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Liver X receptors agonists impede hepatitis C virus infection in an Idol-dependent manner

Jing Zeng, Yang Wu, Qingjiao Liao, Lixia Li [1], Xinwen Chen, Xulin Chen *

State Key Laboratory of Virology, Wuhan Institute of Virology, Chinese Academic of Sciences, Wuhan, Hubei 430071, PR China

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ABSTRACT

Hepatitis C virus (HCV) is a major human pathogen that causes many serious diseases, including acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma. Treatments for this virus are inadequate, and improved antiviral therapies are necessary. Although the precise mechanisms regulating HCV entry into hepatic cells are still unknown, the low-density lipoprotein receptor (LDLR) has been shown to be essential for entry of infectious HCV particles. Liver X receptors (LXR) were recently reported to control LDLR expression through the regulation of the expression of the Idol (inducible degrader of the LDLR) protein, which could trigger the ubiquitination and degradation of LDLR. In this study, we analyzed the antiviral effect of Idol in vitro. The results demonstrated that Huh7.5.1 cells that exogenously expressed Idol were resistant to HCV infection. Next, the treatment of HCV-infected Huh7.5.1 cells with either synthetic LXR agonists (GW3965 or T0901317) or the natural LXR ligand 24(S),25-epoxycholesterol inhibited HCV infection in a dose-dependent manner. Furthermore, a combination of LXR agonists and HCV RNA replication inhibitors exerted additive effects against HCV, as revealed by isobologram analysis. In conclusion, our data indicate that molecules such as LXR agonists, which could stimulate the expression of Idol, represent a new class of potential anti-HCV compounds, and these compounds could be developed for therapeutic use against HCV infection, either as a monotherapy, or in combination with other anti-HCV drugs.

1. Introduction

Approximately 170 million people worldwide are estimated to be infected with hepatitis C virus (HCV), which is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Lauer and Walker, 2001). No vaccines to date have been effective in preventing HCV infection, and HCV vaccines will continue to be difficult to develop because of the different genotypes of the virus and its ability to change, or mutate, during infection. The current standard of care for HCV patients is a combination of pegylated interferon plus ribavirin, and recently boceprevir and telaprevir, which are HCV protease inhibitors, were approved for the treatment of chronic hepatitis C genotype 1 infection. Sustained virological response (SVR) rates approaching 75% can now be expected for genotype 1 patients who take the combination therapy of pegylated interferon, ribavirin and an HCV protease inhibitor (Nelson, 2011). However, despite several pharmaceuticals targeting the viral nonstructural proteins NS3/4A and NS5B that are in clinical trials or in clinical use (Manns et al., 2007), concerns remain regarding the toxicity of these compounds and the development of antiviral resistance to these drugs (Shimakami et al., 2009; Susser et al., 2011). Hence, new therapeutic strategies are urgently required to develop more new drugs acting on different targets and to treat patients who do not respond to the current standard of care.

The rapidly increasing understanding of the HCV entry process has led to a growing appreciation for the potential of HCV entry as a target for further antiviral therapies. However, the initial steps of HCV infection within target cells, primarily human hepatocytes, are not completely understood. Several cellular proteins have been identified as potential candidates for HCV entry receptors, including the low-density lipoprotein receptor (LDLR) (Agnello et al., 1999; Monazahian et al., 1999), the scavenger receptor B type I (SR-B I) (Scarselli et al., 2002), CD81 (Pileri et al., 1998), claudin-1(Evans et al., 2007), occludin (Liu et al., 2009), the C-type lectins L-SIGN and DC-SIGN (Lozach et al., 2004; Pöhlmann et al., 2003), heparin sulfate (Barth et al., 2003), and the asialoglycoprotein receptor (Saunier et al., 2003). Recently, a few cellular factors, including phospholipid scramblase 1 (Gong et al., 2011), cholesterol-uptake receptor Niemann-Pick C1-like 1 (NPC1L1) (Ray, 2012), epidermal growth factor receptor (EGFR) and Ephrin receptor A2 (EphA2) (Lupberger et al., 2011), were identified as novel HCV co-receptors. Among those factors, expression of human CD81 and occludin is

* Corresponding author. Address: Wuhan Institute of Virology, Chinese Academy of Sciences, 44 Xiao Hong Shan Zhong Qu, Wuchang District, Wuhan, Hubei 430071, PR China. Tel.: +86 27 87198772; fax: +86 27 87198466.
E-mail address: chenxl@wh.iov.cn (X. Chen).
[1] Current address: Institute of Physiological Chemistry and Pathobiochemistry, University of Muenster, Muenster, Germany.

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sufficient to allow HCV infection of fully immunocompetent inbred mice (Dorner et al., 2011).

The HCV particle is known be complex with very low-density lipoproteins (VLDL). This association with VLDL allows the virus to bind hepatocyte via the lipoprotein receptor-LDLR (Agnello et al., 1999). Nascent VLDL particles released into plasma are not ligands for LDLR. However, the VLDL can be converted to intermediate density lipoproteins (IDL) upon processing by lipoprotein lipase which can inhibit HCV infection in vitro (Andréo et al., 2007). The IDL can be further converted to LDL (low density lipoproteins) by hepatic lipase. The IDL and LDL associated forms of HCV particles can be taken up by LDLR in hepatic cells (Chappell and Medh, 1998). An anti-LDLR antibody inhibited the binding and/or internalization of HCV from infected sera by at least 60% (Germi et al., 2002). In addition, an excess of LDL, also inhibited the binding and/or internalization of HCV to the same extent. In agreement with these results, HCV RNA in highly infectious serum was predominantly found in fractions with low density (1.06 g/mL), which represent densities of VLDL/LDL, whereas HCV RNA in less infectious plasma was found in fractions with higher density (1.17 g/mL), which represent densities of lipoprotein-free particles (Nielsen et al., 2006; Petit et al., 2005). Since LDLR controls the uptake of circulating cholesterol principally through the internalization of lipoproteins, LDLR has significant implications as a co-receptor for HCV entry.

The liver X receptors, LXRα and LXRβ, belong to the super family of nuclear hormone receptors, and they are ligand-activated transcription factors by binding to the LXR elements of target genes (Lewis and Rader, 2005). Recently, a newly identified transcriptional target of the liver X receptors (LXR), the E3 ubiquitin ligase Idol (inducible degrader of the LDLR), has been demonstrated to post-transcriptionally regulate the LDLR expression level (Zelcer et al., 2009). Idol, also known as Mir or Mylip, was originally identified as a protein with a FERM (4.1 band, ezrin, radixin and moesin) homology domain at the N-terminus and a RING Zinc finger ubiquitin ligase domain at the C-terminus (Bretscher et al., 2002). Idol triggers the ubiquitination of the LDLR on its cytoplasmic domain, which targets it for degradation. LXRs are involved in the regulation of cholesterol and lipid metabolism, and target genes such as ATP binding cassette transporter A1 (ABCA1) and ABCG1 promote the efflux of cellular cholesterol and maintain whole body sterol homeostasis (Costet et al., 2000; Sabol et al., 2005). Oxidized cholesterol derivatives (oxysterols) were identified as natural LXR agonists (Janowski et al., 1996). Several synthetic agonists have also been reported, such as (3-{3-[(2-chloro-3-trifluoromethyl-benzyl)-(2,2-diphenyl-ethyl)-amino]-propoxy}-phenyl)-acetic acid (GW3965) and *N*-(2,2,2-trifluoro-ethyl)-*N*-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phe-nyl]-benzenesulfonamide (T0901317).

In the current study, we demonstrated that the stimulation of Idol expression via an eukaryotic expression vector in susceptible Huh7.5.1 cells blocks infection by the HCV strain JFH1. Furthermore, both synthetic and natural LXR agonists exert a potent Idol-dependent inhibitory effect on HCV entry. These results suggest that the LXR-Idol-LDLR pathway may be amenable for interventions and serve as a novel target for anti-HCV drug development.

2. Materials and methods

2.1. Cell culture

Huh7.5.1 human liver cells (kindly provided by Dr. F.V. Chisari) were cultured in Dulbecco's modified Eagle's media (DMEM, GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS, Gibco). The SGR-JFH1-Luc-5AGFP stable replicon cell line (derived from Huh7.5.1 cells) was maintained in DMEM supplemented with 10% FBS and 500 μg/mL of geneticin (G418, Calbiochem). All cells were grown at 37 °C in 5% CO_2.

2.2. Agents, antibodies and plasmids

The synthetic LXR agonists (GW3965 and T0901917), cyclosporine A (CsA) and MK-7009 were purchased from Sigma–Aldrich. The endogenous LXR agonist 24(S),25-epoxycholesterol (24,25EC) was purchased from Enzo Life Sciences. The antibodies used in this study were rabbit polyclonal antibodies against mylip (Abcam), LDLR (Caymam), SR-B1 (Novus) and mouse monoclonal antibodies against NS3 (kindly provided by Prof. Chenyang Li), CD81 (BD Pharmingen), Flag (Sigma and Beyotime), and GAPDH (Thermo). The eukaryotic expression plasmid pFLAG-CMV2-Idol was generated to express Idol with an N-terminal flag tag. The cDNA fragment containing the Idol open reading frame was amplified with the following primers: 5′-CGAAGCTTATGCTGTGTTATGTGAC GAG-3′ (forward), 5′-CCCGAATTCTTAGATTACAGTCAGATTGAG-3′ (reverse). The cDNA fragment was amplified by PCR from Huh7.5.1 cDNA and then cloned into the HindIII and EcoRI sites of the pFLAG-CMV2 plasmid (Sigma).

