2D). Shortening of tS-ODN4 to 16–11 bp resulted in lower inhibitory activity (<75%) and further shortening to 8 bp in almost no inhibitory activity (<30%) (Fig. 2B and C).

3.2. Inhibition of HCV gene expression by tS-ODN4_13 in Huh7 cells containing a HCV subgenomic replicon

In order to test the antisense effects of the selected 17 bp sequence in cell culture, inhibition experiments were performed using Huh7 cells containing a HCV subgenomic replicon of genotype 1b (Con1/Luc-ubi-neo/ET). Using lipofectamine as transfection reagent, tS-ODN4_13 showed a significant inhibition of HCV translation of about 50% 48 h after cell transfection with 4 μM tS-ODN4_13 compared to mismatch tS-ODN4_13K (Fig. 3A), demonstrating the inhibitory capacity of the chosen 17mer ODN sequence in the replicon system.

3.3. Inhibition of HCV translation by bile acid conjugated phosphorothioate ODN in vitro

After the results in the above experiments, the 17mer tS-ODN was chosen for further bile acid conjugation. As shown in Fig. 3B, taurocholate (T), cholate (C) and cholesterol (X) conjugated antisense tS-ODN4_13 showed strong inhibition rates of 5′NCR-dependent luciferase expression comparable to the inhibitory capacity of

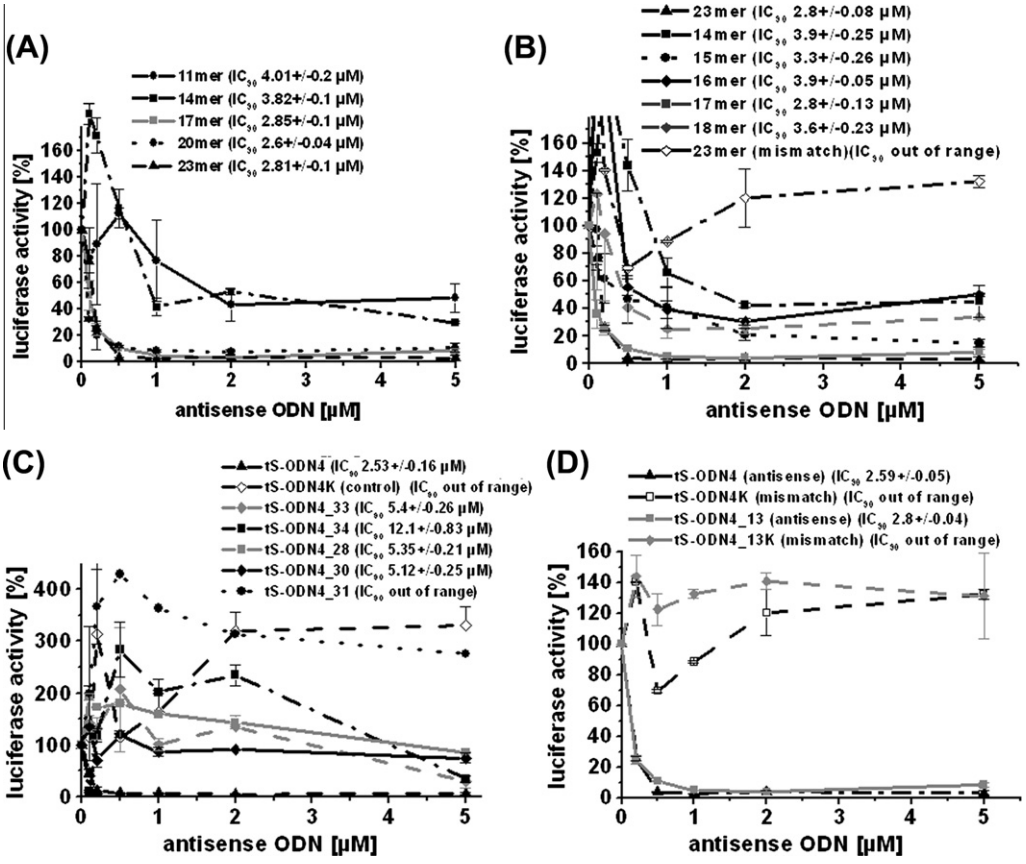

Fig. 2. (A–D) Inhibition of HCV-translation by shortened phosphorothioate modified ODN in an *in vitro* transcription/translation assay. The percentage mean values and SD of three independent experiments are given. The IC_{90} (concentration of ODN, which inhibited the luciferase activity by 90%) was calculated for each ODN.

