Hepatitis C virus infection mediates cholesteryl ester synthesis to facilitate infectious particle production

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Cholesterol is a critical component of the hepatitis C virus (HCV) life cycle, as demonstrated by its accumulation within infected hepatocytes and lipoviral particles. To cope with excess cholesterol, hepatic enzymes ACAT1 and ACAT2 produce cholesteryl esters (CEs), which are destined for storage in lipid droplets or for secretion as apolipoproteins. Here we demonstrate in vitro that cholesterol accumulation following HCV infection induces upregulation of the ACAT genes and increases CE synthesis. Analysis of human liver biopsy tissue showed increased ACAT2 mRNA expression in liver infected with HCV genotype 3, compared with genotype 1. Inhibiting cholesterol esterification using the potent ACAT inhibitor TMP-153 significantly reduced production of infectious virus, but did not inhibit virus RNA replication. Density gradient analysis showed that TMP-153 treatment caused a significant increase in lipoviral particle density, suggesting reduced lipidation. These data suggest that cholesterol accumulation following HCV infection stimulates the production of CE, a major component of lipoviral particles. Inhibition of CE synthesis reduces HCV particle density and infectivity, suggesting that CEs are required for optimal infection of hepatocytes.

INTRODUCTION

Left untreated, hepatitis C virus (HCV) infection becomes chronic in over 70% of individuals and increases the likelihood of cirrhosis and hepatocellular carcinoma (Alter, 1997). These virus-mediated events are often the result of modification of host lipid synthesis, localization and trafficking, which creates an environment favourable for HCV replication. As a result, chronic HCV infection is twice as likely to induce hepatic steatosis as other chronic liver diseases such as hepatitis B virus (HBV) infection (Czaja et al., 1998). Hepatic steatosis in HCV infection correlates with the degree of fibrosis, and is also associated with a lower sustained virological response rate to treatment with interferon and ribavirin (Hourigan et al., 1999; Poynard et al., 2003). In contrast to steatosis in the setting of non-alcoholic fatty liver disease, HCV induced hepatic lipid accumulation is accompanied by a decrease in circulating lipid, particularly the serum cholesterol fraction that is associated with low density lipoprotein (LDL) and apolipoprotein B (Serfaty et al., 2001; Siagris et al., 2006).

Our understanding of the mechanisms by which HCV alters host lipid metabolism has been significantly improved since the development of the JFH1 HCV cell culture model. This model produces infectious virus particles and allows study of the entire HCV replication cycle in vitro (Wakita et al., 2005). It is known that HCV circulates in modified very low density lipoprotein (VLDL)-like particles termed lipoviral particles (LVPs), which contain much more cholesteryl ester than other comparable viruses (Merz et al., 2011). HCV binding and entry rely on multiple cellular receptors, including the lipid receptors LDL receptor (LDLr), scavenger receptor BI (SR-BI) and the Niemann–Pick C1-like 1 receptor (Agnello et al., 1999; Sainz et al., 2012; Scarselli et al., 2002), which facilitate viral infection of hepatocytes through their interactions with the lipid components of LVPs. Following infection, an increase and redistribution of intracellular lipid content occurs in parallel with viral protein expression. In particular, NS4B, a viral non-structural protein, has been shown to induce endoplasmic reticulum (ER) derived membrane remodelling, termed the membranous web (Egger et al., 2002). This web is primarily composed of cholesterol rich, detergent resistant double membrane vesicles in close proximity to lipid droplets, and is considered the site of...
viral replication and particle assembly (Aizaki et al., 2004; Bartenschlager et al., 2011; Paul et al., 2013).

Like other members of the Flaviviridae, HCV-mediated alterations of intracellular membranes provide a platform for viral replication, particle assembly and immune evasion, but likely take a toll on the host cell (Miller & Krijnse-Locker, 2008). Excess cholesterol required to generate the HCV membranous web can significantly alter membrane fluidity, protein localization and signalling, causing toxicity to the infected hepatocyte (Tabas, 2002). One mechanism by which hepatocytes respond to excess intracellular cholesterol is by adding a fatty acid ester to the hydroxyl group of cholesterol, creating less toxic cholesteryl esters (CEs) that can be stored as lipid droplets or exported in lipoproteins (van der Wulp et al., 2013). Cholesterol esterification is mediated by the enzymes acyl-CoA cholesterol acyltransferase (ACAT) 1 and 2. While both ACATs are expressed in the liver, ACAT2 mediates cholesterol esterification prior to VLDL incorporation and secretion, and is upregulated by cholesterol loading (Lee et al., 2000, 2005; Pramfalk et al., 2007).