2.3. HCV RNA synthesis, transfection, and preparation of HCV viral stock

Infectious HCV virus was produced in hepatocytes as previously described (Wu et al., 2011). Briefly, the linearized plasmids pJFH1 (a kind gift from Takaji Wakita) and pJFH1-Luc-5AGFP (Wu et al., 2011) were used as templates for in vitro RNA transcription using a MEGAscript™ T7 kit (Ambion) according to the manufacturer's protocol. Huh7.5.1 cells were electroporated with the synthesized HCV RNA (10 μg), and virus containing supernatants were collected when an obvious cytopathic effect was observed. The multiplicity of infection (MOI) was calculated on the basis of virus titer as measured by focus forming U/mL.

2.4. Effect of the expression of an exogenous human Idol gene on HCV infection in Huh7.5.1 and on HCV replication in SGR-JFH1-Luc-5AGFP cells

Huh7.5.1 cells were transfected with the pFLAG-Idol plasmid using lipofectamine™ 2000, with the pFLAG-CMV2 plasmid serving as a control. At 48 h post-transfection (hpt), cells were infected with either the HCV virus JFH1-Luc-5AGFP or JFH1 at an MOI of 0.2. At 8 h post-infection (hpi), the medium was replaced with fresh complete DMEM, and the cells were maintained for 3 days before being used for the measurements. To measure the production of infectious virions, supernatants from cells infected with JFH1-Luc-5AGFP were used to re-infect the naïve Huh7.5.1 cells. At 72 hpi, the cells were subjected to a luciferase assay. A similar procedure was used for the SGR-JFH1-Luc-5AGFP cells. The cells were transfected with different concentrations of the pFLAG-Idol plasmid, and the pFLAG-CMV2 plasmid served as a control. At 48 hpt, cells were processed for fluorescence microcopy analysis, luciferase assays and Western blotting analysis.

2.5. Effect of the treatment of LXR agonists on HCV infection in Huh7.5.1 and on HCV replication in SGR-JFH1-Luc-5AGFP cells

Huh7.5.1 cells were treated with the specified concentrations of LXR agonists (GW3965, T0901917 or 24,25EC) for 4 h and then infected with either the JFH1-Luc-5AGFP or JFH1 virus at an MOI of 0.2. At eight hpi, cells were incubated in fresh complete DMEM containing the same concentration of agents as the pretreatment

group, and the cells were used for measurements at 72 hpi. To measure the production of infectious viruses, the diluted supernatants of those cells infected with JFH1-Luc-5AGFP were used to reinfect naïve Huh7.5.1 cells. At 4 hpi, fresh medium was supplied. The cells were then subjected to a luciferase assay at 72 hpi. To test the inhibitory effects of LXR agonists on the HCV replicon system, SGR-JFH1-Luc-5AGFP cells were incubated with the specified concentrations of LXR agonists for 48 h and then subjected to detection.

2.6. Western blotting

The cells were lysed in RIPA lysis buffer and boiled for 10 min before separation by SDS–PAGE. The resolved proteins were electrotransferred to immobilon-P polyvinylidene difluoride membranes (Millipore). The membranes were blocked for 1 h in Tris-buffered saline (TBS)–5% skim milk, followed by incubation with primary antibodies overnight at 4 °C. The membranes were then washed with TBS-0.1% Tween-20 and incubated with HRP-conjugated goat anti-mouse or anti-rabbit antibodies (Thermo). The signals were detected using the Super signal West Pico chemiluminescent substrate (Pierce) and an AlphaEase FC Imaging System (Alpha Innotech Corporation).

2.7. Flow cytometry analysis

After trypsinzation, transfected Huh7.5.1 cells were fixed with Formalin Solution (formaldehyde 4%, Sigma) and blocked with paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4)–3% BSA. Cells were then incubated 1 h at 4 °C with antibodies. After rinsing with PBS, cells were incubated with FITC labeled secondary antibody for 45 min at 4 °C, then washed with PBS. Cells stained only with the secondary antibody were used as negative control. Labeled cells were analyzed using a FACS Beckman EPICS ALTRA2.

2.8. Fluorescence microcopy analysis

Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min, permeabilized with 0.3% Triton X-100 in PBS for 30 min and stained with 1 μg/mL of diamidino-2-phenylindole (DAPI, Sigma–Aldrich). Cells were washed twice, and the fluorescence was detected by an inverted fluorescence microscope (Zeiss Axio Observer A1).

2.9. Indirect immunofluorescence assay

Cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After being washed three times with PBS, cells were incubated in blocking buffer (PBS containing 3% BSA, 0.3% Triton X-100 and 10%FCS) for at least 30 min and then in binding buffer (PBS containing 3% BSA and 0.3% Triton X-100) with MAbs against Flag (Beyotime) at a dilution of 1:100 for 1 h at room temperature. After three washes with PBS, cells were incubated with TRITC-conjugated goat anti-mouse IgG at a 1:100 dilution with binding buffer for 1 h at room temperature. Cell nuclei were stained by DAPI. Stained samples were then examined with a Leica TCS SPII confocal microscope.

2.10. Luciferase assay

Cells were harvested and subjected to a luciferase assay using the Renilla luciferase assay system (Promega) according to the manufacturer's instructions. Briefly, cells were washed twice with PBS and then lysed with 1× luciferase cell culture lysis buffer in each culture vessel (20 μL/well for a 96 well plate or 100 μL/well for a 24 well plate). Aliquots of cell lysates (20 μL) were mixed with 100 μL of luciferase assay reagent, and the luciferase activity was determined using the GloMax®20/20 detection system (Promega).

2.11. Cytotoxicity assay

The Huh7.5.1 cells were plated in 96-well plates at a density of 8×10^3 cells/well. Twenty-four hours later, the culture were replaced with complete DMEM containing serially diluted compounds and then maintained for 72 h. Cytotoxicity was evaluated using the CellTiter-Glo luminescent cell viability assay (Promega). CellTiter-Glo (100 μl) reagent was added to the cells and incubated for 10 min at room temperature. The luminescent signal was recorded using an EnVision (Perkin-Elmer) microplate reader.

2.12. Drug combination studies

The combination study between LXR agonists and HCV RNA replication inhibitors (MK-7009 or CsA) was evaluated by luciferase assay as described above. Exponentially growing cells were seeded in 96 well plates at densities of 8×10^3 cells/well and allowed to attach for 24 h. Cells were exposed to serially diluted concentrations (three replicates) of HCV RNA replication inhibitors (MK-7009 or CsA) and GW3965 either alone or in combination. Four hours after treatment with the agents, cells were infected with the reporter virus JFH1-Luc-5AGFP and incubated for 72 h. The extent of the effect of the combination treatment was analyzed by the isobole method (Berenbaum, 1989). Fractional inhibitory concentrations (FICs) are calculated by dividing the EC_{50} of drug A with a fixed overlay of drug B by the EC_{50} of drug A alone (the *x*-coordinate). The *y*-coordinate is the fixed concentration of drug B divided by the EC_{50} of drug B alone. The FIC index is the sum of a pair of FICs. Conventions have been adopted that define a FIC index of $\leqslant 0.5$ as synergy, a FIC index of $\geqslant 4.0$ as antagonism, and a FIC index between 0.5 and 4.0 as an additive effect (Odds, 2003).

3. Results

3.1. Over-expression of Idol decreases the LDLR expression level in Huh7.5.1 cells

We initially verified whether the addition of exogenous Idol expression would reduce LDLR expression in Huh7.5.1 cells, which are highly permissive cells for HCV infection. To introduce exogenous Idol, an expression vector for N-terminally flag tagged Idol was transfected into Huh7.5.1 cells. As shown in Fig. 1, the LDLR protein level was reduced in a dose-dependent manner in correlation with the increase of expression of either the Idol-FLAG fusion protein or total intracellular Idol. To examine whether the effect of Idol was selective on LDLR, we analyzed the expression levels of another HCV co-receptor SR-B I. Similar to LDLR, SR-B I is also involved in cholesterol transportation. Hepatic SR-B I is essential for the delivery of cholesterol esters from HDL to hepatocytes. There was no visible loss of SR-B I expression in the Idol expressing cells compared with cells transfected with the control vector (Fig. 1A). The expression levels of the two tight junction proteins claudin-1 and occludin, which are also HCV co-receptors, were also not affected by the over-expression of Idol in Huh7.5.1 cells (data not shown). We further examined the effect of idol expression on LDLR cell surface expression levels. FACS analysis revealed that there was an obvious loss of LDLR in idol transfected cells when compared with cells transfected with the control vector. Whereas, the expression of both cell surface SR-B I and CD81 were not affected by the over-expression of Idol (Fig. 1B). In sum, our data