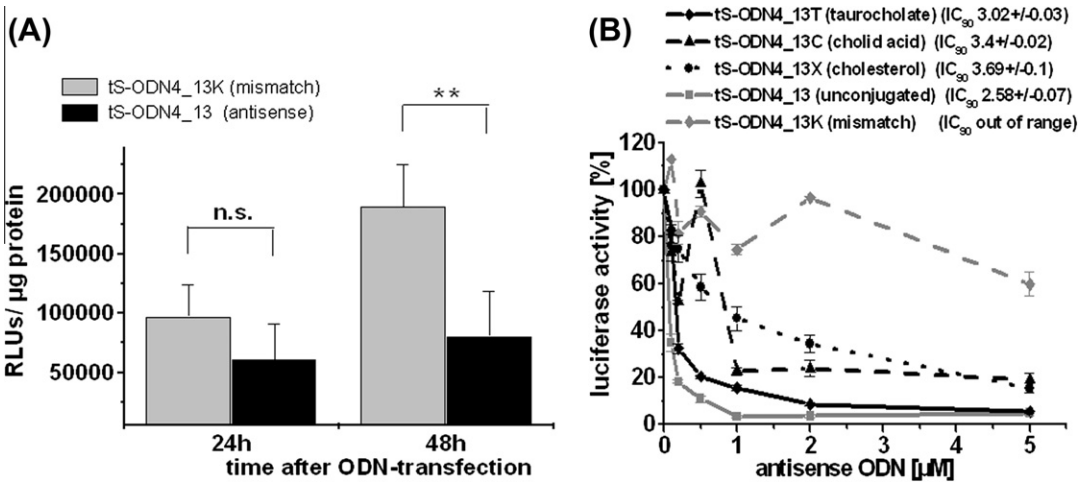

Fig. 3. (A) Inhibition of HCV translation in Huh7 cell clone Con1/Luc-ubi-neo/ET containing the HCV subgenomic replicons of genotype 1b by phosphorothioate modified antisense tS-ODN4_13 (17mer). Luciferase was measured 24 and 48 h after ODN transfection with lipofectamine. Mean and SE are shown. (B) Inhibition of HCV-translation by bile acid conjugated ODN in an *in vitro* transcription/translation assay. The IC_{90} (concentration of ODN, which inhibited the luciferase activity by 90%) was calculated for each ODN.

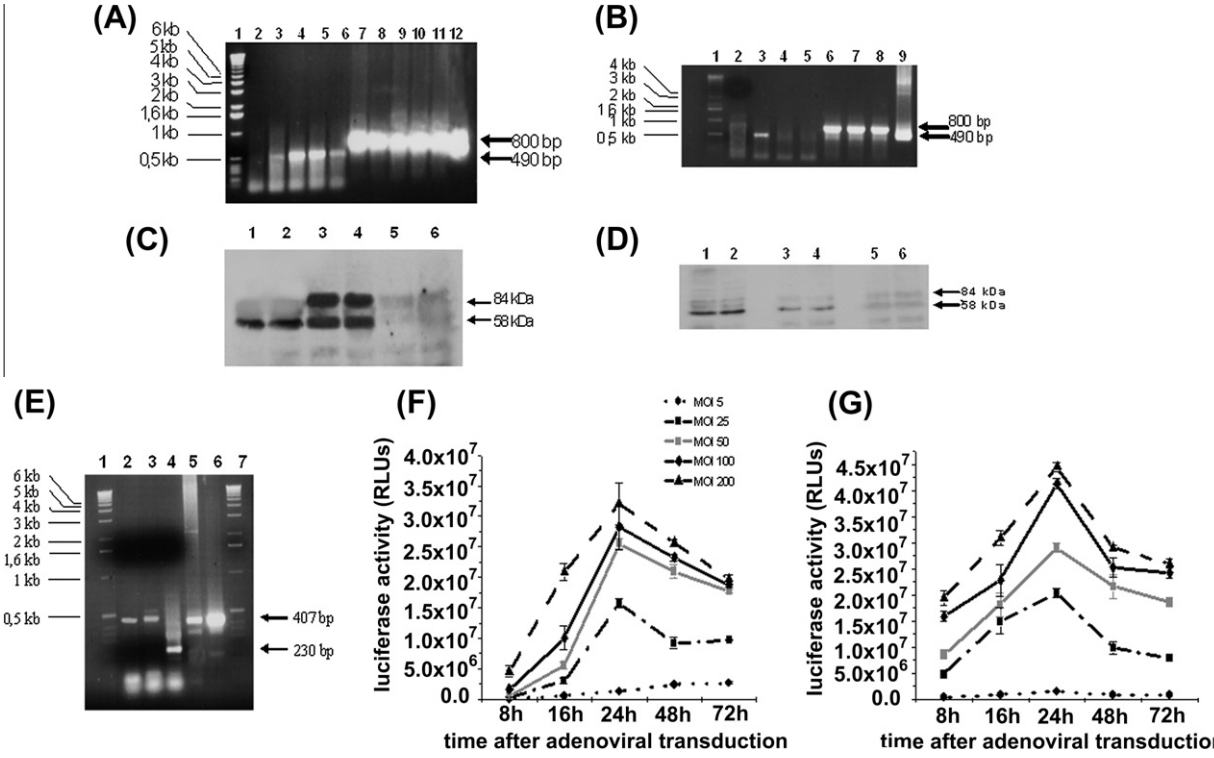

Fig. 4. (A) Transcription of OATP1B1 in HepG2-OATP1B1 cells (RT–PCR). Lane 1: marker, lane 2: 911 cells, lane 3: HepG2-cells, lane 4: HepG2OATP1B1 cells (clone I), lane 5: HepG2OATP1B1 cells (clone II), lane 6: HepG2OATP1B1 cells (clone III), lanes 7–11: beta actin, lane 12: OATP1B1 using plasmid pcDNA3.1/V5-HisOATP-C. (B) Transcription of OATP1B1 in CCL13-OATP1B1 cells (RT–PCR). Lane 1: marker, lane 2: 911 cells, lane 3: CCL13OATP1B1 cells (clone A), lane 4: CCL13OATP1B1 cells (clone B), lane 5: CCL13 cells, lanes 6–9: beta actin controls, lane 10: OATP1B1 using plasmid pcDNA3.1/V5-HisOATP-C. (C) OATP1B1-expression in HepG2-OATP1B1 cells (Western blot). Lanes 1–2: HepG2-cells. Lane 3: HepG2OATP1B1 (clone I). Lane 4: HepG2OATP1B1 cells (clone II). Lanes 5–6: 911 cells. (D) OATP1B1-expression in CCL13-OATP1B1 cells (Western blot). Lanes 1–2: CCL13OATP1B1 (clone A); lanes 3–4: CCL13 cells; lanes 5–6: 911 cells. (E) Transcription of HCV5′NCR in hepatoma cells after adenoviral transduction with Ad-GFP-NCRluc (RT–PCR). Lanes 1/7: marker; lane 2:CCL13OATP1B1 cells; lane 3: HepG2OATP1B1-cells, lane 4: HepG2OATP1B1, lane 5: plasmid pAdTrackCMVNCRluc, lane 6: plasmid pCMVNCRluc. (F) Luciferase activity in HepG2-OATP1B1 and (G) CCL13-OATP1B1 after transduction with Ad-GFP-NCRluc, respectively.

