Up-regulation of the ATP-binding cassette transporter A1 inhibits hepatitis C virus infection.


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Abstract

Hepatitis C virus (HCV) establishes infection using host lipid metabolism pathways that are thus considered potential targets for indirect anti-HCV strategies. HCV enters the cell via clathrin-dependent endocytosis, interacting with several receptors, and virus-cell fusion, which depends on acidic pH and the integrity of cholesterol-rich domains of the hepatocyte membrane. The ATP-binding Cassette Transporter A1 (ABCA1) mediates cholesterol efflux from hepatocytes to extracellular Apolipoprotein A1 and moves cholesterol within cell membranes. Furthermore, it generates high-density lipoprotein (HDL) particles. HDL protects against arteriosclerosis and cardiovascular disease. We show that the up-regulation of ABCA1 gene expression and its cholesterol efflux function in Huh7.5 hepatoma cells, using the liver X receptor (LXR) agonist GW3965, impairs HCV infection and decreases levels of virus produced. ABCA1-stimulation inhibited HCV cell entry, acting on virus-host cell fusion, but had no impact on virus attachment, replication, or assembly/secretion. It did not affect infectivity or properties of virus particles produced. Silencing of the ABCA1 gene and reduction of the specific cholesterol efflux function counteracted the inhibitory effect of the GW3965 on HCV infection, providing evidence for a key role of ABCA1 in this process. Impaired virus-cell entry correlated with the reorganisation of cholesterol-rich membrane microdomains (lipid rafts). The inhibitory effect could be reversed by an exogenous cholesterol supply, indicating that restriction of HCV infection was induced by changes of cholesterol content/distribution in membrane regions essential for virus-cell fusion. Stimulation of ABCA1 expression by GW3965 inhibited HCV infection of both human primary hepatocytes and isolated human liver slices. This study reveals that pharmacological stimulation of the ABCA1-dependent cholesterol efflux pathway disrupts membrane cholesterol homeostasis, leading to the inhibition of virus–cell fusion and thus HCV cell entry. Therefore besides other beneficial roles, ABCA1 might represent a potential target for HCV therapy.

Introduction

Hepatitis C virus (HCV) infection affects 3% of the world population and is major cause of chronic liver disease with severe hepatic consequences such as steatosis, cirrhosis and hepatocarcinoma. Recently, numerous direct acting anti-viral drugs (DDA) have been introduced, which target essential viral functions. These new treatments represent a significant step forward compared to standard Pegylated IFN-α-ribavirin therapy. DDA are mainly inhibitors of NS3/NS4 HCV protease, and others drugs are under development that target the NS3B polymerase or NS5A that also play essential roles in HCV replication [1]. However, these DDA still have side effects and induce the manifestation of drug-resistance [2]. Novel treatments targeting host cell molecules involved in various steps of the HCV life cycle (such as cyclophilin A, microRNA-122, or phosphatidylinositol-4-kinase III alpha) have been proposed for novel anti-HCV approaches (and these are called “indirect acting anti-viral drugs”, IAAD), to prevent the onset of antiviral resistance and to cure infection with all HCV genotypes [1,3].

HCV is an enveloped virus of the Flaviviridae family (genus Hepacivirus) with a single-stranded positive RNA genome of 9.6 kb.
The viral genome encodes a polyprotein of 3,000 amino acids, which is cleaved by the host and viral proteases into structural proteins (the capsid protein and two envelope glycoproteins E1 and E2), P7 and several non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) involved in genome replication and virus assembly [3,4].

Lipids and lipoproteins are essential for the HCV life cycle [5,6,7]. HCV has developed lipoprotein-dependent mechanisms for cell entry [8,9,10], replication regulated by fatty acids [11], virus morphogenesis that is linked to lipid droplets [12] and the assembly and release of infectious virions via the VLDL (very low density lipoprotein) formation and secretion pathway [13,14]. Consequently, HCV circulates in the plasma of infected patients in association with VLDL and LDL (low-density lipoprotein), Concomitantly, HCV infection is the feature of Tangier disease, characterized by a severely impaired lipoprotein metabolism [15,16].

The relationships between lipid metabolism and HCV are complex and intriguing. The expression of host genes involved in biosynthesis, degradation or transport of intracellular lipids is altered upon HCV infection [17,18]. Steatosis and insulin resistance associated with the metabolic syndrome increase fibrosis progression and reduce the response to the IFN-α-ribavirin treatment. Moreover, a high baseline LDL level has been shown to be the best predictor of a sustained virologic response, whereas low lipid levels correlate with steatosis, progressing fibrosis and non-response to treatment [19].

Altogether, these observations reflect the important role of lipids in the HCV life cycle. Therefore, host factors involved in cholesterol/lipid metabolism might represent potential targets for HCV strategies, with only limited possibilities for escape mutations to develop [20,21] and allowing treatment of patients infected with genotype 3 HCV [1].

Cholesterol is an important structural component of biological membranes and is essential for the uptake of many viruses. HCV cell entry requires cholesterol homeostasis and intact cholesterol-rich membrane microdomains [22]. Indeed perturbation of the alignment/packaging of cholesterol in lipid membranes increases the energy barrier required for virus-cell entry via fusion mechanisms [23].

