



SKI-1/S1P inhibition: A promising surrogate to statins to block Hepatitis C virus replication

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ABSTRACT

Hepatitis C virus (HCV) is often associated with steatosis, cirrhosis and hepatocellular carcinoma (HCC). Statins (HMG-CoAR inhibitors) have been shown to exert an antiviral effect *in vitro*, principally on replicon harboring cells, but the effect of their use alone *in vivo* remains controversial. In clinical trials, when used in combination with the standards of care (SOC), they led to an increased proportion of sustained virological responder (SVR). Here we investigated the implication of SKI-1/S1P, a master lipogenic pathways regulator upstream of HMG-CoAR, on different steps of HCV life cycle. We compared the HCV antiviral effect of the most potent SKI-1/S1P small molecule inhibitor (PF-429242) with a set of two statins on different steps of the viral life cycle, and showed that SKI-1/S1P inhibitor blocked HCVcc (strain JFH-1) RNA replication ($EC_{50} = 5.8 \mu M$) more efficiently than statins. Moreover, we showed that PF-429242 could reduce lipid droplets accumulation in Huh7 cells. Interestingly, PF-429242 dramatically reduced infectious particles production ($EC_{90} = 4.8 \mu M$). Such inhibition could not be achieved with statins. SKI-1/S1P activity is thus essential for viral production and its inhibition should be considered for antiviral drug development.

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1. Introduction

The hepatitis C virus (HCV) is known to be a prominent factor for the onset of end-stage liver diseases such as liver cirrhosis and hepatocellular carcinoma (HCC) (Di Bisceglie, 1997). World-wide, approximately 170 million patients are chronically infected, and it has been estimated that over 300,000 individuals die annually from HCV infection. The recent addition of HCV protease inhibitors to the standard treatment consisting of pegylated interferon- α (PEG-IFN) and ribavirin has significantly increased viral clearance in patients (Hezode et al., 2009; Manns et al., 2012). However, 15–30% of patients still do not respond to treatments and severe side effects are common in treated individuals (Fried et al., 2002).

Many studies have highlighted the close relationship between HCV life cycle and lipids metabolism (Herker and Ott, 2011). Statins, HMG-CoA reductase inhibitors that are widely used in human to lower cholesterol levels (Carroll et al., 2005), have been tested for

HCV-antiviral properties. *In vitro*, several statins have shown potent antiviral activity in cells harboring HCV replicon, but lower effect were obtained in cell infected with HCVcc (Delang et al., 2009; Ikeda et al., 2006). Because of their low toxicity profile *in vivo*, statins have been studied in humans. While their antiviral properties alone remains controversial (Bader et al., 2008; Harrison et al., 2010; Milazzo et al., 2009; O'Leary et al., 2007), their use in combination with PEG-IFN and ribavirin seems to have positive effect (Harrison et al., 2010). The discrepancy observed in human could be partially explained by the wide antiviral effectiveness of different statins. However the divergence between various statins in their ability to inhibit HCV *in vitro* is still not understood, emphasizing the complex interrelation of HCV lifecycle and lipids metabolism.

Because of the variable and limited antiviral effects obtained with statins so far, we sought to investigate the antiviral potency of an upstream regulator of cholesterol and fatty acid metabolic pathways. Thus, we concentrated on the eighth member of the protein convertase family, namely subtilisin/kexin-isoenzyme-1 (SKI-1) or site-1 protease (S1P), now designated as SKI-1/S1P (Seidah and Prat, 2007). This pyrolysine-like serine protease is a membrane-bound enzyme, which after autocatalytic activation cleaves a defined set of Golgi-associated membrane-bound transcription factors (Seidah and Prat, 2007). SKI-1/S1P plays a key role in regulating cholesterol and fatty acids pathways by cleaving SREBP-1a, -1c, and -2 (Fig. 1) (reviewed in Horton et al., 2002). Additionally,

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; SOC, standards of care; SKI-1/S1P, subtilisin/kexin-isoenzyme-1/site-1 protease; SREBP, sterol regulatory-element binding protein; LD, lipid droplet; VLDL, very low density lipoprotein; PEG-IFN- α , pegylated interferon- α .

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SKI-1/S1P activates ATF6 (Zhang and Kaufman, 2004), a key regulator of ER stress known to be up-regulated in HCV infected cells (reviewed in Tardif et al., 2005). Given the close relationship between HCV life cycle, lipogenic pathways and ER stress, we hypothesized that SKI-1/S1P might be a relevant target, which if inhibited would impair viral propagation. We thus compared the inhibition potencies of PF-429242, a potent SKI-1/S1P cell permeable small molecule inhibitor (Hawkins et al., 2008), with pravastatin and simvastatin, at different steps of the virus life cycle.

2. Materials and methods

2.1. Cells culture and virus

Huh-7 and Huh7.5 cells were maintained in medium A [Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 100 units/ml of penicillin/streptomycin]. Cells were infected with JFH-1 virus at an MOI of 0.02 and maintained in medium A for 3-to-4 weeks prior to use in downstream assays. Drugs were added to cells in Medium B [medium A with 5% FBS] or Medium C [Medium A with 5% LipoProtein-Deficient Serum (LPDS-Biomedical Technologies)].

2.2. Chemicals

PF-429242, a SKI-1/S1P inhibitor kindly provided by Pfizer (Hawkins et al., 2008), was resuspended in DMSO at 80 mM. Pravastatin sodium salt hydrate and simvastatin (Sigma-Aldrich) were resuspended at 50 mM in H₂O and 40 mM in DMSO, respectively.

2.3. Quantification of cellular mRNA and HCV RNA by RT-qPCR

A summary of the primers used in this study is presented in [Supplementary Table 1](#). Nanodrop-normalized levels of total RNA were reverse transcribed with iScript cDNA synthesis Kit (Biorad). cDNA were used for qPCR using Ssofast Evagreen supermix or Ssofast probe Supermix for cellular mRNA and HCV RNA quantification, respectively. Results were analyzed using the comparative Ct method. For quantification of HCV RNA in supernatant, extracted viral RNA was reverse transcribed using M-MLV polymerase (Promega). cDNA was then used for qPCR with Ssofast probe supermix.

2.4. Cell viability assay

Cells seeded in a 96-well plate were exposed to serial dilutions of drugs in medium B for 72 h. Cell viability was then assayed using the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega).

2.5. Western blot analysis

Cells were lysed in 1% NP40, 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, Complete™ protease inhibitor (Roche). Lysates were normalized for total protein content using the BCA protein assay kit (Pierce). Proteins were then resolved by 12% SDS-PAGE and HCV NS3 protein was detected using a rabbit anti-NS3 antibody (1/1000) (gift from Dr. Olivier Nicolas) and a HRP-conjugated goat anti-Rabbit (Jackson Laboratories). β-actin was detected using the mouse monoclonal AC-15 antibody (Invitrogen) at a dilution of 1/2000.