unconjugated tS-ODN4_13 *in vitro*. Maximum suppression of HCV translation (>90%) was achieved with the taurocholate conjugated ODN tS-ODN4_13T at 5 µM (IC_{90} 3.02 ± 0.03 µM). tS-ODN4_13T was also the most effective bile conjugated ODN at the lowest concentration tested (0.5 µM), leading to an inhibition of almost 80% ($p < 0.0001$). Cholate and cholesterol-coupled ODN (tS-ODN4_13C and tS-ODN4_13X) reached their maximum inhibition rates of 70% at 5 µM and showed no inhibitory effects at 0.5 µM (IC_{90} 3.4 ± 0.02 µM and IC_{90} 3.69 ± 0.1 µM, respectively).

3.4. Generation of OATP1B1 overexpressing hepatoma cells

In HCC cells, a decreased expression of bile acid transporters, such as OATP, has been described compared to hepatocytes

(Kullak-Ublick et al., 1996). Consistently, we observed a reduced uptake of FITC-marked tS-ODN4_13T of <50% in Huh7 cells containing a HCV subgenomic replicon of genotype 1b (not shown). Thus, inhibitory effects of taurocholate conjugated tS-ODN4_13 could not be evaluated properly in Huh7 cells containing a HCV subgenomic replicon.

Therefore, in order to test the inhibitory potential and the specific bile acid transporter uptake of taurocholate coupled tS-ODN4_13T in cell culture, different hepatoma cells (HepG2 and CCL13) were stably transfected to overexpress the OATP1B1 transporter. The transcription of OATP1B1 was verified as a strong amplification of a 490 bp product by RT–PCR (Fig. 4A and B). OATP1B1-expression was also strongly detected by Western blot analysis, as two bands of 84 and 60 kDa in both OATP1B1 overexpressing cell lines compared to parental HepG2 and CCL13 cells (Fig. 4C and D) (Cui et al., 2003; Konig et al., 2000). A stable transfection of Huh7 containing a HCV subgenomic replicon with a second plasmid containing the OATP1B1-gene was rather difficult and induced strong deviations in the transgene expression of both constructs (not shown).

3.5. Construction of a NCRluc-encoding adenovirus vector

To quantify the inhibition of HCV translation by antisense ODN in cell culture, we generated an adenovirus encoding HCV5′NCR fused to the luciferase gene as reporter vector (Ad-GFP-NCRluc). Forty-eight hours after transduction of HepG2-OATP1B1 and CCL13-OATP1B1 cells with Ad-GFP-NCRluc, target-RNA (407 bp) was confirmed by RT-PCR (Fig. 4E). The 5′NCR-dependent luciferase activity was dose-dependent, reaching a peak 24 h after adenoviral transduction for all tested MOI (Fig. 4F and G).

3.6. Inhibition of 5′NCR-dependent HCV gene expression by taurocholate conjugated phosphorothioate ODN in HepG2-OATPB1 cells

Stably transfected HepG2-OATP1B1 cells were transduced with Ad-GFP-NCRluc (MOI 50). Six hours later, 2–6 μM tS-ODN4_13T were added to the cell supernatant without any transfection reagent. GFP-expression served to normalize transgene luciferase expression. As shown in Fig. 5, despite the strong adenoviral transgene expression of HCVNCR-luciferase in HepG2-cells, tS-ODN4_13T reduced luciferase activity 24 h after ODN-treatment in a dose-dependent manner reaching inhibition rates of 31.9 ± 0.9% with 2 μM ODN, 61.7 ± 2.3% with 4 μM ODN and of 70.6 ± 4.9%, with 6 μM ODN compared to the mismatch control ($p = 0.032$, $p = 0.012$ and $p = 0.0037$, respectively). In contrast, no significant inhibition of 5′NCR-dependent luciferase activity was detected after addition of unconjugated tS-ODN4_13, indicating the OATP-selective cell delivery of taurocholate conjugated ODN.

3.7. Intracellular uptake of taurocholate-conjugated ODN by liver-derived cells

To analyze intracellular uptake of taurocholate coupled ODN, FITC-labeled tS-ODN4_13T were used for flow cytometry. As shown in Fig. 6A and B, an about twice as high uptake of FITC-tS-ODN4_13T was measured in OATP1B1-overexpressing HepG2 cells (>90% of cells) compared to parental HepG2 cells (44% of cells). Moreover, intensity of fluorescence was approximately 1log higher in HepG2-OATP1B1 cells than in HepG2 cells, indicating an enhanced concentration of taurocholate conjugated ODN/cell. Surprisingly, FITC-non-conjugated ODN were taken up by a relatively high percentage of HepG2-OATP1B1 cells (about 75%). Yet, intensity of fluorescence was much lower compared to taurocholate conjugated ODN, explaining the failure of inhibition of unconjugated ODN in HepG2 cells.

As expected, taurocholate conjugated tS-ODN were not taken up by a number of tested non-hepatic cell lines, such as A549 cells (lung carcinoma) or 911 cells (retinoblastoma), suggesting a selective delivery of taurocholate coupled tS-ODN into liver-derived cells (Fig. 6C and D).

It has been shown that certain non-hepatic tissues also possess transporters for bile acids, notably the kidney and the bowel (Kagedahl et al., 1997). Correspondingly, FITC-labeled taurocholate tS-ODN were detected in approximately 30% of HEK-293 cells (kidney-derived cells), and in about 35% of HT29-cells (colorectal adenocarcinoma cells) (Fig. 3E and F).