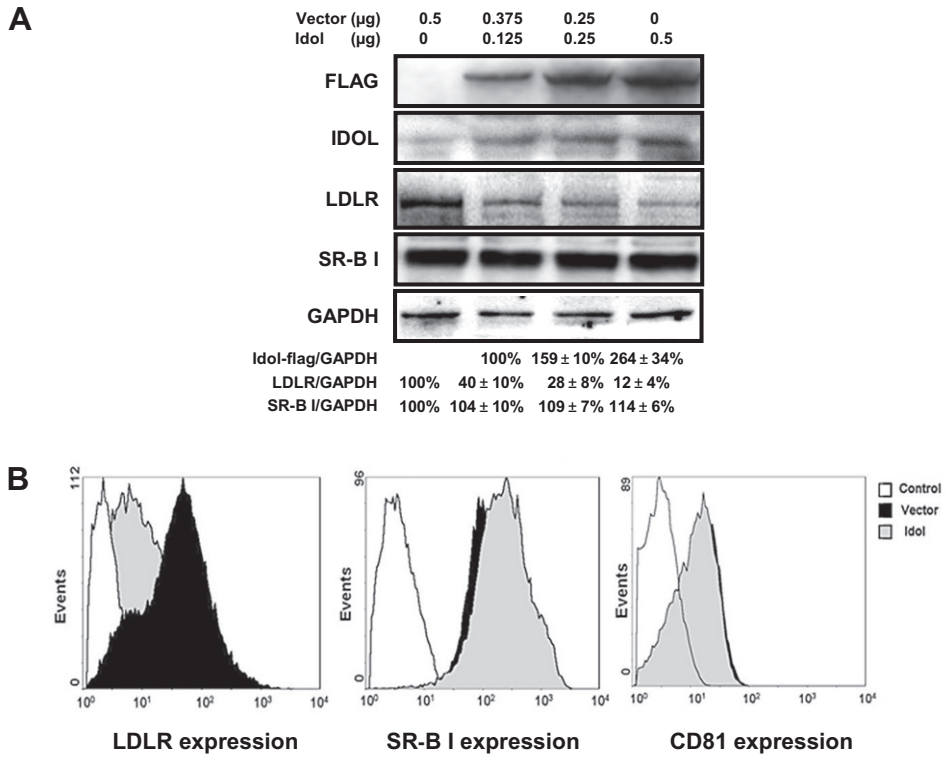

Fig. 1. Over-expression of Idol decreases LDLR expression level in Huh7.5.1 cells. (A) Huh7.5.1 cells were transfected with increasing amounts (0, 0.125, 0.25, 0.5 μg) of a plasmid expressing exogenous Idol tagged with flag. Forty-eight hpt, cells were harvested for Western blot analysis. Both the exogenous Idol and the intracellular Idol were detected using an anti-flag monoclonal antibody or anti-Idol polyclonal antibody, respectively. These are representative data of at least three independent experiments. (B) Huh7.5.1 cells were transfected with plasmid expressing exogenous Idol tagged with flag or control vector. Forty-eight hpt, cells were used for FACS analysis. Cell surface expression of LDLR, SR-B I and CD81 was analyzed by FACS using antibodies against LDLR, SR-B I and CD81, and secondary antibody conjugated with FITC. These are representative data of three independent experiments.

indicate that Idol specifically down-regulates the expression level of the key receptor LDLR that is necessary for productive HCV infection in Huh7.5.1 cells.

3.2. Over-expression of Idol inhibits HCV infection in the HCVcc system

Since exogenous Idol expression leads to decreased expression of LDLR, we determined whether the over-expression of Idol inhibits HCV infection in vitro. Huh7.5.1 cells that were transfected with either the Idol expression vector or the control plasmid pFLAG-CMV were infected with the HCV dual reporter virus JFH1-Luc-5AGFP (Wu et al., 2011) at an MOI of 0.2. Because the dual reporter gene expression levels of this virus were correlated with the viral replication level, we detected both GFP fluorescence intensity and hRluc activity 3 dpi to evaluate the level of HCV replication. As shown in Fig. 2A, the GFP fluorescence intensity was detected to visualize viral replication in situ. The expression of Idol was detected by indirect immunofluorescence assay. The foci of NS5A-GFP dramatically reduced in the cells transfected with Idol-expressing plasmid compared with cells transfected with the control plasmid. Actually at the dosage of the transfection (0.5 μg Idol-expressing plasmid) we used, almost all of the cells expressing Idol had no foci expressing NS5A-GFP, indicating that HCV infection is almost completely inhibited specifically in cells that overexpress Idol. As shown in Fig. 2B, the level of hRLuc activity was decreased in a dose-dependent manner in correlation with the increase of Idol expression, indicating that the over-expression of Idol severely disrupted viral replication. No significant changes in cell proliferation were observed when cells were transfected with different ratios of the Idol expression vector and the control vector, suggesting that the inhibitory effect was not a result of cytotoxicity caused by the over-expression of Idol. The supernatants from the transfected Huh7.5.1 cells were analyzed by infecting naïve Huh7.5.1 cells to determine the production of infectious viruses. Idol expression strongly impeded the secretion or infectivity of HCV JFH1-Luc-5AGFP particles in a dose-dependent manner (Fig. 2C). Next, we confirmed the finding that Idol inhibits HCV infection of the wild-type JFH1 virus. As shown in Fig. 2D, the levels of the non-structural HCV protein NS3 were decreased in a dose-dependent manner in JFH1-infected Huh7.5.1 cells that expressed exogenous Idol compared with cells transfected with the empty vector. These results suggest that the over-expression of Idol strongly inhibits HCV infection in the HCVcc system.

3.3. Over-expression of Idol does not affect HCV RNA replication in the replicon system

To investigate whether Idol expression plays a role in regulating HCV replication by a mechanism other than inhibiting entry, HCV replication was examined using the SGR-JFH1-Luc-5AGFP stable replicon cell line established by our laboratory (unpublished). The cell line was derived from the Huh7.5.1 cell line by transfecting the cells with a subgenomic replicon of HCV containing the HCV non-structural proteins (from NS3 to NS5B) and two reporter genes. One of the reporter genes is a humanized Renilla luciferase gene (hRluc), which is inserted downstream of the HCV 5′ NTR, and the other is enhanced green fluorescent protein gene (EGFP), which is fused to the NS5A protein, and both reporters facilitate the detection of HCV replication. The SGR-JFH1-Luc-5AGFP cells were transfected with increasing concentrations of the Idol expression vector, and then, the intracellular levels of HCV replication were measured at 2 dpt. Both the hRluc activity and GFP fluorescence intensity were not significant altered by increased Idol expression (Fig. 3A and Supplementary Fig. 1). Western blot analysis

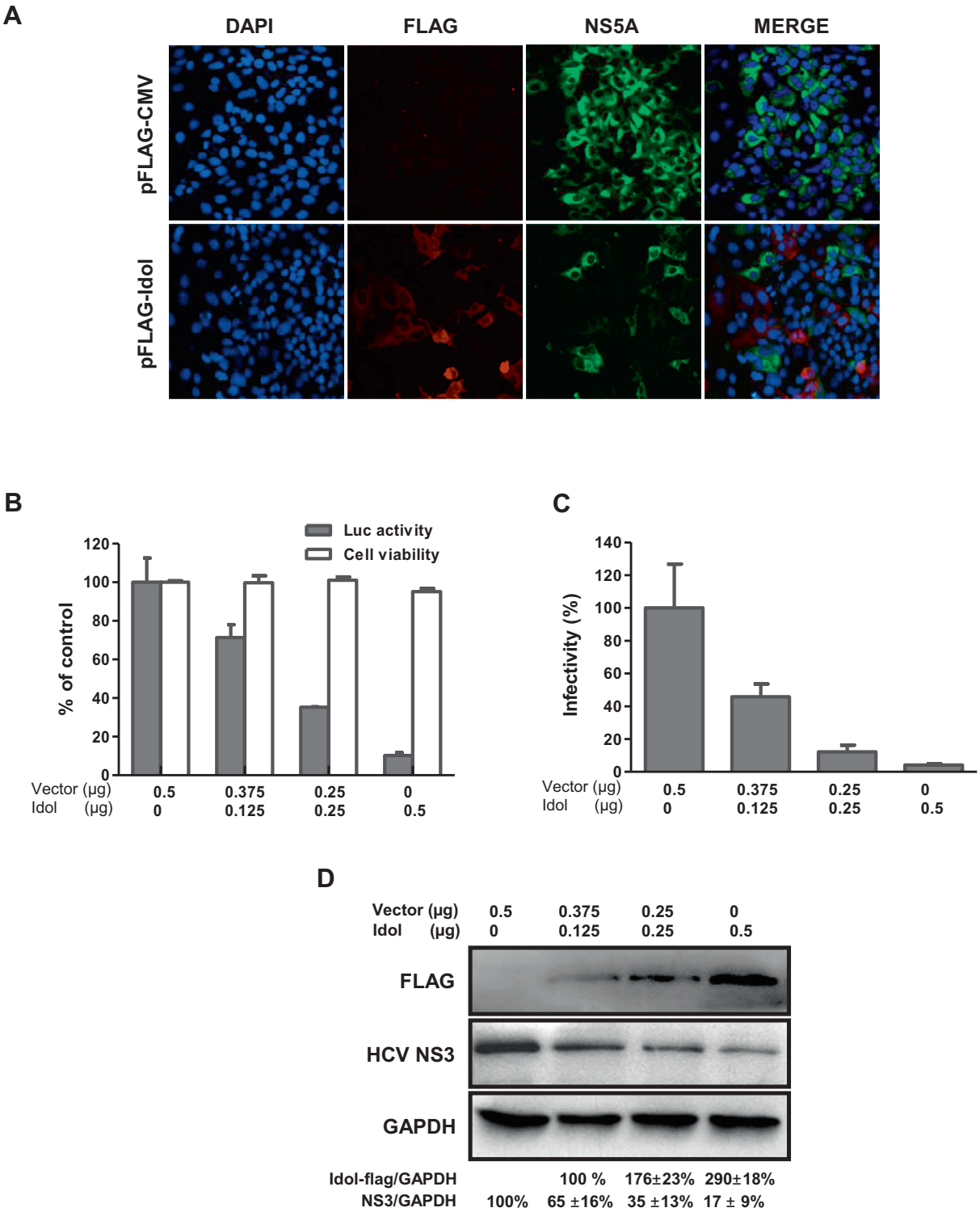

Fig. 2. Over-expression of Idol inhibits HCV infection in a dose-dependent manner. (A) Huh7.5.1 cells were transfected with 0.5 μg the Idol expression plasmid. Two days later the cells were infected with the dual reporter virus JFH1-Luc-5AGFP. Seventy-two hpi, cells were used for indirect immunofluorescence assay. NS5A-GFP (green signal) indicated foci of infected cells. Flag (red signal) indicated the expression of exogenous Idol. The nuclei were stained with DAPI (blue signal). Representative fields are shown. (B) Huh7.5.1 cells were transfected with increasing amounts of the Idol expression plasmid. Two days later the cells were infected with the dual reporter virus JFH1-Luc-5AGFP. Seventy-two hpi, cells were used for luciferase assay. Cell viability tested by the CellTiter-Glo luminescent assay and luciferase activity (RLU) were calculated relative to Huh7.5.1 cells transfected with the control vector, which were arbitrarily set to 100%. Results are expressed as the mean ± standard deviations (error bars) for three independent experiments. (C) Supernatants of cells in B were used to inoculate naïve Huh7.5.1 cells. After 8 h of incubation, the media were replaced. Mean values and the standard deviation of three independent experiments are shown. (D) The same transfection procedure was performed as described in B, except that the wild-type JFH1 virus was used in the infection procedure. Then, the cells were used for Western blot analysis.

confirmed these observations by showing that NS3 levels were not altered by increased Idol expression after normalization of the proteins with the levels of GAPDH (Fig. 3B). These results suggest that Idol expression does not affect HCV RNA replication in this replicon system.

