Understanding the molecular mechanism of host-based statin resistance in hepatitis C virus replicon containing cells.

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ABSTRACT

A number of statins, the cholesterol-lowering drugs, inhibit the *in vitro* replication of hepatitis C virus (HCV). In HCV-infected patients, addition of statins to the earlier standard of care therapy (pegIFN- α and ribavirin) resulted in increased sustained virological response rates. The mechanism by which statins inhibit HCV replication has not yet been elucidated. In an attempt to gain insight in the underlying mechanism, hepatoma cells carrying an HCV replicon were passaged in the presence of increasing concentrations of fluvastatin. Fluvastatin-resistant replicon containing cells could be generated and proved ~8-fold less susceptible to fluvastatin than wild-type cultures. The growth efficiency of the resistant replicon containing cells was comparable to that of wild-type replicon cells. The fluvastatin-resistant phenotype was not conferred by mutations in the viral genome but is caused by cellular changes. The resistant cell line had a markedly increased HMG-CoA reductase expression upon statin treatment. Furthermore, the expression of the efflux transporter P-gp was increased in fluvastatin-resistant replicon cells (determined by qRT-PCR and flow cytometry). This increased expression resulted also in an increased functional transport activity as measured by the P-gp mediated efflux of calcein AM. In conclusion, we demonstrate that statin resistance in HCV replicon containing hepatoma cells is conferred by changes in the cellular environment.

Keywords: statins, hepatitis C virus, resistance, HMG-CoA reductase, efflux

1. INTRODUCTION

Hepatitis C virus (HCV) infection is a major health burden with over 130 million people infected worldwide. Chronic HCV infection can evolve to liver cirrhosis, liver fibrosis and eventually hepatocellular carcinoma, and is the leading cause for liver transplantations in the western world. Treatment response has been markedly improved by the approval of several direct-acting antivirals (DAA) i.e. sofosbuvir and dasabuvir (respectively a nucleoside and a non-nucleoside polymerase inhibitor), NS5A inhibitors (daclatasvir, ledispavir and ombitasvir) and the protease inhibitors (telaprevir, boceprevir, simeprevir and paritaprevir) [1]. With other new and highly efficacious DAAs approaching market approval, future prospects for the treatment of patients with chronic HCV infection are highly favorable.

Statins are the second most commonly prescribed drugs worldwide and are mainly used for the treatment of hypercholesterolemia by direct inhibition of HMG-CoA reductase (HMGCR). HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis and catalyzes the conversion of HMG-CoA to mevalonate in the liver. Mevalonate is also an essential precursor for the production of isoprenoids such as geranylgeranyl pyrophosphate. Despite the nearly excellent safety profile of statins, side-effects are reported with myopathy and subsequent rhabdomyolysis as the most important ones [2,3].

Besides their cholesterol-lowering effect, statins have been reported to have *in vitro* antiviral activity against a variety of viruses, such as HIV-1 [4–6], poliovirus [7], cytomegalovirus [8], dengue virus [9] and respiratory syncytial virus [10]. In a recent review the possibility of statin administration to reduce mortality in the case of an influenza pandemic has also been discussed [11]. Several statins were shown to inhibit the replication of subgenomic HCV replicons [12,13] and to suppress RNA

replication of infectious HCV in cell culture [14]. The mechanism by which statins inhibit HCV replication is still unknown. It was shown that the anti-HCV activity of statins is not due to the inhibition of cholesterol synthesis but due to the inhibition of geranylgeranylation of cellular proteins [15]. Geranylgeranylation is a post-translational modification that covalently attaches geranylgeranyl to cellular proteins to facilitate their membrane association. The F-box/leucine-rich repeat protein 2 (FBL2) was identified as a geranylgeranylated host protein that is crucial for HCV RNA replication [16]. FBL2 was shown to interact with the HCV protein NS5A and this interaction depended on the geranylgeranylation of FBL2. Another recent study suggested that fluvastatin inhibited HCV replication partly by stabilizing microtubule polymers and preventing microtubule disassembly leading to microtubule bundling and defects in cell division [17]. Studies in HCV-infected patients revealed a modest activity or even a lack of antiviral effect of statins as monotherapy, but in combination with the earlier standard of care (pegIFN- α and ribavirin) a significant increase in sustained virological response rates was observed [18].

Host-targeting antivirals such as statins are believed to have a high barrier to resistance [1]. We previously showed that statins can delay or even prevent the development of resistant HCV variants when combined with a non-nucleoside polymerase inhibitor *in vitro* [13]. No statin-resistant variants were observed in these experiments. However, it was shown before that viruses can develop resistance against host-targeting antivirals. For example, HCV subgenomic replicons resistant to the cyclophilin inhibitor alisporivir were reported [19]. One mutation in NS5A (D320E) conferred a low-level resistance to alisporivir (3.9-fold), probably by reducing the need for cyclophilin A-dependent isomerisation of NS5A. On the other hand, ribavirin-resistant HCV replicon cells proved to have a cellular resistance mechanism

consisting of a decreased uptake of ribavirin by ENT1 transporters [20]. Here, we wanted to determine whether fluvastatin-resistant HCV replicon containing cells can be selected. In such case these resistant variants will be a tool to study mechanisms of statin resistance and to unravel the mechanism of action of the anti-HCV activity of statins. We were indeed able to select a fluvastatin-resistant HCV subgenomic replicon containing cell line that proved cross-resistant to simvastatin and atorvastatin. Interestingly, the observed statin-resistant phenotype was not associated with the viral genome, but with changes in the host cell. Our data furthermore revealed that the statin-resistant replicon cells had a markedly increased HMG-CoA reductase expression, especially upon statin treatment. Also the expression and functional transport activity of the statin efflux transporter P-gp was increased in the statin-resistant cell line. As intolerance to statins is nowadays increasingly reported in patients [21,22] and little is known about the underlying mechanism of this intolerance, this study thus provides important information on possible mechanisms of statin resistance *in vitro*.