To determine the importance of cholesterol esterification in the HCV life cycle, we examined the accumulation of lipids in HCV-infected hepatocytes in vitro and the resulting expression of cholesteryl esterifying genes. We observed marked accumulation of cholesterol and CE in HCV-infected cells, and increased expression of ACAT2. We confirmed increased ACAT2 expression in liver biopsies from humans with HCV infection, especially genotype 3. Using a specific ACAT inhibitor, we then demonstrated reduced production of infectious viral particles from HCV-infected cells. This suggests that cholesterol esterification is an important process in the formation of infectious LVPs, and identifies ACAT2 as a novel antiviral target against HCV.

RESULTS

HCV infection stimulates accumulation of triglycerides and cholesterol

To examine the effect of HCV infection on triglyceride (TG) and cholesterol accumulation in hepatocytes, total lipid was extracted from HCV (JFH1) and mock infected Huh-7 cells, normalized to total protein content, and measured by a colorimetric assay. As demonstrated in Fig. 1(a), all lipid species examined were upregulated in JFH1-infected cells, with increased TG, total and free cholesterol, but a preferential ~50% increase in CE. To determine whether certain lipid species accumulate preferentially in the cytoplasm of JFH1-infected cells, fractionation followed by lipid quantification was performed. As for whole cell extracts, all lipids measured were significantly increased in the cytoplasm of JFH1-infected cells (Fig. 1b), particularly CEs. The high ratio of cytoplasmic to whole cell lipid accumulation suggests that TG and CE accumulate preferentially in the cytoplasm, where they are stored within lipid droplets, key organelles for HCV replication (Table 1).

Cholesterol accumulates in proximity to the HCV replication complex

To examine the cellular distribution of TG and cholesterol following JFH1 infection, immunofluorescence labelling was undertaken. Huh-7 cells were exposed to virus-containing medium from JFH1-infected cells and the infection allowed to progress for 2 h to allow virus infection, after which the medium was changed. After 48 h, cells were immune labelled with antibodies against the HCV NS5A protein, BODIPY 493/503 dye to label neutral lipid, and filipin to label cholesterol. A significant accumulation of filipin staining for cholesterol was observed only in HCV-infected cells, which also stained positive for NS5A (Fig. 2a). Cholesterol stained in a multi-vesicular pattern in close proximity to the nucleus, as has been described previously for the membranous web (Romero-Brey et al., 2012). To ascertain the origin of the membranous accumulation, JFH1-infected cells were stained with the Golgi marker Golgi protein 54 and the ER marker calreticulin, in combination with NS5A and filipin (Fig. 2b). Accumulated cholesterol within infected cells preferentially co-localized with the ER marker calreticulin, which is consistent with previous reports suggesting that the membranous web originates from the ER (Egger et al., 2002). Both dsRNA and NS5A localized to the cholesterol-rich perinuclear region, suggesting the presence of RNA replication, and consistent with this being the membranous web (Fig. 2c). Because the membranous web is also believed to be the site of lipoviral particle formation, staining of neutral lipid and core protein was performed. Lipid droplets from HCV-infected cells were coated by the HCV core protein and cholesterol (Fig. 2d) and also demonstrated close interaction with NS5A (Fig. 2a). It is likely that a close association between core protein, NS5A, lipid droplets and cholesterol is required for viral replication and particle production, as has been published previously (Bouant et al., 2007; Miyarani et al., 2007). Furthermore, the accumulation of cholesterol surrounding lipid droplets in infected cells may indicate that excess cholesterol is becoming esterified and transported into lipid droplets, where it contributes to HCV-mediated lipid droplet accumulation and resulting steatosis. Further experiments are nonetheless required to ascertain the fate of the accumulated cholesterol within the membranous web.

Cholesterol esterifying enzymes are upregulated in HCV-infected cells

To prevent the toxic accumulation of cholesterol within cells, cholesterol is esterified and accumulates within lipid droplets for storage and/or export in lipoproteins (van der Wulp et al., 2013). We hypothesized that cholesterol accumulation resulting from JFH1 infection and formation of the membranous web may become toxic, stimulating esterification of excess cholesterol. In the liver, cholesterol
is esterified by the enzymes ACAT1 and ACAT2, and de-esterified by lysosomal acid lipase (LIPA). To determine whether the accumulation of cholesterol as a result of JFH1 infection results in an increase in cholesterol esterifying enzymes, qPCR was performed on Huh-7 and JFH1-infected cells. As demonstrated in Fig. 3(a), JFH1-infected cells demonstrated a 1.5-fold increase in ACAT1 and LIPA and a fourfold increase in ACAT2.

