Statins potentiate the *in vitro* anti-hepatitis C virus activity of selective HCV inhibitors and delay or prevent resistance development.

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Running Title: Statins and STAT-C

Keywords: combination, viral clearance, escape mutants, cholesterol, STAT-C.

Footnotes

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Abbreviations : HCV, hepatitis C virus; IFN, interferon; NS, nonstructural; RdRp, RNA-dependent RNA polymerase; LDL, low density lipoprotein; SR-BI, scavenger receptor class B type I; VLDL, very low density lipoprotein; HMG CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; HIV, human immunodeficiency virus; JFH-1, Japanese fulminant hepatitis-1; STAT-C, specifically targeted antiviral therapy for HCV; FIC, fractional inhibitory concentration; C_{max}, maximum plasma concentration.

Abstract

Statins are 3-hydroxyl-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors used for the treatment of hypercholesterolemia. It was recently reported that statins inhibit *in vitro* hepatitis C virus (HCV) RNA replication. We here report that, of 5 statins studied, mevastatin and simvastatin exhibit the strongest *in vitro* anti-HCV activity, lovastatin and fluvastatin have moderate inhibitory effects and pravastatin is devoid of an antiviral effect. Combination of statins with interferon-α (IFN) or HCV NS5B polymerase or NS3 protease inhibitors results in an additive antiviral activity in short-term (3 days) antiviral assays. Neither statins, at a concentration of 5-fold their EC_{50} value, nor polymerase, protease inhibitors or IFN-α, at concentration of 10- or 20- fold their EC_{50} value, were able to clear cells from their replicon following 4 or 6 consecutive passages of antiviral pressure. However, the combination of HCV polymerase or protease inhibitors with mevastatin or simvastatin resulted in an efficient clearance of the cultures from their replicon. In colony formation experiments, mevastatin reduced the frequency or prevented the selection of HCV replicons resistant to the non-nucleoside inhibitor HCV-796. ***Conclusion :*** A combination of specific HCV inhibitors with statins may result in a more profound antiviral effect and may delay or prevent the development of resistance to such inhibitors.

Introduction

Hepatitis C virus (HCV) is a positive single-stranded RNA virus and a member of the *Hepacivirus* genus within the *Flaviviridae* family. Worldwide approximately 170 million people (or almost 3% of the global population) are chronically infected with HCV. Chronically infected patients are at increased risk of developing liver cirrhosis and hepatocellular carcinoma (1). In Western countries, infection with HCV is the most common cause of liver transplantation. Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) and ribavirin. This therapy is only effective in 50-60% of infected patients and is associated with serious side effects (2). There is thus an urgent need for more selective, potent and better tolerated therapies for chronic hepatitis C.

Most antiviral drugs that are currently in clinical trials are inhibitors of the viral polymerase or serine protease. The design of antiviral drugs that inhibit the function of the HCV protease and polymerase therefore appears to be logic. The first HCV NS3/4A serine protease inhibitor to enter clinical trials was BILN-2061 (3). The most clinically advanced investigational inhibitors of the HCV protease are telaprevir (VX-950), boceprevir (SCH 503034), ITMN-191 and TMC-435350. Both nucleoside and non-nucleoside inhibitors of the HCV RNA-dependent RNA polymerase (RdRp) have been reported (4). Nucleoside HCV polymerase inhibitors act as premature chain terminators following conversion to their 5'-triphosphate metabolite by competition and incorporation in the viral genome. 2'-*C*-methylcytidine was the first nucleoside HCV inhibitor to enter clinical studies. Development of this compound has been discontinued because of modest antiviral efficacy along with significant gastrointestinal side effects (5). Various non-nucleoside inhibitor classes of the HCV RdRp, including benzimidazoles, thiophene-based carboxylic acids, benzothiadiazines and others have been reported (6). On the other hand, host factors that are essential for efficient viral replication may also be good antiviral targets. The non-immunosuppressive cyclophilin binding molecule Debio-025 is a potent inhibitor of HCV replication and has shown excellent efficacy in phase I and phase II clinical trials (7).

HCV requires elements of the cholesterol and fatty-acid biosynthetic pathways for efficient replication (8). Lipoprotein receptors (LDL receptor, SR-BI receptor) have been reported to be involved in HCV entry. Nevertheless, the role of the LDL-receptor in HCV entry is still uncertain (9). Cholesterol metabolism is also required for the assembly of VLDL particles. These lipoprotein particles have been shown to complex with HCV virions in serum. Statins, which are used to treat hypercholesterolemia, inhibit 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver. HMG-CoA reductase catalyzes the conversion of HMG-CoA to mevalonic acid. Besides its cholesterol-lowering effect, statins have been reported to exhibit antiviral activities against a variety of viruses. Antiviral activity was reported against HIV-1 (10-12), poliovirus (13), cytomegalovirus (14) and respiratory syncytial virus (15). Recently, statins were shown to inhibit the replication of subgenomic HCV-1b replicons (16) and to suppress RNA replication of JFH-1 HCV (17). The precise mechanism of the anti-HCV activity of statins has not yet been defined. Recent studies suggest that the anti-HCV activity of statins may result from inhibition of geranylgeranylation of cellular proteins rather than the inhibition of cholesterol synthesis (8;18). Geranylgeranylation is a post-transcriptional modification that covalently attaches geranylgeranyl to various cellular proteins to facilitate their membrane association. These geranylgeranyl groups are isoprenoids synthesized in the cholesterol biosynthesis pathway. More recently, FBL2 has been reported to be a host target for geranylgeranylation. Geranylgeranylation of FBL2 appears to be critical for HCV replication because the association between FBL2 and NS5A, an interaction that is a prerequisite for HCV replication, depends on geranylgeranylation of FBL2 (19).

A major concern for successful anti-HCV therapy is the rapid emergence of drug resistance to selective HCV inhibitors (20;21). Combination therapy of drugs with a different mode of action will most likely be necessary to delay or prevent the development of drug resistance. We here report on the effect of combining statins with selective HCV antivirals.