2. MATERIAL AND METHODS

2.1 Cells

Huh-7 cells containing subgenomic genotype 1b HCV replicon I₃₇₇/NS3-3'/wt (Huh 9-13) [23] as well as the highly permissive cell clone Huh-7 Lunet have been described before [24]. Cells were cultured in Dulbecco's modified Eagle's Medium (DMEM; Gibco, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS; Integro, Zaandam, The Netherlands), 1x non-essential amino acids (Gibco, Merelbeke, Belgium), 100 IU/ml penicillin (Gibco, Merelbeke, Belgium), 100 µg/ml streptomycin (Gibco, Merelbeke, Belgium) and 1 mg/ml Geneticin (G418; Gibco, Merelbeke, Belgium). Cell cultures were maintained at 37°C in an atmosphere of 5% CO₂.

2.2 Compounds

Fluvastatin, atorvastatin and simvastatin were purchased from Cayman Chemicals (Huissen, The Netherlands). GTI-298, 25-hydroxycholesterol and doxorubicin were purchased from Sigma-Aldrich (Bornem, Belgium). Non-nucleoside inhibitor VX-222 [25] and NS5A inhibitor BMS-790052 [26] were purchased from Selleck Chemicals (Huissen, The Netherlands). Protease inhibitor VX-950 and nucleoside polymerase inhibitor 2'-C-methylcytidine were synthesized as reported earlier [13]. Compound stock solutions were prepared in DMSO.

2.3 Antiviral assay with Huh 9-13 replicon containing cells

Huh 9-13 replicon containing cells were seeded at a density of 5×10^3 cells per well in a 96-well cell culture plate in a total volume of 100 µL complete DMEM without G418. After 24 hours of incubation at 37°C (5% CO₂) serial dilutions of the test compounds in complete DMEM were added in a total volume of 100 µL. After 3 days of incubation at 37°C, cell culture medium was removed and monolayers were washed

once with phosphate buffered saline. Cells were lysed with 100 μ L Cells-to-cDNA lysis buffer (Ambion, Cambridgeshire, United Kingdom) following manufacturer's instructions. HCV replicon RNA levels were determined by a reverse transcription quantitative polymerase chain reaction. EC₅₀ values were calculated as the concentration of a compound that caused a 50% reduction in HCV RNA levels compared to that of the untreated control.

2.4 Reverse transcription quantitative polymerase chain reaction

To quantify HCV RNA, a 25 μ L reverse transcription polymerase chain reaction (RT-qPCR) was set up containing 12.5 μ L One step RT-qPCR mastermix (Eurogentec, Seraing, Belgium), 6.3 μ L H₂O, 5 μ L total cellular RNA extract and 300 nmol/L neo-forward primer [5'-CCGGCTACCTGCCCCATTC-3'], 300 nmol/L neo-reverse primer [5'-CCA GATCATCCTGATCGACAAG-3'] and 300 nmol/L neo-probe [5'-FAM-ACATCGCAT CGAGCGAGCACGTAC-TAMRA-3']. The RT step was performed at 48°C for 30 minutes, denaturation at 95°C for 15 minutes, and subsequent PCR amplification of 40 cycles of denaturation at 94°C for 20 seconds and annealing and extension at 60°C for 1 minute in a 7500 Fast Real-Time PCR system (Applied Biosystems, Branchburg, New Jersey, USA). To quantify HMG-CoA reductase, SREBP2, breast cancer resistance protein (BCRP) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels, the 20x PrimeTime HMGCR mix (Integrated DNA technologies, Heverlee, Belgium), the SREBP2 TaqMan gene expression assay (Hs01081784_m1, Life technologies, Gent, Belgium), the BCRP TaqMan gene expression assay (Hs01053790_m1, Life technologies, Gent, Belgium) and the GAPDH Control kit (Eurogentec, Seraing, Belgium) were used, respectively. The reverse transcription step was performed at 50°C for 30 minutes, denaturation at 95°C for 10 minutes, and subsequent PCR amplification of 40 cycles of denaturation

at 95°C for 15s and annealing and extension at 60°C for 45s. The mRNA levels of efflux transporters P-gp and MRP2 were determined as follows. 1 µg of total RNA was used for cDNA synthesis using the Superscript III First-Strand synthesis kit (Invitrogen, Gent, Belgium). RT-qPCR was performed with the Platinum SYBR green qPCR Supermix-UDG (Invitrogen, Gent, Belgium) with a ViiA7™ Real-Time PCR system (Applied Biosystems, Branchburg, New Jersey, USA). The denaturation step was performed at 95°C for 5 minutes, and subsequent PCR amplification of 40 cycles of denaturation at 95°C for 15s and annealing and extension at 60°C for 45s. The primers used for RT-qPCR were: P-gp/MDR1 forward 5'-CTAATGCCGAACACATTGGA-3' and reverse 5'-CAG TCGCTTTATTTCTTTGCC-3', MRP2 forward 5'-CGATATACCAATCCAAGCCTC-3' and reverse 5'-GAATTGTCACCCTGTAAGAGTG-3', GAPDH forward 5'-TCAAGAAGGTGGTGAAGCAGG-3' and reverse 5'-ACCAGGAAATGAGCTTGACAAA-3'.

2.5 In vitro selection of fluvastatin-resistant replicon containing cells

Huh 9-13 replicon containing cells were seeded at a density of 3×10^5 cells in a 25 cm² T-flask in complete DMEM containing 1 mg/mL G418, 10% fetal bovine serum and a starting concentration of the EC₅₀ value of fluvastatin. The cells were cultured until 90% confluency was reached and were maintained at the same concentration for (at least) 3 passages. At the fourth passage, the concentration was increased by two-fold. This process was repeated until a maximum concentration was reached of 15 µM. Due to cytotoxicity the concentration of fluvastatin could not be increased further.

2.6 Growth efficiency of wild-type and fluvastatin-resistant replicon containing cells

To determine the growth efficiency in absence or presence of statins, the cells were seeded at a density of 5×10^3 cells per well in a 96-well transparent cell culture plate

in the absence or presence of fluvastatin and in complete DMEM without geneticin. Cells were counted at different time points post seeding (24h, 48h, 72h) with the Coulter particle counter (Z1-Beckman Coulter, Analis sa, Suarlée, Belgium). The average cell count of 3 independent experiments over time was calculated to generate a growth curve.