Hepatocytes play a vital role in cholesterol homeostasis, acquiring cholesterol by synthesis via the mevalonate pathway or by LDL-R mediated endocytosis. Cholesterol is exported from hepatocytes together with triglycerides through the VLDL secretion pathway [24]. However, a major regulator of cellular cholesterol and phospholipid homeostasis is the ABCA1 transporter. ABCA1 is an integral trans-membrane protein that moves phospholipids and free cholesterol across the cell membrane to combine them with lipid-free ApoA1, which is also synthesised in the liver, to form nascent HDL particles [25,26]. ABCA1 is highly expressed in the liver and tissue macrophages. Nevertheless, the liver ABCA1 pathway appears to generate most (70–80%) plasma HDL [27]. ABCA1 exports cholesterol exclusively at the cell surface [28]. Free cholesterol in nascent HDL particles is subsequently converted to cholesterol esters by the lecithin:cholesterol acyltransferase (LCAT). The absence of functional ABCA1 is the feature of Tangier disease, characterized by a severely impaired lipidation of ApoA1 via the ABCA1 pathway, and very low blood levels of HDL [29].

The modulation of intracellular and membrane cholesterol homeostasis has dramatic effects on the early stages of several viral infections [30,31]. Thus, we hypothesised that stimulation of the ABCA1-mediated cholesterol efflux may influence the course of HCV infection. We provide here the first evidence that pharmacological stimulation of the ABCA1 pathway efficiently inhibits virus-cell entry and decreases virus infection levels in the HCVcc model and in primary human hepatocytes. Our findings highlight ABCA1 as a novel potential target for HCV strategies.

Materials and Methods

Cell Infection with HCVcc

The JFH1 HCV strain (genotype 2a) was kindly provided by T. Wakita. Infectious HCV virions were generated as described [32]. Huh7.5 hepatoma cells (kindly provided by C. Rice) were grown as described [33] and were infected with the virus preparation at 10⁵ PFU/mL (focus forming units per millilitre), at an MOI = 0.001–0.01 for 2 h at 37°C. They were then grown further for the indicated time at 37°C.

Primary human hepatocytes were isolated from two adult liver donors after resection for medical reasons and infected with HCVcc as previously described [34]. To culture liver tissue slices, human samples were obtained from adult HCV, HBV and HIV seronegative patients who underwent liver resection in the absence of underlying liver disease. These samples were infected with HCVcc [35].

Ethics Statement

The French Ministry of Research and Higher Education delivered the authorization N’DC-2008-531 to collect hepatic resection samples from the digestive surgery department and to isolate primary human hepatocytes. The experiments on the human liver slices were performed on surgical samples (from resections of liver metastases), which are considered as “biological waste” and require neither patient nor institutional review board approval. The local Ethics Committee of the Cochin Hospital in Paris, France, approved these experiments.

Stimulation of ABCA1 Expression

Cell toxicity of LXR agonists GW3965 and TO901317 (Sigma) was determined using the CellTiter-Glo luminescent Cell Viability Assay (Promega). To stimulate ABCA1 expression, Huh7.5 cells or primary hepatocytes were treated with 1–10 μM concentrations of the drugs (diluted in DMSO) for 24 h before infection, or with corresponding dilutions of DMSO. Cells were grown for the indicated time and ABCA1 mRNA levels were analysed by qRT-PCR. ABCA1 protein was determined by Western Blot and quantified using the Odyssey Infrared Imaging system. ABCA1 function was measured using cholesterol efflux assay.

Quantitative RT-PCR (qRT-PCR)

HCV RNA was determined as previously described [33]. For measurement of the ABCA1 mRNA, 5'-CCTGACCCGTTGTCTCCC-3' and 5'-TTCTGCGGGATGGTGTCCT-3' primers were used for amplification and 5'-AGATTTGAGCACATTCGTCCTG-3' (Eurogentec) served as an internal probe. The results were normalized by quantification of HPRT1 or GADPH cellular genes using the HPRT1 Taqman Gene Expression Assay (Life Technology Applied Biosystems) or the GAPDH Control Kit (Eurogentec), respectively.

All assays were performed at least in quadruplicate. Error bars on the histograms represent standard deviation of the mean values from at least quadruplicates.

Cholesterol Efflux Assay

Control or drug-treated Huh7.5 cells were cholesterol-loaded using 1 μCi/mL [3H] cholesterol-labelled SVF (10%) for 24 h in DMEM medium. Cells were then incubated in serum-free medium containing 0.2% BSA with or without 1 μM GW3965.
for 16 h. Cellular cholesterol efflux to 25 μg/mL lipid-free ApoA1 (Sigma) was assayed in serum-free medium containing 0.2% BSA for a 4 h chase period, then culture medium was harvested and cleared by centrifugation. Cell-associated radioactivity was determined by extraction in hexane-isopropanol (3:2), evaporation of the solvent and liquid scintillation counting (Wallac TriLux 1450 Microbeta). The percentage of cholesterol efflux was calculated according to the formula = 100×(medium cpm)/(medium cpm−cell cpm). ApoA1-specific cholesterol efflux was determined by subtracting the cholesterol efflux that occurred in apoA1-free medium.

**ABCA1 Silencing**

Stealth siRNA targeting three different zones of human ABCA1 gene (Life Technologies Applied Biosystems) were used. Huh7.5 cells were transfected with 25 nM ABCA1-specific siRNA or with 25 nM scrambled siRNA (Eurogentec), using the RNAi Max reagent (Invitrogen). At 24 h and 48 h after transfection ABCA1 mRNA levels were determined by qRT-PCR, ABCA1 protein by Western Blot and ABCA1 function by cholesterol efflux assay.