3.8. Inhibition of 5′NCR-dependent HCV gene expression by taurocholate conjugated phosphorothioate ODN in CCL13-OATPB1 cells

Similar to the results in HepG2, in CCL13-OATP1B1 cells, taurocholate conjugated antisense tS-ODN4_13T inhibited the 5′NCR-dependent HCV-translation reaching a maximum of specific inhibition by 57.4 ± 0.45% ($p = 0.0042$) with 6 μM tS-ODN4_13T (Fig. 7A). By contrast, no inhibition of 5′NCR-dependent luciferase activity was detected after addition of unconjugated tS-ODN4_13 (not shown).

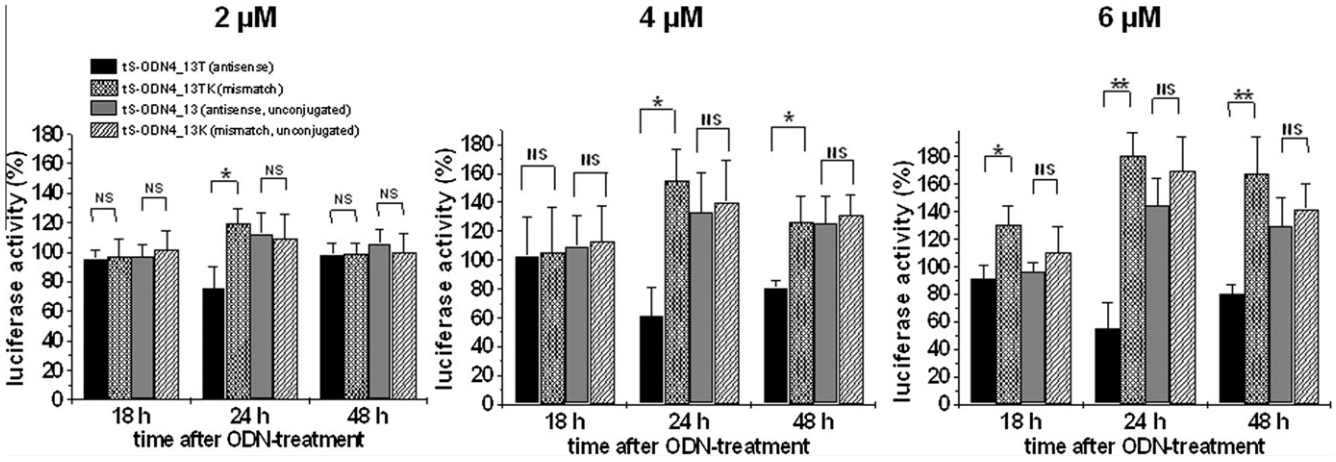

Fig. 5. Inhibition of HCV5′NCR-dependent luciferase gene expression in HepG2-OATP1B1 cells by taurocholate conjugated ODN. Luciferase activity was determined 16, 24 and 48 h after addition of bile acid conjugated antisense tS-ODN4_13T (2, 4 or 6 μM) to the culture medium without any transfection reagent. Mean and SE are shown ($n = 3$).