2.6. HCV titration by foci forming unit (FFU) assay

Titration was performed as previously described (Guevin et al., 2009). Briefly, 10⁴ Huh-7.5 cells seeded in 96-well plates were inoculated for 6 h with 100 μl of pure or 2-fold diluted supernatants. Cells were washed and maintained in medium A for 48-to-72 h, as mentioned in the legends of Fig. 6. Infection was controlled by immunofluorescence microscopy using a rabbit anti-NS5A antibody (gift from Dr. Olivier Nicolas) and a goat anti-rabbit IgG-Alexa Fluor® 488 (Invitrogen). Nuclei were stained with DAPI. The average number of NS5A-positive foci detected was calculated for each sample and the viral titer was reported as a percentage of the non-treated control.

2.7. Analysis of LD concentrations

2.7.1. FACS

Huh-7 cells were seeded in 6-well plates. After overnight incubation at 37 °C in medium A, 5 μM of PF-429242 or simvastatin were added to the cells in medium B for 3 days. Cells were fixed in 4% PFA and stained for virus with a rabbit anti-NS5A antibody (1/500) and a Chicken Anti-Rabbit IgG-Alexa Fluor® 647 (Invitrogen). Cells were then stained for LD with BODIPY® 493/503 (Invitrogen). Cells were assayed on a FACSCalibur flow cytometer (BD Bioscience).

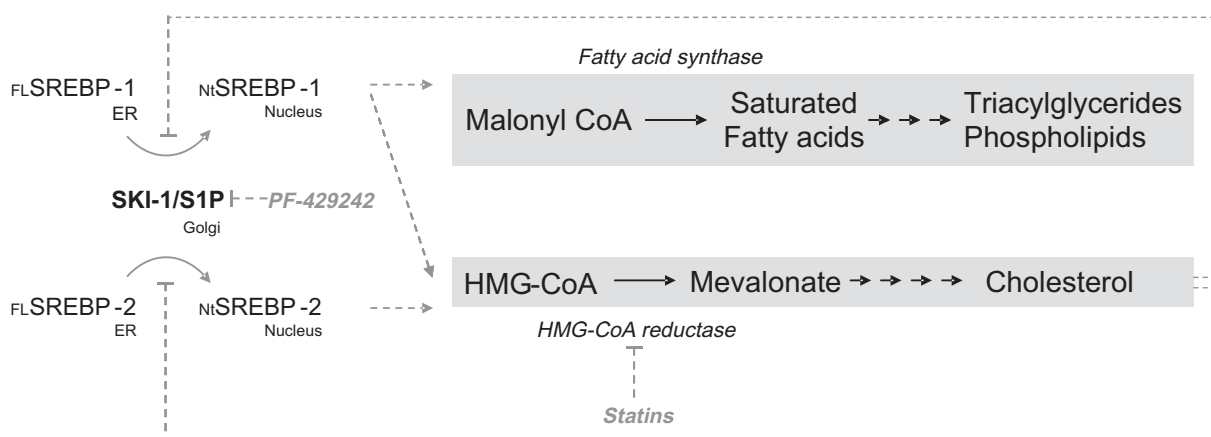


Fig. 1. Schematic overview of lipogenic metabolic pathways. Upon cholesterol accumulation, SREBPs are sequestered to the RE thus reducing lipids synthesis. Upon cholesterol depletion, SREBP-2 and -1 are targeted to the Golgi, and are activated by SKI-1/S1P. They then upregulate cholesterol and fatty acids synthesis. Sites of inhibition by PF-429242 and statins are indicated. Dashed arrows represent feedback regulations (FL, full length; Nt, amino-terminal moiety).

2.7.2. Confocal microscopy

Drug or mock-treated Huh7 cells were fixed and stained with Bodipy and DAPI and analyzed on a Biorad confocal microscope.

3. Results

3.1. Efficiency and specificity of the SKI-1/S1P inhibitor in Huh-7 cells

PF-429242 was developed by Pfizer as an alternative to statins to treat dyslipidemia and other associated pathologies (Hawkins et al., 2008). Since it has been shown that HCV replication is intimately linked to lipids metabolism (Herker and Ott, 2011), we sought to investigate a putative HCV-antiviral effect of this compound. Because of Huh-7-restricted tropism of the JFH-1 virus strand (Wakita et al., 2005; Zhong et al., 2005), we first had to confirm the inhibitory properties of PF-429242 in this cell line. Cells were exposed to increasing concentrations of PF-429242 and tested for SREBPs targets genes activity. The SKI-1/S1P inhibitor specifically decreased the expression of SREBP-2 and -1 target genes in a dose dependent manner (Fig. 2). A maximal inhibition of ~75% was obtained for HMG-CoAR, FASn and LDL-R genes. BIP/Grp78 gene expression, regulated by SKI/S1P-dependent ATF6 cleavage, did not reach 50% inhibition. SKI-1/S1P and ATF6 were used as controls and as expected, their expression was not altered by PF-429242. Altogether, these data indicate that PF-429242 can effectively downregulate cholesterol and triacylglycerides synthesis pathways, as well as the ER stress response in Huh-7 cells. We then sought to evaluate the PF-429242 long term inhibitory effect on lipogenic pathways. When added to cells, the SKI-1/S1P inhibitor reduced HMG-CoAR gene expression for at least 5 days (Fig. 3A). Statin inhibition of HMG-CoAR is known to trigger cholesterol depletion (Briel et al., 2005). In response to this depletion, SREBPs are targeted to the Golgi where they are activated by SKI-1/

S1P. SREBPs then act as transcription factors that increase expression of target genes (Fig. 1). To further characterize the SKI-1/S1P inhibition, Huh-7 cells were treated with PF-429242 and/or simvastatin. As anticipated from the regulation pattern of the lipogenic pathways (Fig. 1), and from previous studies (Murthy et al., 2005; Ness et al., 1996), simvastatin induced an increase in HMG-CoAR and LDL-R mRNA concentrations (~2.3- and ~1.5-fold, respectively) (Fig. 3B). Pravastatin induced a comparable increase in gene expression, with a more potent FASn gene activation (Fig. 3C). Again, PF-429242 decreased the expression of SREBPs-regulated genes (Fig. 3B). Combination of PF-429242 and simvastatin induced a comparable pattern, indicating the dominance of the SKI-1/S1P inhibitor on gene regulation (Fig. 3B). Toxicity was assessed by exposing Huh-7 cells to increasing concentrations of PF-429242, simvastatin or pravastatin for 3 days. No effect was observed for up to 120 μ M pravastatin, whereas comparable toxicity ($CC_{50} \approx 90 \mu$ M) was measured for simvastatin and PF-429242 (Fig. 3D). Nevertheless, toxicity was negligible at the <20 μ M concentrations that efficiently modulated lipid gene expression.