**Cell–to-cell Fusion Assay**

The assay was performed as described previously [36]. HEK293T kidney cells (ATCC CRL-1573) were cultured in DMEM supplemented with 10% FBS, 1% non-essential amino acids, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells (2.5×10⁵ cells/well) were seeded in 35 mm 6-well tissue culture dishes 24 h before transfection) were co-transfected using the calcium phosphate reagent with a plasmid encoding HCV envelope proteins (H77 strain genotype 1a) or Chikungunya envelope encoding-plasmid (ChikV) issued from ChikV E3E1E2 plasmid from the Réunion infectious clone and with an HIV-1 LTR (long terminal repeat) luciferase reporter plasmid kindly provided by Francoise Bex. After 12 h, transfected HEK293T cells were detached with 0.53 mM EDTA (Invitrogen) and co-cultured (5×10⁴ cells/well) with Huh7.1-Tat indicator cells (5×10⁴ cells/well). Co-cultured cells were then incubated with 1 μM of GW3965 or with DMSO. After 24 h, the cells were washed with serum-free DMEM, incubated for 3 min in either pH 7 or pH 5 buffer, (containing 130 mM NaCl, 15 mM sodium citrate, 10 mM MES and 5 mM Hepes), then washed with serum-free DMEM. Cells were next incubated with 1 μM of GW3965 or with DMSO for 48 h. Luciferase activity was measured using a luciferase assay kit (Promega). The experiments were performed several times and results interpreted using student t-test.

**Analysis of Green Fluorescent Protein-Folate Receptor Cell Distribution by Fluorescence Microscopy**

Huh7.5 cells were transfected with 0.5 μg of DNA encoding a fusion protein, where GFP is fused to the Glycosylphosphatidylinositol-anchor attachment signal of folate receptor (GFP-FR) [37,38]. Four hours post-transfection cells were treated with 1 μM GW3965 or with a drug solvent (DMSO) that was renewed at 24 h post-transfection. Two days after transfection cells were fixed with 4% PFA containing 0.2% glutaraldehyde. Fluorescence of GFP-FR was visualised with a Zeiss Axioplan 2 microscope at 488 nm (x63 objective).

**Quantification of mRNA of Genes Involved in Lipid Metabolism**

Total RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel). Reverse transcription and real time qPCR assays were performed as previously described [39] and mRNA levels were normalized compared to those of housekeeping genes (18S, human delta-aminolevulinate synthetase, human alpha-tubulin, human heat shock protein 90 kDa alpha and human hypoxanthine phosphoribosyltransferase 1). Data were expressed as a fold-change in mRNA expression relative to control values.

**Determination of Intracellular Lipids**

Quantification of total cellular triglyceride mass was performed as previously described [40]. Free and esterified cholesterol mass was quantified using the Amplex Red cholesterol assay kit (Molecular Probes) [41].

**Cholesterol Replenishment**

Control or GW3965-treated Huh7.5 cells were incubated with 20 μg/mL cholesterol:Membrane β Cyclodextrine D (MβCD) complexes (Sigma) for 1 h at 37°C. Cells were extensively washed and analysed for their lipid/cholesterol content or infected with HCV.

**Virus Binding Assay**

GW3965 - treated or control Huh7.5 cells were washed with cold medium and incubated with 100 μL of HCV preparation for 2 h at 4°C. Cells were extensively washed to remove the unbound virus, total RNA was extracted and HCV RNA was determined by qRT-PCR.

**HCV Replicon**

Huh7 cells harbouring the HCV JFH-1 sub-genomic replicon were kindly provided by J. McLachlan and grown as described [42]. Replicon cells were treated with 1 μM GW3965 diluted in DMSO for 72 h. Every 24 h the cell culture medium was replaced by fresh medium that contained the drug at the same concentration. As a control, replicon cells were treated in the same way with 0.66 μM cyclosporine A (CsA, Sigma) a known HCV replication inhibitor. Total RNA was extracted from cells every 24 h and HCV RNA assessed by qRT-PCR.

**Determination of HCV Infectivity**

An infectivity assay was performed using Huh7.5 cells seeded in 96-well plates and incubated with serially (ten-fold) diluted HCVcc preparations. After 72 h cells were fixed with 4% paraformaldehyde for 30 min at room temperature and the infected foci were visualized by In-Cell Western assay using human anti-HCV serum and DyLight™ 800 labelled goat anti-Human IgG (KPL, Inc., MD, USA). Fluorescent foci were detected in infected cells using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA). Infectivity was expressed as focus forming units/ml (ffu/ml).

**Ultracentrifugation through an Iodixanol Gradient**

Centrifugation was carried out as previously described [33]. A discontinuous 5–50% iodixanol density gradient (OptiPrep) was prepared in a buffer that contained 40 mM HEPES, 270 mM NaCl and 10 mM KCl. The supernatants from infected cells were concentrated using a Vivaspin concentrator (Vivasience) and centrifuged in the gradient for 24 h at 38,000 rpm at 4°C in an SW41Ti rotor of a Beckman ultracentrifuge. Fractions (450 μl) were collected and analysed for the presence of HCV RNA by qRT-PCR. HCV core protein, Apolipoprotein E (ApoE) and Apolipoprotein B (Apo B) were determined by ELISA assays (Mabtech AB, France), and HCV core was quantified by the
Alternatively 50 nM control siRNA was employed. After 24 h expression was silenced with 50 nM SR-BI specific siRNA. Scavenger Class B type I Receptor (SR-BI) pathway, SR-BI gene labelled with [\textsuperscript{3}H] normolipidemic plasma [43] was assayed during a 4 h chase freeze-thawing and sonication, samples of 50 g total protein were washed with FACS buffer, fixed with 1% paraformaldehyde and incubated with primary antibodies: monoclonal antibody anti-CD81, rabbit anti-LDL-R antibody (Abcam, Cambridge, UK), or rabbit polyclonal anti SR-BI (kindly provided by T. Huby) for 30 min at 4°C, washed and incubated with APC-conjugated anti-mouse or FITC-conjugated anti-rabbit antibody (BD Pharmigen) for 30 min at 4°C. Cells stained with only secondary antibodies were used as negative controls. Cells were washed with FACS buffer, fixed with 1% paraformaldehyde and analysed by FACSCalibur (BD Bioscience) using FlowJo software (Three stars). A total of 25,000 to 50,000 events were collected per sample.