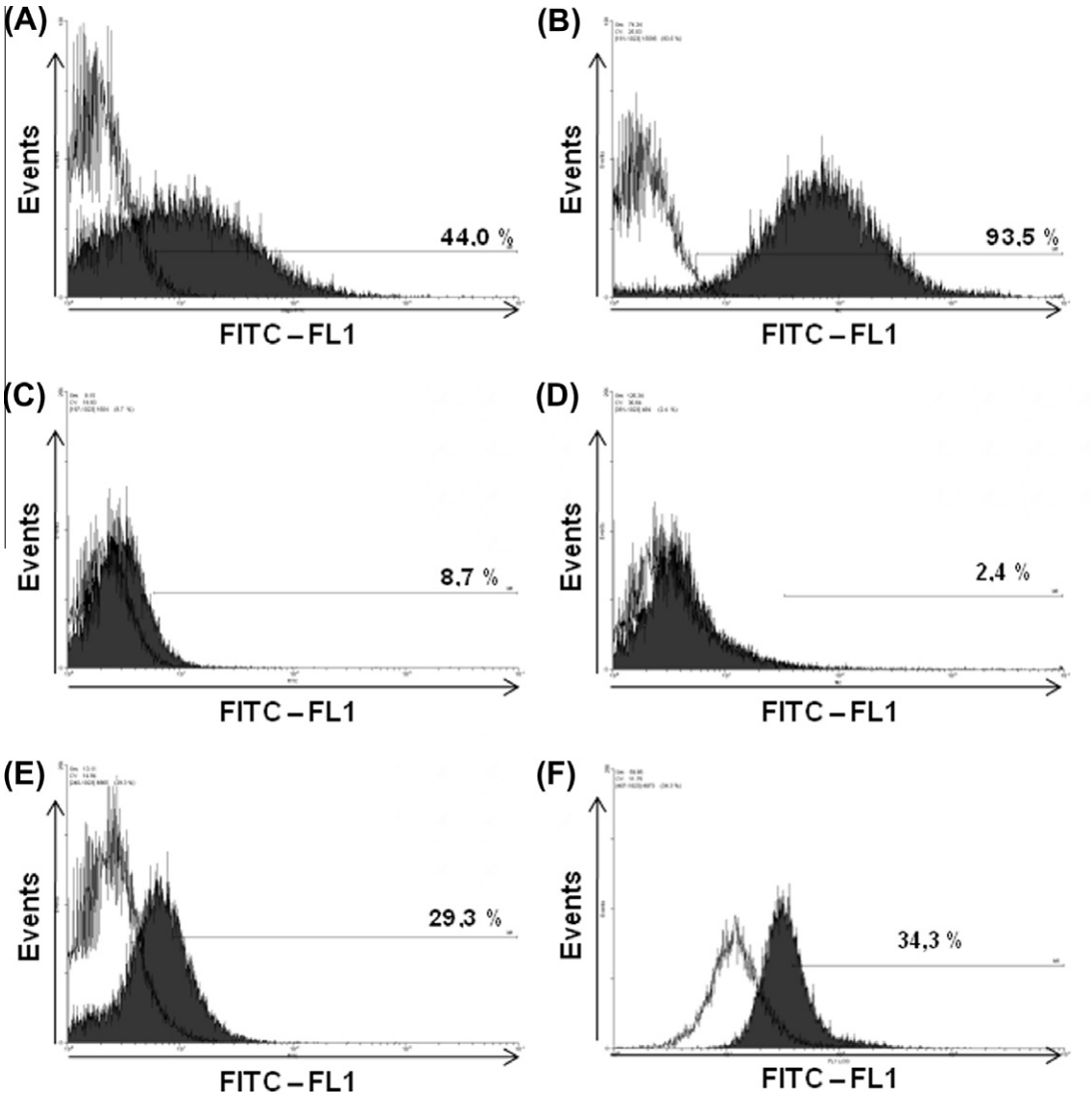

Fig. 6. Uptake of FITC-labeled taurocholate conjugated tS-ODN in different cell lines. Flow cytometry of cells was performed after addition of 2 μM of FITC-labeled ODN to the cells. (A) Uptake of FITC-labeled ODN by HepG2 cells, (B) HepG2OATP1B cells, (C) A549 cells, (D) 911 cells, (E) HEK293 cells and (F) HT29 cells.

3.9. Inhibition of HCV gene expression by lipofection of unconjugated phosphorothioate ODN in HepG2- and CCL13-OATPB1 cells

Only after using a transfection reagent (lipofectamine), a moderate specific inhibition of HCV translation of approximately 35% could be achieved with unconjugated tS-ODN4_13 (4 μM) in CCL13-OATP1B1 cells ($p = 0.018$), (Fig. 7B). In HepG2-OATP1B1 cells, despite the use of lipofectamine, no decrease of luciferase activity was detected (Fig. 7C).

3.10. Uptake of taurocholate-conjugated ODN by primary human hepatocytes

Using FITC-labeled taurocholate conjugated ODN or unconjugated ODN, the uptake of ODN could be detected and analyzed in hepatocytes by fluorescence microscopy. As shown in Fig. 8A–E, FITC-tS-ODN4_13T were highly taken up by primary human hepatocytes (40–50% of cells were positive) compared to unconjugated ODN (less than 10% of the cells).

To test the competitive effect of bile acid on the uptake of taurocholate conjugated ODN, taurocholate at 4 μM concentration was added in addition to ODN to the cell culture medium. As shown in Fig. 8E, FITC-taurocholate-conjugated ODN were still taken up by a high percentage of hepatocytes when cultured at the same time with taurocholate, indicating the high efficiency of hepatocytes to take up bile acids and the potential of bile acids as possible carriers for delivering oligonucleotides to the liver.

In hepatoma cells or primary human hepatocytes, we did not observe any toxicity regarding cell viability after the addition of taurocholate conjugated ODN in the culture medium at the used concentrations.

3.11. Inhibition of HCV gene expression by antisense bile acid conjugated ODN in vivo

For *in vivo* evaluation of bile acid conjugated oligonucleotides tS-ODN4_13T, we used the adenovirus encoding 5′NCR-luciferase fusion RNA (Ad-GFP-NCRluc). After i.v. application of Ad-GFP-NCR-luc (5×10^7 pfu/mouse), a high HCV luciferase expression could be tracked in the liver by whole body imaging bioluminescence. For inhibition experiments, mice were i.v.-treated with 20 mg ODN/kg body weight. As shown in Fig. 8, tS-ODN4_13T showed a trend to higher inhibition of NCR-dependent luciferase of about 57%, compared to mismatch ODN. No toxicities regarding weight loss,

