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Open-label, ascending dose, prospective cohort study evaluating the antiviral efficacy of Rosuvastatin therapy in serum and lipid fractions in patients with chronic hepatitis C

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SUMMARY

HMG CoA reductase inhibition suppresses *in vitro* HCV replication through depletion of cellular sterol proteins such as geranylgeraniol. Our aims were to prospectively evaluate the changes in serum and lipid fraction HCV RNA with Rosuvastatin in non-responder (NR) patients with CHC. A total of 11 patients with CHC genotype-1 received Rosuvastatin at 20 mg qd (weeks 0–4), 40 mg qd (weeks 5–12), with 4 week follow up. Lipid fractions were separated by a sucrose density gradient ultracentrifugation, HCV RNA determined at wks 0, 2, 4, 8, 12, 16 in serum, and in selected very low- (VLDF) to high-density (HDF) lipid fractions. A reduction in LDL and total cholesterol (TC) was not accompanied by significant decline in HCV RNA. At baseline, there was an inverse correlation between HDL and HCV RNA ($r = -0.45$, $P = 0.036$). At 20 mg, there was correlation between change (Δ) in TG and HCV RNA ($r = 0.75$, $P = 0.007$), ALT and TC ($r = -0.64$, $P = 0.03$) and LDL ($r = -0.67$, $P = 0.02$). At 40 mg, TG maintained a positive correlation with HCV RNA ($r = 0.65$, $P = 0.03$). There was a group difference for HCV RNA in relation to lipid fractions ($P = 0.04$) but not study time intervals ($P = 0.17$); mean log HCV RNA was greater in VLDF compared to HDF (5.81 ± 0.59 vs 5.06 ± 0.67 , $P = 0.0002$) with no other differences to study time intervals ($P = 0.099$). Short-term Rosuvastatin monotherapy is not associated with significant changes in serum or lipid fraction HCV RNA in NR patients. HCV co-localizes with the lowest density lipid fractions in serum.

Keywords

cholesterol; HMG CoA reductase; lipoprotein; lipoviroparticle; statin

INTRODUCTION

At present, there are essentially no approved alternative treatment options for patients that are intolerant or fail to respond to interferon-based therapy [1]. A number of interesting *in vitro* and clinical observations have indicated a potential role for host lipid metabolism in HCV replication. The low-density lipoprotein receptor (LDLR) has been proposed as a cell entry co-receptor [2,3]. LDLR expression correlates with HCV RNA levels[4], and single nucleotide alterations in the LDLR may influence responses to antiviral therapy or development of chronic infection [5]. Prior studies have noted genotype-specific differences in baseline cholesterol levels that are predictive of virologic response [6–9].

Cholesterol and fatty acid synthesis pathways also appear to influence HCV replication *in vitro* [10]. HCV viral replication is dependent upon cellular membrane constituents such as the mevalonate-derived non-sterol isoprenoids, farnesyl and geranylgeranyl pyrophosphate for assembly and release of the HCV polyprotein. *In vitro* depletion of mevalonate may be achieved through 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibition. A pivotal study noted that high-dose lovastatin was able to inhibit HCV replication in Huh7 cell lines, and that this effect was mediated through inhibition of geranylgeranylation of host cellular proteins [11]. Recent *in vitro* studies have demonstrated that other statin compounds, such as fluvastatin, also have strong inhibitory effects in an HCV genotype 1b replicon assay, with further synergistic inhibition when given in combination with pegylated IFN [12] or emerging small molecule inhibitors[13]. However, a pilot study in 10 patients with CHC treated with a relatively low-dose atorvastatin 20 mg daily as monotherapy for 12 weeks indicated no effect on HCV RNA levels [14]. In contrast, another study noted a transient and modest viral decline in one-half of patients with CHC treated with fluvastatin 80 mg daily [15]. Prior studies have not evaluated differences in HCV and lipoprotein co-localization before and during statin therapy. Although direct HCV inhibitory effect may not be achievable with statins *in vivo*, favourable changes in the host lipoprotein profile could still be important, as the HCV replication complex is dependent on VLDL assembly and secretion [16], and circulates in serum bound to Apolipoprotein B and E containing low-density lipoviro particles [17]. Therefore, potential changes in HCV RNA in lipid fractions may have been overlooked in prior clinical studies evaluating the antiviral efficacy of statin therapy. Thus, our aims in this study were to prospectively evaluate the changes in serum HCV RNA levels within lipid fractions in patients with CHC, receiving the potent lipid-lowering agent Rosuvastatin for short-term HMG CoA reductase inhibition.