2.7 Cell cycle analysis

The cells were seeded in a 75 cm² tissue culture flask in 20 mL of complete DMEM. After 24 hours of incubation different dilutions of the test compounds in complete DMEM were added. Colchicine (0.02 µM) was used as a positive control. After 24 hours (colchicine) or 3 days (test compounds), the cells were trypsinized. 10⁶ cells were resuspended in 0.5 mL pre-chilled PBS and transferred to 4.5 mL ice cold 70% ethanol and kept on ice. After fixation on 4°C, the cells were resuspended in 1 mL propidium iodide staining solution (0.1% v/v Triton X-100, 100 µg/mL DNase-free RNase A, 10 µg/mL propidium iodide (Sigma-Aldrich, Bornem, Belgium) in PBS). After incubation for 30 min at room temperature, the cell cycle was analyzed using a FACScalibur flow cytometer (BD Biosciences, Erembodegem, Belgium). The fluorescence intensity was measured using the FL2 channel (excitation wavelength: 488 nm/emission wavelength: 564-606 nm). Using CellQuest software (BD Biosciences, Erembodegem, Belgium), a FL2-W/FL2-A dot plot was generated and the single cell population was gated. 10 000 events within the gate were collected. Subsequently, a FL2-A histogram of the gated population was drawn and the percentage of cells within each cell cycle phase was calculated using a marker in CellQuest. The average percentage of cells per phase was calculated based on 3 independent experiments.

2.8 Clearance of fluvastatin-resistant replicon containing cells

Fluvastatin-resistant replicon cells were passaged for six consecutive times in complete DMEM in the presence of 4 μ M 2'-C-methylcytidine and 100 IU/mL IFN- α in 25 cm² tissue culture flasks. The cells were cultured until 90% confluency was reached and were maintained at the same concentration for six passages. To confirm that no replicon RNA was left in these cells after six passages in the presence of the compounds, cleared cells were seeded in a 25 cm² T-flask in complete DMEM supplemented with 1 mg/mL G418 to assess cell death. The absence of replicon RNA was also confirmed by real-time quantitative polymerase chain reaction.

2.9 Stable transfection of Huh-7 Lunet cells and cleared fluvastatin-resistant replicon cells

Huh-7 Lunet cells were stably transfected with wild-type (WT) Huh 9-13 RNA and fluvastatin-resistant RNA using DMRIE-C (Promega, Leiden, The Netherlands). Cleared fluvastatin-resistant replicon cells were stably transfected with RNA isolated from WT Huh 9-13 replicon cells. In brief, RNA was extracted from Huh 9-13 cells and fluvastatin-resistant replicon cells using the RNeasy mini kit (Qiagen Benelux, Venlo, Netherlands). Huh-7 Lunet cells or cleared fluvastatin-resistant replicon cells were seeded at a density of 2×10^5 cells in a 6-well cell culture plate in complete DMEM. After incubation of 24 hours, cells were transfected with a mixture of 12 μ l DMRIE-C, 1 ml OptiMEM (Gibco, Merelbeke, Belgium) and 10 μ g of total RNA. After 4 hours, the transfection mixture was removed and complete DMEM was added to the cells. The next day, the cell culture medium was removed and complete DMEM with 1 mg/mL G418 was added. Following four weeks of G418 selective pressure, stable cell lines were obtained.

2.10 Sequencing

Total cellular RNA was extracted from 5×10^5 WT or fluvastatin-resistant replicon cells using the RNeasy mini kit (Qiagen, Venlo, The Netherlands). A RT-PCR was conducted following manufacturer's instructions (OneStep RT-PCR kit; Qiagen, Venlo, The Netherlands) with specific HCV primers [26] or HMGCR primers (5'–GAGGATCCAA GGATTCTGTAGC-3' en 5'–TGTTCCAGTTCAGAACTGTCGG-3'). The PCR product was purified using the PCR purification kit (Qiagen, Venlo, The Netherlands). A sequencing reaction contained: 2 µl BigDye terminator, 1 µl sequencing buffer, 1 µl primer (5 µM) and a certain amount of DNA and H₂O depending of the fragment length. Primers used for sequencing of the HCV RNA were described before [27]. The sequencing program contained of a denaturation step of 2 minutes at 96°C, and 25 cycles of denaturation (30s at 96°C), annealing (15s at 50°C) and extension (4 min at 60°C). After ethanol precipitation of the samples, sequences were detected using the BigDye terminator v. 3.1 sequence system (Applied Biosystems, Branchburg, New Jersey, USA).

2.11 Western blot

WT and fluvastatin-resistant replicon containing cells were cultured in the absence or presence of 5 µM fluvastatin for 24h. Cells were lysed in M-PER lysis buffer (ThermoScientific, Erembodegem, Belgium), denatured and loaded onto a 4-20% SDS-PAGE gel. After separation and transfer to a PVDF membrane, immunoblotting was performed to detect HMG-CoA reductase using a monoclonal mouse antibody (1:100, Santa Cruz, Heidelberg, Germany) and β-actin as endogenous control with a monoclonal mouse antibody (1:10000, ThermoScientific, Erembodegem, Belgium). The secondary anti-mouse HRP antibody (Santa Cruz, Heidelberg, Germany) was used at a dilution of 1:1000. Bound antibodies were visualized using the ECL Prime kit (Amersham, Diegem, Belgium) and the BioRad imaging system.