EXPERIMENTAL PROCEDURES

Cells and replicon constructs

Huh 7 cells containing subgenomic HCV replicons I_{389}luc-ubi-neo/NS3-3'/5.1 (Huh 5-2) and

I_{377}/NS3-3'/wt (Huh 9-13) have been described before (22;23). Cells were cultured in Dulbecco's

modified Eagle's Medium (DMEM; Gibco, Merelbeke, Belgium) supplemented with 10% heat-

inactivated fetal bovine serum (Integro, Zaandam, The Netherlands), 1x non-essential amino

acids, 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 250 µg/ml Geneticin® (G418;

Gibco) for Huh 5-2 cells and 1 mg/ml G418 for Huh 9-13 cells. Cell cultures were maintained at

37°C in an atmosphere of 5% CO_2. Replicons resistant to the protease inhibitor BILN-2061, the

nucleoside polymerase inhibitor 2'-*C*-methylcytidine, or the non-nucleoside polymerase inhibitor

HCV-796 were generated by selective pressure.

HCV inhibitor compounds

Mevastatin, simvastatin, lovastatin and pravastatin were purchased from Sigma-Aldrich (Bornem,

Belgium). Fluvastatin was purchased from Cayman Chemical (Huissen, The Netherlands).

Recombinant IFN-α 2b (Intron® A) was purchased from Schering Plough (Kenilworth, NJ, USA).

HCV NS5B polymerase inhibitors 2'-*C*-methylcytidine, R1479, benzothiadiazine GSK-4 and

benzofuran HCV-796 and NS3 protease inhibitors VX-950 and BILN-2061 were synthesized as

described before (24).

Antiviral assay with Huh 5-2 cells or Huh 9-13 cells

Antiviral assays were performed as described before (25). Briefly, cells were seeded at a density

of $5x10^3$ cells per well (for Huh 9-13 cells) or $6.5 x10^3$ cells per well (for Huh 5-2 cells) in a 96-well

cell culture plate in complete DMEM. Following incubation of 24 hours at 37°C (5% CO_2), serial

dilutions of the test compounds in complete DMEM were added in a total volume of 100 µL. For

the Huh 5-2 cells, luciferase activity was determined after 3 days of incubation by using the

Steady-Glo luciferase assay system (Promega, Leiden, The Netherlands). For Huh 9-13 cells,

replicon RNA levels were determined by a reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Antiviral assay with JFH-1/CS-N6 HCV$_{cc}$

Huh 7-5 cells were seeded at a density of $5x10^3$ cells per well in a 96-well cell culture plate in complete DMEM. Following incubation of 24 hours at 37°C (5% CO_2), serial dilutions of the test compounds in complete DMEM were added in a total volume of 100 μL. Cells were then inoculated with 100 μL diluted culture media containing infectious HCV JFH-1/CS-N6 (2.7×10^5 HCV-RNA copies)(26). Intracellular HCV RNA levels were determined after 3 days of incubation by a reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Reverse transcription quantitative polymerase chain reaction

Reverse transcription polymerase chain reactions (RT-qPCR) were performed as described before (25). Primers used for detection of HCV replicon RNA were : 5'-CCG GCT ACC TGC CCA TTC-3' (forward primer), 5'-CCA GAT CAT CCT GAT CGA CAA G-3' (reverse primer) and 5'-FAM-ACA TCG CAT CGA GCG AGC ACG TAC-TAMRA-3' (probe). Primers used for detection of HCV$_{cc}$ RNA were : 5'-ACG CAG AAA GCG CCT AGC CAT GGC GTT AGT A-3' (forward primer), 5'-TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG-3' (reverse primer) and 5'-FAM-TGG TCT GCG GAA CCG GTG AGT ACA CC-TAMRA-3' (probe).

Cytostatic assay

Cytostatic assays were performed as described before (25). Briefly, cells were seeded at a density of $5x10^3$ cells per well (for Huh 9-13 cells) or $6.5 x10^3$ cells per well (for Huh 5-2 cells) in a 96-well cell culture plate in complete DMEM. After 24 hours of incubation at 37°C serial dilutions of the test compounds in complete DMEM were added. After 3 days of incubation at 37°C, cell viability was determined using the MTS/PMS method (Promega).

Other antiviral assays

Antiviral assays with herpes simplex virus-1 (KOS strain and TK$^-$ KOS ACV^r), herpes simplex virus-2 (G strain) [*Herpesviridae*], vaccinia virus [*Poxviridae*], vesicular stomatitis virus [*Rhabdoviridae*], coxsackie virus B4 [*Picornaviridae*], respiratory syncytial virus, para-influenza-3 virus [*Paramyxoviridae*], reovirus-1 [*Reoviridae*], sindbis virus, chikungunya virus [*Alphaviridae*], influenza A virus (H1N1 and H3N2), influenza B virus [*Orthomyxoviridae*], Punta Toro virus [*Bunyaviridae*], Yellow fever virus (YFV-17D) and bovine viral diarrhea virus (BVDV) [*Flaviviridae*] were carried out as reported before (27).

Combination antiviral assay

The effects of drug-drug combinations were evaluated using the method of Prichard and Shipman (28). In brief, the theoretical additive effect is calculated from the dose-response curves of individual compounds by the equation Z=X+Y(1-X) where X and Y represent the inhibition produced by the individual compounds and Z represents the effect produced by the combination of compounds. The theoretical additive surface is subtracted from the actual experimental surface, resulting in a horizontal surface that equals the zero plane when the combination is additive. A surface that lies higher than 20% above the zero plane indicates a synergistic effect of the combination and a surface lower than 20% below the zero plane indicates antagonism. The antiviral assays were performed in a similar way as described for Huh 5-2 cells except that the compound dilutions were added in a checkerboard format. Combination studies for each pair of compounds were performed in triplicate.

Clearance rebound assay

Huh 9-13 cells were seeded at a density of $3x10^5$ cells in complete DMEM with 1 mg/ml G418 in 25 cm² T-flasks. After 24 hours of incubation at 37°C (5% CO_2) cell culture medium was removed and replaced by complete DMEM without G418 containing either no antiviral compound or containing one or two antiviral compounds at a fixed concentration. Mevastatin and simvastatin were used at concentrations of 5-fold their EC_{50} value, 2'-*C*-methylcytidine, HCV-796 and IFN-α at 10-fold their EC_{50} value and BILN-2061 at 20-fold its EC_{50} value. Concentrations of particular

compounds were selected such that the concentration was not cytostatic but still able to result in at least 2-log reduction in replicon replication following two passages of treatment. When 90% confluency was reached, cells were trypsinized after which $3x10^5$ cells were seeded in a new 25 cm² T-flask in complete DMEM with the same concentration of compound(s); $1.5x10^5$ cells from each flask were lysed in RLT buffer. In total, cells were passaged 6 consecutive times in the presence or absence of compound(s) and in the absence of G418. Following every 2 passages (P2, P4 and P6), cells of each flask were passaged for 3 consecutive times in the presence of 1 mg/ml G418 (rebound phase). Again, $3x10^5$ cells were seeded in new 25 cm² T-flasks and $1.5x10^5$ cells from each flask were lysed in RLT buffer. After collecting all samples, RNA was extracted and the replicon RNA content was measured by real-time quantitative polymerase chain reaction.