METHODS

Patient selection

This was a prospective, open-label, ascending dose cohort study in adult patients infected with CHC genotype 1 that were either non-responders or intolerant to current standard-of-care IFN-based therapy. The study was conducted at a single centre, with patient enrolment between February 2005 and June 2006. Patients with CHC that were naïve to prior lipid-lowering therapy were required to have compensated disease with detectable HCV RNA on at least two occasions during a 28-day screening period. Exclusion criteria included (i) Patients with CHC with other co-morbid states such as autoimmune disease, alcoholic liver disease, HBV or HIVco-infection, renal impairment (serum creatinine $>1.5 \times \text{ULN}$), hepatic decompensation; (ii) biochemical abnormalities such as elevated creatine kinase (CK), Alanine aminotransferase (ALT) $> 3 \times \text{ULN}$, low total serum cholesterol ($<80 \text{ mg/dL}$); (iii) evidence for primary (familial hypercholesterolaemia, combined hyperlipidaemia) or secondary dyslipidaemias (including hypothyroidism, diabetes mellitus, obesity, renal impairment, nephrotic syndrome, or drugs such as thiazide diuretics, retinoids and

corticosteroids) and (iv) known hypersensitivity and/or myopathy (CK > 10 × ULN with muscle symptoms) to previous lipid-lowering therapy.

The study was conducted in accordance with the ethics principles of the Declaration of Helsinki and was consistent with Good Clinical Practice Guidelines. Each patient provided written informed consent, and the study was approved by the Duke University Institutional Review Board.

Study procedures

Study eligible patients had a 28-day screening period with laboratory and clinical evaluation. Rosuvastatin 20 mg daily was provided for 4 weeks followed by 40 mg for a further 8 weeks, and a posttreatment follow-up of 4 weeks. Standard fasting laboratory and clinical assessments were performed at weeks 0, 2, 4, 8, 12 and 16. Blood samples were stored at -70°C for HCV RNA quantification in serum and in lipid fractions by the HCV Superquant assay (NGI Labcorp, Los Angeles, CA, USA). Serum samples from weeks 0, 2, 4, 8, 12 and 16 were used for sucrose density gradient lipid fractionation. Briefly, a 9-mL sucrose gradient prepared in a Sorvall PA Ultracrimp tube (Thermo Scientific, Waltham, MA, USA) using equal volumes of 10–60% sucrose solutions in 10% increments layering the gradient from highest to lowest density; 800 μL of serum was carefully applied to the gradient, and after crimping, the sample tubes were centrifuged (TH-641 swing bucket rotor, Sorvall RC-6 ultracentrifuge, 25 000 rpm, 4°C) for 16 h. To collect individual fractions, the top of the tube was removed and 650- μL fractions were manually extracted from top to bottom for a total of 15 fractions per serum sample. After a pilot set of study samples from one patient was analysed for HCV RNA in all 15 fractions, samples were sent for HCV RNA quantitation in specific lipid fractions 1 (very low density), 3, 5 (low), 7 (intermediate) and 15 (highest density) from the remaining 10 patients. These particular fractions were selected for HCV RNA quantification as they had shown the greatest change in HCV RNA between study timepoints in the pilot sample.

Statistical analysis

The primary outcome parameter was a decline of $-1 \log_{10}$ HCV RNA (copies/mL) during Rosuvastatin therapy. Secondary measures included a change in liver aminotransferases (AST and ALT) and changes in lipid fraction HCV RNA levels. Baseline clinical characteristics are descriptively summarized for categorical data and as median (percentile) or mean (standard deviation) for non-parametric and parametric data, respectively. Only patients that completed all study assessments were included in the final analysis. At $\alpha = 0.05$, we estimated that nine patients would be required to detect a $1 \log_{10}$ HCV RNA decline at a power of at least 90%. Spearman's Rho pairwise correlation was used for dose–response assessments and Fisher's exact test for group differences. A mixed linear model permitting data to exhibit correlation and non-constant variability was used to determine difference in least square mean total cholesterol, LDL, HDL and serum triglycerides between baseline and higher doses. Generalized Estimating Equations (GEE) methodology was used to explore the effect of time and Rosuvastatin dose and lipid fraction on co-localization of HCV RNA in the fractions over time [18]. This dataset is a longitudinal dataset, in which subjects are measured for their HCV RNA levels at different points in time that correspond to no treatment (week 0), 20 mg Rosuvastatin (at weeks 2 and 4), 40 mg Rosuvastatin (at weeks 8 and 12) and a follow-up visit at week 16 within varying levels of lipid fractions that are fixed for all 11 subjects at fractions 1, 3, 5, 7 and 15. The 5-level lipid fraction was entered as a categorical covariate after adjusting for best-fitting intra-subject correlation structure, and using time as a continuous variable in the model. Significance was assessed at the 0.05 level, and all analyses were performed by SAS version 8.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Patient characteristics