2.12 SREBP2 immunofluorescence

WT Huh 9-13 and fluvastatin-resistant replicon containing cells were grown to subconfluency in 8-well Lab-Tek™ chambered cover glass systems (VWR, Haasrode, Belgium). Following 24h of incubation at 37°C, the cells were treated with 5 µM of fluvastatin. Cells were fixed 24h after addition of fluvastatin with 4% paraformaldehyde and permeabilized with PBS/0.1% Triton X-100. Following blocking with 4% BSA in PBS, the cells were stained with the anti-SREBP2 antibody (1:100; BD Pharmingen, Erembodegem, Belgium) for 1h and next with the goat anti-mouse antibody Alexa 488 (1:1000; Life Technologies, Gent, Belgium) for 1h in the dark. Consequently, cells were stained using CellMask™ Deep Red stain (Life Technologies, Gent, Belgium) and DNA was counterstained with DAPI in mounting medium (Invitrogen, Gent, Belgium). All images were collected with a Leica TCS SP5 confocal microscope employing a HCX PL APO 63x (NA 1.4)/oil immersion objective. Alexa Fluor® 488 was detected using the excitation line of 488 nm (Argon Laser) and DAPI was detected using the excitation line of 405 nm (pulsed diode laser). Blue emission was detected between 410-480 nm and green emission was detected between 493-565 nm. CellMask™ Deep Red was imaged employing 633 nm excitation and emission detection between 638-720 nm.

For quantification of the cellular SREBP2 protein content, wild-type or fluvastatin-resistant replicon containing cells were cultured in different wells of a chambered cover glass, and then stained for SREBP2 as described above. Each well was imaged at least three times at different positions in a random order. The experiment was repeated two times. The fluorescence in the green channel was quantified on a per cell basis employing the Imaris v.7.6.5 image analysis software (Bitplane, Zurich, Switzerland). The mean pixel intensity per cell values was normalized to compare

different experiments. A total of 216, 191, 184 and 257 cells were analysed for wild-type and fluvastatin-resistant cells treated with or without fluvastatin, respectively. Statistical significance was determined with an unpaired two-tailed Student's t-test.

2.13 Flow cytometry of efflux transporters

Cell surface expression of P-glycoprotein (P-gp) was monitored following recovery of the cells after trypsinization. Approximately 1×10^6 cells were fixed in PBS containing 2% formaldehyde for 30 min and blocked in PBS + 3% FBS for 15 min at 4°C. Cells were stained for 1h at 4°C with an anti-P-gp PE (BD Biosciences, Erembodegem, Belgium) at a dilution of 5 µg/ml in PBS + 3% FBS. Cells were washed twice in PBS and incubated for 1h at 4°C with a goat anti-mouse Alexa 488 antibody (1:400; Life technologies, Gent, Belgium) in PBS + 3% FBS. All samples were analyzed immediately using a FACSCalibur™. 10 000 events were collected and the healthy cell population was gated for analysis with CellQuest software (BD Biosciences, Erembodegem, Belgium). The net fluorescence intensity was determined by subtracting the mean background fluorescence intensity from the mean fluorescence intensity.

2.14 P-gp efflux assay

A calcein AM efflux assay was used to study the functional activity of P-gp [28]. Calcein acetoxymethyl ester (AM), a non-fluorescent compound, rapidly traverses the cell membrane and is converted to a fluorescent form (calcein free acid) by cellular esterases. Calcein-AM is effluxed from cells by P-gp before its intracellular conversion. When calcein-AM efflux is blocked by an agent that interferes with P-gp (e.g. verapamil), fluorescent calcein free acid rapidly accumulates, providing a measure of P-gp function. Briefly, replicon containing cells were trypsinized, resuspended in complete DMEM and left at room temperature for 2h. Next, cells

were counted and 5×10^5 cells were resuspended in PBS + 2% FBS. Cells were incubated with or without 100 μ M verapamil (Acros Organics, Geel, Belgium), an inhibitor of P-gp [29], for 20 min at room temperature. Next, 250 nM of calcein AM (Sigma-Aldrich, Bornem, Belgium) was added to the cells. Following incubation for 20 min at 37°C, cells were washed two times with PBS + 2% FBS. Intracellular fluorescence intensity was measured using a FACScalibur™ flow-cytometer at excitation wavelength 488 nm and emission wavelength 564-606 nm. The P-gp activity factor (PAF) was calculated as follows: $PAF = 100 \times (NFI_I - NFI_0) / NFI_I$ with NFI_I the netto fluorescence intensity in presence of verapamil and NFI_0 the netto fluorescence intensity in absence of verapamil.

3. RESULTS

3.1 Characterization of the fluvastatin-resistant phenotype.

Fluvastatin-resistant HCV subgenomic replicon containing cells were generated by culturing the cells in the presence of increasing concentrations of fluvastatin. The replicon containing cell line thus obtained proved ~8-fold less susceptible to fluvastatin than the wild-type (WT) cultures (EC_{50} : $22 \pm 3 \mu\text{M}$ versus $2.7 \pm 0.5 \mu\text{M}$) (Figure 1A, Table 1). The fluvastatin-resistant replicon containing cells also proved cross-resistant to simvastatin and atorvastatin (12- and 4.1-fold, respectively) (Figure 1B). On the other hand, HCV direct-acting antivirals and a geranylgeranyltransferase inhibitor (GTI-298) retained their antiviral activity against the resistant replicon cells.

To further characterize the statin-resistant replicon cell line, the growth characteristics were compared to those of WT replicon cells. Both cell lines exponentially grew in the absence of fluvastatin with comparable efficiency (Figure 2A). Treatment with $15 \mu\text{M}$ fluvastatin for 72h resulted in a decrease in the average growth of WT replicon cells as compared to the untreated control (UTC), but this difference was not significant. In contrast, treatment with $25 \mu\text{M}$ fluvastatin markedly inhibited the growth of the WT replicon cells. The cell growth of the fluvastatin-resistant replicon cells was not hampered by fluvastatin at any concentration tested.

Because it was recently reported that fluvastatin (at a concentration of $5 \mu\text{M}$) caused cell cycle arrest at the G1/S checkpoint in HCV subgenomic replicon cells and in naïve Huh 7.5 cells [17], the impact of fluvastatin on the cell cycle of both WT and fluvastatin-resistant replicon cells was analysed using propidium iodide staining measured by flow cytometry. Colchicine ($0.02 \mu\text{M}$) was used as a positive control because it inhibits microtubule polymerization and, as a consequence, mitosis. Indeed, a significant cell cycle arrest at G2/M was observed due to the treatment with

colchicine in both cell populations (Figure 3). WT and fluvastatin-resistant replicon cells resulted in a comparable distribution of cells in the different phases of the cell cycle. In WT cells treated with 15 μ M fluvastatin, a small but significant decrease in the G2/M phase was observed. As a consequence, more cells were expected to be in the G0/G1 phase but the observed difference was not significant (Figure 3). No cell cycle arrest at the G1/S checkpoint was observed in either cell population at this concentration of fluvastatin (and lower). However, when increasing the concentration up to 25 μ M, the WT replicon cells showed a significant arrest in the G0/G1 phase. This was in agreement with microscopical observations showing a cytotoxic effect of this concentration and with the results shown in Figure 2B. In contrast, the cell cycle of the fluvastatin-resistant replicon cells was not influenced by 25 μ M of fluvastatin and no signs of cytotoxicity were observed by microscopy.