**Duolink Proximity Ligation Assay**
Duolink assay (Sigma-Aldrich) based on proximity ligation technology (PLA) was used to determine the co-localisation of HCV receptors CLDN1 and SR-BI relative to CD81 (<40 nm) in drug-treated or solvent-treated cells. Monoclonal antibodies to CD81 (Abcam, Cambridge, UK), and rabbit anti-CLDN1 (Abcam, Cambridge, UK) and rabbit polyclonal anti SR-BI (kindly provided by T. Huby) were used in the assay. After signal amplification the slides were examined using fluorescence Zeiss axioplan 2 microscope (x63 objective) at 598 nm. The obtained images were quantitatively analysed using Duolink Image Tool software.

**Determination of SR-BI Function**
To investigate cholesterol efflux to HDL via the human Scavenger Class B type I Receptor (SR-BI) pathway, SR-BI gene expression was silenced with 50 nM SR-BI specific siRNA. Alternatively 50 nM control siRNA was employed. After 24 h incubation at 37°C, SR-BI knocked-down and control cells were labelled with [\textsuperscript{3}H] cholesterol in the presence or absence of 1 \textmu M GW3965. Cholesterol efflux to 50 g/mL HDL isolated from normolipidemic plasma [43] was assayed during a 4 h chase period in the presence or absence of 1 \textmu M GW3965.

**Western Blot**
Cells were lysed with a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol and 1% Triton X-100. After freeze-thawing and sonication, samples of 50 \mu g total protein were heated for 10 min at 95°C in a sample buffer containing SDS and reducing agent and subjected to electrophoresis in 3–8% Tris-Acetate polyacrylamide. Proteins were then transferred to nitrocellulose membranes and blocked with 5% skim milk in PBS containing 0.1% Tween 20. Blots were then reacted with rabbit anti-CLDN1, rabbit anti-OCLN, rabbit anti-pan Cadherin (Abcam Cambridge, UK); rabbit anti-ABCA1 (Novus Bio); or mouse anti-NPC1 (Santa Cruz) as primary antibodies, followed by DyLight 680 conjugated anti-Mouse IgG or DyLight 800 conjugated anti-Rabbit IgG (WWR, France). Blots were quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, NE, USA).

**Results**
**GW3965 Up-regulates ABCA1 Gene Expression and Cholesterol Efflux Function**
Liver X receptors (LXR\textalpha and LXR\textbeta) are ligand-activated transcription factors, which act as cholesterol sensors and control hepatic cholesterol and fatty acid homeostasis [44,45]. GW3965 is a synthetic compound (LXR agonist) that up-regulates ABCA1 [46]. No appreciable cytotoxicity of the drug for Huh 7.5 cells was observed at a concentration range of 0.08–10 \textmu M (Figure 1A). Treatment of Huh7.5 cells with 1 \textmu M GW3965 for 24 h increased ABCA1 mRNA levels up to 5-fold (Figure 1B). Up-regulation of the ABCA1 gene expression raised ABCA1 protein production (Figure 1C) and enhanced free cholesterol efflux to ApoA1 (Figure 1D). Studies of the kinetics of ABCA1 stimulation showed progressive increase of ABCA1 gene expression until a plateau was reached after 16–24 h (Figure 1E), and gradually augmented cholesterol efflux to ApoA1 (Figure 1F). Thus, 24 h of treatment of cells with the drug was used in further experiments to raise ABCA1 levels.

**Stimulation of ABCA1 Inhibits HCV Infection**
Infection with HCV did not modify ABCA1 gene expression in Huh7.5 cells during 72 h cell growth (not shown). To assess whether stimulation of ABCA1 expression and physiological function would influence the HCV life cycle, Huh7.5 cells were pre-treated with 1 \textmu M GW3965 for 24 h to raise ABCA1 levels and then infected with HCV. Up-regulation of ABCA1 decreased intracellular HCV RNA levels (Figure 2A) at 24 h post infection and significantly reduced the virus production. This was evidenced by the decrease of HCV RNA in the cell supernatant (Figure 2B), which corroborated with a decrease of the concentration of HCV core antigen by 100 fold (not shown). Continuous GW3965 treatment for 7 days substantially reduced the amount of HCV RNA detected in the cell supernatant in parallel with the increase of ABCA1 expression in virus-producing cells (Figure 2C). TO901317, another synthetic LXR agonist known to be a potent inducer of ABCA1 [47,48], inhibited HCV infection to the same extent as GW3965 did (not shown). No further decrease of infection was obtained when the two drugs were used simultaneously or in higher concentrations (up to 50 \mu M). These observations suggested that GW3965 and TO901317 inhibited HCV infection by similar mechanisms.

**ABCA1 Plays a Key Role in the Inhibition of HCV Infection**
Activation of LXRs by their agonists such as GW3965 or TO901317 enhances ABCA1 gene expression but also may affect the expression of other genes that regulate lipid metabolic pathways [45]. Indeed, transcriptomic profiling of Huh7.5 cells treated with GW3965 revealed modified mRNA levels of several genes involved in hepatic lipid metabolism: increased mRNA levels of ABCA1 and ABCG1, nuclear LXR\textalpha receptor, a sterol regulatory element binding protein-1c (SREBP-1c), fatty acid synthase (FAS) and phospholipid transfer protein (PLTP). The treatment had no significant effect on mRNA levels of fatty acid transporter CD36 and ApoA1 mRNA (Figure 3).