Combined resistance selection

Huh 9-13 cells were seeded at a density of $7,2x10^4$ cells in a 12-well plate in complete DMEM containing 1 mg/mL G418 and in the presence of mevastatin (1 µM, 5 µM and 10 µM) or HCV-796 (20 nM, 100 nM and 200 nM) or a combination of both in a matrix format. When 90% confluency was reached, cells were trypsinized after which $7,2x10^4$ cells were seeded in a new 12-well plate in complete DMEM with the same concentration of antiviral(s). After approximately 3 weeks of selection, HCV-796 resistant colonies developed. Cultures were either fixed with ethanol and stained with 1% methylene blue or expanded to obtain sufficient cells for subsequent pheno- and genotype characterization.

Sequence analysis of the NS5B gene

Total cellular RNA was extracted using the RNeasy minikit (Qiagen, Venlo, The Netherlands) and subjected to RT-PCR using primers NS5B-F [5'-TGCTTTGACTCAACGGTCAC-3'] (corresponding to nucleotides 6649 to 6668 of accession number AJ242652) and NS5B-R [5'-TGTAACCAGCAACGAACCAG-3'] (7629 to 7648 of accession number AJ242652). Nucleotide

sequences were determined by automated sequencing using BigDye terminator v. 3.1 (Applied

Biosystems).

RESULTS

Antiviral activity of statins in HCV subgenomic replicon cells

The effect of lovastatin, mevastatin, simvastatin, fluvastatin and pravastatin on *in vitro* HCV

replication was evaluated in genotype 1b Con1 HCV subgenomic replicons (Huh 5-2). Lovastatin,

mevastatin, simvastatin and fluvastatin inhibited HCV replicon replication (measured as luciferase

signal) in a dose-dependent manner (Table 1, Figure 1). These 4 statins were roughly equipotent

[with EC_{50} values of 1.9, 1.3, 1.5, 1.8 µM], whereas pravastatin was devoid of an inhibitory effect

on HCV replicon replication as was expected (16;29). The anti-HCV activity of statins was not the

result of a cytostatic effect; the 50% cytotoxic concentrations of lovastatin, mevastatin,

simvastatin and fluvastatin were respectively 60 µM, 34 µM, 32 µM and 44 µM, thus resulting in

selectivity indices of 32, 23, 21 and 24 (Table 1).

The anti-HCV activity was confirmed in Huh 9-13 replicon containing cells by means of qRT-PCR

(Table 1). Mevastatin and simvastatin exhibited the strongest anti-HCV activity, whereas

fluvastatin and lovastatin had moderate inhibitory effects and pravastatin was, akin to the

situation in Huh 5-2 replicon containing cells, devoid of antiviral activity. The antiviral activity of

statins was however less effective in the HCV_{cc} model than in the subgenomic replicon model

(Table 1).

It was next studied whether statins inhibit the replication of other members of the *Flaviviridae*

family, ie flaviviruses (yellow fever virus 17P) and pestiviruses (bovine viral diarrhea virus). No

activity was observed against these viruses nor against a selection of unrelated viruses

(chikungunya virus, sindbis virus, coxsackie B4 virus, punta toro virus, para-influenza-3 virus,

respiratory syncytial virus, vesicular stomatitis virus, influenza A virus [H1N1 and H3N2],

influenza B virus, reovirus-1, vaccinia virus, herpes simplex virus 1 and 2).

Combination of mevastatin or simvastatin with selective HCV inhibitors or IFN-α

Drug resistant variants develop readily against most selective inhibitors of HCV replication. Combination therapy of drugs with different modes of action will most likely be necessary to delay or prevent the development of viral escape mutants. Therefore the combined antiviral effects of mevastatin with either IFN-α or selective HCV polymerase inhibitors (R1479, GSK-4 benzothiadiazine and HCV-796) or a protease inhibitor (VX-950) were studied in checkerboard format. Combinations were analyzed by the method of Prichard and Shipman (Figure 2) (28). Overall the combined activity of mevastatin with IFN-α or with selective HCV polymerase and protease inhibitors was additive. Combinations of simvastatin with the various HCV inhibitors resulted also in an additive antiviral effect (data not shown).

HCV replicon clearance and rebound

The effect of statin-containing combinations was studied in clearance-rebound experiments. Huh 9-13 replicon containing cells were cultured for 6 consecutive passages in the presence of mevastatin or simvastatin, alone or in combination with IFN-α, BILN-2061, 2'-*C*-methylcytidine or HCV-796 (in the absence of neomycin pressure). IFN-α, BILN-2061 2'-*C*-methylcytidine and HCV-796, at concentrations of 10- or 20-fold their EC_{50} values, resulted already after one passage in a pronounced decrease (~2 $\log_{10}$) in HCV RNA content (Figure 3A-B). For simvastatin and mevastatin, at a concentration of 5-fold their EC_{50} value, 3 passages of drug pressure were needed before the HCV RNA content of the cells dropped below 3% (simvastatin) or 1% (mevastatin) of the untreated control. To study whether the mono- and combination therapies were able to clear the cells from their replicon following 6 passages of antiviral pressure, the antiviral compounds were omitted from the culture medium and cells were cultured under the selective pressure of G418 for another 3 consecutive passages. In such case that antiviral therapy is able to clear the replicon from the cultures, cells will not be able to proliferate when cultured in the presence of G418. Cells that still carry the replicon will be able to survive under these conditions. Although monotherapy with either statins or IFN-α, BILN-2061, 2'-*C*-methylcytidine, HCV-796 was able to reduce replicon levels by 2.5 to 5 $\log_{10}$ after passage 6, none of the cultures were cleared from their replicon under these conditions. However, cultures

that contained statins in combination with IFN- α or another STAT-C inhibitor were completely

cleared from their replicon. It was next studied at which passage these combination resulted in

complete clearance. The combination of 2'-*C*-methylcytidine and simvastatin, the combination of

IFN- α and simvastatin and the combination of BILN-2061 and mevastatin cleared cells from their

replicon after 4 passages of antiviral pressure; the combination of the benzofuran HCV-796 and

simvastatin was able to clear cells after only 2 passages of antiviral pressure (Figure 3C).