To determine whether upregulation of ACAT2 was HCV genotype specific, it was examined both in vitro using JFH1 chimaeras containing genotype 1b or 3a core (Fig. 3b), and in vivo using liver biopsy tissue from patients infected with HCV genotype 1 or 3, or HBV as control (Fig. 3c). Interestingly, ACAT2 was upregulated approximately 1.5-fold in cells infected with both genotype 1b and 3a core JFH1 chimaeras, but slightly more with genotype 3a. Human liver infected with HCV genotype 3 demonstrated approximately twofold upregulation of ACAT2, compared with HBV-infected liver \( (P<0.05) \). HCV RNA was slightly higher in liver tissue from genotype 1 than genotype 3-infected patients, confirming that increased ACAT2 expression in genotype 3-infected patients was not due to higher viral load (Fig. S1, available in the online Supplementary Material). Finally, the effect of genotype 1b and 3a core protein expression was compared using baculovirus expression vectors. We observed a non-significant trend towards higher expression of ACAT2 in cells expressing genotype 3a core (Figs S2–4).

Inhibition of cholesterol esterification impairs production of infectious virus

Apart from being a major component of the LVP (Merz et al., 2011), little is known about the role of CEs in the HCV life cycle. To explore this, we inhibited cholesterol esterification with a potent ACAT1/2 inhibitor, TMP-153, and examined its effects on HCV infection and/or production of infectious HCV particles.

To confirm the effects of the ACAT inhibitor TMP-153 on cholesterol esterification, Huh-7 cells were treated with 10 or 25 nM TMP-153 for 72 h, or mock treated. Total lipid was extracted and cholesterol content was measured and normalized to total protein. Fig. 4(a) demonstrates a dose-dependent effect of TMP-153 on cholesterol esterification, with a significant reduction at the 25 nM concentration. Interestingly, no effect on total cholesterol and TG was found, as measured by biochemical assay and immunofluorescent staining. Cleaved caspase-3 and -7 were also examined to ensure that ACAT inhibition did not cause cell apoptosis. Compared with mock treatment, 25 nM TMP-153 did not significantly increase apoptosis in JFH1-infected cells (data not shown).

To determine whether cholesterol esterification affects secretion of viral RNA and infectious virus particles, JFH1-infected cells were treated with 25 nM TMP-153 for 72 h, then the culture medium was collected and analysed for...
HCV RNA (by qPCR) and virus infectivity, using naive Huh-7 cells and the TCID\textsubscript{50} (50% tissue culture infectious dose) assay. Fig. 4(b) demonstrates that inhibition of cholesterol esterification did not affect total virus export, as measured by HCV RNA copies in the medium, but reduced infectivity by 50%. Conversely, pre-treating Huh-7 cells with 25 nM TMP-153 for 72 h had no effect on susceptibility of the cells to infection, as measured by TCID\textsubscript{50} and intracellular HCV RNA copies (Fig. 4c). These data suggest that inhibition of cholesterol esterification alters the composition of the LVP, rendering it less infectious.

To determine whether the TMP-153-mediated reduction in viral infectivity was associated with an alteration in virus density, JFH1-containing medium from TMP-153 and mock-treated cells was applied to a sucrose density gradient. TMP-153 treatment resulted in a modest reduction in secreted virus, particularly at lower density, as measured by qPCR (Fig. 5a). This was associated with a significant reduction in viral infectivity of $>75\%$ of the lowest density fractions surrounding 1.05 g ml$^{-1}$ (Fig. 5b). Specific infectivity of each fraction was next calculated by dividing the relative infectivity by HCV RNA. A shift of infectivity towards a higher density was observed following TMP-153 treatment, suggesting a reduction in LVP lipidation (Fig. 5c). Together, these data suggest that inhibiting cholesterol esterification does not reduce the overall export of virus, but prevents the addition of CEs to LVPs, thus increasing their density while making them less infectious (see Fig. S5 for total infectivity and viral RNA quantification).