3.2 The observed statin resistance is conferred by cellular changes.

The entire coding region of the HCV subgenomic replicon RNA extracted from the fluvastatin-resistant replicon cells was sequenced and compared to the WT replicon genome. Several mutations were identified in the replicon genome, most of which were located in NS5A (data not shown). To study whether these mutations conferred phenotypic resistance to statins, naïve Huh-7 Lunet cells were stably transfected with RNA derived from the fluvastatin-resistant cells by lipofection. Following four weeks of G418 selective pressure, stable cell lines were obtained containing the RNA of both WT and fluvastatin-resistant cells. During the G418 selection period, the cells transfected with the RNA of the fluvastatin-resistant cells were cultured either in the presence or absence of 5 μ M of fluvastatin.

Following the generation of these stably transfected cell lines, the antiviral activity of fluvastatin was evaluated by qRT-PCR. The HCV RNA replication in the resistant

transfected cells cultured in the absence of fluvastatin was as efficiently inhibited by statins as in cells that had been transfected with WT HCV RNA (Figure 4A-B). In contrast, the EC₅₀ value of fluvastatin in the resistant transfected cells cultured in the presence of fluvastatin was 3-fold higher as compared to the EC₅₀ value for WT (EC₅₀= 11 ± 2.2 µM vs. 3.8 ± 1.4 µM, respectively; p<0.05). These data may suggest that there is a modest contribution of the viral genome to the resistant phenotype. However, the mutations identified in the viral genome of the fluvastatin-resistant transfected cells were identical regardless of the presence/absence of fluvastatin in the medium during cell culturing (data not shown). Hence, the mutations identified in the viral genome are most likely not responsible for the drug-resistant phenotype. It may therefore be assumed that the low-level of statin resistance observed in the resistant transfected cell line probably emerged during the selection process due to the presence of fluvastatin which resulted in yet unknown cellular changes.

To address the contribution of such cellular changes to the resistant phenotype, the fluvastatin-resistant replicon cells were cleared from their replicon (using interferon-α and 2'-C-methylcytidine) and were next stably transfected with WT HCV replicon RNA. Following stable transfection, these cells were cultured either in the absence or presence of 5 µM fluvastatin. The resulting replicon cell lines proved both to be less susceptible to fluvastatin (4.9- and 5.5-fold for cells cultured in absence of presence of fluvastatin, respectively) suggesting that the fluvastatin-resistant phenotype is, at least partially, caused by a cellular resistance mechanism (Figure 4C-D).

3.3 HMGCR expression is upregulated in statin-resistant replicon cells.

Because statins reduce cholesterol synthesis by inhibition of HMG-CoA reductase, a logic explanation for the resistant phenotype would be an increase in HMG-CoA reductase expression. Therefore, the HMGCR mRNA expression was studied by

qRT-PCR on total RNA extracted from WT and fluvastatin-resistant replicon containing cells cultured in the absence or presence of 5 μ M fluvastatin for 24h. The basal levels of HMGCR mRNA proved to be 1.9-fold higher in the resistant cells than in WT cells (Figure 5A). Following a 24h treatment with 5 μ M of fluvastatin, the expression of HMGCR mRNA was upregulated in both cell lines as expected. Interestingly, the upregulation of HMGCR mRNA expression in the fluvastatin-resistant replicon cells was markedly higher as compared to the upregulation in WT replicon cells (3.9 ± 0.8 fold vs 1.7 ± 0.04 fold, respectively) (Figure 5A). The increased expression of HMG-CoA reductase in the statin-resistant replicon cells upon statin treatment was also confirmed at the protein level by Western blotting (Figure 5B-C). As single nucleotide polymorphisms in the HMGCR gene have been shown to contribute to variations in statin response in patients [30,31], the coding regions of the HMGCR gene of WT and fluvastatin-resistant replicon cells were sequenced. No mutations were identified in the HMGCR gene in the statin-resistant replicon cells (data not shown).

The cholesterol synthesis pathway is tightly regulated by a feedback system to maintain cholesterol levels in the cell. The nutritional control of this pathway occurs at the transcriptional level by sterol regulatory element-binding proteins (SREBPs) [32]. SREBP-2 is more specific for controlling cholesterol biosynthesis than the other SREBPs. Therefore, the mRNA levels of SREBP2 were studied by qRT-PCR on total RNA extracted from WT and fluvastatin-resistant replicon containing cells cultured in the absence or presence of 5 μ M fluvastatin for 24h. GAPDH mRNA levels were measured as endogenous control. No upregulation of SREBP2 mRNA was observed in the fluvastatin-resistant replicon cells as compared to WT replicon cells (Figure 5D). To study the localization of SREBP2 in the WT and statin-resistant cells upon

statin treatment, immunofluorescence staining was employed using an anti-SREBP2 antibody that detects both immature and mature SREBP2 forms. Representative images of all studied conditions are shown in Figure 5E. Employing the Imaris v.7.6.5 image analysis software, the cellular SREBP2 protein content was quantified on a per cell basis. Treatment with fluvastatin induced a slight increase in SREBP2 expression in WT cells, especially in the cytoplasm ($p < 0.001$). Interestingly, the untreated statin-resistant cells had a higher SREBP2 expression in the cytoplasm as compared to WT UTC (1.3-fold, $p < 0.001$) and treatment with statins resulted in an increase of 1.5-fold when compared to WT UTC ($p < 0.001$, Figure 5F). Study of the distribution of total SREBP2 between nucleus and cytoplasm for each cell showed that treatment with fluvastatin resulted in a shift of SREBP2 pools to the cytoplasm in WT HCV replicon containing cells (Figure 5G). A similar shift to the cytoplasm, albeit not statistically significant, was observed in the untreated statin-resistant HCV replicon containing cells. Interestingly, treatment with statins in the statin-resistant cells did not result in changes in the SREBP2 distribution when compared to the untreated statin-resistant cells.