Susceptibility of various drug resistant replicons to mevastatin

It was studied whether statins retain their antiviral activity against HCV replicons that are resistant

to several selective HCV inhibitors. To this end BILN-2061[res], 2'-*C*-methylcytidine[res] and HCV-

796[res] replicon containing cells were used. Mevastatin proved equipotent against replicons

resistant to the NS5B polymerase (2'-*C*-methylcytidine, HCV-796) and NS3 protease (BILN-2061)

inhibitors as against wild-type replicons (Table 2).

Combined resistance selection

It was next studied whether statins may delay or prevent the emergence of escape variants

against the non-nucleoside polymerase inhibitor HCV-796. Huh 9-13 replicon containing cells

were cultured in the presence of G418 and mevastatin (1, 5 or 10 µM), HCV-796 (20, 100 or 200

nM), or combinations of mevastatin and HCV-796. Following three weeks of culturing, cells were

fixed and stained with methylene blue (Figure 4). Mevastatin, at concentrations of 1, 5 or 10 µM,

did not reduce replicon replication to sufficiently low levels to render the culture sensitive to G418.

At a concentration of 20 µM mevastatin (that is near to the cytostatic concentration of 34 µM),

replicon containing cells did not survive in the presence of G418 (data not shown). Replicon

containing cells that were cultured in the presence of 100 or 200 nM HCV-796 (25- and 50-fold

the EC_{50}) went through a "crisis" after which colonies developed. The HCV-796 resistant

phenotype of the colonies that developed in the presence of HCV-796 was confirmed (Table 3).

Genotyping of these replicons revealed that mutation C445F was responsible for this drug

resistant phenotype (data not shown). In contrast, mevastatin escape mutants did not develop.

Replicon from several conditions [1 μM mevastatin, 200 nM HCV-796, 1 μM mevastatin + 200 nM HCV-796, 5 μM mevastatin + 20 nM HCV-796] proved equally sensitive to mevastatin as wild-type replicon. On the other hand, replicon containing cells that were cultured in the presence of 200 nM HCV-796 and 1 μM mevastatin were found to be resistant to HCV-796. Mutation M414I was responsible for this drug resistant phenotype (data not shown) (30).

The average number of colonies formed for each condition in two independent experiments is presented in Table 4. When HCV-796 was combined with mevastatin, the frequency of colony formation was markedly reduced. For example, at a HCV-796 concentration of 100 nM and a mevastatin concentration of 5 μM, on average only 2 colonies (0.003% of control) developed. Mevastatin is thus able to delay (at concentrations of 1 μM and 5 μM) or prevent (at a concentration of 10 μM) the emergence of HCV-796 resistant replicon.

DISCUSSION

We report on the combined anti-HCV activity of mevastatin and simvastatin with IFN-α or with

HCV NS5B polymerase or NS3 protease inhibitors. Mevastatin and simvastatin were selected for

this combination experiments since these statins exhibited the strongest antiviral activity when

used alone. The combined effect of mevastatin or simvastatin with IFN-α or with selective HCV

inhibitors was first studied in regular 3-day antiviral assays. All combinations resulted in an overall

additive antiviral activity. An additive activity is to be expected with compounds that do (likely) not

interfere with each others' metabolism or mechanism of action. Others reported recently that *in*

vitro combinations of IFN-α with simvastatin, fluvastatin or pitavastatin resulted in a synergistic

activity (16;17;29). It must be emphasized however that in these studies combinations were

analyzed using the FIC method and that minimal FIC values of ~0.5 were observed which

suggests a subsynergistic rather than a synergistic activity. Pronounced synergistic activities

should result in lower FIC values. For example, we reported earlier that mycophenolic acid, the

active component of the immunosuppressive drug mycophenolate mofetil, markedly potentiate

the anti-herpes virus activities of acyclovir and other guanine-based nucleoside analogues and

we explained the biochemical mechanism responsible for this potentiating effect. In this case, FIC

values of ~0.1 were calculated (31).

A short-term antiviral assay may not necessarily predict the antiviral effect of either single

compounds or combinations thereof during long-term treatment. We therefore studied the anti-

HCV activity of statins alone or in combination with either IFN-α, a HCV NS3 protease or a HCV

polymerase inhibitor in so-called "clearance-rebound" experiments. Replicon containing cells

were cultured for 6 consecutive passages in the presence of mevastatin alone or in combination

with IFN- α or a selective HCV inhibitor and in the absence of neomycin selection pressure

(clearance phase). Following every 2 passages (passage 2, 4, 6) a rebound condition was

included. Despite the fact that no synergistic anti-HCV activity was observed in regular short-term

antiviral combination assays, all statin-containing combinations were able to clear cells from their

replicon. The combination of simvastatin with the benzofuran polymerase inhibitor HCV-796 did

so after only 2 passages of combined drug pressure. "Clearance-rebound" experiments may possibly have predictive value for estimating the potential of drugs (or combinations thereof) to clear liver cells from replicating virus and may better mimic the real-life situation than a 3-day antiviral combination assay. Our data also indicate that regular short-term antiviral combination assays (in replicon based systems) may not necessarily predict "synergistic" antiviral effects that develop following long-term culture. It may be important to further assess the potential value of such combination strategies in animal models. The human liver-uPA-SCID mouse may be well suited for this purpose (32).

Our data also demonstrate that statins may delay or prevent the development of drug resistant variants. These findings can be important, because it is known that the fidelity of the viral replication machinery of HCV is low, therefore enabling the virus to quickly develop resistance mutations for compounds targeting viral enzymes (4). Antiviral compounds that target cellular factors generally select less readily for drug resistant variants than those inhibiting viral proteins since cellular factors are independent of the viral escape via genetic mutations caused by the RNA-dependent-RNA polymerase (33).