**DISCUSSION**

Following HCV infection, cholesterol accumulates in the liver, where it performs a number of roles to facilitate HCV replication and LVP production. Promising antiviral effects of HMG-CoA reductase inhibitors (statins) in vitro (Ye et al., 2003) were unfortunately not replicated in clinical trials (Forde et al., 2009), and so shifted the development of antivirals away from targeting cholesterol metabolism. However, understanding the mechanisms by which HCV uses hepatic cholesterol to replicate and assemble new virus may inform the development of novel drugs that inhibit specific cholesterol functions exploited by HCV.

In agreement with recently published data, we demonstrated that HCV infection in vitro results in a significant
intracellular accumulation of cholesterol and TG (Woodhouse et al., 2010). We found an accumulation of cholesterol in close association with viral NS5A and dsRNA, suggesting that it is highly concentrated at the location of viral replication. The cholesterol accumulation is likely occurring within the cholesterol rich, detergent resistant lipid rafts which characterize the membranous web (Aizaki et al., 2004). We then demonstrated a close association between cholesterol, lipid droplets and core protein in JFH1-infected cells. This may represent the site of virus particle assembly within the membranous web, where core protein located on lipid droplets assembles and encapsidates newly replicated viral RNA (Counihan et al., 2011). Incorporation of newly formed virus capsids into LVPs may therefore involve addition of CE by ACAT2, which is responsible for the

lipidation of apolipoproteins within the lumen of the ER, to form VLDL (Temel et al., 2007). For HCV LVPs to maintain a lipid composition similar to that of LDL or VLDL (Merz et al., 2011), a significant intrahepatic accumulation of lipid would be required, especially CEs, which provide almost half the lipid within LVPs (Merz et al., 2011). Interestingly, LVPs possess even more lipid than lipoproteins of the same density, to compensate for the additional viral protein in the particles (Scholtes et al., 2012), further increasing the need for CE. Therefore, in HCV-infected hepatocytes, cholesterol that accumulates in the membranous web to facilitate viral replication may also assist in particle formation, through the esterification of excess cholesterol and its incorporation into LVPs. PTEN downregulation following HCV infection in vitro was recently shown to facilitate the accumulation of

Fig. 3. Upregulation of cholesterol esterifying enzymes in JFH1-infected cells. The expression of cholesterol esterifying enzymes was quantified in uninfected and JFH1-infected cells by qPCR. JFH1-infected Huh-7 cells express significantly more ACAT1, ACAT2 and LIPA than uninfected cells. Due to its elevated expression following JFH1 infection, intergenotypic ACAT2 expression was examined in vitro using JFH1 core chimaeras, and in vivo in liver biopsy tissue. Both genotype 1 and 3 core chimaeras demonstrated a significant ~1.5-fold increase in ACAT2 expression (b) whereas only genotype 3 infected liver biopsy tissue demonstrated a twofold increase in ACAT2 compared with HBV biopsy tissue (c) (*P<0.05, **P<0.01). No significant difference in viral RNA was observed between genotype 1 and 3 patients (Fig. S1). Error bars, ± SEM.
CE, consistent with our observations, and so may also contribute to ACAT2 upregulation in HCV-infected hepatocytes (Peyrou et al., 2013).

We have shown that ACAT2, the enzyme that mediates the esterification of cholesterol destined for secretion in VLDL (Lee et al., 2000), was increased fourfold in HCV-infected cells. Unlike ACAT1, ACAT2 induction is mediated by elevated hepatic cholesterol, which may explain its high expression in HCV-infected hepatocytes (Pramfalk et al., 2007). Elevated CE production, accompanied by reduced microsomal triglyceride transfer protein (MTP) activity, lipid breakdown and VLDL secretion, may explain the accumulation of CE in infected hepatocytes (Harris et al., 2011; Mirandola et al., 2006; Perlemuter et al., 2002). CEs then likely contribute to the accumulation of lipid droplets observed in HCV-infected hepatocytes, consistent with the increased cytoplasmic lipid content we observed.

Similar to ACAT2, ACAT1 and LIPA were significantly upregulated in JFH1-infected Huh-7 cells, although to a lesser extent. Their respective roles in cholesterol esterification and de-esterification suggest increased cholesterol ‘remodelling’ in HCV-infected cells, which may facilitate virus replication and particle assembly (Fig. 6).