3.4. Synthetic LXR agonists decrease LDLR expression in Huh7.5.1 cells

GW3965 and T0901317 (Fig. 4A) are two non-steroid synthetic LXR agonists that are commonly used in experimental studies. T0901317 activates both LXRα and LXRβ, but GW3965 has a greater affinity for LXRβ than for LXRα. Peter Tontonoz et al. demonstrated that synthetic LXR agonists (GW3965 and T0901317) decreased the levels of the LDLR protein in HepG2 cells by binding to the LXR elements in the Idol gene promoters and inducing the transcription of Idol (Zelcer et al., 2009). We tested the ability of LXRs to modulate the LDLR protein levels in Huh7.5.1 cells, the susceptible cell line for HCV infection. The cells were treated with GW3965 or T0901317 at the specified doses for 12 h, and then, the Huh7.5.1 cells were harvested for Western blot analysis. As shown in Fig. 4B, levels of the Idol protein were increased after treatment with LXR agonists. There was also a reduction of the HCV receptor LDLR in a dose-dependent manner. Notably, the level of LDLR was reduced to an almost undetectable level when T0901317 was used at a concentration of 1 μM. However, the LXR agonists had no significant effects on the expression of SR-B I, CD81, claudin-1 and occludin, which suggested that the effect on LDLR is specific. Treatment of the Huh7.5.1 cells with both GW3965 and T0901317 showed no toxicity at doses used in this study (Fig. 4C).

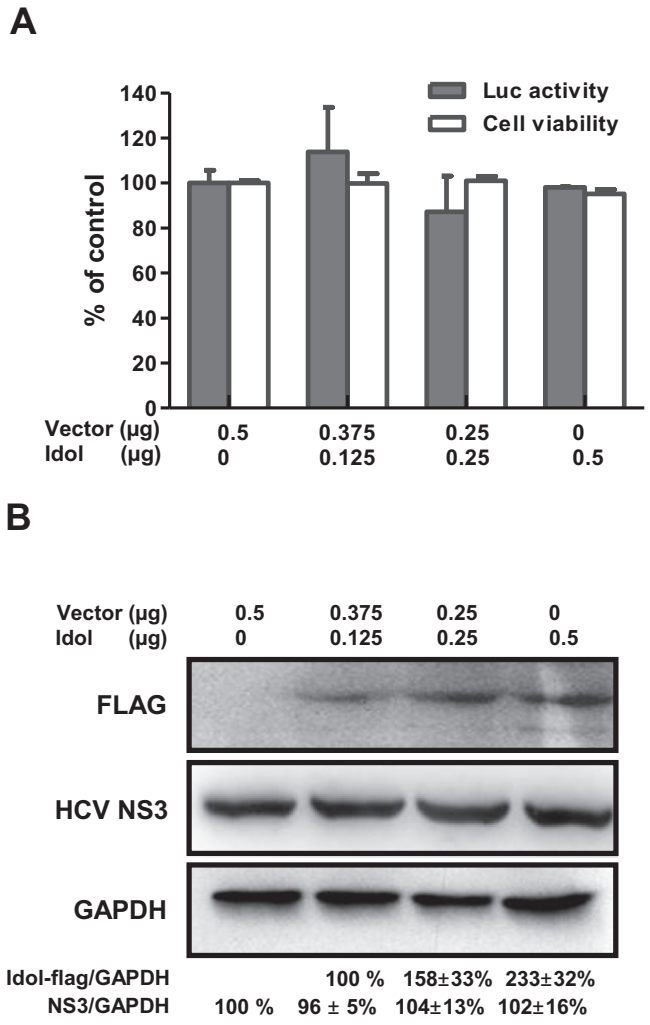

Fig. 3. Over-expression of Idol does not affect HCV RNA replication in a replicon system. SGR-JFH1-Luc-5AGFP cells were transfected with increasing amounts of the plasmid expressing exogenous Idol tagged with flag. Forty-eight hpt, cells were used for cell viability assay and luciferase assay (A) or Western blot analysis (B). Over-expression of Idol showed no effect on HCV RNA amplification in this replicon system.

3.5. Synthetic LXR agonists block HCV entry but do not interfere with HCV RNA replication

In addition to its effect on LDLR degradation by increasing Idol expression, LXRs control the expression of some other genes involved in the pathways of cholesterol and fatty acid metabolism (Beltowski, 2008). As the elements of the cholesterol and fatty-acid-biosynthetic pathways are required for HCV RNA replication (Kapadia and Chisari, 2005), LXR agonists might have a different activity (inhibitory/stimulatory) on distinct phases of the viral replication cycle. To determine whether treatment with LXR agonists would interfere with HCV infection, Huh7.5.1 cells were first inoculated with the dual reporter virus JFH1-Luc-5AGFP at an MOI of 0.2 in the presence of GW3965 or T0901317. At seventy-two hpi, the cells were used for measurements. As shown in Fig. 5A, treatment of the cells with LXR agonists decreased intracellular hRLuc activity in a dose-dependent manner. The foci of NS5A-GFP were also decreased after GW3965 or T0901317 treatment (Supplementary Fig. 2). Furthermore, the infectious virus that was released from the cells was assayed by re-infection of naïve Huh7.5.1 cells with the supernatants, and a dose-dependent inhibition of infection was observed (Fig. 5B). The EC_{50} value of the GW3965 inhibition of HCV infection was 121 nM and the CC_{50} was 39.3 μM, resulting in a selective index of 325. The values of EC_{50} and the CC_{50} of T0901317 were 164 nM and 18.9 μM, respectively, resulting in a selective index of 115. To exclude the possibility that the reporter genes may interfere with the antiviral responses to the drug treatment, the two LXR agonists were tested on Huh7.5.1 cells infected with the wild-type JFH1 virus, and a dose-dependent reduction of NS3 protein expression was observed by immunoblot analysis. Thus, our data strongly demonstrate that the synthetic LXR agonists GW3965 and T0901317 significantly decrease HCV infectivity.

The decrease of HCV infectivity after LXR agonist pretreatment could be due to an effect either on JFH1 RNA replication or on JFH1 viral entry since the stimulation of the LXR pathway can activate multiple elements which could affect the HCV infection cycle. To exclude the possibility that the synthetic LXR agonists might affect HCV RNA replication, the SGR-JFH1-Luc-5AGFP cells were treated with different doses of GW3965 or T0901317, and the intracellular levels of HCV replication were measured 2 days post-treatment. Compared with interferon-alpha, which inhibits HCV RNA replication as measured by luciferase assay (Fig. 6A), both GW39655 and T0901317 showed no effects on HCV RNA replication (Fig. 6A and B). Our data are consistent with previous findings that T0901317 had no effect on HCV RNA replication (Kapadia and Chisari, 2005). To further confirm the inhibition of virus entry by LXR agonists, we administered GW3965 and T0901317 at different time intervals during the early phase of infection. As shown in Fig. 6C, LXR agonists are only effective when used shortly before (−4 to 0 h), or during infection (0–4 h) in decreasing HCV infectivity. After the cell is fully infected (4–24 h after infection), as predicted, application of the LXR agonists have no effects. Taken together, these results suggest that LXR agonists only affect the entry process. Notably, addition of the drug shortly before the infection followed by withdrawl exactly before the inoculation, the virus infectivity was decreased, suggesting that LXR agonists act on the cell rather than the virion.