We therefore investigated whether ABCA1 activation was responsible for the inhibition of HCV infection by knocking down the ABCA1 gene. We used three siRNAs that target different regions of ABCA1 gene or a mixture of them to reduce ABCA1 expression. All approaches gave similar rates of ABCA1 reduction (60–80%) as compared to scrambled siRNA. Knocking down of the ABCA1 gene in Huh7.3 cells (Figure 4A) significantly reduced the production of the ABCA1 protein (Figure 4B) and impaired
cholesterol efflux to ApoA1 (Figure 4C) as compared to a control siRNA. To assess whether ABCA1 stimulation was responsible for the inhibition of infection, Huh7.5 cells were transfected with ABCA1 siRNA to knock down its expression (or with scrambled siRNA) and then treated for 24 h with 1 mM GW3965 or solvent. Cells were subsequently infected with HCV and infection levels were measured by measuring intracellular HCV RNA at 24 h after infection. Consistent with the requirement of ABCA1 stimulation to induce the inhibitory effect by GW3965 treatment, the drug did not decrease infection levels in ABCA1-silenced cells (Figure 4D). These results provided evidence that the inhibition of infection was mediated by the over-expressed and fully functional ABCA1.

GW3695 Treatment Inhibits HCV Cell Entry, but does not Impair other Steps of the Virus-cell Cycle

To determine which step of the HCV infection was affected by GW3965 treatment, we analysed the kinetics of activity of this compound by adding the drug at different time points of the virus cycle (depicted in Figure 5). When cells were pre-treated with GW3695 before virus inoculation, and the drug was maintained

Figure 1. GW3965 treatment up-regulates ABCA1 expression and its cholesterol efflux function. (A) Cell toxicity of GW3965. Huh7.5 cells were cultured in the presence of indicated concentrations of the drug for 24 h. The luminescent signal is expressed in luminescence units (RLU). (B) Up-regulation of ABCA1 mRNA expression by GW3965 treatment. Huh7.5 cells were treated for 24 h with 1 μM GW3695 or drug solvent (DMSO). Then ABCA1 mRNA was determined by qRT-PCR. (C) ABCA1 protein production in drug-stimulated Huh7.5 cells. Cells were treated for 24 h with 1 μM GW3695 and analysed by Western blot (shown in the insert). Protein content in the ABCA1 band (220 kDa) in GW3695-(GW) and DMSO-(solv) treated cells was quantified relative to the calnexin band using the Odyssey Infrared Imaging System. (D) GW3965 stimulation promotes ABCA1-mediated cholesterol efflux to ApoA1. Huh7.5 cells were labelled with [3H] cholesterol then incubated with GW3965 or drug solvent. ABCA1-dependent [3H] cholesterol efflux was assayed by comparing cell-associated and free radioactivity. (E) Kinetics of ABCA1 gene expression following stimulation of cells with GW3965. Huh7.5 cells were treated with 1 μM GW3695 for the indicated time and ABCA1 mRNA was determined by qRT-PCR. Results were expressed as relative values compared to ABCA1 expression in cells treated with drug solvent. (F) Kinetics of cholesterol efflux in cells stimulated with GW3695. Huh7.5 cells were labelled with [3H] cholesterol for 24 h, and incubated for an additional 16 h with 1 μM GW3695 or drug solvent. ABCA1-dependent [3H] cholesterol efflux was assayed in the presence of ApoA1 and either GW3695 or solvent for the indicated period of time.

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Figure 2. Stimulation of ABCA1 inhibits HCV infection. (A) Reduction of intracelluar HCV RNA levels in cells that over-express ABCA1. Huh7.5 cells were pre-treated with 1 μM GW3695 then infected with HCV. Cells were grown for a further 24 h, total RNA was extracted and intracellular HCV RNA was determined by qRT-PCR. Results are expressed as the percentage of HCV RNA relative to that in cells treated with drug solvent prior to infection. (B) Decrease of HCV RNA levels in the supernatant collected from drug-stimulated cells. Huh7.5 cells were pre-treated with 1 μM GW3695 then infected with HCV. After a further 72 h, HCV-RNA in the culture medium was determined by qRT-PCR. Results are expressed as the percentage of HCV RNA secreted from drug-treated cells compared to solvent-treated cells. (C) Effect of GW3695 treatment on long-term HCV infection. Huh 7.5 cells were pre-treated with 1 μM GW3695, infected with HCV and grown for up to 7 days in the presence of the drug. ABCA1 mRNA was determined by qRT-PCR every 24 h and results are expressed as a fold-increase of ABCA1 mRNA compared to solvent-treated cells (grey bars). HCV RNA in the cell supernatant was measured at the same time points by qRT-PCR (line curves for GW3695 treated [filled triangles] or control [filled squares] cells) and is expressed in International Units (IU).

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To assess whether ABCA1 stimulation was responsible for the inhibition of infection, Huh7.5 cells were transfected with ABCA1 siRNA to knock down its expression (or with scrambled siRNA) and then treated for 24 h with 1 μM GW3695 or solvent. Cells were subsequently infected with HCV and infection levels were measured by measuring intracellular HCV RNA at 24 h after infection. Consistent with the requirement of ABCA1 stimulation to induce the inhibitory effect by GW3695 treatment, the drug did not decrease infection levels in ABCA1-silenced cells (Figure 4D).

These results provided evidence that the inhibition of infection was mediated by the over-expressed and fully functional ABCA1.

GW3695 Treatment Inhibits HCV Cell Entry, but does not Impair other Steps of the Virus-cell Cycle

To determine which step of the HCV infection was affected by GW3695 treatment, we analysed the kinetics of activity of this compound by adding the drug at different time points of the virus cycle (depicted in Figure 5). When cells were pre-treated with GW3695 before virus inoculation, and the drug was maintained
during infection, intracellular HCV RNA levels decreased at 24 h, 48 h or 72 h post-infection (Figure 5). No inhibition of infection was observed when the drug was added concomitantly with the virus, without cell pre-incubation. These observations provided evidence that GW3965 had no direct detrimental effect on virus structure and infectivity, membrane composition, or fusion events and suggested that raising ABCA1 levels was required to affect infection. Furthermore, no inhibitory effect was noted when the drug was applied at different time points post-infection (Figure 5) implying that pre-treatment of cells with the drug stimulating ABCA1 impaired virus entry, but did not affect later steps of the virus life cycle.