Using HCV genotype 1/3 core chimaeras and human liver biopsy samples, we confirmed that ACAT2 expression is increased more with genotype 3 infection than genotype 1. Consistent with this, genotype 3 infection is associated with more steatosis and the induction of genes promoting lipid synthesis (Negro, 2010). Perturbations in cholesterol
synthesis and trafficking are also more pronounced in genotype 3-infected individuals, where steatosis is inversely associated with serum cholesterol (Clark et al., 2012; Hui et al., 2002). The genotype specific effect of HCV on CE formation is, we believe, a novel finding, which provides insight into the pro-lipogenic mechanisms of genotype 3 infection. Of note, we observed less ACAT2 upregulation in Huh-7 cells infected with chimaeric JFH1 virus than with wild-type JFH1, but this is probably due to the reduced fitness of the chimaeric viruses, which replicate ~1 log less efficiently (data not shown). The absence of ACAT2 upregulation in core expressing HepG2 cells (Fig. S4) suggests that viral replication, and perhaps the formation of the cholesterol-rich membranous web, is required to induce its expression.

Finally, the importance of cholesterol esterification in the HCV life cycle was examined by inhibiting ACAT1 and ACAT2 using the inhibitor TMP-153. TMP-153 is a potent ACAT inhibitor that does not affect hepatic TG content, and thus is unlikely to affect other members of the membrane-bound O-acyltransferase family, such as DGAT1, particularly at the low doses used in these experiments (Sugiyama et al., 1995). To ensure that reduced hepatic CE content did not induce an increase in cholesterol receptors, thereby increasing HCV infection, Huh-7 cells were treated with TMP-153, or mock treated, prior to infection. TMP-153 pre-treatment did not affect HCV infection, suggesting that there was no modification of HCV receptor expression or distribution. This agrees with previous reports showing no effect of ACAT inhibition on LDLr expression, although a mild increase in SR-BI has been observed in hamsters (Milliat et al., 2001; Yu-Poth et al., 2004).

Interestingly, inhibition of ACAT activity in JFH1-infected cells did not affect virus RNA replication (data not shown) or total export of virus, but resulted in reduced production of low density virus, with a significant loss of infectivity. It

![Fig. 5. TMP-153 treated Huh-7 cells secrete less low density virus. JFH1-infected Huh-7 cells were treated with 25 nM TMP-153 or mock-treated and the medium was collected after 72 h. Secreted virus was separated by density using a sucrose gradient, and individual fractions were tested for viral RNA by qPCR and for infectivity by TCID_{50}. Viral RNA and infectivity for each fraction were calculated as a percentage of mock-treated total RNA and infectivity, respectively. The infectious dose of JFH1 virus derived from Huh-7 cells treated with TMP-153 was significantly decreased at lower densities (a), with little effect at higher densities. Conversely, total viral secretion, as measured by viral RNA (b), was slightly increased at intermediate densities but decreased at very low density, suggesting that inhibiting esterification resulted in production of less infectious, higher density virus particles. Relative infectivity, as measured by dividing infectivity by RNA (c), was increased at higher densities following TMP-153 treatment, suggesting that particles were de-lipidated as compared with mock-treated cells. (*P<0.05, **P<0.01). Error bars, ±SEM.](image-url)
has been well documented that low density, highly lipidated HCV LVPs are more infectious than high density virus (Merz et al., 2011; Nielsen et al., 2006). Our data agree with the observed reduction in VLDL secretion in ACAT2 knockout mice (Lee et al., 2005). Because esterified cholesterol is packaged into VLDL via MTP, it is not surprising that ACAT inhibition reduces virus particle secretion without affecting RNA replication, similar to inhibition of MTP (Huang et al., 2007; Icard et al., 2009). Interestingly, we observed a large peak of viral RNA at high density (~1.2 g ml⁻¹) for both mock and TMP-153 treated cells. This could represent naked or capsidated RNA, but may simply be an artefact of the PEG precipitation of virus. Further purification of virus would be required to clarify this.

ACAT inhibitors have now entered clinical trials, principally for the treatment of coronary heart disease. Pactimibe, a non-selective inhibitor of ACAT1 and ACAT2, showed initial promise for the treatment of coronary heart disease, but was disappointing in larger clinical trials (Meuwese et al., 2009; Nissen et al., 2006). Specific inhibitors of ACAT2 have now been developed, and may show more potential for treatment of atherosclerosis and fatty liver disease (Alger et al., 2010). Our data suggest that specific inhibitors of ACAT2 may be the most effective to treat HCV, particularly for genotype 3 infection. The oral ACAT2 inhibitor pyripyropene A has demonstrated cholesterol lowering activities in mice, suggesting promise for clinical development of this class of drugs (Ohshiro et al., 2011).