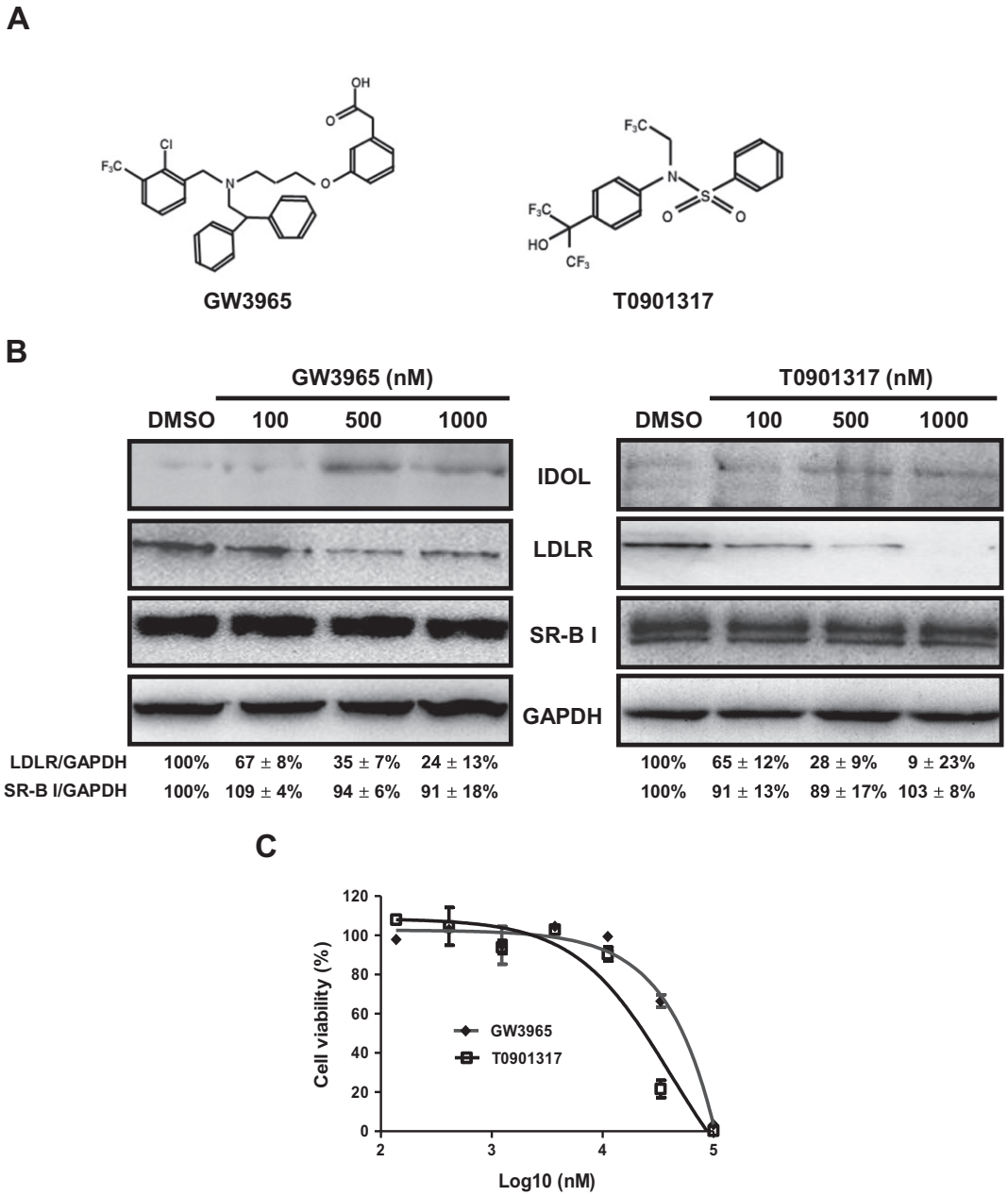

Fig. 4. Synthetic LXR agonists decrease the LDLR expression level in Huh7.5.1 cells. (A) Chemical structure of GW3965 (left) and T0901317 (right). (B) Huh7.5.1 cells were plated and treated with the specified amounts of GW3965 (left) or T0901317 (right) for 12 h and then harvested for Western blot analysis of the expression of Idol, LDLR and SR-B I. (C) The cytotoxicity of GW3965 or T0901317 in Huh7.5.1 cells was determined by the CellTiter-Glo luminescent assay. All the results are representative of at least three independent experiments.

In sum, these results suggest that synthetic LXR agonists impede HCV infection primarily through an inhibition of viral entry. The agonists inhibit HCV viral entry via a signaling-dependent down-regulation of LDLR expression. However, the synthetic LXR agonists do not interfere with other processes of HCV lifecycle.

3.6. The natural LXR agonist 24(S),25-epoxycholesterol inhibits HCV infection

Oxysterols are thought to be natural ligands for LXR. There is now compelling evidence that endogenous 24(S),25-epoxycholesterol (24,25EC) that is produced in a shunt of the same mevalonate pathway that produces cholesterol is a bona fide LXR ligand (Wong et al., 2008). Therefore, we tested whether the natural LXR agonist 24,25EC could also inhibit the infectivity of HCV. As expected, 24,25EC caused a dose-dependent inhibition of HCV replication and the infectivity (Fig. 7B). The EC_{50} of 24,25EC on HCV infection was 233 nM. There was no obvious cell toxicity when Huh7.5.1 cells were treated with 24,25EC at concentrations up to 10 μM (data not shown), resulting in a selective index over 43. This result strengthens our conclusion that the stimulation of the liver X receptors leads to an inhibition of HCV infection.

3.7. LXR agonists have an additive effect on HCV infection in combination with MK-7009 and CsA

Since synthetic LXR agonists strongly inhibited HCV infectivity, the LXR could be a potential target for anti-HCV drugs. Combina-

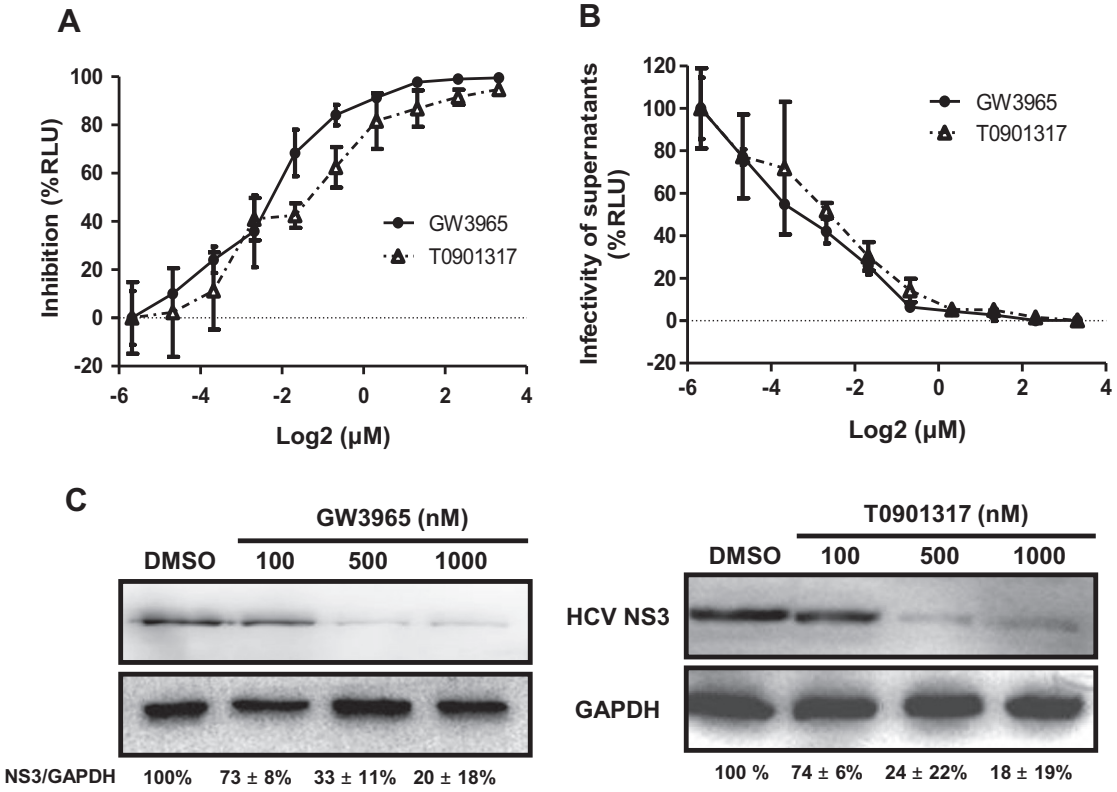

Fig. 5. Synthetic LXR agonists inhibit HCV infection in a dose-dependent manner. Huh7.5.1 cells were treated with the specified amounts of GW3965 or T0901317 for 4 h prior to infection with JFH1-Luc-5AGFP or JFH1. At 72 hpi, HCV infection was strongly inhibited. (A) The inhibition experiment was performed using JFH1-Luc-5AGFP reporter virus. Seventy-two hpi, cells were lysed and processed for a luciferase assay. (B) The supernatants of these cells were used to infect naïve Huh7.5.1 cells as described in Section 2. The infectivity was determined by quantification of luciferase activity and normalized to the control. (C) The inhibition experiment was performed using the wild-type JFH1 virus. Cells were harvest for Western blot analysis 72 hpi. All the values are the average of three independent experiments.

tion therapy targeting HCV infection could delay the emergence of drug-resistant virus. To test whether LXR agonists could be combined with other antiviral compounds targeting HCV replication in an interferon-free regimen, we tested the synthetic LXR agonists with two established anti-HCV drugs. MK-7009 is a drug that directly targets the HCV protease (NS3/4A), and cyclosporine A (CsA) inhibits RNA replication via the HCV host factor cyclophilin. The combination study was conducted using a checkerboard setup where dose–response curves were generated for the HCV RNA replication inhibitors (MK-7009 or CsA) either alone or in combination with GW3965 (Fig. 8A) and were used to determine the EC_{50} values for each drug alone or in the presence of the fixed concentration of the second drug. Isobologram analysis (Berenbaum, 1989) and the fractional inhibition concentration index (FIC index) (Odds, 2003) were used to determine whether the combination of MK-7009 + GW3965 and CsA + GW3965 exert a synergistic, additive or antagonistic effect on the inhibition of HCV infection. As shown in Fig. 8B, the *x*-axis and *y*-axis represent the fractional inhibitory concentrations (FIC) of MK-7009 (or CsA) and GW3965, respectively. The calculated effects of the combination therapy, shown by closed five-point stars that are all located near the additive line, or by the calculated FIC indexes that are all fit between 0.5 and 4, indicate that both the MK-7009 + GW3965 and CsA + GW3965 combinations are additive effects on the inhibition of HCV infection. Another LXR agonist T0901317 worked in a similar pattern as GW3965 in the combination treatment with MK-7009 or CsA (Supplementary Fig. 3). It was speculated that MK-7009 + T0901317 and CsA + T0901317 also exerted additive effects on anti-HCV infection. These data indicate that synthetic LXR agonists could efficiently be used in HCV combination therapy.

4. Discussion

Our study provides strong evidence that LXR is a promising target for anti-HCV drug development. The over-expression of Idol, whose transcription is controlled by LXR, could significantly suppress HCV infection by down-regulating LDLR level without interfering with HCV RNA replication. Furthermore, LXR agonists, including synthetic ligands (GW3965 and T0901317) and the natural ligand (24,25EC), strongly interfere with HCV infection through a mechanism of Idol triggered degradation of the LDLR. Moreover, the LXR agonists could be efficiently used with HCV RNA replication inhibitors in combination treatment.