3.4 Are statin efflux transporters involved in the resistant phenotype?

Since drug efflux is the main mechanism responsible for resistance to chemotherapeutics, we next investigated the possible role of statin efflux transporters in the mechanism of statin resistance. Statins are reported to be a substrate of many transporters, e.g. P-glycoprotein transporter (P-gp/MDR1), multidrug resistance-associated protein 2 (MRP2) and breast cancer resistance protein (BCRP) [33–35]. Therefore, the mRNA expression of P-gp, MRP2 and BCRP was determined in total RNA extracted from both cell lines by qRT-PCR with GAPDH as endogenous control gene. Only the mRNA levels of P-gp were slightly increased in the fluvastatin-

resistant replicon cells (1.7 ± 0.2 fold, $p < 0.001$) (Figure 6A). No difference was observed in the levels of MRP2 or BCRP. P-gp protein expression was next monitored by means of flow cytometry was performed. Although P-gp expression at the plasma membrane was 5-fold upregulated on the fluvastatin-resistant replicon cells, this difference was not statistically significant due to variability in P-gp protein levels measured in the WT replicon cells ($p > 0.05$) (Figure 6B).

To study the functional activity of P-gp, a previously described calcein AM efflux assay was employed [28]. In this assay, calcein acetoxymethyl ester (AM), a non-fluorescent compound that is effluxed from cells by P-gp, is used. Calcein AM rapidly enters the cell and is converted to a fluorescent form (calcein free acid) by cellular esterases. This fluorescent form cannot be effluxed by P-gp. When calcein-AM efflux is blocked by an agent that interferes with P-gp (e.g. verapamil), fluorescent calcein free acid rapidly accumulates, providing a measure of P-gp function. The efflux of calcein AM was determined in both WT and fluvastatin-resistant replicon cells. The P-gp activity factor in the fluvastatin-resistant replicon cells proved to be 2.5 ± 0.3 fold higher as compared to the activity in WT replicon cells ($p < 0.001$) (Figure 6C), demonstrating that the increased P-gp levels also resulted in an enhanced P-gp mediated efflux of calcein AM.

P-gp transporters are ubiquitously expressed in human cells and export lipophilic cytostatic drugs, such as doxorubicin, out of the cell. It was shown previously that overexpression of P-gp can result into an increased resistance against the toxic effects of doxorubicin [36]. The CC_{50} value of doxorubicin should thus be higher in cells with elevated expression or activity of P-gp. However, no difference in cytotoxicity of doxorubicin could be determined ($p > 0.05$) between WT and fluvastatin-

resistant replicon cells (CC_{50} : $0.3 \pm 0.1 \mu\text{M}$ vs. $0.2 \pm 0.06 \mu\text{M}$, respectively) (Figure 6D).

DISCUSSION

Statins are one of the most commonly prescribed drugs worldwide and are considered to be effective and safe. However, nearly a third of statin-treated patients do not meet the lipid-lowering goals. The exact prevalence of statin resistance is difficult to assess due to the relatively frequent occurrence of such symptoms independent from statin use, but is estimated around 20% based on observational studies [21,22]. The mechanisms underlying this resistance to statins are still unclear. For this reason, and because host-targeting antivirals such as statins are believed to be less prone to HCV resistance development [1], we set out to study whether the development of statin resistance in HCV replicon cells is possible and, if so, to gain more insight into the mechanism of resistance. To generate statin-resistant replicons, HCV replicon containing cells were passaged in the presence of increasing concentrations of fluvastatin. The fluvastatin-resistant HCV replicon cell line generated by this method proved cross-resistant to other statins. The growth efficiency of this resistant cell line was similar to WT replicon cells. Experiments to investigate the role of the HCV genome and the cellular environment in the resistant phenotype indicated that the observed resistance to statins was due to cellular changes and not to changes in the viral genome.

Since statins are inhibitors of the HMG-CoA reductase, a logic explanation for the resistant phenotype would be an increase in HMG-CoA reductase expression. Other statin-resistant cell lines were previously reported to have an increased expression of the HMG-CoA reductase gene. Especially in resistant cell lines that were generated in medium containing delipidated serum, an extensive increase in HMG-CoA reductase up to 500-fold was observed [37,38]. In a lovastatin-resistant CHO cell line generated in serum-containing medium, the levels of HMGCR enzyme were also

elevated [39]. A similar observation was made with the fluvastatin-resistant HCV replicon cells. On basal level, the increase in HMGCR expression in the resistant replicon cells was modest but significant as compared to WT replicon cells. However, upon statin treatment, the HMGCR expression was markedly increased in the resistant replicon cells. These data thus suggest that the increased HMGCR expression is (at least partially) responsible for the statin-resistant phenotype. It is however worthy to note that statin resistance and levels of HMG-CoA reductase activity have been reported to be not directly correlated [40]. On the other hand, it was recently shown in a mouse model that inflammation increased mRNA levels and protein expression of HMG-CoA reductase, SCAP and SREBP2 in the mouse kidney, causing 'kidney statin resistance' [41], suggesting that this mechanism could be relevant in statin-resistant patients. Indeed, in the statin-resistant replicon cells slightly increased SREBP2 levels were observed. Whether this upregulation contributed to the resistant phenotype, will need more study.

Single nucleotide polymorphisms in several genes (HMGCR, APOE13, human anion transporting polypeptide C (OATP-C), mitochondrial unfolded protein response activator (ATFS-1)) have also been associated with reduced effectiveness of statin therapy in patients [42–44]. This was also shown in a lovastatin-resistant CHO-K1 cell line, in which a mutation in HMGCR was identified that enhanced the affinity of the enzyme for HMG-CoA [45]. However, no mutations in the HMGCR gene were observed in the fluvastatin-resistant HCV replicon cells.

