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Short communication

Statins can exert dual, concentration dependent effects on HCV entry *in vitro*

Matthieu Blanchet [a], Quoc-Tuan Le [a], Nabil G. Seidah [b], Patrick Labonté [a, *]

[a] INRS-Institut Armand-Frappier, Institut National de la Recherche Scientifique, Laval, Canada
[b] Laboratory of Biochemical Neuroendocrinology, Institut de Recherches Cliniques de Montréal, Affiliated to the Université de Montréal, Montréal, Canada

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ABSTRACT

Statins are used daily by a large and increasing number of individuals worldwide. They were initially designed as 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMG-CoAR) inhibitors to treat patients with hypercholesterolemia. Recent studies on HCV chronically infected individuals have suggested that their use *in vivo* in combination with PEG-IFN and ribavirin favor the sustained viral response (SVR). Herein, we describe the effects of a set of statins on HCV entry and on HCV key entry factors *in vitro*. Our results suggest that all tested statins exert a proviral effect through the upregulation of LDLR. Interestingly, at higher concentration, we also provide evidence of a yet unknown competing antiviral effect of statins (except for pravastatin) through the downregulation of CLDN-1. Importantly, this work enlightens the blunt proviral effect of pravastatin at the entry step of HCV *in vitro*.

HCV chronic infection affects approximately 170 million individuals worldwide and have been a healthcare burden for decades. Infected individual, if not treated, are likely to develop steatosis, cirrhosis, and ultimately hepatocellular carcinoma (Di Bisceglie, 1997). In recent years tremendous efforts have been made to develop efficient direct acting antiviral (DAA) molecules with over 95% sustained viral response (SVR) (Pawlotsky et al., 2015). In addition, studies have also been conducted to identify antiviral drugs that target cellular pathways, known to be genotype-independent and to trigger less resistant variants than DAA (Provencher et al., 2004). HCV has been shown to strictly depend on cellular lipid metabolism at virtually every step of its lifecycle (Alvisi et al., 2011). In the serum of infected patients, HCV virions are associated with VLDL-like vesicles to form highly infectious particles referred to as lipoviroparticles (LVP) (Andre et al., 2002; Boyer et al., 2014; Merz et al., 2011). This association allow the LVP to interact with cellular receptors specific for the VLDL moiety of LVP, namely LDLR, SR-B1 and NPC1L1 (Zeisel et al., 2013). HCV genome replication has been shown to rely on the lipidation (geranylgeranylation) of FBL-2 (Kapadia and Chisari, 2005; Wang et al., 2005) and on the presence of intracellular lipid rafts-like microdomains (Aizaki et al., 2004; Shi et al., 2003). Furthermore, lipid droplets that accumulate in infected cells are strictly required for virions maturation (Popescu et al., 2014). Accordingly, molecules that alter the overall lipids homeostasis such as statins could severely affect the HCV lifecycle.

Statins have been initially developed the treat patients with hypercholesterolemia, and are used daily by a large and increasing number of individuals worldwide. Given the known effects of statins on cellular lipid metabolism, a better characterization of their putative antiviral and/or proviral effect during HCV infection is needed. This is of particular interest in regard to the large number of asymptomatic HCV-infected individual unaware of their

Abbreviations: HMG-CoAR, 3-hydroxy-3-methylglutharyl-coenzyme A reductase; PEG-IFN, pegylated interferon; SVR, sustained viral response; LDLR, low density lipoprotein receptor; NPC1L1, Niemann–Pick C1-like 1; CLDN-1, claudin-1; DAA, direct acting antiviral; LVP, lipoviroparticles; LD, lipid droplets; SR-B1, scavenger receptor-B1; OCLN, occludin; EGFR, epidermal growth factor; VLDL, very low density lipoprotein; SKI-1/S1P, subtilisin/kexin-isoenzyme-1/site-1 protease; S2P, site-2 protease; SREBP, sterol regulatory-element binding protein; PCSK9, proprotein convertase subtilisin/kexin type 9; LPDS, lipoprotein-deficient serum.

* Corresponding author. INRS-Institut Armand-Frappier, Institut National de la Recherche Scientifique, 531 Boulevard des Prairies, Laval, H7V 1B7, Canada.
E-mail address: patrick.labonte@iaf.inrs.ca (P. Labonté).

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infection that used statin daily. To date, with the exception of pravastatin, statins have been shown to impair HCV genome replication *in vitro* (Blanchet et al., 2012; Delang et al., 2009), So far, no consensus has been reached concerning the propensity of statins alone to impair HCV propagation *in vivo* (Bader et al., 2008; O'Leary et al., 2007). However their use in combination with PEG-IFN and ribavirin seems to favor the sustained viral response (SVR) (Butt et al., 2015).

At the molecular level statins inhibit the function of the 3-hydroxy-3-methylglutharyl-coenzyme A reductase (HMG-CoAR), thus impairing the synthesis of mevalonate and ultimately cholesterol (Fig. 1). In response to cholesterol depletion, the sterol responsive element binding protein (SREBP) is targeted to the Golgi in its precursor/inactive form where it is activated by two consecutive cleavages by subtilisin/kexin-isozyme-1/site-1 protease (SKI-1/S1P) and site-2 protease (S2P) (Ye and DeBose-Boyd, 2011). Following activation, the released active moiety of the sterol responsive element binding protein (SREBP), referred to as NtSREBP, is targeted to the nucleus where it activates the transcription of SRE and/or E-box promoted genes. Among these genes are HMG-CoAR, whose activation triggers neo-synthesis of cholesterol, and LDLR along with NPC1L1, which allow the retrieval of cholesterol from the extracellular milieu. PCSK9, the 9th member of the proprotein convertase family, is also transcriptionally activated in a SREBP-dependant manner, and has been shown to be able to reduce the levels of LDLR (Attie and Seidah, 2005; Seidah et al., 2014), and, under specific conditions, CD81 (Blanchet et al., 2015; Labonte et al., 2009).