Besides LDLR, Idol can induce the degradation of two other members of LDLR family, VLDLR and ApoER2 (Hong et al., 2010). Thus we cannot exclude the possibility that Idol may trigger ubiquitination and degradation of HCV co-receptors other than LDLR, which may also contribute to the inhibition of HCV entry.

Accumulating evidence suggests that HCV is associated with lipoproteins, leading to the recognition of HCV as an LVP (lipoviro-particles) (André et al., 2002). LDLR has been implied to play an indispensable role in HCV infection on the basis of the well-documented interaction between HCV particles and lipoproteins. The involvement of the LDLR in HCV infection provides a new approach to therapy in the future. Previous studies have explored the feasibility of neutralizing HCV infection based on the use of anti-LDLR antibodies or peptides (Molina et al., 2007). Since the depletion of LDLR from Huh7.5.1 cells by Idol had a direct effect on the susceptibility of the cells to infection by HCV (Fig. 2), the ubiquitin ligase Idol has clearly entered the field of molecular medicine as a possible anti-HCV molecule. Because Idol is a rather unstable

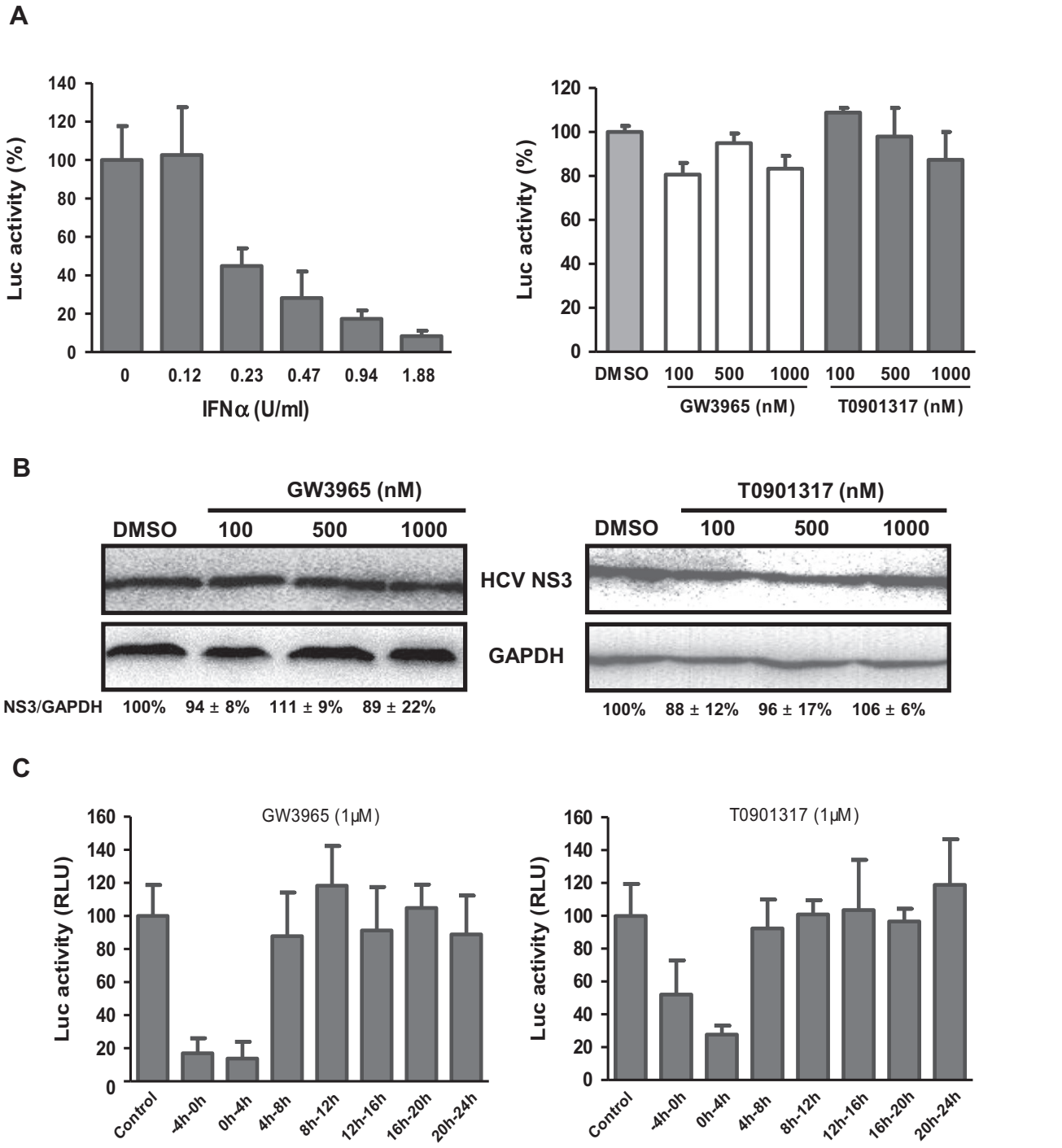

Fig. 6. Synthetic LXR agonists do not affect HCV replication. (A) SGR-JFH1-Luc-5AGFP cells were plated and treated with the specified amounts of GW3965 or T0901317 for 48 h. Cells were harvested and subjected to luciferase assay. IFN-α was used as a positive control in this experiment (left). (B) Western blot analysis of the expression of HCV NS3 in cells under the same treatment as in A. (C) Huh-7.5.1 cells were incubated for 4 h (0–4 h) with JFH1-Luc-5AGFP virus. Before, during or after the infection processes, the drug (GW3965 or T0901317) was added and maintained for 4 h, then withdrawed. Viral replication levels were determined by luciferase assay. Mean values of three independent experiments including the standard deviation are shown.

protein, upregulation of Idol could be accomplished by increasing its mRNA expression levels or enhancing protein stability in a screen for small molecular compounds. However, purely increasing Idol expression levels may lead to hypercholesterolemia as a result of lower hepatic levels of LDLR. Thus, a combination therapy or a kind of compound aimed at up-regulating Idol and concurrently lowering the plasma cholesterol level might be appropriate. Fortunately, the ligands of LXR could achieve this goal.

Because both the synthetic and natural LXR agonists could efficiently suppress HCV entry by indirectly promoting LDLR degradation (Figs. 5 and 8), LXR agonists show strong potential as anti-HCV drug targets. One would not need to worry about the accumulation

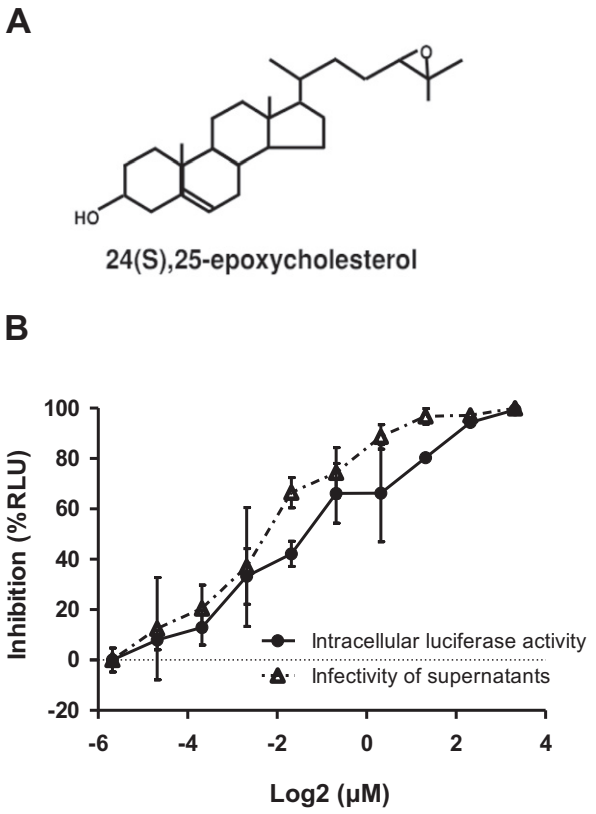

Fig. 7. The natural LXR agonist 24(S),25-epoxycholesterol inhibits HCV infection in a dose-dependent manner. (A) Chemical structure of 24(S),25-epoxycholesterol. (B) Huh-7.5.1 cells were inoculated with the JFH1-Luc-5AGFP reporter virus in the presence of increasing concentrations of 24(S),25-epoxycholesterol. Infected cells were lysed three days later, and the luciferase activity was determined. And the supernatants of these cells were used to infect naïve Huh7.5.1 cells as described in Section 2. The inhibition of the supernant virus infectivity was determined by luciferase assay. Results are expressed as the mean ± standard deviations (error bars) for three independent experiments.

of cholesterol in the plasma due to decreased hepatic LDLR. The primary indication for LXR agonists is treatment of hypercholesterolemia and atherosclerosis. The response to excess cellular cholesterol is mediated by LXR via triggering various adaptive mechanisms protecting the cell from cholesterol overload. LXR agonists up-regulate the expression of transporters involved in the cholesterol pathway, such as ABCA1 and ABCG1, that participate in the process of reverse cholesterol transport (the transport of excess cholesterol from peripheral tissue to the liver). This excess cholesterol in the liver is then excreted in the bile as unchanged cholesterol or after conversion to bile acids (Jamroz-Wisniewska et al., 2007; Wojcicka et al., 2007). In addition to inducing reverse cholesterol transport, LXR could also limit the cellular cholesterol content by increasing bile acid production and inhibiting intestinal cholesterol absorption (Repa et al., 2000). Several studies have shown that LXR agonists reduce atherosclerotic lesions in animal models. One study has demonstrated that if mice are injected with macrophages previously loaded with titrated amounts of cholesterol, and then the mice are treated with GW3965 for 10 days, fecal excretion of a cholesterol tracer is markedly higher and excretion of titrated bile acids tends to be higher than in vehicle-treated animals (Naik et al., 2006). These results clearly indicate that LXR agonists would be ideal candidate targets for HCV antiviral drug development, possibly without causing severe side effects due to the degradation of LDLR.