To assess whether the treatment influenced the initial virus attachment to the cell surface, or later steps in virus-cell entry, Huh7.5 cells were pre-treated with GW3965 for 24 h to stimulate ABCA1 expression. The virus was then added and incubated with cells for 2 h at 4°C. After washings, the cell-bound HCV was quantified by qRT-PCR. These experiments showed that over-expression of ABCA1 after GW3965 treatment (Figure 6A) did not affect virus binding to the cell surface (Figure 6B). Hence, the inhibitory effect induced by over-expressed ABCA1 concerned virus entry events after the binding step.

No inhibition of infection was observed when the drug was added at several time points (2 h, 4 h and 6 h) after infection (Figure 5), suggesting that ABCA1 stimulation does not impair virus replication. This was further confirmed using the sub-genomic HCV replicon model, which permits studies of HCV replication mechanisms without the expression of HCV structural proteins. HCV RNA replication was not affected by GW3965 treatment for 72 h (Figure 6C), but was inhibited, in a time dependent manner by cyclosporine A, known inhibitor of HCV replication, used here as a control (Figure 6D).

Increased ABCA1 expression did not affect mechanisms of assembly/secrection of virus particles. Indeed, when infected cells were continuously treated with GW3965 and the cell medium collected every 24 h (to prevent the effect of ABCA1 stimulation...
on virus-cell entry) no difference in concentration of HCV core antigen was observed in supernatants collected at 48 h or 72 h post infection, from drug-treated compared to non-treated cells (Figure 6E). The infectivity of virus particles in these cell supernatants was also very similar (Figure 6F).

Collectively, these data demonstrated that stimulation of ABCA1-mediated cholesterol efflux inhibited cell entry step, after virus attachment to the cell surface. However the treatment did not affect other steps of the HCV life cycle and the infectivity of secreted virus particles was unchanged. Accordingly, a decrease of virus production in ABCA1 over-expressing cells was a consequence of reduced virus-cell entry.

Analysis of HCV Particles Produced in GW3965-treated Cells

The increased cellular cholesterol efflux in GW3965 cells did not affect levels of ApoB secreted to the cell supernatant (data not shown) and only slightly affected the total (Figure 7A) and free cholesterol (Figure 7B) content in Huh7.5 cell cultures. Instead, the treatment reduced the concentration of cholesteryl esters (Figure 7C) and increased cellular triglycerides (Figure 7D), as a result of well-known stimulation of lipogenesis via LXRs. These changes might potentially affect physical characteristics of the virus particles produced in drug-treated cells.

We therefore investigated the properties of virus particles produced in cells continuously stimulated with GW3965 by centrifugation in a discontinuous 5–50% iodixanol gradient. Since significantly less virus was produced and secreted from drug-stimulated compared to non-stimulated cells (shown in Figure 2B) the cell culture medium was concentrated before ultracentrifugation. Nevertheless, in spite of quantitative differences in the levels of virus production, overall properties of viruses produced in the presence or absence of the drug were very similar (Figure 8A and B). In both gradients HCV RNA was detected in two peaks at a density of 1.1 and 1.12 g/ml. The low-density virus peak co-localised with ApoB and partially with HCV core antigen, which was more abundant in the high density RNA peak.

Thus, stimulation of ABCA1 expression upon GW3965 treatment did not significantly change either infectivity (shown in Figure 6F) or physicochemical properties of the virus particles produced (Figure 8A and B) regardless of the influence of ABCA1 on virus-cell entry.
was not due to changes in the overall expression of major HCV (data not shown).

ABCA1 over-expression did not reduce SR-BI specific cholesterol transfer activity to HDL that is required for HCV cell entry [50]. Furthermore, Duolink Proximity Ligation Assay (data not shown). Furthermore, DMSO is shown in (a); that in cells pre-treated for 24 h with 1 µM GW3965 does not Change the Expression of HCV Receptors.

GW3695 does not Change the Expression of HCV Receptors

We further assessed whether the inhibition of HCV cell entry by ABCA1 over-expression was associated with changes in expression of major HCV receptors. Flow cytometry analyses demonstrated that GW3965 stimulation did not change the global expression levels of CD81, SR-BI, and LDL-R in Huh7.5 cells (Figure 9A). Equally, Western Blot analyses (Figure 9B) confirmed that the expression of NPC1, CLDN1 and OCLN were not modified by the treatment. In addition, ABCA1 stimulation did not modify the cell surface distribution of CD81 relative to SR-BI, or CD81 relative to CLDN1, which interact at the plasma membrane forming HCV receptor complexes [22,49]. The distance between these pairs of receptors was determined to be <40 nm by the Duolink Proximity Ligation Assay (data not shown). Furthermore, ABCA1 over-expression did not reduce SR-BI specific cholesterol transfer activity to HDL that is required for HCV cell entry [50].

Hence, impaired HCV entry following ABCA1 over-expression was not due to changes in the overall expression of major HCV receptor molecules, or distribution of receptors known to co-localise. It was equally not due to reduced SR-BI cholesterol transfer activity to HDL.

Stimulation of ABCA1 Affects Virus-cell Fusion

ABCA1 exerts an important influence on the plasma membrane structure, moving cholesterol within the membrane, leading to disorganisation of cholesterol-rich raft domains [51,52,53,54,55]. These changes might impair virus-cell fusion mechanisms. Using a previously developed assay [36], we found that indeed increased ABCA1 expression upon GW3965 stimulation inhibited cell fusion induced by HCV envelope proteins (*p<0.03), whereas Chikungunya envelope-induced fusion (a control) was not significantly affected (Figure 9C).