Since replicons lack viral structural proteins, the antiviral effect of statins must be targeted at the viral replication complex. Because statins inhibit biosynthesis of cholesterol, LDL receptors expression is upregulated at the cell membrane, leading to an increased uptake of LDL particles in hepatocytes. Since HCV infection is partially dependent on the LDL receptor, statin-treatment may theoretically increase HCV infectivity. We observed that statins proved indeed less potent in the HCV_{cc} model than in the subgenomic replicon model. Our preliminary experiments revealed however that (i) there was a tendency towards less efficient uptake of HCV pseudoparticles in hepatoma cells that had been treated with mevastatin (34 % inhibition at a concentration of 10 μM) and that (ii) statin treatment did not markedly affect binding of HCV_{cc} to hepatoma cells and that (iii) there was a tendency towards less efficient internalization of HCV_{cc} in cells that had been treated with statins (48% inhibition at a concentration of 10 μM mevastatin) [data not shown]. It is

thus rather unlikely that the reduced potency of statins in the infectious HCV_{cc} system as compared to the replicon system can be explained by an increased binding/uptake of HCV in statin-treated cells.

The potential antiviral activity of statins was studied in patients chronically infected with HCV. Neither atorvastatin (after conventional 12-week therapy) (34) nor rosuvastatin (35) resulted in a reduction in viral load. The most plausible explanation for the discrepancy between the *in vitro* activity and the lack of clinical efficacy is, as was suggested by the authors, is that the plasma concentrations were likely below the levels employed in cell culture (34). In a recently published study, fluvastatin was found to inhibit HCV RNA replication in HCV infected patients (36). The drug was well tolerated and, at relatively low doses (20-80 mg daily), resulted in a transient reduction in viral load (-0.5 to -1.75 $\log_{10}$, 2-5 weeks); higher doses of fluvastatin did not reduce viral load. The authors hypothesize that fluvastatin acts by binding to lipo-viro-particles (LVP) and that the result of this attachment would interfere with the ability of the virus to modulate the immune system of the host (36). There is today obviously no compelling evidence that statins, used in monotherapy, may result in a marked reduction in HCV load in chronically infected patients. Also ribavirin exhibits a very limited antiviral activity when used in monotherapy. The combination of ribavirin and (pegylated) IFN-α results however in a more than additive effect in HCV infected patients. It has been suggested that a disturbance of the kinetics of viral replication brought about by IFN-α is required to create a condition where a weak antiviral drug, such as ribavirin, can exert substantial antiviral activity (37). Possibly, the combination of a statin with the current standard therapy or with one or more potent STAT-C inhibitor(s) may result in a more than additive effect, as suggested by the clearance-rebound experiments reported in the present study. The potential use of statins in HCV infected patients may thus lie in combination therapy. Although plasma levels of statins are usually rather low [C_{max} (ng/ml) based on a 40 mg oral dose; lovastatin: 10-20 ng/ml, pravastatin: 45-55 ng/ml, simvastatin: 10-34 ng/ml, fluvastatin: 448 ng/ml] (38), the replication of HCV occurs predominantly in the liver. Concentrations of statins in the liver may therefore be more relevant than plasma concentrations. Although the thesis that statin

concentrations are likely much higher in the human liver than in plasma, is widely accepted, there

are, to the best of our knowledge, no studies published in which concentrations of statins in the

human liver have been determined. In rats, the liver concentration of lovastatin is 15 to 18-fold

higher than the concentration in blood and other tissues (39-41). If one would assume that liver

concentrations of statins in the human liver may also be ≈ 15-fold higher than in plasma, a very

rough estimation would suggest concentrations of 0.75-15 µM in the human liver (depending on

the pharmacokinetic profile of the specific statin). Statin doses as high as 2 to 8-fold the currently

used standard doses can be safely used to treat hypercholesterolemia (42). Hence, higher dosing

may be expected to result in liver concentrations that are sufficiently high to inhibit HCV

replication, in particular when combined with STAT-C inhibitors. Since statins target cellular

factors, the genetic barrier to resistance is expected to be (relatively) high (which is corroborated

by our unpublished findings). Furthermore, since we here demonstrate that mevastatin delays or

even prevents the development of HCV-796 escape mutants, the combination of STAT-C

inhibitors with statins may also have the potential to delay or even to prevent resistance

development in the clinical setting. In summary, the combination of statins with several selective

HCV inhibitors result in a pronounced antiviral effect in cell culture and prevents or delays the

emergence of drug variants resistant to the STAT-C inhibitor. Statins may have the potential to (i)

increase the efficacy of current or future HCV therapy and (ii) delay the development of