A cellular resistance mechanism in HCV replicon containing cells was previously reported for ribavirin. The ribavirin resistance in these cells was caused by a decreased uptake of ribavirin by ENT1 transporters [20,46,47]. As changes in drug uptake/efflux are also regularly observed for i.e. chemotherapeutics as a mechanism

of cellular resistance, it was therefore hypothesized that the cellular resistance to statins might also be related to a difference in expression and activity of statin membrane transporters. To study this hypothesis, the mRNA levels and protein expression of statin efflux transporters was determined for WT and fluvastatin-resistant replicon cells. Only for P-gp a significant increase in mRNA levels was observed between WT and resistant cells. Although the upregulation of P-gp proteins was not statistically significant, the P-gp efflux activity proved to be more pronounced on the fluvastatin-resistant replicon cells. However, the lack of resistance to the toxic effect of doxorubicin, a substrate of P-gp, suggested that the observed increase in P-gp expression and activity may not result in a biological relevant effect and may hence not or only slightly contribute to the statin-resistant phenotype. On the other hand, it was shown previously that the calcein efflux assay is more rapid and significantly more sensitive than conventional assays that are based on the accumulation of doxorubicin [48]. This may explain why the moderate increase in P-gp activity observed in the statin-resistant cells by the calcein efflux assay did not result in an increased CC_{50} value in the doxorubicin cytotoxicity assay. Furthermore, it has been suggested, but also contradicted, that P-gp transporters and cholesterol biosynthesis are related and as a consequence the resistant phenotype might have upregulated P-gp expression to compensate the inhibition of cholesterol biosynthesis by statins [49–51]. This might lead to an equal concentration in the cell of P-gp substrate, e.g. doxorubicin and statins. Evidence also exists that an increase in P-gp expression may be a direct consequence of the exposure of cells to chemotherapeutic/cytotoxic agents [52]. The expression of P-gp in a number of human cell lines can be induced in response to cellular damage by cytotoxic drugs regardless of whether the drugs are transported by P-gp [53]. Whether these

'nonspecific' changes in P-gp contribute to clinical drug resistance remains unclear. Interestingly, similar observations regarding P-gp upregulation were made for alisporivir [19]. In HCV replicon containing cells resistant to alisporivir, a 9-fold increase in P-gp expression was observed as compared to WT replicon cells. Whether these cellular changes played any role in the *in vitro* resistance to alisporivir was not studied. It is thus possible or presumable that the upregulation of P-gp transporters in the fluvastatin-resistant replicon cell line is a non-specific response to the statin treatment.

In conclusion, we showed that HCV replicon containing cells are able to acquire statin resistance and that the resistant phenotype is due to changes in the cellular environment. Statin resistance is increasingly reported in patients, but the mechanisms underlying this resistance remain elusive. Whether HMGCR or P-gp expression is upregulated in statin-intolerant patients, has, to the best of our knowledge, not been studied yet. It will be interesting to explore whether the mechanisms of statin resistance observed in *in vitro* models are also contributing to the resistance to statins in patients.

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FIGURE LEGENDS

Figure 1. Phenotype of fluvastatin-resistant HCV replicon containing cells.

A) Dose-dependent inhibition of HCV replication by fluvastatin in WT (black) and fluvastatin-resistant replicon containing cells (grey). HCV RNA levels were determined by RT-qPCR. Data shown are the averages $\pm$ SD of at least three independent experiments. **B)** Fold-resistance values of statins, GTI-298 and HCV DAA on fluvastatin-resistant replicon containing cells. The fold-resistance was calculated as the ratio of the EC_{50} value in fluvastatin-resistant replicon cells to the EC_{50} value in WT replicon cells. Data shown are average values $\pm$ SD of at least three independent experiments.

Figure 2. Growth efficiency of WT and fluvastatin-resistant replicon containing cells.

A) Growth curves of WT (black) and fluvastatin-resistant (grey) replicon containing cells in the absence of fluvastatin and geneticin. Cells were counted at different time points post cell seeding (24h, 48h, 72h). Data shown are average values $\pm$ SD of at least three independent experiments. **B)** Percentage of cell growth of WT and fluvastatin-resistant replicon containing cells in comparison to untreated control cells in the presence of 1 μ M (white), 5 μ M (light grey), 15 μ M (dark grey) or 25 μ M (black) of fluvastatin at 72h post cell seeding. Data shown are average values $\pm$ SD of at least three independent experiments.

Figure 3. Cell cycle analysis of WT and fluvastatin-resistant replicon containing cells upon fluvastatin treatment.

WT and fluvastatin-resistant replicon containing cells were either untreated or cultured in the presence of 5 μ M, 15 μ M or 25 μ M of fluvastatin for 72h and consequently stained with propidium iodide (PI) to analyze cell cycle by flow

cytometry. The percentages of cell population are calculated for different stages of the cell cycle: subG1 (black), G0/G1 (dark grey), S (light grey) and G2/M (white). Colchicine (0.02 μ M) was used as a positive control for cell cycle arrest at G2/M. Data shown are the average values of three independent experiments. * $p < 0.05$ compared to UTC.

Figure 4. The statin-resistant phenotype is not associated with the viral genome. **A)** Scheme explaining the stable transfection experiment. **B)** Dose-response curves for inhibition of HCV replication by fluvastatin in Huh-7 Lunet cells stably transfected with RNA isolated from WT (black) or fluvastatin-resistant replicon cells, cultured in the presence (RES + FLV, dark grey) or absence (RES – FLV, light grey) of fluvastatin during G418 selection. HCV RNA was quantified by RT-qPCR and is expressed as the percentage of HCV RNA of UTC. Data are mean values $\pm$ SD for at least three independent experiments. **C)** Fold-resistance values of fluvastatin and VX-950 on the stably transfected Huh-7 Lunet cells with RNA isolated from WT (black) or fluvastatin-resistant replicon cells, cultured in the presence (RES + FLV, dark grey) or absence (RES – FLV, light grey) of fluvastatin. The fold-resistance is calculated as the ratio of the EC₅₀ value in the fluvastatin-resistant replicon cells to the EC₅₀ value in WT replicon cells.