Eleven patients completed study procedures with 12 weeks of Rosuvastatin therapy. Another three patients commenced therapy, but were not included in final analysis in this small study, as they either discontinued the drug during study because of nausea at week 1 ($n = 1$), and ALT $> 5 \times$ ULN at week 4 ($n = 1$), or were lost to follow-up at week 12 ($n = 1$). Study patients were typical of our tertiary centre CHC non-responder referral cohort and were mostly middle-aged men with HCV genotype-1 infection and ALT elevated $1-2 \times$ ULN (Table 1).

Serum lipid changes during therapy

There was a significant decline in mean serum total cholesterol from baseline for 20 mg (174.81 ± 26.89 vs 122.0 ± 18.89 mg/dL; $P < 0.0001$) and 40 mg Rosuvastatin (174.81 ± 26.89 vs 114.25 ± 22.40 mg/dL; $P < 0.0001$), with a return to baseline at 4-weeks post-treatment follow-up. Likewise, there was a decline in LDL from baseline during both the 20 mg (95.73 ± 26.65 vs 53.28 ± 16.76 mg/dL; $P < 0.0001$) and 40 mg dose intervals (95.73 ± 26.65 vs 45.21 ± 19.15 ; $P < 0.0001$). There was a slight decline in HDL for 20 mg dose interval (53.36 ± 12.07 vs 49.76 ± 13.09 ; $P = 0.03$), but no further significant changes in HDL levels were noted for the 40 mg or follow-up time points. During therapy, there was a significant decline from baseline in serum triglyceride levels for 40 mg Rosuvastatin (128.41 ± 65.91 vs 90.02 ± 43.90 mg/dL, $P = 0.006$), but not for the 20 mg dose. There were no significant differences in lipid levels between the 20 and 40 mg dosing intervals (Table 2).

Serum lipids and HCV RNA

Only one patient achieved a single time point decline in HCV RNA from baseline of >1 log at week 12 of therapy (8.33 vs 6.48 HCV RNA $\log_{10}$ copies/mL), with a return to baseline at 4 weeks posttreatment follow-up (8.11 HCV RNA $\log_{10}$ copies/mL) (Fig. 1). Interestingly, in this patient, there was an early significant decline in total cholesterol (223 vs 97 mg/dL) and LDL (140 vs 41 mg/dL), but not HDL (40 vs 37 mg/dL) by week 4 of therapy. However, there was no significant change from baseline in ALT at week 12 (68 vs 73 iu/mL) that accompanied the decline in HCV RNA.

At baseline, there was a trend towards an inverse association between HDL and HCV RNA (Spearman's $\rho = -0.45$, $P = 0.036$), but no further associations were observed for HDL during therapy or at follow-up. At the 20 mg dosing interval, there was a positive correlation between change from baseline () in TG and HCV RNA ($\rho = 0.75$, $P = 0.07$), and a negative correlation between ALT and total cholesterol ($\rho = -0.64$, $P = 0.03$) and LDL ($\rho = -0.67$, $P = 0.02$). This positive correlation between TG and HCV RNA was maintained during the 40 mg dosing interval ($\rho = 0.65$, $P = 0.03$), but there was no association between ALT and cholesterol or LDL at this dose. No significant associations were observed at follow-up, when serum lipids had essentially reverted to baseline values.

Lipid fractions and HCV RNA

There was a group difference for HCV RNA in the five measured lipid fractions ($\chi^2 = 10.01$, DF 4, $P = 0.04$), with overall co-localization in the lowest density fraction 1 compared to highest density fraction 15 (5.81 ± 0.59 vs 5.06 ± 0.67 $\log_{10}$ copies/mL; $P = 0.0002$.) There were no significant differences in HCV RNA between other fractions (figure 2). Also, there were no significant changes in HCV RNA levels in lipid fractions over time ($P = 0.177$), or in lipid fraction co-localization ($P = 0.099$) during the study period. There were no corresponding changes in individual lipid fraction HCV RNA for the single patient that

achieved a week 12 decline of >1 log HCV RNA in serum from baseline (mean lipid fraction HCV RNA = 6.38 vs 6.22 log₁₀ copies/mL, $P = \text{NS}$).