Recently, the stimulation of the LXR pathway has been reported to also inhibit HIV-1 replication (Morrow et al., 2010). LXR agonists suppressed HIV-1 replication in both cell types and inhibited HIV-1 replication in ex vivo cultured lymphoid tissue and in RAG-hu mice infected in vivo. The mechanism of this effect of LXR agonists appeared to be the stimulation of ABCA1 expression. Hence, the stimulation of the LXR pathway could be employed as a new strategy to discover broad-spectrum antiviral drugs for at least HCV, HIV-1, as well as HCV/HIV-1 co-infection.

HCV infection is a complex disease. Drugs now are being developed for patients who have failed in the treatment with pegylated interferon and ribavirin, such as specifically targeted antiviral therapy (STAT) drugs for HCV enzymes and their host factors. Of these, inhibitors of NS3/4A and NS5B and their co-factors are the most promising therapeutic molecules since these enzymes play a highly strategic role in the virus life. However, treatments with antiviral molecules directed against NS3/4 or NS5B generally lead to the emergence of mutants with reduced sensitivity to this treatment though the new polymerase inhibitor PSI-7977 was reported to have high barrier to resistance. Considering the inherent mutability and the great intra-strain variability of HCV, the permanent eradication of HCV will require creative and multipronged measures. Our study demonstrated that combination of LXR agonists with HCV RNA inhibitors showed a strong additive inhibition on HCV infection (Fig. 7 and Supplementary Fig. 3). The combined use of two or more drugs with different mechanisms of action cannot only potentially reduce the incidence of drug resistance but also present other advantages, such as having potentially greater potency, better clinical efficacy, dose-sparing, and less toxicity and side effects.

The utility of LXR agonists as anti-HCV therapeutics might be limited by the fact that stimulation of LXR also up-regulates the sterol regulatory element-binding protein-1c (SREBP-1c), which induces enhanced triglyceride synthesis in the liver, resulting in hypertriglyceridemia (Chisholm et al., 2003). However, this problem could be resolved by using a selective LXR modulator that is less involved or not at all involved in lipogenesis. Interestingly, GW3965, is one such serviceable modulator, which could raise HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia (Miao et al., 2004). Recently, another synthetic steroidal LXR agonist *N*,*N*-dimethyl-3b-hydroxy-cholenamide (DMHCA) has been reported to reduce atherosclerotic lesion formation in apoE-null mice without increasing hepatic triglyceride levels (Kratzer et al., 2009). Other than the selective synthetic LXR agonists, the natural ligand, 24,25EC, could also solve this problem. The stimulating effect on SREBP-1c expression via LXR is counteracted by suppressing the activation of SRBEP-1c by detaining the SREBP precursor in the ER and preventing its activation in Golgi (Rowe et al., 2003). Since the oral administration of 24,25EC is not an option because it could be hydrolyzed in the stomach, inhibitors of OSC (2,3-oxidosqualene:lanosterol cyclase sterol), a microsomal enzyme in both the cholesterol biosynthetic pathway and the alternative oxysterol synthetic pathway that could highly increase 24,25EC synthesis in vivo (Rowe et al., 2003), might be used as an anti-HCV drug in clinical therapy.

In conclusion, our results demonstrate a potent anti-HCV effect of LXR agonists. These drugs affect HCV infection by blocking HCV entry, the first and critical step of the HCV lifecycle, resulting in significant suppression of HCV infection. This in vitro study also emphasizes the essential role of Idol in the regulation of HCV infectivity. And the stimulation or stabilization of Idol expression might also be an effective approach to inhibit HCV entry.

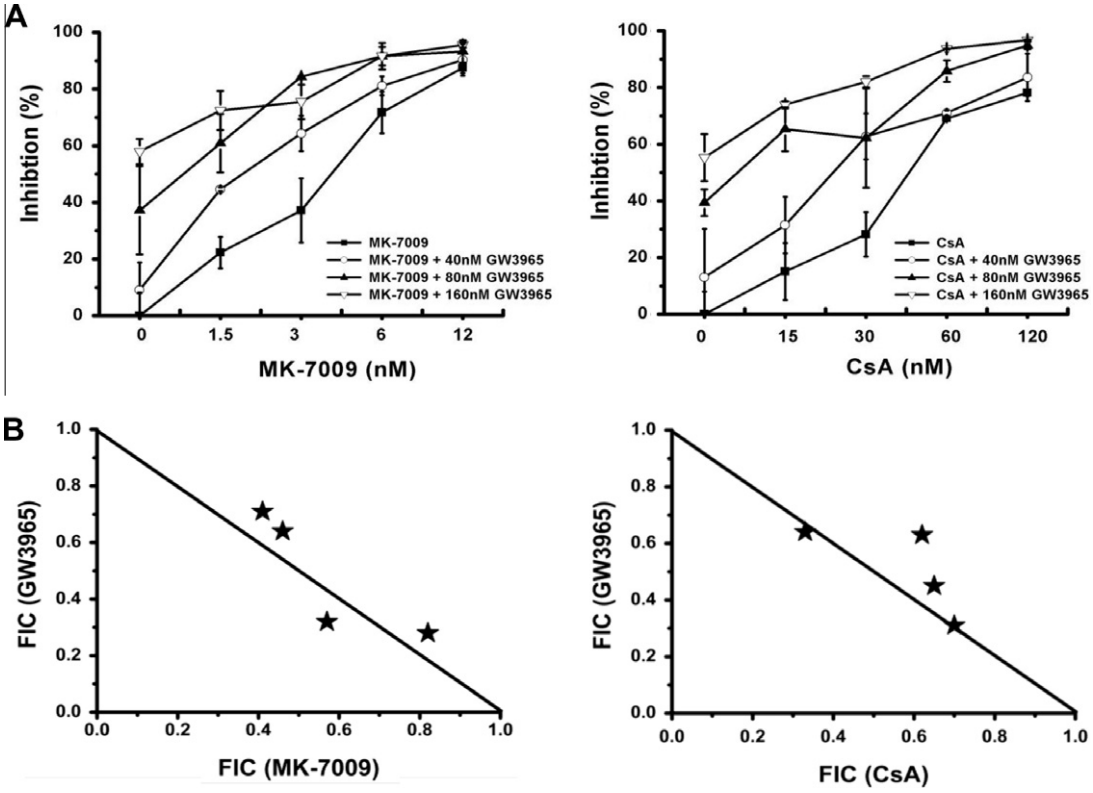

Fig. 8. Additive inhibition of HCV infection by MK-7009 or CsA combined with the LXR agonist GW3965. Huh7.5.1 cells were treated with different concentrations of MK-7009 or CsA, alone or in combination with GW3965 at different concentrations. Then, cells were inoculated with the JFH1-Luc-5AGFP reporter virus, maintained for 72 h and subjected to the luciferase activity assay. (A) The dose-dependent curves of the combination of MK-7009 with GW3965 (left) and CsA with GW3965 (right) on HCV infection inhibition are shown. A representative experiment from three independent repetitions with standard deviation of the mean is shown. (B) EC_{50} isobologram of the combination of MK-7009 with GW3965 (left) or CsA with GW3965 (right) is shown. The oblique line indicates the expected additive line. The calculated combination effects, shown by the closed five-point stars, are all located nearby the additive line. The FIC indexes fluctuated around 1, which is between the range of 0.5 and 4, indicating additive inhibition of HCV infection by MK-7009 or CsA combined with GW3965.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.06.004.

References

Agnello, V., Ábel, G., Elfahal, M., Knight, G.B., Zhang, Q.-X., 1999. Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proc. Natl. Acad. Sci. USA 96, 12766–12771.

André, P., Komurian-Pradel, F., Deforges, S., Perret, M., Berland, J.L., Sodoyer, M., Pol, S., Bréchot, C., Paranhos-Baccalà, G., Lotteau, V., 2002. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J. Virol. 76, 6919–6928.

Andréo, U., Maillard, P., Kalinina, O., Walic, M., Meurs, E., Martinot, M., Marcellin, P., Budkowska, A., 2007. Lipoprotein lipase mediates hepatitis C virus (HCV) cell entry and inhibits HCV infection. Cell. Microbiol. 9, 2445–2456.

Barth, H., Schäfer, C., Adah, M.I., Zhang, F., Linhardt, R.J., Toyoda, H., Kinoshita-Toyoda, A., Toida, T., van Kuppevelt, T.H., Depla, E., von Weizsäcker, F., Blum, H.E., Baumert, T.F., 2003. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. J. Biol. Chem. 278, 41003–41012.

Bełtowski, J., 2008. Liver X receptors (LXR) as therapeutic targets in dyslipidemia. Cardiovasc. Ther. 26, 297–316.

Berenbaum, M.C., 1989. What is synergy? Pharmacol. Rev. 41, 93–141.

Bretscher, A., Edwards, K., Fehon, R.G., 2002. ERM proteins and merlin: integrators at the cell cortex. Nat. Rev. Mol. Cell. Biol. 3, 586–599.

Chappell, D.A., Medh, J.D., 1998. Receptor-mediated mechanisms of lipoprotein remnant catabolism. Prog. Lipid Res. 37, 393–422.

Chisholm, J.W., Hong, J., Mills, S.A., Lawn, R.M., 2003. The LXR ligand T0901317 induces severe lipogenesis in the db/db diabetic mouse. J. Lipid Res. 44, 2039–2048.

Costet, P., Luo, Y., Wang, N., Tall, A.R., 2000. Sterol-dependent transactivation of theABC1 promoter by the liver X receptor/retinoid X receptor. J. Biol. Chem. 275, 28240–28245.