resistance against STAT-C inhibitors.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. **Anti-HCV effect of statins in Huh 5-2 cells.** Effect of lovastatin, mevastatin, simvastatin, fluvastatin and pravastatin on HCV replicon replication in Huh 5-2 cells (measured as luciferase signal; bars) and on the proliferation of exponentially growing cells (diamonds). Data are expressed as percentage of untreated controls (UTC) and are mean values $\pm$ SD for at least three independent experiments.
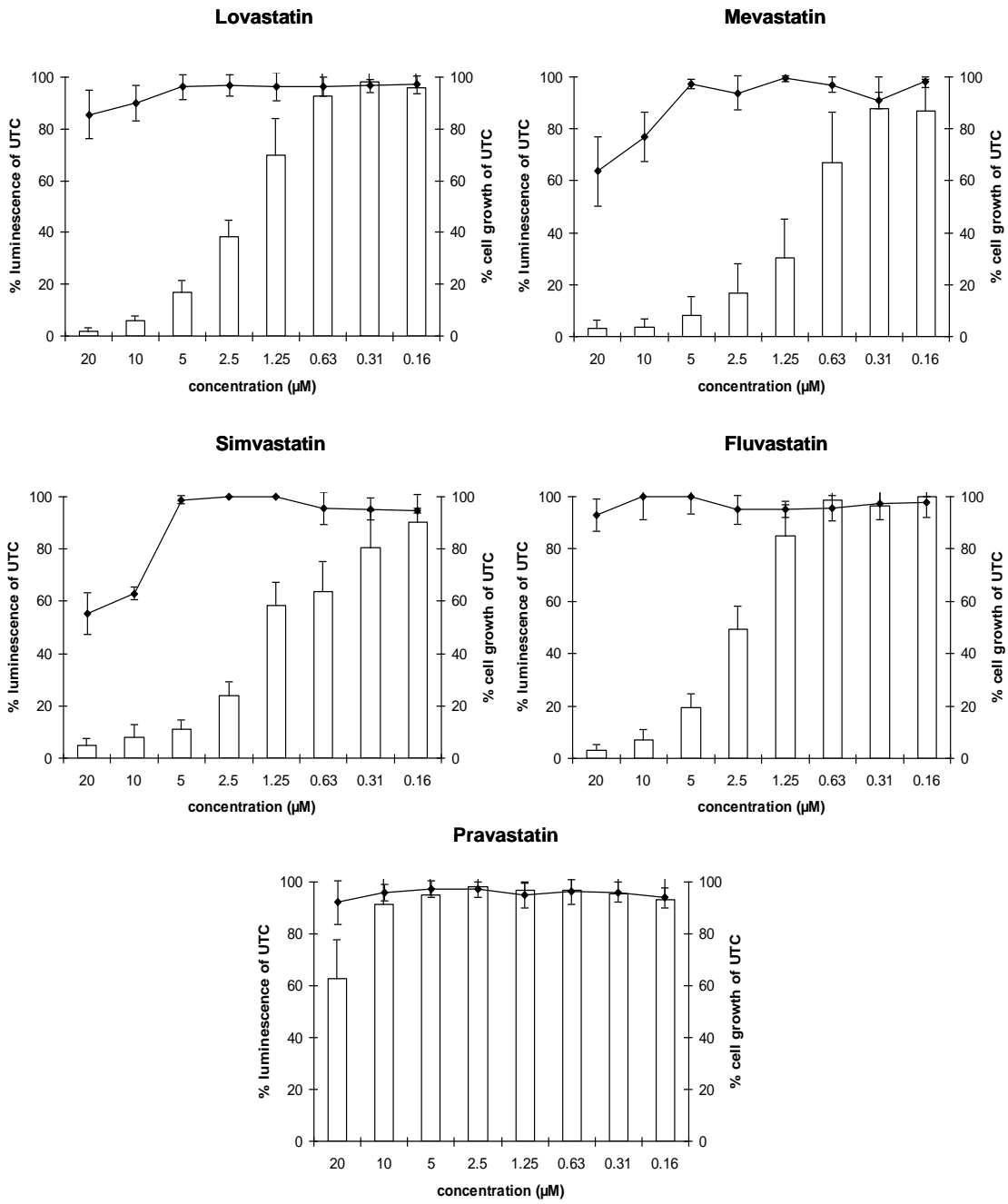
Figure 2. Antiviral effect of the combination of IFN-α or selective HCV inhibitors with statins in short-term (3 days) antiviral assays. IFN-α (A), VX-950 (B), HCV-796 (C), GSK-4 benzothiadiazine (D) and R1479 (E) (X-axis) and mevastatin (Y-axis). Data were analysed using the method of Prichard and Shipman. The different colors represent different ranges of values: turquoise: -40% to -20%, burgundy: -20% to 0%, red: 0% to 20%, blue: 20% to 40%. The 0 plane on the Z-axis represents an additive effect. A surface that is higher than 20% above the zero plane indicates a synergistic effect of the combination, a surface lower than 20% below the zero plane indicates antagonism. Data are mean values for three independent experiments.

Figure 3. Effect of statin-containing combinations on the clearance of replicons from Huh 9-13 cells. (A) Clearance phase. Cells were treated for 6 consecutive passages with mevastatin (8 μM) /simvastatin (10 μM) [blue], BILN-2061 (0.3 μM), IFN-α (50 IU/ml), 2'-C-methylcytidine (4 μM), HCV-796 (0.1 μM) [green] or the combination of one of these selective HCV inhibitors with either mevastatin or simvastatin [black] in the absence of G418 selective pressure. **(B) Percentage inhibition for each condition expressed in $\log_{10}$ values. (C) Rebound phase.** During the rebound passages, antiviral compounds were omitted from the culture medium but cells were again cultured under selective pressure of G418 (1000 μg/ml). Three rebound passages of passage 2, 4 and 6 are depicted. Data are expressed as percentage of untreated controls (UTC).
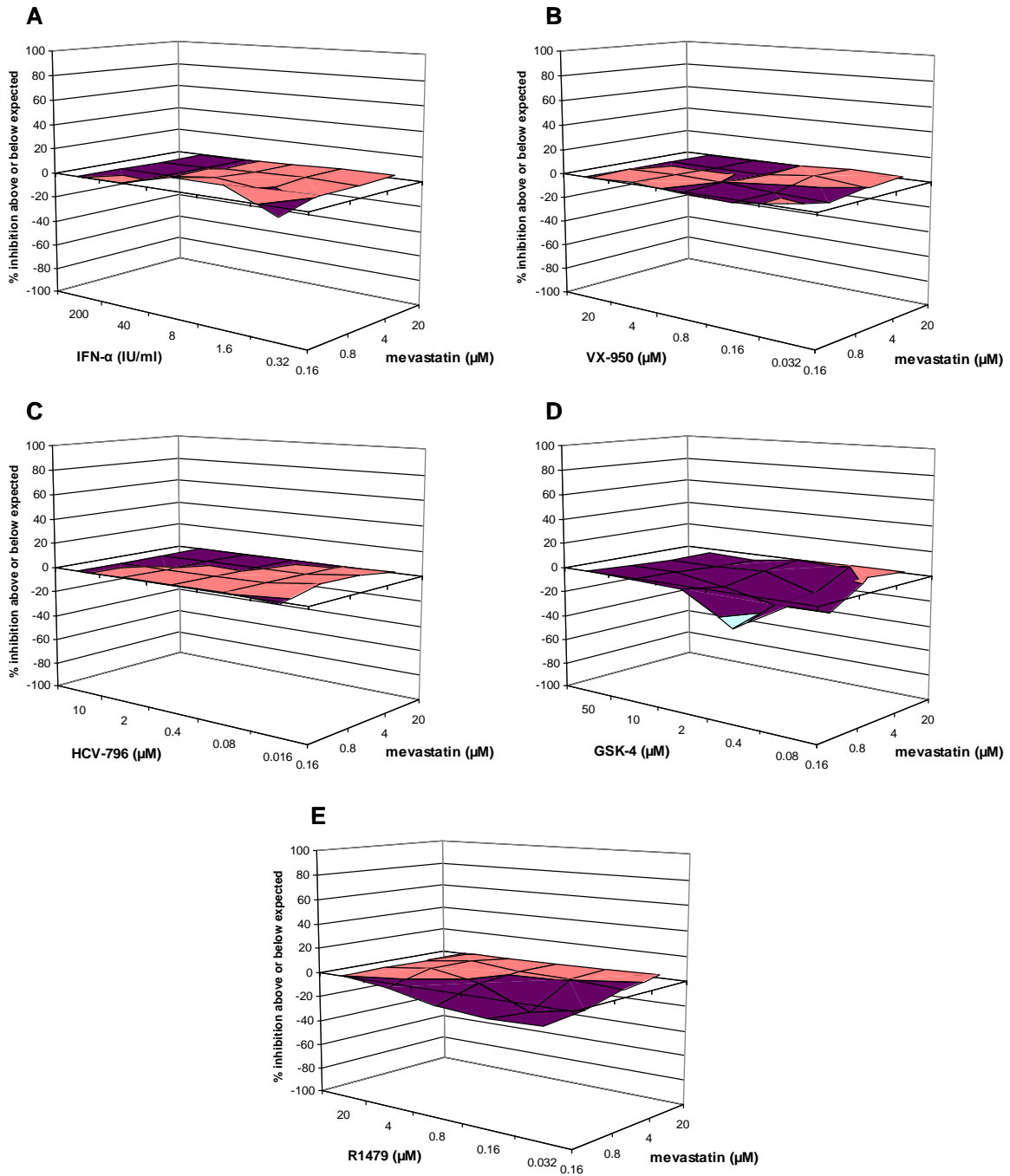
Figure 4. Effect of mevastatin on the selection of HCV-796 resistant mutants. Huh 9-13 cells were cultured in the presence of G418 and of various concentrations of mevastatin and/or HCV-796 for 3 weeks. The result of a representative colony formation assay experiment is depicted. The passage number at the moment of fixation is given in the corner of each well. Comparable data were obtained in an independent experiment. Mean data from both experiments are presented in Table 3 and 4.