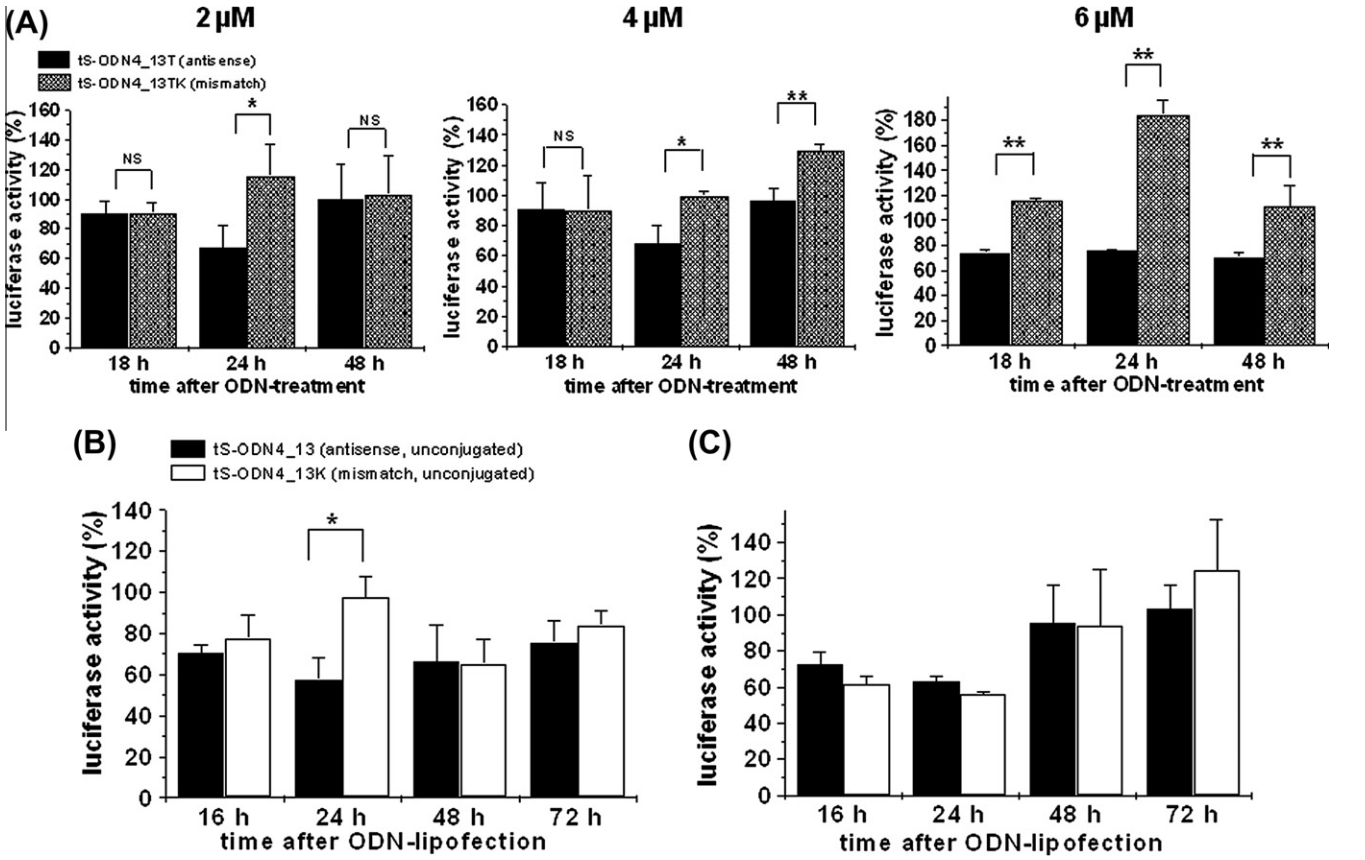

Fig. 7. (A) Inhibition of HCV5′NCR-dependent luciferase gene expression by taurocholate conjugated ODN in CCL13-OATP1B1 cells. (B) Inhibition of HCV5′NCR-dependent luciferase gene expression in HepG2-OATP1B1 (C) and CCL13-OATP1B1 cells by unconjugated antisense tS-ODN4_13. Unconjugated tS-ODN4_13 were transfected using lipofectamine. Luciferase activity was determined 16, 24, 48 and 72 h later. Mean and standard error are shown ($n = 3$).

occurrence of gastrointestinal symptoms or elevation of aminotransferases were observed (data not shown).

4. Discussion

In this study, we show that taurocholate conjugated antisense oligonucleotides (ODN) complementary to the 5′NCR of the HCV genome allow selective ODN uptake into liver-derived cells and specific inhibition of HCV translation indicating a novel strategy to improve antisense approaches with ODN for the control of HCV infection.

The highly conserved 5′NCR of the HCV genome necessary for the HCV translation/replication has been shown to be an ideal target for antisense approaches, such as antisense RNA, RNA interference (RNAi), ribozymes or ODN (Alt et al., 1995; Gonzalez-Carmona et al., 2006, 2011; Guerniou et al., 2007; Sen et al., 2003). Phosphorothioate modified ODN are the first generation of antisense drugs entering clinical trials for the treatment of patients with chronic HCV infection which show acceptable properties for drug development (McHutchison et al., 2006). In the past, we reported that a 23-base phosphorothioate ODN (tS-ODN4) complementary to the nucleotides 326–348 of HCV5′NCR was highly efficient in reducing viral translation *in vitro* (Alt et al., 1995). However, a major limitation of antisense technology – whether ODN, ribozymes or siRNA – is the tissue delivery. Phosphorothioate ODN are poorly incorporated into cells. Several strategies, such as liposomes, poly(ethylenglycol) (PEG)-conjugates, nanoparticles, electroporation, as well as cholesterol conjugates or cell-penetrating peptides, have been explored for tissue or cell delivery of antisense molecules. However, these compounds either carry considerable cytotoxicity or their tissue specificity remains limited (De Rosa and La Rotonda, 2009; Delogu et al., 2009; Oh and Park, 2009; Stewart et al., 2008). Conjugation of drugs as well as small peptide molecules and ODN by bile acid carriers has been described to augment oral bioavailability, uptake and selectivity of drug action, specifically in the liver (Lischka et al., 2003; Swaan et al., 1997; Barbara et al., 2006; Richards et al., 2006; Tolle-Sander et al., 2004). Moreover, bile acids appear to stabilize ODN against degradation (Petzinger et al., 1999). Thus, in this work, different bile acid conjugated phosphorothioate modified antisense ODN were generated and tested in order to achieve selective ODN uptake into the liver and more specific inhibition of HCV gene expression.

Reduction of the number of nucleotides can favor uptake of ODN in hepatocytes after conjugation with bile acids, while at the same time, shortened ODN can lose their inhibitory efficacy. Therefore, we analyzed several shortened ODN derived from the 23mer tS-ODN4 for their remaining antisense effects *in vitro*. Despite nucleotide reduction, a 17mer phosphorothioate modified ODN (tS-ODN4_13) complementary to nt 342–326 of the HCV genome achieved almost a complete block of the 5′NCR-dependent luciferase expression (95%). Moreover, tS-ODN4_13 was also effective in reducing HCV gene expression in Huh7 cells containing a subgenomic HCV replicon (Lohmann et al., 1999). This effect was HCV sequence-specific, since the mismatch ODN did not inhibit HCV translation. Further shortening of the 23mer tS-ODN4 considerably reduced the inhibitory capacity of ODN. The specific hybridization of single-stranded ODN to the complementary messenger RNA sequence by Watson–Crick base-pairing rules is a key mechanism of ODN blocking of the protein translation (Weiss et al.,