3.2. PF-429242 is superior to statins to block HCVcc genome replication

FASn pathway was shown to be also instrumental in HCV genome replication and is not downregulated by statins (Kapadia and Chisari, 2005; Yang et al., 2008). We sought to investigate the benefit of SKI-1/S1P inhibition because of its ability to regulate both SREBP-2 and -1 dependent pathways. Virtually 100% HCV-infected cells from Fig. 4A were treated with increasing concentrations of the drug for 3 days and tested for HCV genome replication and HMG-CoAR mRNA concentration, as a control for drug efficiency. Pravastatin and simvastatin were used as references. As expected, pravastatin was competent for HMG-CoAR protein inhibition, as shown by the 1.5-fold increased HMG-CoAR mRNA level

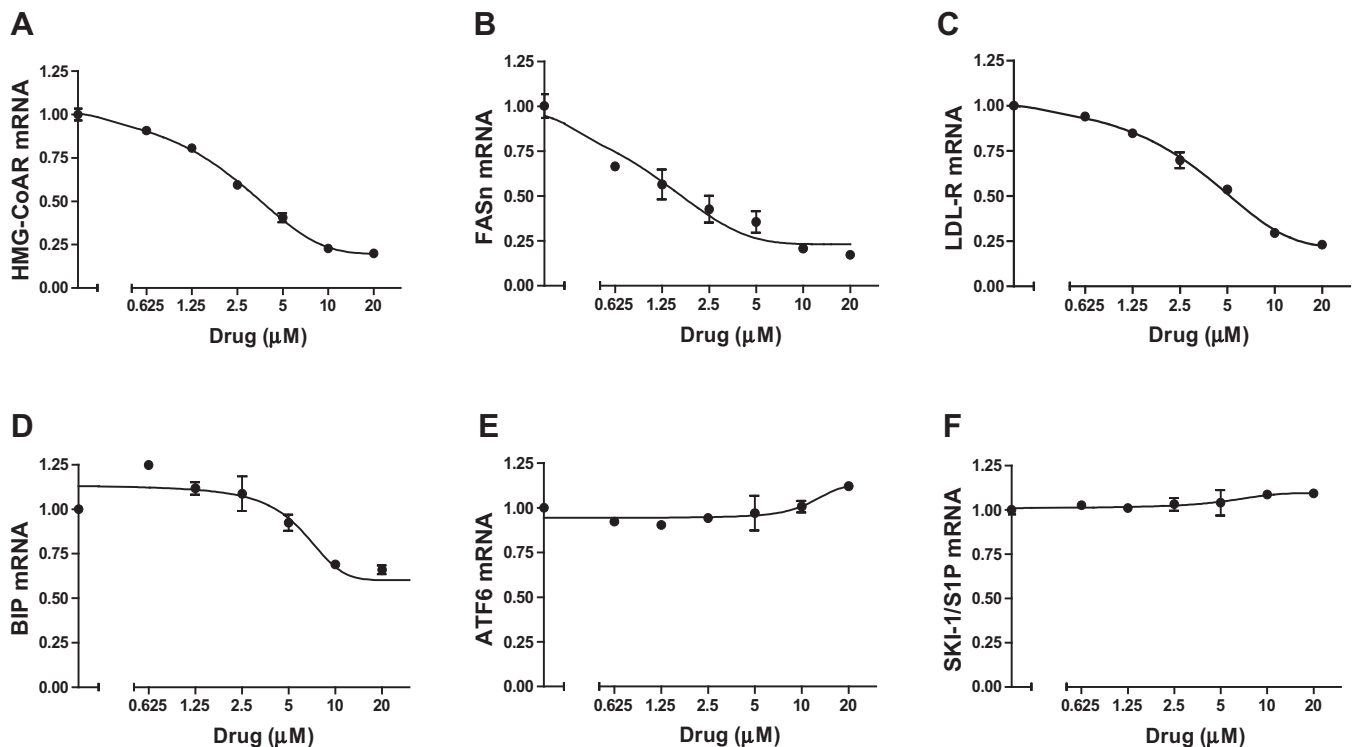


Fig. 2. Effect of SKI-1/S1P inhibitor PF-429242 on the expression of SREBPs target genes in Huh-7 cells. Cells were treated with increasing concentration of PF-429242 in medium C for 24 h. HMG-CoAR (A), FASn (B), LDL-R (C) and BIP (D) genes activity was then measured by RT-qPCR as described in the material and method section. ATF6 (E) and SKI-1/S1P (F) were used as control genes.

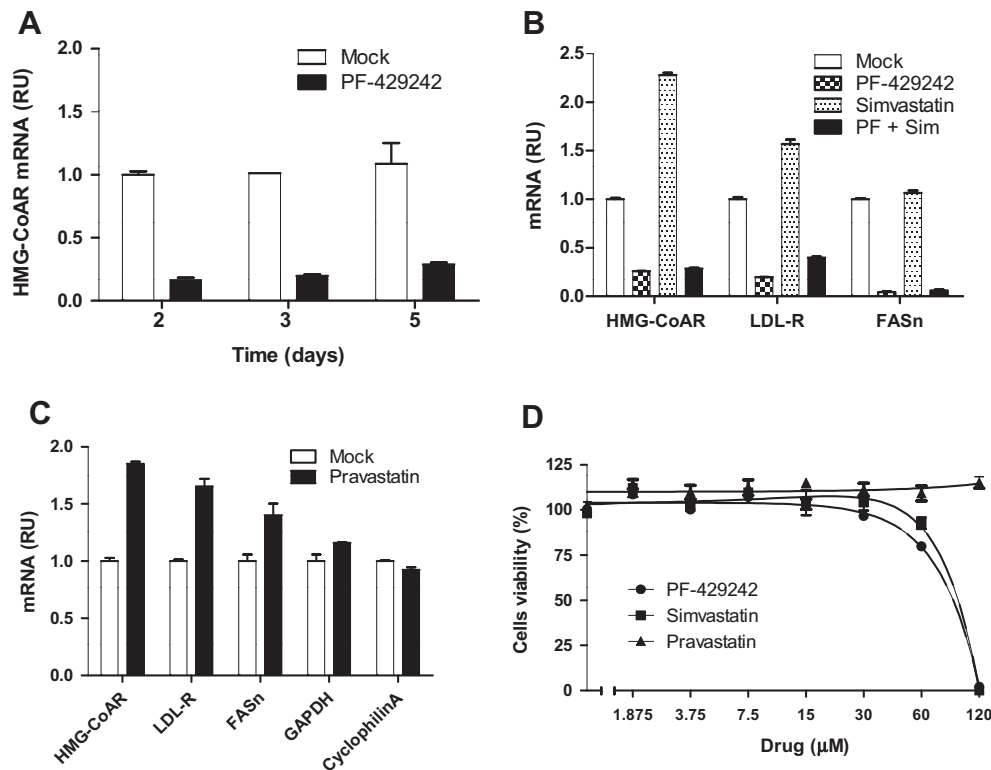


Fig. 3. Comparative regulation of lipogenic pathways by pravastatin, simvastatin, and PF-429242 in Huh-7 cells. (A) Cells were incubated with 20 μ M PF-429242 for 2, 3 and 5 days in medium C. Total RNA was extracted and HMG-CoAR mRNA was quantified by RT-qPCR. (B) Huh-7 cells were exposed to 15 μ M of PF-429242 and/or simvastatin for 24 h in medium C. Total RNA was extracted and HMG-CoAR, LDL-R, and FASN mRNA were quantified by qPCR. (C) Huh-7 cells were exposed to 40 μ M pravastatin for 6 h in medium C. After total RNA extraction, HMG-CoAR, LDL-R and FASN mRNA were quantified by qPCR. (D) Cells were exposed to increasing concentrations of PF-429242, simvastatin, or pravastatin for 72 h in medium B. Cell viability was then assessed using an MTS assay as described in Section 2.