Figure 1. Anti-HCV effect of statins in Huh 5-2 cells.

Figure 2. Antiviral effect of the combination of IFN-α or selective HCV inhibitors with statins in short-term (3 days) antiviral assays.

Figure 3. Effect of statin-containing combinations on the clearance of replicons from Huh

9-13 cells.

A Mevastatin + BILN-2061

B

	Log$_{10}$ inhibition		
P1	2.0	0.3	1.6
P2	3.1	3.0	2.6
P3	3.4	2.6	4.0
P4	3.5	3.5	4.1
P5	3.6	3.5	5.3
P6	3.6	3.7	5.5
	BILN-2061		
	Mevastatin		
	Mevastatin + BILN-2061		

C

Simvastatin + IFN-α

	Log$_{10}$ inhibition		
P1	1.6	0.3	2.3
P2	3.1	1.1	4.2
P3	3.6	1.8	5.4
P4	3.7	1.6	5.0
P5	3.9	1.5	5.2
P6	4.1	2.5	5.4
	IFN-α		
	Simvastatin		
	Simvastatin + IFN-α		

Simvastatin + 2'-*C*-methylcytidine

	Log$_{10}$ inhibition		
P1	2.2	0.3	2.4
P2	5.1	1.1	4.4
P3	5.9	1.8	5.7
P4	5.4	1.6	5.0
P5	5.0	1.5	5.2
P6	5.0	2.5	6.3
	2'-*C*-methylcytidine		
	Simvastatin		
	Simvastatin + 2'-*C*-methylcytidine		

Simvastatin + HCV-796

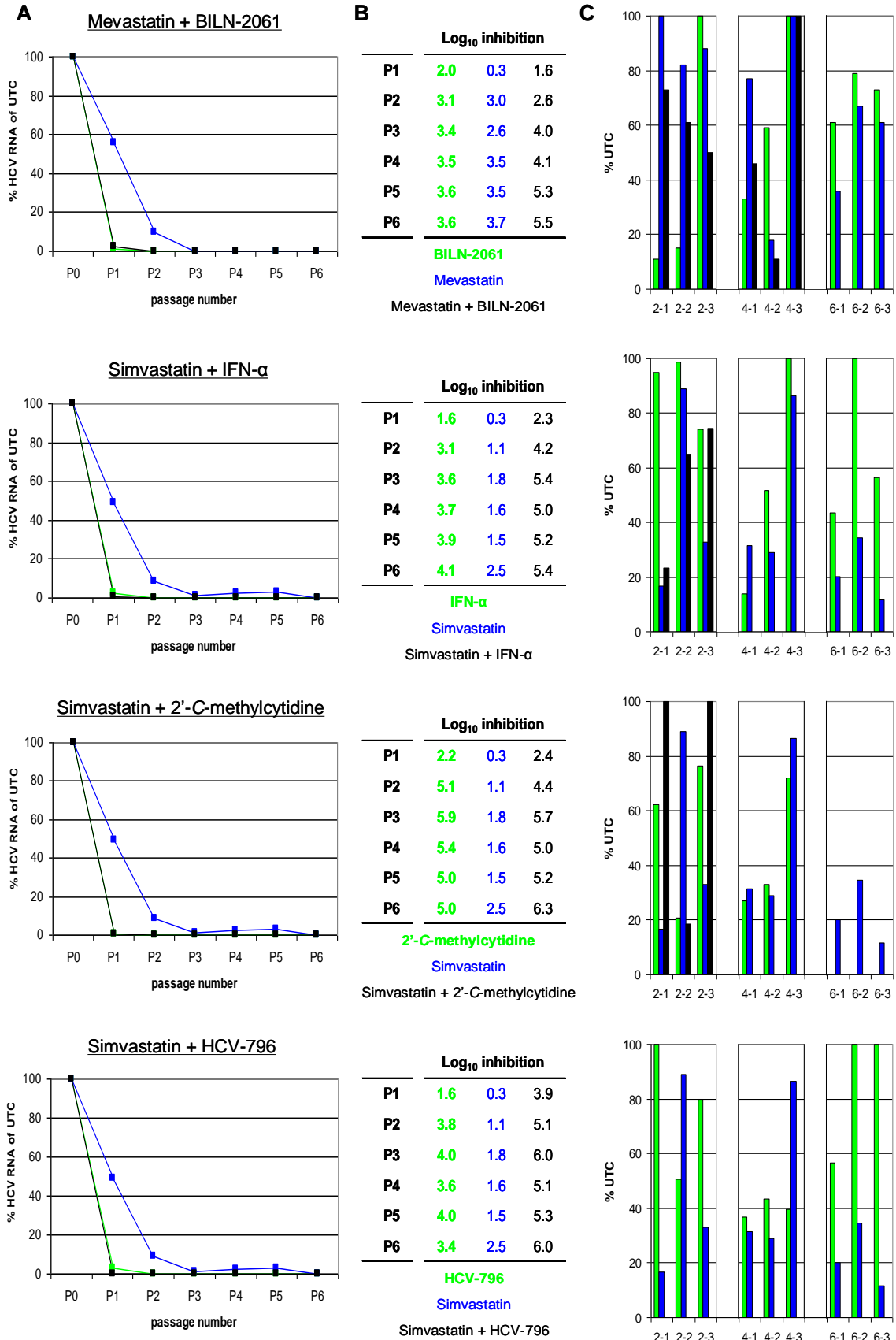|	Log$_{10}$ inhibition		
P1	1.6	0.3	3.9
P2	3.8	1.1	5.1
P3	4.0	1.8	6.0
P4	3.6	1.6	5.1
P5	4.0	1.5	5.3
P6	3.4	2.5	6.0
	HCV-796		
	Simvastatin		
	Simvastatin + HCV-796		