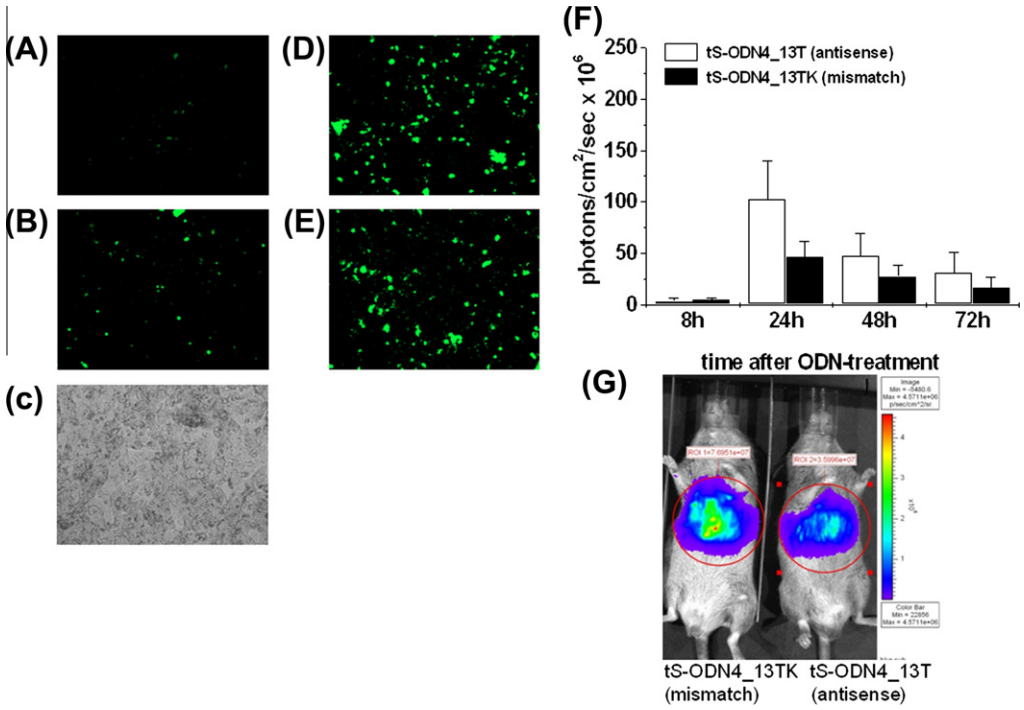

Fig. 8. Uptake of FITC-labeled ODN in primary hepatocytes. Fluorescence microscopy was performed 8 h after addition of 4 μM of FITC-coupled ODN to the hepatocytes. (A) Autofluorescence of primary hepatocytes, (B) detection of non-conjugated FITC-marked ODN, (C) light microscopy of primary hepatocytes after addition of taurocholate-coupled ODN, (D) detection of taurocholate conjugated FITC-marked ODN and (E) detection of taurocholate conjugated FITC-marked ODN incubated with 4 μM taurocholate (competition). The depicted images were made with 10× magnification. (F) Inhibition of HCV5′NCR-dependent luciferase gene expression by taurocholate conjugated ODN *in vivo*. 5×10^7 pfu/mouse Ad-NCRluc was injected i.v. Two hours later, 20 mg/kg body weight ODN was injected i.v. Luciferase activity was determined 8, 24, 48 and 72 h later. White bars: mismatch tS-ODN4_13TK; black bars: antisense tS-ODN4_13T. (G) Representative image of light emitted from two mice 24 h after i.v. injection of tS-ODN4_13TK (left) or tS-ODN4_13T (right).

1999). Therefore, longer antisense sequences of nucleotides display increased nucleotide binding capacity and are more specific to its target, which explains the lower inhibitory effects of shortened ODN from tS-ODN4. The 17mer ODN, tS-ODN4_13, was the least effective inhibitory sequence and was therefore chosen for further bile acid conjugation.

One of the most important findings of this work was to show that bile acid conjugation was feasible and did not affect the antisense capacity of ODN. While cholate and cholesterol conjugations induced only a slightly reduction of the inhibitory capacity of tS_ODN4_13, taurocholate conjugation exhibited no negative effects at all on the inhibitory capacity of the 17mer ODN, reaching similar antisense capacity and inhibition rates of HCV-translation (>90%) than the unconjugated ODN *in vitro*.

To demonstrate the antisense properties and the selective cell uptake of taurocholate conjugated ODN due to bile acid transport in cell culture, cell lines with an adequate expression of bile acid transporters are necessary. However, hepatoma cells are known to have a down-regulation of bile acid transports (Cui et al., 2003; Kullak-Ublick et al., 1996), explaining the poor uptake of FITC-marked taurocholate conjugated tS-ODN4_13 by Huh7 cells containing HCV replicons (not shown). The organic anion transporting polypeptides (OATPs) are a family of transporter proteins responsible for transmembrane transport of diverse endogenous and exogenous compounds. In contrast to other OATP members localized in a variety of tissues (liver, kidney, brain and intestine) OATP1B1 has been found selectively in hepatocytes (Kullak-Ublick et al., 1996; Tamai et al., 2000). It is the most important OATP for liver-specific bile salt uptake (Meier and Stieger, 2002; Trauner and Boyer, 2003) and was therefore chosen to generate stably transfected hepatoma cells for this study. For completion of the test system, an adenoviral vector (Ad-GFP-NCRluc) able to express the HCV-5′NCR sequence fused to the luciferase gene was generated. For this fusion construct, we previously demonstrated that expression of luciferase is related to IRES activity of HCV5′NCR providing a sensitive tool for quantification of the antisense effects towards HCV5′NCR (Alt et al., 1995). An adenoviral system offers a high transgene expression in hepatoma cells, allowing rapid screening and quantification of the 5′NCR-dependent HCV gene expression.