(Fig. 4D). Interestingly, a slight (~ 1.2 -fold) raise in FASN gene activation was also observed at higher concentrations of pravastatin (Fig. 4E). In accordance with previous studies (Delang et al., 2009), pravastatin had no effect on HCV genome replication and simvastatin reduced HCV genome replication with an $EC_{50} \sim 17 \mu$ M (Fig. 4B). Surprisingly and contrary to pravastatin, the activation of HMG-CoAR and FASN genes by simvastatin was progressively decreased at high concentrations. Noteworthy, at simvastatin concentrations $>10 \mu$ M we consistently observed a reduction of β -actin content (Fig. 4C) and an important alteration in cell morphology (Supplementary Fig. 1). Interestingly, these observations have already been made by others in the case of myoblasts (Matzno et al., 1997). In contrast, treatments with PF-429242 decreased HCV RNA replication by approximately 75% in the 15–30 μ M range, with an EC_{50} of $\sim 5.8 \mu$ M, emphasizing its superiority compared to simvastatin. Additionally, viral protein levels correlated with HCV RNA concentrations (Fig. 4C and B, respectively).

3.3. SKI-1/S1P inhibition reduces lipid droplets levels, but HCV partially counteracts this effect

Beyond the need of lipids for genome replication, HCV infectious particle production depends on lipid droplets (LD) (Miyazaki et al., 2007), and on FASN gene expression (Yang et al., 2008). Interestingly, the size of LD has also been shown to be dependent on FASN expression (Kurokawa et al., 2010). Given the differential regulations exerted by the drugs on lipids metabolism, we sought to compare their effects on LD synthesis. Huh-7 cells were treated with drugs, and stained with Bodipy. Cells treated with PF-429242 exhibited $>50\%$ decrease in LD levels (Fig. 5A and B), supe-

rior to that observed with simvastatin and pravastatin. Noteworthy, the virus was able to raise LD concentration regardless of drugs treatments (Fig. 5C).

3.4. The SKI-1/S1P inhibitor PF-429242 but not simvastatin reduces the infectivity of the virus in the supernatant of HCV-infected Huh-7 cells

Based on the above data, we hypothesized that PF-429242 could have a more dramatic effect than simvastatin on the production of infectious particles. Results showed a drastic reduction ($EC_{50} \approx 2 \mu$ M, $EC_{90} \approx 4.8 \mu$ M) of infectivity for HCV virus supernatants obtained from cells treated with PF-429242 (Fig. 6A and B). Up to 15 μ M simvastatin did not markedly impair infectious particles production. Fig. 6C shows the same results from a distinct experiment.

4. Discussion

Persistent HCV infection induces major liver pathologies. Among other symptoms, infection can lead to steatosis (Clement et al., 2009). First thought to be an infection side effect, studies have highlighted the close relationship between lipids metabolism and HCV life cycle (Herker and Ott, 2011). HCV viral proteins stimulate lipogenic pathways (Oem et al., 2008; Park et al., 2009; Waris et al., 2007; Xiang et al., 2010) and HCV entry has been shown to rely on virus-specific and lipid receptors. LDL-R gene expression is upregulated upon SREBP-2 activation. In counterpart, PCSK9, another gene activated by SREBP-2, has been shown to reduce expression of LDL-R and CD81 at the cell surface (Labonte et al., 2009). Additionally, Claudin-1 expression, a co-receptor of HCV,

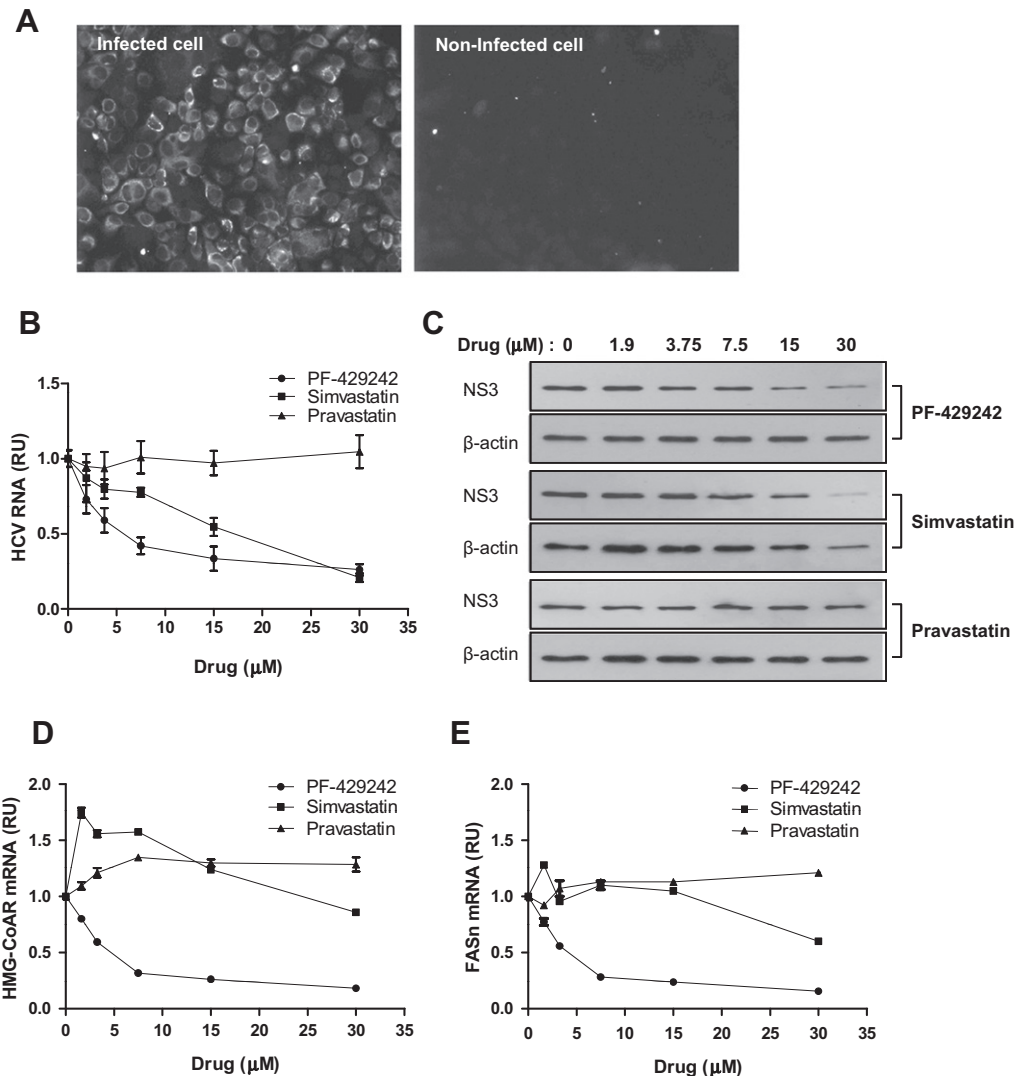


Fig. 4. Effect of Lipogenic pathways inhibitors on HCV genome replication and polyprotein synthesis. (A) Huh-7 were infected at an MOI of 0.02 for 6 h and maintained in medium A for 3-to-4 weeks to reach virtually 100% infection (left panel) as monitored by immunofluorescence microscopy after staining of NS5A protein as described in Section 2. Non-infected cells are shown on the right panel. Such HCV-infected Huh-7 cells were treated for 72 h with increasing concentrations of PF-429242, simvastatin or pravastatin in medium B. Cellular total RNA was extracted and tested for HCV RNA (B), HMG-CoAR (D) and FASN mRNA (E) concentrations by RT-qPCR as described in Section 2. Cells from the same experiment were tested for intracellular viral NS3 protein and β-actin content by western Blot (C).