Figure 4. Effect of mevastatin on the selection of HCV-796 resistant mutants.

Table 1. *In vitro* anti-HCV activity of various statins.
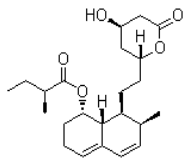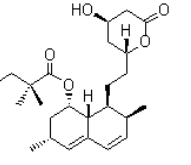
			Huh 5-2	Huh 9-13	HCV_{cc}
Lovastatin		EC_{50}	1.9 ± 0.3	7.4 ± 1.3	> 30
		EC_{90}	7.4 ± 1.3	17 ± 0.3	--
		CC_{50}	60 ± 12	> 20	88 ± 3.6
Mevastatin		EC_{50}	1.3 ± 1.0	2.2 ± 1.1	23 ± 4.1
		EC_{90}	4.7 ± 1.8	5.8 ± 2.8	--
		CC_{50}	34 ± 3.6	30 ± 4.7	110 ± 2.3
Simvastatin		EC_{50}	1.5 ± 0.6	2.9 ± 0.3	19 ± 1.5
		EC_{90}	6.7 ± 3.9	8.5 ± 0.2	--
		CC_{50}	32 ± 5.9	19 ± 4.4	> 120
Fluvastatin		EC_{50}	1.8 ± 1.5	4.2 ± 1.5	24 ± 4.1
		EC_{90}	14 ± 2.4	17 ± 1.6	--
		CC_{50}	44 ± 4.8	> 20	> 120
Pravastatin		EC_{50}	> 20	> 20	--
		EC_{90}	> 20	> 20	--
		CC_{50}	> 100	> 20	--

Table 2. Replicons resistant to polymerase or protease inhibitors remain susceptible to mevastatin.

Cell type/compound	EC_{50} (µM) (fold change[b])			
	BILN-2061	2'-*C*-methylcytidine	HCV-796	Mevastatin
WT	0.0094 ± 0.007 (1.0)	0.43 ± 0.1 (1.0)	0.0034 ± 0.001 (1.0)	1.7 ± 0.2 (1.0)
BILN-2061[R]	**1.3 ± 0.5 (138)**	0.29 ± 0.2 (0.7)	0.0048 ± 0.0002 (1.4)	3.2 ± 0.8 (1.9)
2'-*C*-methylcytidine[R]	0.037 ± 0.03 (3.9)	**18 ± 2.9 (42)**	0.0057 ± 0.0003 (1.7)	1.6 ± 0.6 (0.9)
HCV-796[R]	0.0055 ± 0.002 (0.6)	0.47 ± 0.3 (1.1)	**63 ± 12 (18.529)**	2.6 ± 0.7 (1.5)

a. Data are mean values ± SD for at least three independent experiments.

b. Fold change in EC_{50} is when compared to the EC_{50} for wild-type Huh 9-13 replicon

Table 3. Effect of mevastatin on the selection of HCV-796 resistant mutants.

A   Fold changes in sensitivity to HCV-796

fold change of EC_{50}	CC	20 nM HCV-796	100 nM HCV-796	200 nM HCV-796
CC	1	6,5	17	59 ± 23
1 µM mevastatin	--	--	--	38 ± 13
5 µM mevastatin	--	7,3	nc	nc
10 µM mevastatin	--	nc	nc	nc

B   Fold changes in sensitivity to mevastatin

fold change of EC_{50}	CC	20 nM HCV-796	100 nM HCV-796	200 nM HCV-796
CC	1	--	--	1,8
1 µM mevastatin	1,2	--	--	1,3
5 µM mevastatin	--	1,1	nc	nc
10 µM mevastatin	--	nc	nc	nc

Fold change in EC_{50} is calculated by comparison with EC_{50} for wild-type Huh 9-13 replicon.

nc, no colonies; --, not determined.

Table 4. Frequency of resistant-colony formation following combination selection.

Selection	Mean number of colonies ± SD[a]	Frequency (%)[b]
mevastatin (1 µM)	monolayer	100
mevastatin (5 µM)	monolayer	100
mevastatin (10 µM)	monolayer	60
HCV-796 (20 nM)	monolayer	100
HCV-796 (100 nM)	128 ± 5	0,179
HCV-796 (200 nM)	30 ± 17	0,042
HCV-796 (20 nM) + mevastatin (1 µM)	monolayer	100
HCV-796 (20 nM) + mevastatin (5 µM)	146 ± 6	0,204
HCV-796 (20 nM) + mevastatin (10 µM)	3 ± 1	0,004
HCV-796 (100 nM) + mevastatin (1 µM)	85 ± 1	0,119
HCV-796 (100 nM) + mevastatin (5 µM)	2 ± 1	0,003
HCV-796 (100 nM) + mevastatin (10 µM)	0	0
HCV-796 (200 nM) + mevastatin (1 µM)	5 ± 1	0,007
HCV-796 (200 nM) + mevastatin (5 µM)	1 ± 1	0,001
HCV-796 (200 nM) + mevastatin (10 µM)	0	0

a. Following selection for three consecutive weeks, remaining colonies were stained with

methylene blue and counted. Data shown are mean values ± SD of two independent experiments.

b. The frequency was determined as number of colonies/initial number of cells x 100.