Figure 5. The statin-resistant phenotype is associated with cellular factors.

A) Scheme explaining the clearance and transfection protocol. **B)** Dose-response curves for inhibition of HCV replication by fluvastatin in cleared WT (black) and fluvastatin-resistant replicon cells stably transfected with RNA isolated from WT replicon cells, cultured in the presence (cRES+FLV, dark grey) or absence (cRES-FLV, light grey) of fluvastatin during G418 selection. HCV RNA was quantified by RT-qPCR and is expressed as the percentage of HCV RNA of UTC. Data are mean

values $\pm$ SD for at least three independent experiments. * $p < 0.05$ compared to WT. **C)** Fold-resistance values of fluvastatin and VX-950 on the stably transfected cleared cells with RNA isolated from WT replicon cells, cultured in the presence (cRES+FLV, dark grey) or absence (cRES-FLV, light grey) of fluvastatin. The fold-resistance is calculated as the ratio of the EC_{50} value in the fluvastatin-resistant replicon cells to the EC_{50} value in WT replicon cells.

Figure 6. HMG-CoA reductase expression is upregulated in fluvastatin-resistant replicon containing cells upon statin treatment.

A) HMGCR mRNA expression. HMGCR mRNA levels in WT and fluvastatin-resistant replicon cells cultured in the absence (black) or presence (grey) of 5 μ M of fluvastatin for 24h. HMGCR mRNA levels were determined by relative RT-qPCR. GAPDH mRNA was measured as endogenous control. Data are mean values $\pm$ SD for at least three independent experiments. * $p < 0.05$ compared to WT UTC. $^{\dagger}p < 0.05$ compared to fluvastatin-resistant UTC. **B-C) HMG-CoA reductase protein expression.** HMG-CoA reductase protein levels were determined with (grey) or without (black) fluvastatin treatment by Western blotting for WT and fluvastatin-resistant replicon containing cells. Data are mean values $\pm$ SD of two independent experiments. A representative western blot is depicted in panel C. **D) SREBP2 mRNA expression.** RT-qPCR was performed on total RNA extracted from WT and fluvastatin-resistant replicon containing cells cultured in the absence (black) or presence (grey) of 5 μ M fluvastatin for 24h. GAPDH mRNA was measured as endogenous control. Data presented are average values $\pm$ SD of two independent experiments. **E-G) Confocal imaging of SREBP2.** **E)** SREBP2 (green) was visualized in WT and fluvastatin-resistant replicon cells cultured in the absence or presence of 5 μ M of fluvastatin for 24h using confocal fluorescence microscopy. DNA

was stained with DAPI (blue). **F)** Fold of the average SREBP2 fluorescence intensity in the nucleus (light grey) and cytoplasm (dark grey) of WT and fluvastatin-resistant replicon cells as compared to WT UTC. Data are average values $\pm$ SD of at least 60 cells per condition in two independent experiments. * $p < 0.001$ compared to WT UTC. **G)** Percentage of total intracellular SREBP2 expression quantified in the nucleus (light grey) or in the cytoplasm (dark grey) of WT and fluvastatin-resistant replicon cells cultured in the absence or presence of 5 μ M of fluvastatin for 24h. Data are average values $\pm$ SD of at least 60 cells per condition in two independent experiments. * $p < 0.001$ compared to WT UTC. $^{\dagger}p < 0.001$ compared to fluvastatin-resistant UTC.

Figure 7. The expression of statin efflux transporters in WT and fluvastatin-resistant replicon containing cells.

A) mRNA expression of efflux transporters. P-gp, MRP2 and BCRP mRNA levels were determined by relative RT-qPCR on total RNA extracted from WT (black) and fluvastatin-resistant (grey) replicon containing cells. GAPDH mRNA was measured as endogenous control. Data are mean values $\pm$ SD for at least three independent experiments. *** $p < 0.001$ compared to WT. **B) Pg-p protein expression.** Protein expression of Pg-p as measured by flow cytometry on WT (black) and fluvastatin-resistant (grey) replicon containing cells. Data are mean values $\pm$ SD for at least three independent experiments. **C) P-gp mediated efflux of calcein AM.** Values were calculated as the fold verapamil-inhibitable calcein AM efflux measured after 20 min in fluvastatin-resistant replicon containing cells as compared to WT. P-gp activity factor = $100 \times (NFI_I - NFI_0) / NFI_I$ with NFI_I the netto fluorescence intensity in presence of verapamil and NFI_0 the netto fluorescence intensity in absence of verapamil. *** $p < 0.001$ compared to WT. **D) Doxorubicin toxicity.** The cytotoxicity of

doxorubicin was determined on WT (black) and fluvastatin-resistant (grey) replicon cells as described before [13]. Data shown are mean values $\pm$ SD for at least three independent experiments.

Figure 1.

Figure 2.

A

B

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Table 1. EC₅₀ values of statins and other inhibitors of HCV replication in WT and fluvastatin-resistant replicon containing cells.

	WT	Fluvastatin-resistant	
	EC ₅₀ in μ M	EC ₅₀ in μ M	Fold-resistance
Fluvastatin	2.7 $\pm$ 0.5	22 $\pm$ 3.0	8.0
Simvastatin	2.8 $\pm$ 1.1	34 $\pm$ 8.1	12
Atorvastatin	4.3 $\pm$ 0.2	18 $\pm$ 3.4	4.1
GTI-298	2.8 $\pm$ 0.3	1.7 $\pm$ 0.3	0.6
VX-950	0.08 $\pm$ 0.01	0.03 $\pm$ 0.01	0.4
BMS-790052 (nM)	0.004 $\pm$ 0.002	0.007 $\pm$ 0.0008	1.6
2'-C-methylcytidine	0.55 $\pm$ 0.1	0.98 $\pm$ 0.6	1.8
VX-222 (nM)	2.0 $\pm$ 0.3	1.2 $\pm$ 0.3	0.6
IFN-α 2a (IU/ml)	2.8 $\pm$ 2.9	7.9 $\pm$ 7.6	2.8

Data are mean values $\pm$ SD for at least 3 independent experiments.