Safety

Two patients discontinued Rosuvastatin therapy during the study period. One patient had nausea in the first week that was possibly related to study medication, and another patient developed asymptomatic elevation in his liver transaminases at week 4 (ALT = 357, AST = 250 iu/mL, ULN = 60 iu/mL) from baseline (ALT = 124, AST = 87 iu/mL) that was likely related to study drug. Following discontinuation of study drug in this patient, liver transaminases gradually returned to baseline values over several weeks. There were no other significant laboratory abnormalities or clinical events noted during the study period.

DISCUSSION

Although Rosuvastatin has not been evaluated for *in vitro* antiviral efficacy, we chose this compound based on its favourable safety profile and greater potency for lowering LDL cholesterol compared to other HMG CoA reductase inhibitors in the same class [19]. However, this ascending dose open-label study of Rosuvastatin monotherapy for 12 weeks did not show any significant effect on serum or lipid fraction HCV RNA levels in non-responder patients with CHC genotype 1 despite significant reduction in serum lipids. There were associations between serum HCV RNA and HDL at baseline, and with change in triglyceride levels during therapy. A single time point HCV RNA decline of >1 log at week 12 that was not accompanied by a biochemical response or a significant change in the corresponding lipid fraction likely reflected an aberrant result because of sample-related issues.

The absence of primary antiviral efficacy for Rosuvastatin monotherapy, compared to *in vitro* studies of statins, likely results from an inability to achieve an equivalent *in vivo* drug concentration. OR6 reporter assay cell lines demonstrated effective viral inhibition for fluvastatin at 5 $\mu\text{mol/L}$ [12]. However, the mean peak plasma concentration of Rosuvastatin following a 40 mg dose in healthy volunteers is in the 20 ng/mL range at 3–5 h [20]. This is equivalent to 0.05 $\mu\text{mol/L}$ or 100-fold less drug concentration compared to *in vitro* studies. Rosuvastatin is relatively hydrophilic compared to other statin compounds, resulting in high-affinity uptake in hepatocytes and lower 50% inhibitory concentration (IC_{50}) for blocking intrahepatic sterol synthesis [21,22]. Although the liver concentration of Rosuvastatin is likely to be several fold higher compared to plasma [23], statin doses that are tolerated in humans are unlikely to result in adequate depletion of mevalonate and cellular farnesyl and geranylgeranyl pyrophosphates. Interestingly, statin compounds such as pravastatin, that have limited interaction with the cytochrome P450 unlike other compounds in the same class, have not demonstrated *in vitro* antiviral activity despite HMG CoA reductase inhibition [12,13]. For HCV lipid fraction analysis, we used a sucrose density gradient, although iodixanol is an alternative isosmotic non-ionic density gradient medium that has been used in prior studies evaluating HCV and lipovirion interaction [24].

Our results are comparable to a previous study that noted no significant change in HCV RNA levels in eight patients with CHC with elevated total cholesterol following administration of atorvastatin 20 mg daily for 12 weeks [14]. Our study cohort had a comparable body mass index of 29, but lower baseline total cholesterol and LDL levels. However, despite achieving a greater reduction in LDL to 45 mg/dL (compared to 100 mg/dL in the atorvastatin study), there was no effect on HCV RNA levels. This decline in total cholesterol and LDL demonstrates compliance to study drug, but serum HDL levels did not change significantly during the study period, despite previous data indicating greater increases in HDL for Rosuvastatin compared to atorvastatin [25]. Another study evaluated

fluvastatin, in varying doses from 20 to 320 mg daily for up to 12 weeks, in a Veterans CHC cohort of 33 predominantly male patients with variable genotype and prior history of IFN-based treatment [15]. Using fold-decline analysis, transient HCV RNA reductions from baseline were observed in one-half of patients that received up to 80 mg fluvastatin. Although one patient achieved a single time point $1.75 \log_{10}$ HCV RNA decline at a lower dose of 20 mg fluvastatin daily, this effect did not appear to be maintained despite continued therapy.

There are theoretical concerns with statin therapy regarding enhanced infectivity of hepatocytes through up-regulation of LDLR. Although *in vitro* studies have indicated an association between LDLR and serum-derived HCV RNA entry into cell lines, there is no evidence of an association with HCVcc and increased intrahepatic HCV replication [3]. A recent study of fluvastatin 80 mg daily for 4 weeks in HIV-HCV co-infected patients noted a higher mean HCV RNA level in the fluvastatin group compared to controls [26]. Although we did not evaluate LDLR expression on mononuclear cells or hepatocytes, our study did not find an association between LDL and HCV RNA either at baseline or with change in LDL levels during statin therapy.