These observations suggested the impact of the increased cholesterol efflux on specific membrane rearrangements required for HCV-induced fusion.

GW3965 Treatment Affects the Organization of Cholesterol Enriched Membrane Microdomains

ABCA1 disorganises cholesterol-rich raft microdomains and redistributes cholesterol/spingolipids from raft to non-raft domains making it available for ApoA1 and facilitating HDL production [51,52,53,54,55] Supplying cells that over-express ABCA1 with exogenous cholesterol should redistribute membrane cholesterol between these domains and thus inverse the effect of ABCA1 [56].

To assess whether ABCA1-stimulated inhibition of HCV entry might be reversed by the cholesterol supply, we incubated cells that over-expressed ABCA1 with cholesterol/MβCD complexes. Cholesterol/MβCD treatment increased total cellular cholesterol content (Figure 9D) and completely reversed the inhibitory effect of ABCA1 stimulation on HCV cell entry (Figure 9E).

These data suggested that the increased cholesterol efflux via ABCA1 induced remodelling of the cholesterol-rich lipid raft microdomains and accordingly affected virus-induced fusion. The inhibitory effect could be reversed by an exogenous cholesterol supply, providing evidence that restriction of HCV infection was induced by changes of cholesterol content/distribution in membrane regions essential for virus-cell entry.

To confirm that indeed GW3965 treatment affected cholesterol-enriched membrane microdomains (lipid rafts) we analysed the plasma membrane distribution of a raft-associated Glycosylphosphatidylinositol-anchor attachment signal of the folate receptor (GFP-FR). Glycosylphosphatidylinositol-membrane (GPI)-anchored proteins are cholesterol-dependent for their plasma membrane organization and thus can be used as lipid raft markers [37,57]. Fluorescence microscopy analysis demonstrated that, while in untreated cells GFP-FR was homogenously distributed at the plasma membrane, in GW3965-treated cells GFP-FR expression was drastically different from the control (Figure 9 F and G). Thus, ABCA1 stimulation affected the organization of cholesterol enriched membrane microdomains.

GW3965 Inhibits HCV Infection of Primary Human Hepatocytes

Since Huh7.5 cells currently used as a model for studies of HCV infection have several differences in lipid metabolism compared to primary human hepatocytes, we investigated the effect of ABCA1 up-regulation on HCV infection of human hepatocytes in primary culture [34] or HCV infection of human liver slices [35], the two available models that are permissive for a full HCV life cycle and display normal lipid/lipoprotein metabolic pathways.
First, hepatocytes obtained from two liver donors were pre-treated with various concentrations of GW3965 (2–10 μM). Cells were then infected with HCVcc and grown for 24 h or 48 h and HCV RNA was determined by qRT-PCR. As shown in Figure 10 (A–B) ABCA1 up-regulation was associated with a dose dependent decrease of intracellular HCV RNA levels.

A substantial inhibition of HCV infection was also observed in the model of primary human liver slices cultures. Cultures of human liver slices were established with differentiation status and were treated with 5 μM and 10 μM concentrations of GW3965 or with drug solvent, for 24 h before infection with HCVcc. These drug concentrations were selected on the basis of results showing...
that they were not toxic for cells and were required to raise ABCA1 levels.

HCV RNA levels decreased at least by two logs in drug-treated cells, compared to non-treated cells, while ABCA1 gene expression concomitantly increased 2.5 fold over the time course of the experiment (Figure 10 C and D).

Altogether these data provided evidence that pharmacological stimulation of ABCA1 expression efficiently inhibited HCV infection of primary hepatocytes.

Discussion

In this work, we provide the first demonstration that pharmacological stimulation of ABCA1 gene expression and its cholesterol efflux function with the LXR agonist GW3965 impaired HCV infection, acting on virus protein mediated-host cell fusion. The inhibitory mechanism was associated with re-organisation of cholesterol-rich membrane microdomains (lipid rafts). Stimulation of ABCA1 expression also efficiently inhibited infection of human hepatocytes in primary culture (the natural HCV target cell) and isolated human liver slices.

LXR agonists increase ABCA1 expression but also modulate several other genes that regulate lipid metabolic pathways [58,59]. Indeed, we found that GW3965 affected the expression of several genes involved in hepatic lipid metabolism. Nevertheless, silencing of the ABCA1 gene and reducing its expression of several genes involved in hepatic lipid metabolism.

LXRs are ligand-activated transcription factors, which maintain cholesterol homeostasis. LXRs increase ABCA1 expression and cholesterol efflux function with the LXR agonist GW3965 [58]. Indeed, we found that GW3965 inhibited the expression of several genes involved in hepatic lipid metabolism. Nevertheless, silencing of the ABCA1 gene and reducing its expression has no effect on cholesterol efflux function with the LXR agonist GW3965 [58]. Indeed, we found that GW3965 inhibited the expression of several genes involved in hepatic lipid metabolism. Nevertheless, silencing of the ABCA1 gene and reducing its expression has no effect on cholesterol efflux function with the LXR agonist GW3965.
its binding affinity for certain viral envelope proteins. Cholesterol-rich microdomains are implicated in the entry of many virus species such as Ebola and Marburg viruses, Vaccinia virus, murine hepatitis virus, lymphocytic choriomeningitis virus and Herpes Simplex Virus. Indeed, cholesterol dispersion or reorganisation of lipid microdomains using drugs such as filipin and nystatin reduced virus entry, although virion attachment was unaffected. Since all these viruses enter host cell via cholesterol-rich microdomains, these observations suggested drug action on a fusion step [71].