In summary, we have demonstrated that cholesterol ester production is increased following HCV infection in vitro, likely due to increased ACAT2 expression in response to hepatic cholesterol accumulation. We propose that inhibition of the ACAT enzymes using TMP-153 decreases the density of LVPs by reducing their CE content, and as a result significantly reduces their infectivity. Inhibition of cholesterol esterification therefore provides an attractive strategy for the treatment of HCV infection.

Fig. 6. Cholesterol sequestration facilitates HCV replication. Following HCV infection, viral protein expression induces the formation of a membranous web (1) consisting of an accumulation of ER derived membrane where viral replication takes place. Cholesterol accumulates within the lipid raft component of the replication complex to facilitate viral replication, which can then become toxic at high concentrations (2). To prevent cellular toxicity from excessive cholesterol, ACAT enzymes (particularly ACAT2) add fatty acid chains to cholesterol, producing cholesteryl esters (CE), for storage in lipid droplets or export in lipoproteins (3). In HCV-infected cells, CE is diverted to virus assembly complexes at the lipid droplets, for incorporation into lipoviral particles (4). Treatment with an ACAT2 inhibitor reduces the availability of CE, resulting in the production of higher density virus, which is less infectious (Perlemuter et al., 2002).
novel target for antiviral therapy against HCV. ACAT2 inhibitors may offer potential as adjunct therapy for patients with chronic HCV infection. They may be particularly effective for genotype 3 infection, which is characterized by significant accumulation of hepatic lipid compared with other genotypes, and for which direct acting antivirals have been the least effective (Foster et al., 2011; Lawitz & Gane, 2013).

METHODS

Cell culture, viral RNA production and infection. All JFH1 constructs were maintained in the Huh-7 hepatoma cell line. Cells were maintained in Dulbecco’s minimal essential medium with 10 % fetal bovine serum. Viral RNA was transcribed in vitro using the T7 RiboMAX express large scale RNA production system (Promega) and transfected into Huh-7 cells. Cells harbouring full-length JFH1 and chimaeric JFH1 constructs containing different genotype core proteins were maintained for a maximum of six passages to minimize viral mutation. See the Supplementary data for methods of baculovirus-mediated core expression in HepG2 cells.

Lipid extraction and quantification. Triglyceride and cholesterol were extracted and quantified using a Wako-E triglyceride kit and Wako total cholesterol kit, respectively. Briefly, lipid was extracted from mock and JFH1-infected Huh-7 cells using a lipid extraction reagent containing 60 % tert-methyl alcohol, 20 % methanol and 20 % Triton X-100. The mixture was spun at 1060 g to pellet any cell debris. The supernatant was removed and nitrogen gas was used to concentrate the lipid emulsion. Lipid assays were then performed according to the manufacturer’s specifications. Prior to lipid extraction, a subset of cells were removed for protein estimation using a Bio-Rad DC protein assay kit.

Cell fractionation. To isolate cytoplasm from JFH1 and Huh-7 cells, approximately 2 x 10^7 cells were suspended in MTE buffer (270 mM d-mannitol, 10 mM Tris, 0.1 mM EDTA, pH 7.4) and sonicated. Nuclei and mitochondria were removed by centrifugation at 2080 g and the supernatant was loaded onto a 1.3 M sucrose cushion and centrifuged at 4 °C at 21000 g for 1 h. Lastly, the cytoplasm and floating lipid droplets were removed from the supernatant above the sucrose cushion and the ER band located at the interface of the cushion.

Immunofluorescent cell staining. Cells were fixed in 4 % paraformaldehyde for 10 min at 4 °C then permeabilized with 0.1 % Triton X-100 in PBS containing 2 % FCS. Immunofluorescence was performed using anti-core rabbit antisera R308 (kindly provided by Dr John McLauchlan, MRC-University of Glasgow Centre for Virus Research, UK) (Hope & McLauchlan, 2000), antibody against non-structural protein 5A (NS5A) (kindly provided by Professor Mark Harris, University of Leeds, UK), dsRNA antibody J2 (Scicons), calreticulin antibody (BD Biosciences) and 58 kDa Golgi protein antibody (Abcam). To stain cellular cholesterol and neutral lipid, 20 µM filipin and BODIPY 493/503 dye were used. Cells were examined on a Deltavision deconvolution microscope (Applied Precision).