has been shown to be regulated by FASN (Yang et al., 2008). HCV genome replication has been shown to rely on geranylgeranylation of FBL-2 (Kapadia and Chisari, 2005; Wang et al., 2005), and on the presence of intracellular lipid raft-like microdomains (Aizaki et al., 2004; Shi et al., 2003). Geranylgeranylation of FBL-2 has been shown to depend on SREBP-2 activation only, but the proper composition of lipid rafts may depend on both SREBPs because of the need for cholesterol and phospholipids. Indeed, the FAS pathway was shown to be critical for the replication of the HCV genome (Kapadia and Chisari, 2005; Kim et al., 2010; Yang et al., 2008), and for LD formation (Kurokawa et al., 2010), which is central to HCV assembly. In summary, SKI-1/S1P modulates several molecular mechanism involved in HCV lifecycle and inhibitors of this master regulator should be considered.

Combination of PEG-IFN and ribavirin has proven to be the most potent in treating chronically infected patients. Statins were shown to limit HCV genome replication *in vitro* (Delang et al., 2009). However, the benefit of their sole use in clinical trials remains controversial (Bader et al., 2008; Milazzo et al., 2009; O'Leary et al., 2007). Nevertheless, statins showed positive effects

when used in combination with standard therapy, leading to an increased SVR (Harrison et al., 2010). A possible explanation could be the prevention of drug-resistant variants selection (Delang et al., 2009). Actually, antiviral compounds that target cellular factors have been proven to select less drug-resistant variants than those inhibiting viral proteins (Provencher et al., 2004).

On a molecular basis, limited effects of statins on virus RNA replication could be explained by their side effects on the FAS pathway. As mentioned above, statin-triggered cholesterol depletion also stimulates SREBP-1 activation and thus the FAS pathway (Guzman et al., 1993; Su et al., 2002). Nystatin, a molecule thought to also induce cholesterol depletion and trigger SREBP-1 activation, was shown to even favor HCV genome replication (Su et al., 2002). In the current study, simvastatin but not pravastatin decreased HCV RNA replication. Results obtained with more than 10 μM of simvastatin might be related to an unexpected reduction of FASN gene activity (Fig. 4D), by an as yet unknown non-specific effect. These results should be analyzed with caution, as they were associated with an alteration in β-actin content and cell morphology (Fig. 4B and Supplementary Fig. 1, respectively). The absence of

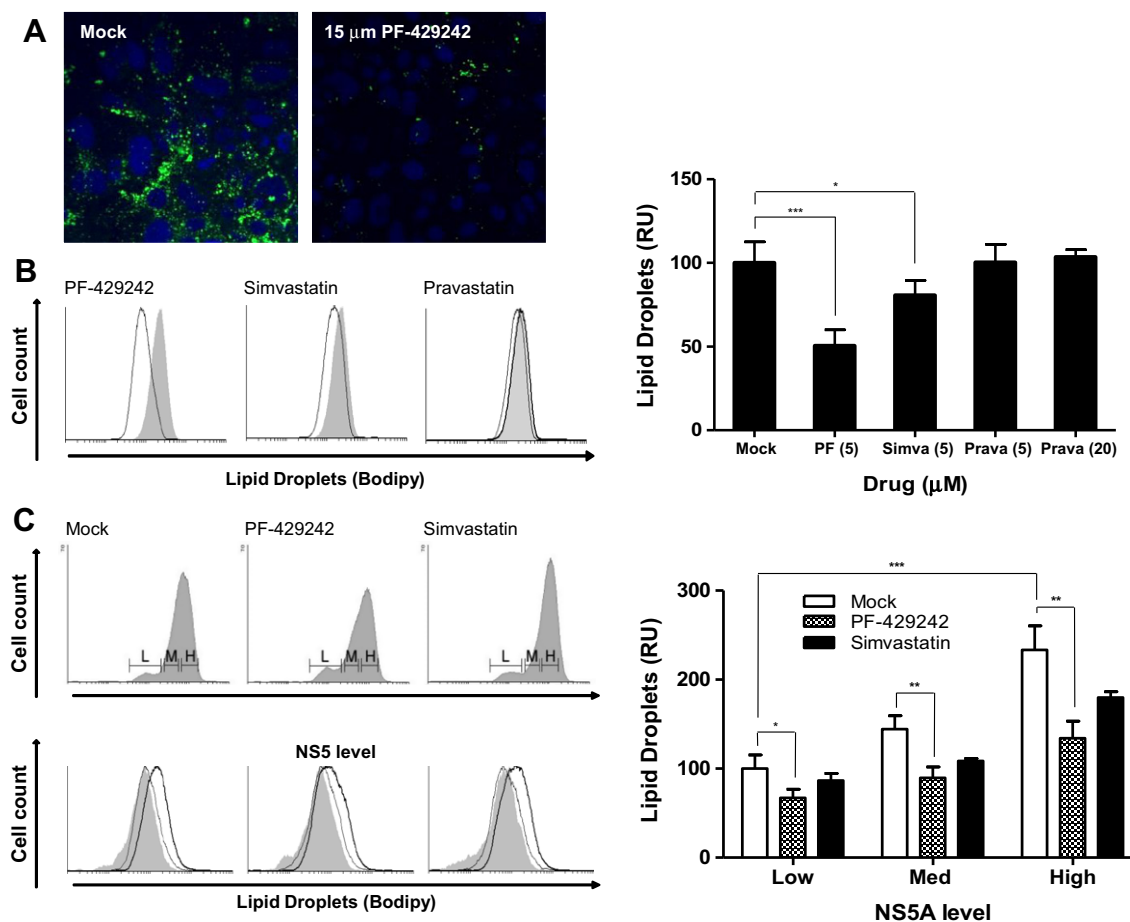


Fig. 5. Effects of drug use and HCV infection rate on the concentration of lipid droplets. (A) Cells were treated with 15 μM PF-429242 for 48 h in medium B. Cells were then fixed and stained with BODIPY 493/503 and DAPI and analyzed by confocal immunofluorescence microscopy. (B) Cells were treated with PF-429242, simvastatin and pravastatin in medium B for 72 h. After fixation and staining with BODIPY 493/503, cells were assayed on a FACSCalibur flow cytometer using the FL2 channel. Mean values and standard deviations from 4 experiments were reported on a histogram (right panel). Gray area, mock; thin line, 5 μM; bold line, 20 μM. (C) Infected cells were treated for 72 h with 5 μM of PF-429242 or simvastatin in medium B. After staining with BODIPY 493/503, a primary monoclonal anti-NS5A antibody and an anti-rabbit IgG-Alexa Fluor® 647, cells were analyzed using a flow cytometer. Population of cells with different infection rates were selected and gated using the FL4 channel, and their respective LD content was estimated using the FL2 channel. Lower panel: gray area, low infection rate (L); thin line, medium infection rate (M); bold line, high infection rate (H). Mean values were reported on an histogram (right panel). Statistical treatments were done using one way analysis of variance “ANOVA” and the “Dunnnett’s” post treatment test (Graphpad Prism 5). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

antiviral effect of pravastatin despite its efficient modulation of HMG-CoAR mRNA expression is intriguing (Figs. 3C and 4), and was described elsewhere (Delang et al., 2009). It is tempting to speculate that the antiviral effect of impaired geranylgeranylation of FBL-2 could be compensated for by the concomitant activation of the FASn pathway (Fig. 4D). This hypothesis was made by others (Su et al., 2002). Given the overall limitations of statins, we wished to target a more central actor of lipogenesis regulation.