Several studies underline the fact that ABCA1 expression does not alter the overall cellular cholesterol content or its subcellular distribution [53]. The current model proposes that ABCA1 exerts an influence on plasma membrane structure, modifying the organisation of cholesterol-rich microdomains by redistributing cholesterol/sphingolipids to non-raft domains that facilitates ApoA1 interaction and efflux [51,53,54,56]. In agreement with this notion, in our study activation of the ABCA1 pathway had only a minor effect on total and free cholesterol content in Huh7.5 cells. Instead, stimulation of ABCA1 modified the cholesterol distribution in cell membranes. Indeed, lipid raft-dependent localisation of GFP-FR in membranes was dramatically changed in GW3965-treated cells. These changes most probably disturbed virus-cell entry, confirming that the integrity of cholesterol-rich membrane microdomains is essential for the initiation of a productive HCV infection.

Supplying a surplus of exogenous cholesterol to GW3965-treated cells overturned the inhibitory effect on HCV cell entry, in accordance with the notion that adding exogenous cholesterol to cells that over-express ABCA1 tightens lipid packaging in raft and non-raft microdomains, while decreasing ABCA1-dependent cholesterol efflux [56].

HCV (JFH-1 strain) infection of the Huh7.5 cell line is currently used for studies of the HCV life cycle and the evaluation of HCV inhibitors. Nevertheless, this in vitro cell culture system, based on transformed and poorly differentiated hepatoma cells, has several
Figure 9. GW3965 treatment does not change the expression of HCV receptors, but affects HCV-cell fusion and modifies lipid raft structure. (A) Analysis of the expression of CD81, SR-BI and LDL-R in Huh7.5 cells treated with 1 μM GW3965 by Flow Cytometry using anti-receptor antibodies. GW3965-stimulated cells are shown by bold lines and non-treated cells by plain lines. Filled histograms represent cells stained with the secondary antibody only. (B) Western Blot assessment of the expression of OCLN, CLDN1 and NPC1 receptor in GW3965-treated cells, compared to solvent-treated cells. Pan Cadherin (pCadh) was used as a loading control. (C) GW3965 treatment inhibits HCV envelope-induced cell fusion. 293T cells that co-express a luciferase marker and the HCV E1–E2 envelope glycoproteins or the Chikungunya virus envelope glycoproteins were co-
cultured with Huh7-Tat indicator cells. Co-cultured cells were incubated with 1 μM GW3965 or DMSO and exposed to pH 5. Luciferase activity was measured 72 h later. Data are presented as the fusion rate in the presence of the drug (black bars) relative to fusion in the absence of drug (gray bars), which was considered as 100%.

Thus, we confirmed our findings in primary culture of human hepatocytes and in human liver slices, which maintain the 3D structure and gene expression of the liver with normal lipid/lipoprotein metabolic pathways. We provide evidence that stimulation of ABCA1 expression with GW3965 inhibits HCV infection in primary hepatocytes even more efficiently than in Huh7.5 cells.

**Figure 10. Over-expression of ABCA1 inhibits HCV infection of primary human hepatocytes and human liver slices.** (A–B) Inhibition of HCV infection of primary human hepatocytes. (A) Primary human hepatocytes were treated with 2–10 μM GW3965 (non-toxic concentrations for cells) or with drug solvent, prior to HCV infection. Twenty-four hours post-infection, ABCA1 mRNA was determined by qRT-PCR and expressed in arbitrary units, taking into account ABCA1 levels in liver cells pre-treated with the drug. (B) GW3965-treated and solvent-treated primary human hepatocytes were inoculated with HCV. After 24 h, intracellular HCV RNA was quantified by qRT-qPCR. The efficiency of infection in drug pre-treated cells was expressed as the percentage of infection compared to solvent-treated cells. (C–D) ABCA1 over-expression inhibits HCV infection of human liver slices. Human liver slices were cultured for 24 h, treated with 5 or 10 μM GW3965 or with DMSO before infection with HCVcc. At 24 h post-infection, total RNA was extracted and ABCA1 mRNA (C) and HCV RNA (D) were quantified by corresponding qRT-PCR assays and expressed as the percentage of RNA compared to the values obtained for solvent-treated cells.

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limitations, especially concerning lipoprotein metabolism [72]. Thus, we confirmed our findings in primary culture of human hepatocytes and in human liver slices, which maintain the 3D structure and gene expression of the liver with normal lipid/lipoprotein metabolic pathways. We provide evidence that stimulation of ABCA1 expression with GW3965 inhibits HCV infection in primary hepatocytes even more efficiently than in Huh7.5 cells.

HIV infection depends on membrane cholesterol for virus assembly, budding as well as cell entry, which also requires lipids
rafts [73]. Strikingly, via its protein Nef, HIV down-regulates ABCA1 expression and impairs cholesterol efflux activity to infect its target cell [74]. ABCA1 overexpression achieved using the TO901317 LXR agonist impaired HIV infection; accordingly, pharmacological stimulation of ABCA1 has been proposed as an approach to inhibit HIV infection [73].

ABCA1-mediated cholesterol efflux leads to the formation of HDL, a lipoprotein preventing arteriosclerosis and cardiovascular disease progression. New drugs are under development that should reduce the side effects found for LXR agonists such as an increase in triglyceride levels [76,77] also observed in our study. Moreover, cholesterol efflux function has been linked to anti-tumour activity, and thus ABCA1 is considered as a tumour-suppressor molecule [78].

Our study suggests that in addition to other known beneficial roles, ABCA1 emerges as a potential target to better control HCV infection.

References


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Author Contributions

Conceived and designed the experiments: AB PM SB MY S. Lebretson S. Lagaye CG FH DL. Analyzed the data: AB PM SB MG LS. Contributed reagents/materials/analysis tools: PR MLM MB SP. Wrote the paper: AB PM.

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