Given these complex regulations we first sought to investigate the overall effect of different statins on HCV viral entry (Fig. 2). To this end, human hepatocyte-derived Huh7.5 cells were exposed to non-toxic concentrations of statins (Supplementary Fig. 1) for 24 h, prior to addition of HCVcc JFH1 virus. After a 6 h exposure to the virus, cells were extensively washed and further cultured for 18 h. Cells were then lysed and analyzed for their content in HCV RNA by RT-qPCR (Taqman) as described elsewhere (Blanchet et al., 2015). Surprisingly, the results revealed that low concentrations of statins (2.5 and 5 μM) correlated with increased levels of HCV RNA, while higher concentrations (20–40 μM) were associated with a significant reduction (Fig. 2, blue bars). Very importantly, the use of pravastatin induced a significant increase in intracellular HCV RNA at all concentrations.

To rule out the contribution of an inhibition of the HCV genome replication by statins in our previous infection experiment, we monitored the effect of a 24 h treatment of Huh7.5 cells harboring a subgenomic replicon (Huh7-SGR-JFH1) with statins (Fig. 2, black bars). With the exception of simvastatin for which a slight reduction was achieved at the highest concentrations, no significant alteration of replication was observed after 24 h treatment (Fig. 2). This is in line with former studies (Blanchet et al., 2012; Delang et al., 2009) in which 72 h exposure was required to achieve a significant antiviral effect on genome replication. Altogether, these results strongly suggested a yet unknown antiviral effect of statins at high concentrations (with the exception of pravastatin). Importantly, they also revealed a blunt proviral effect of pravastatin at all concentrations at the entry step of HCV.

In an attempt to identify the mechanism(s) responsible for viral entry inhibition, we sought to precisely monitor the effect of statins on the expression of HCV entry key-factors (Fig. 3). To this end, Huh7.5 cells were exposed to statins for 24 h prior to analysis by RT-qPCR (SYBR Green). Sequences of primers and protocol have been described elsewhere (Blanchet et al., 2015). As shown in Fig. 3, LDLR, NPC1L1 and PCSK9 mRNA concentrations were globally increased in the presence of statins, especially pravastatin. This is in accordance with the presence of SRE sequences on their promoters (Attie and Seidah, 2005; Li et al., 2009; Pramfalk et al., 2010). However, except for pravastatin, a biphasic modulation of mRNA expression of these genes at drug concentrations of ≥10 μM was observed depending on the type of statin used.

Strikingly, CLDN-1 mRNA concentration was reduced up to ~70% after exposure to simvastatin, atorvastatin, lovastatin, and fluvastatin, likely leading to an antiviral effect of these statins. Very importantly pravastatin did not induce any alteration in CLDN-1 mRNA level. To further assess the relevance of the effects observed on CLDN-1 and LDLR mRNA expression, we took advantage of the primary human hepatocyte (PHH) model. PHH were plated, cultured, and treated with statins as described in the figure legend. Results confirm the activation of LDLR gene transcription

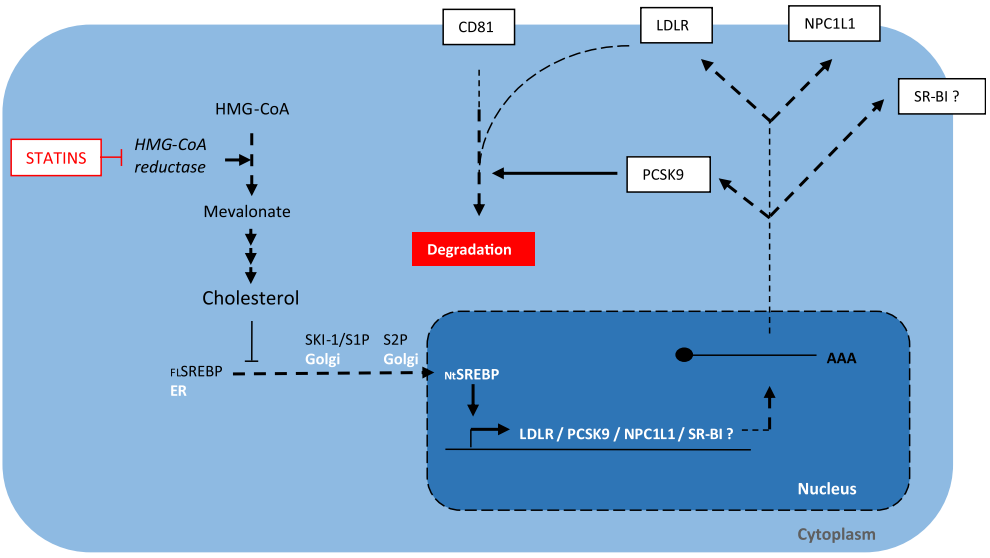

Fig. 1. Schematic representation of known regulation of HCV key entry factors by statins. Through inhibition of HMG-COaR, statins impair cholesterol synthesis. In response to cholesterol depletion, the precursor form of SREBP is targeted to the golgi where it is activated by consecutive cleavage by SKI-1/S1P and S2P. Following activation, the relieved active moiety of SREBP referred to as NtSREBP is targeted to the nucleus where it activates the transcription of SRE and/or E-box promoted genes, including HCV key entry factors. Site of inhibition of statins is indicated. Ability of PCSK9 to favor CD81 and LDLR degradation is depicted (FL, full length; Nt, amino-terminal active moiety).