SKI-1/S1P was a good candidate because of its property to regulate both SREBP-2 and -1 activations (Fig. 1). Hence, we used the most potent cell permeable small molecule inhibitor available to date (PF-429242). For most target genes, a strong reduction (>70%) could be achieved with this compound. However, caspase-3 and -7 are able to cleave SREBPs in a cholesterol independent manner (Pai et al., 1996; Wang et al., 1996), and could thus account for the remaining basal activity of SREBPs target genes in presence of PF-429242 (Fig. 2). We then investigated the effects of SKI-1/S1P inhibition on HCV lifecycle, and first showed a higher efficiency to reduce genome replication (Fig. 4A and B) than simvastatin ($EC_{50} \approx 5.8$ and 17.1 μM, respectively). Contrary to simvastatin, PF-429242 did not induce any alteration in cell shape and actin content regardless of concentration (Supplementary Fig. 1 and

Fig. 4B, respectively). Inhibition of the FAS pathway (Fig. 4D) could account for the stronger inhibition of genome replication with PF-429242 (Kapadia and Chisari, 2005; Kim et al., 2010; Yang et al., 2008). As observed for SREBPs target genes, HCV RNA and proteins could be reduced by ~70%. Upon infection, HCV induces ER stress (Tardif et al., 2005) which leads to sequential activation of caspase-12, -9, and caspase-3 (Zhang and Kaufman, 2004). Caspase-3 potency to activate SREBPs (see above) might limit the antiviral efficiency of PF-429242. Activation of the FAS pathway is also thought to be a major actor of the LD synthesis (Fukasawa, 2010; Kurokawa et al., 2010). LDs form the assembly platform of HCV virions (Herker and Ott, 2011; Miyanari et al., 2007) and are crucial for infectious particles production. We sought to investigate the comparative effects of PF-429242 and statins on LDs concentration. As shown in Fig. 5A and B a significant decrease (about 50%) could be achieved upon PF-429242 addition. Surprisingly, LDs concentration was also reduced by addition of simvastatin, albeit to a lesser extent. No major effect was observed for up to 20 μM pravastatin (Fig. 5B). Several HCV proteins are known to increase SREBP-1 activity (Jackel-Cram et al., 2007; Jackel-Cram et al., 2010; Tsutsumi et al., 2002) and to trigger ER stress, thus raising LDs concentration in infected hepatocytes. In accordance, we showed that

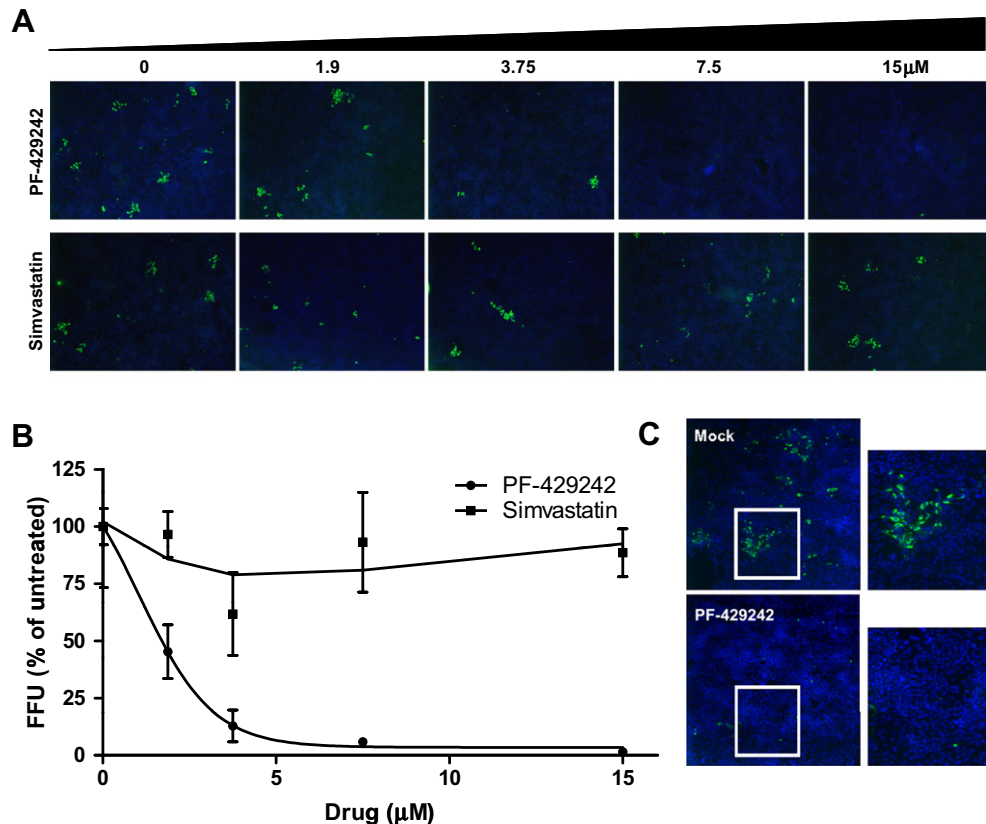


Fig. 6. Effect of PF-429242 on production of infectious particles. (A) Huh-7.5 naïve cells were incubated for 6 h with supernatant from drug-treated HCV-positive cells from Fig. 4, washed with PBS and maintained for 48 h in medium A. Cells were then fixed and stained with DAPI, a primary monoclonal anti-NS5A antibody and an anti-rabbit IgG-Alexa Fluor® 488. Stained cells were then analyzed for the presence of FFU. (B) Quantification from (A). (C) Magnification of Huh-7.5 inoculated for 6 h with supernatant from distinct 3.5 μM PF-429242-treated cells and maintained in medium A for 72 h.