Quantification of mRNA. RNA was extracted from Huh-7 JFH1-infected cells and liver biopsy tissue, using a Qiagen RNeasy kit. Viral RNA was extracted from medium using a Qiagen Qiamp viral RNA Mini kit and cDNA synthesized from 500 ng RNA using the MMLV reverse transcriptase from Promega and random hexamers according to the manufacturer’s protocol. Genes of interest were amplified by quantitative PCR (qPCR), using the Rotor-Gene 3000 or 6000 (Corbett Research). TaqMan primer probes or primer sets combined with SYBR green were used to amplify ACAT1 (GAAGACGGCTGCTAAGTCC, AATGGCTTCAATTCCCTTGC), ACAT2 (GAGACTTACCCTAGGACGCCCT, AGTTCTTGGCCACATAATTCCAC), LIPA (Hs00165098_m1; Applied Biosystems) and 18S RNA (4319413E; Applied Biosystems). To determine the relative expression of genes of interest, standard curves were generated using 10-fold dilutions of cDNA, ensuring all sample values obtained were within the area of the generated curve. All mRNA levels were normalized to 18S rRNA and compared with controls. To standardize the experimental replicates, all treatments were compared with controls, which were normalized to a value of 1.

Patient samples. Liver biopsies were collected from 23 patients chronically infected with HBV, 31 with HCV genotype 1, and 24 with HCV genotype 3. RNA was extracted and gene expression analysed by qPCR, as above. All samples were confirmed histologically to have low fibrosis (Metavir score £1) and steatosis (£grade 1).

TCID50. To determine the amount of infectious virus present in culture media, TCID50 assays were performed as described previously (Shavinskaya et al., 2007). Briefly, filtered JFH1 medium was added to a column of Huh-7 cells within a 96-well plate and diluted fivefold for every column of wells along the length of the plate. Infection was allowed to proceed for 72 h, then cells were fixed with 100 % methanol. Cells were blocked with 2 % FCS in PBS for 30 min and endogenous peroxidase was inactivated by adding PBS containing 0.3 % hydrogen peroxide. N5SA antibody was added at 1:10 000 dilution, then a HRP-conjugated secondary antibody was added at 1:1000 dilution. A HRP substrate (VECTOR NovaRED Substrate kit) was added to the cells to visualize infectious foci. Wells containing at least one infectious centre were counted as positive. Lastly, well counts based on dilution of the virus were entered into a TCID50 calculator to determine the TCID50 of the infectious medium.

Infectivity assay. To supplement the TCID50 assay, infectivity was also examined by qPCR. Approximately 10^4 Huh-7 cells were plated in six-well plates and treated with 25 nM TMP-153, for 72 h. Medium was removed and replaced with filtered medium from JFH1-infected Huh-7 cells (without drug treatment). Virus was allowed to infect cells for 24 h to limit viral replication following infection. RNA was extracted, cDNA was synthesized and qPCRs were then performed.

Concentration and density fractionation of HCV virions. JFH1-infected cells were treated with 25 nM TMP-153 or DMSO as a control, for 3 days. At least 50 ml medium from JFH1-infected cells was gathered and combined with 40 % polyethylene glycol to create an 8 % solution. The virus was precipitated overnight at 4 °C and pelleted by centrifugation at 8000 g for 20 min. The virus pellet was washed and resuspended in PBS and stored at 4 °C until use (<1 week). A sucrose gradient was prepared by layering sucrose solutions from 80 to 10 % sucrose. Precipitated JFH1 was layered on top of the gradient, and tubes were centrifuged at 20000 g for 16 h at 4 °C to separate virus based on particle density. To extract individual fractions of the gradient for TCID50 and reverse transcriptase PCR analysis, a needle was placed at the bottom of the centrifuged tubes and fractions of the sucrose gradient were removed, starting from the densest. Viral RNA extraction from individual fractions was performed using Trizol (Invitrogen). Individual fractions were diluted 1:5 in Dulbecco’s modified Eagle’s medium for TCID50 assays, as high sucrose concentrations resulted in Huh-7 cell death.

Data analysis. Quantitative data were expressed as mean ± SEM. Statistical analysis was performed using Graphpad Prism, comparing control with treated groups. Student’s t-tests were performed to compare individual treatments. Correlation was performed and the r² value determined to identify the relationship between gene expressions.
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