Using FITC-labeled taurocholate conjugated tS-ODN in OATP1B1-overexpressing HepG2-cells, we show that taurocholate conjugation can deliver ODN to liver-derived cells via bile acid transport. In fact, uptake of FITC-labeled taurocholate conjugated tS-ODN in OATP1B1-overexpressing HepG2 cells was about twice as high as in parental HepG2 cells with low or non-existent expression of bile acid transporters. Moreover, taurocholate conjugated tS-ODN were not taken up by non-hepatic cell lines, indicating selective entry of bile acid conjugated ODN only into liver-derived cells with sufficiently high bile acid transporter expression. These results are contrary to earlier findings of Petzinger et al. (1999), who showed that a bile acid conjugated 15mer ODN was not effectively taken up by hepatocytes. The observed moderate uptake of taurocholate conjugated ODN in wild type HepG2 cells as well as in HEK-293 and HT29 cells, suggested a possible contribution of other bile acid uptake transporters. Molecular evidence for the expression of both NTCP and different members of the OATP family in human hepatocellular carcinoma cells has been described (Kullak-Ublick et al., 1996). Furthermore, OATP transporters are expressed about of all in the gastrointestinal tract but also in the kidney, explaining the observations in HEK293 and HT29 cells (Kim, 2003; Kagedahl et al., 1997).

Confirming our hypothesis, the increased uptake of taurocholate coupled ODN in OATP1B1-expressing hepatoma cells was translated into an effective inhibition of the 5′NCRHCV-dependent

translation. By contrast, no decrease of luciferase activity was observed with unconjugated antisense ODN when ODN were used without transfection reagent. Moreover, taurocholate conjugation provided even a more efficient antisense effect on inhibiting HCV translation than use of traditional strategies for ODN-delivery, such as liposome-transfected ODN.

In order to evaluate the possible impact of taurocholate conjugation for the delivery of antisense ODN into the liver we analyzed the uptake of taurocholate ODN in primary human hepatocytes. As expected, FITC-taurocholate-conjugated ODN without any transfection reagent were taken up by a significant higher percentage of hepatocytes than non-conjugated ODN. Several *in vitro* and *in vivo* studies have been shown that bile acids are quickly taken up and secreted into bile again by hepatocytes. Therefore, the exact concentrations of bile acid to which the hepatocytes are exposed are not well-established. In this work, taurocholate-coupled FITC-marked ODN were highly taken up by hepatocytes even when incubated in the presence of low concentrations of taurocholate, which can be used as competitor for OATP transporter. These findings indicate the high efficiency of hepatocytes to take up bile acids and the potential of this approach as efficient carrier for delivering oligonucleotides to the liver.

One of the difficulties in the investigation of new approaches towards the hepatitis C virus is the absence of a suitable small animal model, and only chimpanzees can be used for this purpose. In this work, it was not the primary goal to test the approach *in vivo*. However, a pilot experiment in a mouse model was performed in order to evaluate toxicities of the taurocholate-coupled ODN. For the *in vivo* experiment, adenovirus expressing a HCV-luciferase fusion RNA was used in order to achieve a sufficient transcription of target RNA within the liver. Despite low concentrations of ODN, taurocholate coupled antisense ODN also exhibited a trend towards less expression of the 5′NCR-dependent luciferase activity in the liver, confirming the data in cell culture. However, standard deviations were high between the animals. This was probably due to a host immune response to viral proteins expressed by adenoviral vectors *in vivo*. Therefore, the findings in this artificial small mouse model should be carefully interpreted.

Nevertheless, this experiment provided an important proof-of-concept, namely that taurocholate conjugated antisense ODN have also the potential to inhibit HCV5′NCR-dependent luciferase gene expression. Most importantly, i.v.-application of taurocholate-conjugated ODN did not exhibit any apparent toxicity in the mice at the doses employed.

To our knowledge, HCV-specific antisense taurocholate conjugated phosphorothioate modified ODN have never been used before. Thus, direct comparison of the data obtained for the ODN used here with data from other works is rather difficult. We and others have reported wide differences in inhibitory potency of several antisense compounds in a variety of cell culture systems. In all these studies, inhibition rates of HCV-translation ranged from 50–80%. However, the tested antisense molecules were always introduced into the cells by using different transfection reagents (Alt et al., 1999; Chang et al., 2010) (Fukuma et al., 2003; Heintges et al., 2001). By contrast, in our study we reported about inhibition rates of >70% of HCV-translation by using antisense ODN without any transfection reagent. This inhibitory effect of tS-ODN4_13T was rather considerable since highly hepatotropic adenoviral vectors with a CMV immediate-early promoter achieved strong transgene luciferase levels which are probably much stronger than those found in patients with HCV infection. However, the real impact of such a strategy for the treatment of hepatitis C alone or in combination with current therapies should be tested in clinical trials. More interesting may be the combination with new approaches targeting HCV-promoting host cell factors such as cellular receptors or microRNAs (Lupberger et al., 2011; Lanford et al., 2010).

5. Conclusions

Thus, the results of these experiments are an important proof-of-concept because they indicated that taurocholate conjugation of antisense ODN improves the selective cell uptake and the efficacy of antisense nucleotides in liver-derived cells. Concretely, the tested taurocholate conjugated 17mer ODN complementary to HCV5′NCR showed a selective uptake by liver-derived cells through OATP-mediated transport resulting in specific and effective inhibition of HCV gene expression. This new approach may overcome the challenges derived from use of viral methods or invasive systems such as liposomes for cell delivering of antisense molecules and may represent therefore a promising strategy for the control of hepatitis C infection.

Disclosure

The authors of this study do not have anything to disclose regarding funding from industry or conflict of interest with respect to this manuscript.

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