HCV replication rate correlated with increasing concentrations of LDs but surprisingly, this occurred regardless of drug use (Fig. 5C). We then showed that the SKI-1/S1P inhibitor could dramatically affect the infectivity of the HCV virus in the supernatant of infected cells ($EC_{90} \approx 4.8 \mu\text{M}$), while no marked alteration was obtained with up to 15 μM simvastatin. This discrepancy may be due to the differential effect of drugs on the FASN pathway (and on LD concentration) for the tested concentrations. Indeed, as mentioned above, LDs act as a platform for viral assembly and maturation, and their alteration might explain, at least in part, the loss of infectious particles in cell culture supernatants. Further studies on the structure and the density distribution of secreted virions are needed to confirm a putative impairment in assembly and maturation. Additionally, VLDL synthesis and secretion is instrumental for highly infectious particle production (Chang et al., 2007; Gastaminza et al., 2008; Huang et al., 2007). Depletion of triacylglycerol is known to lead to ApoB co-translational degradation, thus preventing the formation of VLDL (Fisher and Ginsberg, 2002). It is tempting to speculate that SKI-1/S1P would impair VLDL formation and thus lessen HCV particle infectivity. However, to precisely monitor a putative drug-dependant impairment of VLDL assembly/secretion on HCV virions infectivity, further experiments on VLDL-fully competent cells will be required. While we were writing this manuscript a study was published on the anti-viral effect of SKI-1/S1P inhibitors, including PF-429242, on HCV (Olmstead et al., 2012). Based on their data, the authors concluded that the inhibitors were acting on viral entry and infectious particles production by infected cells, but not on viral genome replication. Conversely, using virtually 100% infected cells (Fig. 4A), we showed that inhibition of SKI-1/S1P significantly affected HCV RNA replication. This is in accordance with previous studies using different

approaches which demonstrated the down regulating effect of HMG-CoAR and FASN inhibition on HCV genome replication (Kapadia and Chisari, 2005; Kim et al., 2010; Wang et al., 2005; Yang et al., 2008). Additionally, we showed that beyond the effect on genome replication, cell treatment with PF-429242 dramatically impaired infectious particles production. In conclusion, SKI-1/S1P inhibition reduced HCV genome replication and infectious particles formation much more effectively than statins and represent a promising lead surrogate in association with classical treatments.

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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.2012.05.006>.

References

- Aizaki, H., Lee, K.J., Sung, V.M., Ishiko, H., Lai, M.M., 2004. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. *Virology* 324, 450–461.