Dorner, M., Horwitz, J.A., Robbins, J.B., Barry, W.T., Feng, Q., Mu, K., Jones, C.T., Schoggins, J.W., Catanese, M.T., Burton, D.R., Law, M., Rice, C.M., Ploss, A., 2011. A genetically humanized mouse model for hepatitis C virus infection. Nature 474, 208–211.

Evans, M.J., von Hahn, T., Tscherne, D.M., Syder, A.J., Panis, M., Wolk, B., Hatziioannou, T., McKeating, J.A., Bieniasz, P.D., Rice, C.M., 2007. Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry. Nature 446, 801–805.

Germi, R., Crance, J.-M., Garin, D., Guimet, J., Lortat-Jacob, H., Ruigrok, R.W.H., Zarski, J.-P., Drouet, E., 2002. Cellular glycosaminoglycans and low density lipoprotein receptor are involved in hepatitis C virus adsorption. J. Med. Virol. 68, 206–215.

Gong, Q., Cheng, M., Chen, H., Liu, X., Si, Y., Yang, Y., Yuan, Y., Jin, C., Yang, W., He, F., Wang, J., 2011. Phospholipid scramblase 1 mediates hepatitis C virus entry into host cells. FEBS Lett. 585, 2647–2652.

Hong, C., Duit, S., Jalonen, P., Out, R., Scheer, L., Sorrentino, V., Boyadjian, R., Rodenburg, K.W., Foley, E., Korhonen, L., Lindholm, D., Nimpf, J., van Berkel, T.J.C., Tontonoz, P., Zelcer, N., 2010. The E3 ubiquitin ligase IDOL induces the degradation of the low density lipoprotein receptor family members VLDLR and ApoER2. J. Biol. Chem. 285, 19720–19726.

Jamroz-Wisniewska, A., Wojcicka, G., Horoszewicz, K., Beltowski, J., 2007. Liver X receptors (LXRs). Part II: non-lipid effects, role in pathology, and therapeutic implications. Postepy Hig. Med. Dosw. (Online) 61, 760–785.

Janowski, B.A., Willy, P.J., Devi, T.R., Falck, J.R., Mangelsdorf, D.J., 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 383, 728–731.

Kapadia, S.B., Chisari, F.V., 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc. Natl. Acad. Sci. USA 102, 2561–2566.

Kratzer, A., Buchebner, M., Pfeifer, T., Becker, T.M., Uray, G., Miyazaki, M., Miyazaki-Anzai, S., Ebner, B., Chandak, P.G., Kadam, R.S., Calayir, E., Rathke, N., Ahammer, H., Radovic, B., Trauner, M., Hoefler, G., Kompella, U.B., Fauler, G., Levi, M., Levak-Frank, S., Kostner, G.M., Kratky, D., 2009. Synthetic LXR agonist attenuates plaque formation in apoE−/− mice without inducing liver steatosis and hypertriglyceridemia. J. Lipid Res. 50, 312–326.

Lauer, G.M., Walker, B.D., 2001. Hepatitis C virus infection. New Engl. J. Med. 345, 41–52.

Lewis, G.F., Rader, D.J., 2005. New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ. Res. 96, 1221–1232.

Liu, S., Yang, W., Shen, L., Turner, J.R., Coyne, C.B., Wang, T., 2009. Tight junction proteins claudin-1 and Occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. J. Virol. 83, 2011–2014.

Lozach, P.-Y., Amara, A., Bartosch, B., Virelizier, J.-L., Arenzana-Seisdedos, F., Cosset, F.-L., Altmeyer, R., 2004. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. J. Biol. Chem. 279, 32035–32045.

Lupberger, J., Zeisel, M.B., Xiao, F., Thumann, C., Fofana, I., Zona, L., Davis, C., Mee, C.J., Turek, M., Gorke, S., Royer, C., Fischer, B., Zahid, M.N., Lavillette, D., Fresquet, J., Cosset, F.-L., Rothenberg, S.M., Pietschmann, T., Patel, A.H., Pessaux, P., Doffoel, M., Raffelsberger, W., Poch, O., McKeating, J.A., Brino, L., Baumert, T.F., 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat. Med. 17, 589–595.

Manns, M.P., Foster, G.R., Rockstroh, J.K., Zeuzem, S., Zoulim, F., Houghton, M., 2007. The way forward in HCV treatment [mdash] finding the right path. Nat. Rev. Drug Discov. 6, 991–1000.

Miao, B., Zondlo, S., Gibbs, S., Cromley, D., Hosagrahara, V.P., Kirchgessner, T.G., Billheimer, J., Mukherjee, R., 2004. Raising HDL cholesterol without inducing hepatic steatosis and hypertriglyceridemia by a selective LXR modulator. J. Lipid Res. 45, 1410–1417.

Molina, S., Castet, V., Fournier-Wirth, C., Pichard-Garcia, L., Avner, R., Harats, D., Roitelman, J., Barbaras, R., Graber, P., Ghersa, P., Smolarsky, M., Funaro, A., Malavasi, F., Larrey, D., Coste, J., Fabre, J.-M., Sa-Cunha, A., Maurel, P., 2007. The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. J. Hepatol. 46, 411–419.

Monazahian, M., Böhme, I., Bonk, S., Koch, A., Scholz, C., Grethe, S., Thomssen, R., 1999. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J. Med. Virol. 57, 223–229.

Morrow, M.P., Grant, A., Mujawar, Z., Dubrovsky, L., Pushkarsky, T., Kiselyeva, Y., Jennelle, L., Mukhamedova, N., Remaley, A.T., Kashanchi, F., Sviridov, D., Bukrinsky, M., 2010. Stimulation of the liver X receptor pathway inhibits HIV-1 replication via induction of ATP binding cassette transporter A1. Mol. Pharmacol. 78, 215–225.

Naik, S.U., Wang, X., Da Silva, J.S., Jaye, M., Macphee, C.H., Reilly, M.P., Billheimer, J.T., Rothblat, G.H., Rader, D.J., 2006. Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. Circulation 113, 90–97.

Nelson, D.R., 2011. The role of triple therapy with protease inhibitors in hepatitis C virus genotype 1 naïve patients. Liver Int. 31, 53–57.

Nielsen, S.U., Bassendine, M.F., Burt, A.D., Martin, C., Pumeechockchai, W., Toms, G.L., 2006. Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J. Virol. 80, 2418–2428.

Odds, F.C., 2003. Synergy, antagonism, and what the chequerboard puts between them. J. Antimicrob. Chemother. 52, 1.

Pöhlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G.J., Lin, G., Granelli-Piperno, A., Doms, R.W., Rice, C.M., McKeating, J.A., 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. 77, 4070–4080.

Petit, M.-A., Lièvre, M., Peyrol, S., De Sequeira, S., Berthillon, P., Ruigrok, R.W.H., Trépo, C., 2005. Enveloped particles in the serum of chronic hepatitis C patients. Virology 336, 144–153.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R., Weiner, A.J., Houghton, M., Rosa, D., Grandi, G., Abrignani, S., 1998. Binding of hepatitis C virus to CD81. Science 282, 938–941.

Ray, K., 2012. Hepatitis: NPC1L1 identified as a novel HCV entry factor. Nat. Rev. Gastroenterol. Hepatol. 9, 124.

Repa, J.J., Turley, S.D., Lobaccaro, J.-M.A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R.A., Dietschy, J.M., Mangelsdorf, D.J., 2000. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 289, 1524–1529.

Rowe, A.H., Argmann, C.A., Edwards, J.Y., Sawyez, C.G., Morand, O.H., Hegele, R.A., Huff, M.W., 2003. Enhanced synthesis of the oxysterol 24(S),25-epoxycholesterol in macrophages by inhibitors of 2,3-oxidosqualene: lanosterol cyclase. Circ. Res. 93, 717–725.

Sabol, S.L., Brewer, H.B., Santamarina-Fojo, S., 2005. The human ABCG1 gene: identification of LXR response elements that modulate expression in macrophages and liver. J. Lipid Res. 46, 2151–2167.

Saunier, B., Triyatni, M., Ulianich, L., Maruvada, P., Yen, P., Kohn, L.D., 2003. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. J. Virol. 77, 546–559.

Scarselli, E., Ansuini, H., Cerino, R., Roccasecca, R.M., Acali, S., Filocamo, G., Traboni, C., Nicosia, A., Cortese, R., Vitelli, A., 2002. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J. 21, 5017–5025.

Shimakami, T., Lanford, R.E., Lemon, S.M., 2009. Hepatitis C: recent successes and continuing challenges in the development of improved treatment modalities. Curr. Opin. Pharm. 9, 537–544.

Susser, S., Vermehren, J., Forestier, N., Welker, M.W., Grigorian, N., Füller, C., Perner, D., Zeuzem, S., Sarrazin, C., 2011. Analysis of long-term persistence of resistance mutations within the hepatitis C virus NS3 protease after treatment with telaprevir or boceprevir. J. Clin. Virol. 52, 321–327.

Wojcicka, G., Jamroz-Wisniewska, A., Horoszewicz, K., Beltowski, J., 2007. Liver X receptors (LXRs). Part I: structure, function, regulation of activity, and role in lipid metabolism. Postepy Hig. Med. Dosw. (Online) 61, 736–759.

Wong, J., Quinn, C.M., Gelissen, I.C., Brown, A.J., 2008. Endogenous 24(S),25-epoxycholesterol fine-tunes acute control of cellular cholesterol homeostasis. J. Biol. Chem. 283, 700–707.

Wu, Y., Liao, Q., Yang, R., Chen, X., Chen, X., 2011. A novel luciferase and GFP dual reporter virus for rapid and convenient evaluation of hepatitis C virus replication. Virus Res. 155, 406–414.

Zelcer, N., Hong, C., Boyadjian, R., Tontonoz, P., 2009. LXR regulates cholesterol uptake through Idol-dependent ubiquitination of the LDL receptor. Science 325, 100–104.