- Bader, T., Fazili, J., Madhoun, M., Aston, C., Hughes, D., Rizvi, S., Seres, K., Hasan, M., 2008. Fluvastatin inhibits hepatitis C replication in humans. *Am. J. Gastroenterol.* 103, 1383–1389.
- Briel, M., Nordmann, A.J., Bucher, H.C., 2005. Statin therapy for prevention and treatment of acute and chronic cardiovascular disease: update on recent trials and meta analyses. *Curr. Opin. Lipidol.* 16, 601–605.
- Carroll, M.D., Lacher, D.A., Sorlie, P.D., Cleeman, J.L., Gordon, D.J., Wolz, M., Grundy, S.M., Johnson, C.L., 2005. Trends in serum lipids and lipoproteins of adults 1960–2002. *JAMA* 294, 1773–1781.
- Chang, K.S., Jiang, J., Cai, Z., Luo, G., 2007. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. *J. Virol.* 81, 13783–13793.
- Clement, S., Pascarella, S., Negro, F., 2009. Hepatitis C virus infection: molecular pathways to steatosis, insulin resistance and oxidative stress. *Viruses* 1, 126–143.
- Delang, L., Paeshuyse, J., Vliegen, I., Leyssen, P., Obeid, S., Durantel, D., Zoulim, F., Op de Beeck, A., Neyts, J., 2009. Statins potentiate the in vitro anti-hepatitis C virus activity of selective hepatitis C virus inhibitors and delay or prevent resistance development. *Hepatology* 50, 6–16.
- Di Bisceglie, A.M., 1997. Hepatitis C and hepatocellular carcinoma. *Hepatology* 26, 345–385.
- Fisher, E.A., Ginsberg, H.N., 2002. Complexity in the secretory pathway: the assembly and secretion of apolipoprotein B-containing lipoproteins. *J. Biol. Chem.* 277, 17377–17380.
- Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Goncales Jr., F.L., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N. Engl. J. Med.* 347, 975–982.
- Fukasawa, M., 2010. Cellular lipid droplets and hepatitis C virus life cycle. *Biol. Pharm. Bull.* 33, 355–359.
- Gastaminza, P., Cheng, G., Wieland, S., Zhong, J., Liao, W., Chisari, F.V., 2008. Cellular determinants of hepatitis C virus assembly, maturation, degradation, and secretion. *J. Virol.* 82, 2120–2129.
- Guevin, C., Lamarre, A., Labonte, P., 2009. Novel HCV replication mouse model using human hepatocellular carcinoma xenografts. *Antiviral Res.* 84, 14–22.
- Guzman, M., Cortes, J.P., Castro, J., 1993. Effects of lovastatin on hepatic fatty acid metabolism. *Lipids* 28, 1087–1093.
- Harrison, S.A., Rossaro, L., Hu, K.Q., Patel, K., Tillmann, H., Dhaliwal, S., Torres, D.M., Koury, K., Goteti, V.S., Novello, S., Brass, C.A., Albrecht, J.K., McHutchison, J.G., Sulkowski, M.S., 2010. Serum cholesterol and statin use predict virological response to peginterferon and ribavirin therapy. *Hepatology* 52, 864–874.
- Hawkins, J.L., Robbins, M.D., Warren, L.C., Xia, D., Petras, S.F., Valentine, J.J., Varghese, A.H., Wang, I.K., Subashi, T.A., Shelly, L.D., Hay, B.A., Landschulz, K.T., Geoghegan, K.F., Harwood Jr., H.J., 2008. Pharmacologic inhibition of site 1 protease activity inhibits sterol regulatory element-binding protein processing and reduces lipogenic enzyme gene expression and lipid synthesis in cultured cells and experimental animals. *J. Pharmacol. Exp. Ther.* 326, 801–808.
- Herker, E., Ott, M., 2011. Unique ties between hepatitis C virus replication and intracellular lipids. *Trends Endocrinol. Metab.* 22, 241–248.
- Hezode, C., Forestier, N., Dusheiko, G., Ferenci, P., Pol, S., Goeser, T., Bronowicki, J.P., Bourliere, M., Gharakhanian, S., Bengtsson, L., McNair, L., George, S., Kieffer, T., Kwon, A., Kauffman, R.S., Alam, J., Pawlotsky, J.M., Zeuzem, S., 2009. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N. Engl. J. Med.* 360, 1839–1850.
- Horton, J.D., Goldstein, J.L., Brown, M.S., 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J. Clin. Invest.* 109, 1125–1131.
- Huang, H., Sun, F., Owen, D.M., Li, W., Chen, Y., Gale Jr., M., Ye, J., 2007. Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. *Proc. Nat. Acad. Sci. USA* 104, 5848–5853.
- Ikedo, M., Abe, K., Yamada, M., Dansako, H., Naka, K., Kato, N., 2006. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. *Hepatology* 44, 117–125.
- Jackel-Cram, C., Babiuk, L.A., Liu, Q., 2007. Up-regulation of fatty acid synthase promoter by hepatitis C virus core protein: genotype-3a core has a stronger effect than genotype-1b core. *J. Hepatol.* 46, 999–1008.
- Jackel-Cram, C., Qiao, L., Xiang, Z., Brownlie, R., Zhou, Y., Babiuk, L., Liu, Q., 2010. Hepatitis C virus genotype-3a core protein enhances sterol regulatory element-binding protein-1 activity through the phosphoinositide 3-kinase-Akt-2 pathway. *J. Gen. Virol.* 91, 1388–1395.
- Kapadia, S.B., Chisari, F.V., 2005. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. *Proc. Nat. Acad. Sci. USA* 102, 2561–2566.
- Kim, K., Kim, K.H., Kim, H.Y., Cho, H.K., Sakamoto, N., Cheong, J., 2010. Curcumin inhibits hepatitis C virus replication by suppressing the Akt-SREBP-1 pathway. *FEBS Lett.* 584, 707–712.
- Kurokawa, J., Arai, S., Nakashima, K., Nagano, H., Nishijima, A., Miyata, K., Ose, R., Mori, M., Kubota, N., Kadowaki, T., Oike, Y., Koga, H., Febrario, M., Iwanaga, T., Miyazaki, T., 2010. Macrophage-derived AIM is endocytosed into adipocytes and decreases lipid droplets via inhibition of fatty acid synthase activity. *Cell Metab.* 11, 479–492.
- Labonte, P., Begley, S., Guevin, C., Asselin, M.C., Nassoury, N., Mayer, G., Prat, A., Seidah, N.G., 2009. PCSK9 impedes hepatitis C virus infection in vitro and modulates liver CD81 expression. *Hepatology* 50, 17–24.
- Manns, M.P., Markova, A.A., Serrano, B.C., Cornberg, M., 2012. Phase III results of Boceprevir in treatment naive patients with chronic hepatitis C genotype 1. *Liver Int.* 32 (Suppl 1), 27–31.
- Matzno, S., Yamauchi, T., Gohda, M., Ishida, N., Katsuura, K., Hanasaki, Y., Tokunaga, T., Itoh, H., Nakamura, N., 1997. Inhibition of cholesterol biosynthesis by squalene epoxidase inhibitor avoids apoptotic cell death in L6 myoblasts. *J. Lipid Res.* 38, 1639–1648.
- Milazzo, L., Meroni, L., Galazzi, M., Cesari, M., Caramma, I., Marchetti, G., Galli, M., Antinori, S., 2009. Does fluvastatin favour HCV replication in vivo? A pilot study on HIV-HCV coinfected patients. *J. Viral Hepat.* 16, 479–484.
- Miyazaki, Y., Atsuzawa, K., Usuda, N., Watashi, K., Hishiki, T., Zayas, M., Bartenschlager, R., Wakita, T., Hijikata, M., Shimotohno, K., 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat. Cell Biol.* 9, 1089–1097.
- Murthy, S., Tong, H., Hohl, R.J., 2005. Regulation of fatty acid synthesis by farnesyl pyrophosphate. *J. Biol. Chem.* 280, 41793–41804.
- Ness, G.C., Zhao, Z., Lopez, D., 1996. Inhibitors of cholesterol biosynthesis increase hepatic low-density lipoprotein receptor protein degradation. *Arch. Biochem. Biophys.* 325, 242–248.
- O'Leary, J.G., Chan, J.L., McMahon, C.M., Chung, R.T., 2007. Atorvastatin does not exhibit antiviral activity against HCV at conventional doses: a pilot clinical trial. *Hepatology* 45, 895–898.
- Oem, J.K., Jackel-Cram, C., Li, Y.P., Zhou, Y., Zhong, J., Shimano, H., Babiuk, L.A., Liu, Q., 2008. Activation of sterol regulatory element-binding protein 1c and fatty acid synthase transcription by hepatitis C virus non-structural protein 2. *J. Gen. Virol.* 89, 1225–1230.
- Olmstead, A.D., Knecht, W., Lazarov, I., Dixit, S.B., Jean, F., 2012. Human Subtilase SKI-1/S1P is a master regulator of the HCV lifecycle and a potential host cell target for developing indirect-acting antiviral agents. *PLoS Pathog.* 8, e1002468.
- Pai, J.T., Brown, M.S., Goldstein, J.L., 1996. Purification and cDNA cloning of a second apoptosis-related cysteine protease that cleaves and activates sterol regulatory element binding proteins. *Proc. Nat. Acad. Sci. USA* 93, 5437–5442.
- Park, C.Y., Jun, H.J., Wakita, T., Cheong, J.H., Hwang, S.B., 2009. Hepatitis C virus nonstructural 4B protein modulates sterol regulatory element-binding protein signaling via the AKT pathway. *J. Biol. Chem.* 284, 9237–9246.
- Provencher, V.M., Coccato, E., Lacasse, J.J., Schang, L.M., 2004. Antiviral drugs that target cellular proteins may play major roles in combating HIV resistance. *Curr. Pharm. Des.* 10, 4081–4101.
- Seidah, N.G., Prat, A., 2007. The proprotein convertases are potential targets in the treatment of dyslipidemia. *J. Mol. Med.* 85, 685–696.
- Shi, S.T., Lee, K.J., Aizaki, H., Hwang, S.B., Lai, M.M., 2003. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. *J. Virol.* 77, 4160–4168.
- Su, A.L., Pezacki, J.P., Wodicka, L., Brideau, A.D., Supkova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R.H., Schultz, P.G., Chisari, F.V., 2002. Genomic analysis of the host response to hepatitis C virus infection. *Proc. Nat. Acad. Sci. USA* 99, 15669–15674.
- Tardif, K.D., Waris, G., Siddiqui, A., 2005. Hepatitis C virus, ER stress, and oxidative stress. *Trends Microbiol.* 13, 159–163.
- Tsutsumi, T., Suzuki, T., Shimoike, T., Suzuki, R., Moriya, K., Shintani, Y., Fujie, H., Matsuura, Y., Koike, K., Miyamura, T., 2002. Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. *Hepatology* 35, 937–946.
- Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat. Med.* 11, 791–796.
- Wang, C., Gale Jr., M., Keller, B.C., Huang, H., Brown, M.S., Goldstein, J.L., Ye, J., 2005. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol. Cell* 18, 425–434.
- Wang, X., Zelenski, N.G., Yang, J., Sakai, J., Brown, M.S., Goldstein, J.L., 1996. Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. *EMBO J.* 15, 1012–1020.
- Waris, G., Felmlee, D.J., Negro, F., Siddiqui, A., 2007. Hepatitis C virus induces proteolytic cleavage of sterol regulatory element binding proteins and stimulates their phosphorylation via oxidative stress. *J. Virol.* 81, 8122–8130.
- Xiang, Z., Qiao, L., Zhou, Y., Babiuk, L.A., Liu, Q., 2010. Hepatitis C virus nonstructural protein-5A activates sterol regulatory element-binding protein-1c through transcription factor Sp1. *Biochem. Biophys. Res. Commun.* 402, 549–553.
- Yang, W., Hood, B.L., Chadwick, S.L., Liu, S., Watkins, S.C., Luo, G., Conrads, T.P., Wang, T., 2008. Fatty acid synthase is up-regulated during hepatitis C virus infection and regulates hepatitis C virus entry and production. *Hepatology* 48, 1396–1403.
- Zhang, K., Kaufman, R.J., 2004. Signaling the unfolded protein response from the endoplasmic reticulum. *J. Biol. Chem.* 279, 25935–25938.
- Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. *Proc. Nat. Acad. Sci. USA* 102, 9294–9299.