HDL is a natural ligand of the scavenger receptor class B type 1 (SRB1) that mediates HCV cell entry [27] and could explain the moderate inverse association between HDL and HCV RNA observed in our study at baseline. A recent study in Taiwanese patients infected with CHC genotype 1 and 2 also noted an inverse relationship between HDL and HCV RNA in non-obese individuals [28]. However, statins are also associated with up-regulation of SRB1[29], and we did not observe any significant relationship between HCV RNA and HDL during therapy.

In summary, our study confirms that the role of statins as monotherapy in patients with CHC appears limited. However, there is evidence for *in vitro* synergy between statins, IFN α and emerging HCV NS3 protease and NS5B polymerase inhibitors, along with prevention of resistant variants to these newer therapies [12,13]. Recent data indicate that statins may increase virologic response rates to pegylated IFN and ribavirin therapy in patients with CHC genotype 1b [30]. HCV and lipid interactions may also be different based on viral genotype, and modulation of lipidogenic pathways through statins could have benefits in terms of reducing steatosis. Ongoing clinical studies of statins in combination with current standard-of-care and emerging therapies in HCV infection should provide more information in due course.

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Abbreviations

ALT	Alanine aminotransferase
CHC	chronic hepatitis C
CK	creatinine kinase
GEE	Generalized Estimating Equations

HBV	hepatitis B virus
HCV	Hepatitis C virus
HDF	high-density lipid fractions
HMG	3-hydroxy-3-methyl-glutaryl
IFN	interferon
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
TC	total cholesterol
TG	triglyceride
ULN	upper limit of normal
VLDF	very low-density lipid fractions

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Fig. 1.

HCV RNA change in individual patients based on Rosuvastatin dosing schedule. There were no significant declines in HCV RNA during therapy, except for one patient (bold line) with a 1.85 log decline at 40 mg dosing that may represent a sampling issue, as there was no concurrent decline at this time point in the lipid fraction analysis.

Fig. 2.

Box plot Distribution of HCV RNA in lipid fractions. HCV RNA levels were higher in lowest density (1) compared to highest density (15) fractions, with no significant changes in co-localization of HCV RNA with statin therapy. The box plots summarize the distribution of HCV RNA for all 11 patients at four study intervals. The box extends to the 25th and 75th quantiles, with the median HCV RNA shown as horizontal line within the box. The whiskers (shown as vertical dash lines) extend from the ends of the box to the outermost data point.

Table 1

Baseline patient characteristics

Variable	Value
Age (years)	50.8 ± 4.1
Gender (M/F)	8/3
Race (%)	
Caucasian	6 (55%)
African American	5 (45%)
Genotype 1 (%)	11 (100%)
Log ₁₀ HCV RNA (copies/mL)	7.13 ± 0.61
Total cholesterol (mg/dL)	174.82 ± 26.63
LDL	95.73 ± 26.26
HDL	53.36 ± 12.23
Triglycerides (mg/dL)	128.41 ± 64.11
ALT (U/L)	90.04 ± 53.69
BMI	29.58 ± 4.54

BMI, Body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; ALT, Alanine aminotransferase.

Table 2

Changes in serum biochemistry and HCV RNA with Rosuvastatin therapy

	Baseline*	20 mg [†]	40 mg [‡]	Follow-up [§]
Log ₁₀ HCV RNA (copies/mL)	7.13 ± 0.60	7.11 ± 0.60	7.04 ± 0.57	7.14 ± 0.69
Total Cholesterol (mg/dL)	174.81 ± 26.89	122.0 ± 18.89	114.25 ± 22.40	168.91 ± 25.86
HDL	53.36 ± 12.07	49.76 ± 13.09	51.18 ± 12.32	52.54 ± 13.31
LDL	95.73 ± 26.65	53.28 ± 16.76	45.21 ± 19.15	92.91 ± 26.74
Triglycerides (mg/dL)	128.41 ± 65.91	109.43 ± 77.91	90.02 ± 43.90	118.91 ± 50.94
ALT (IU/mL)	90.04 ± 54.0	86.71 ± 48.92	91.33 ± 58.95	81.82 ± 52.13

HCV RNA, all differences not significant (NS); Total cholesterol * vs †, $P < 0.0001$, * vs ‡, $P < 0.0001$, ‡ vs §, $P < 0.001$; HDL * vs †, $P = 0.03$; LDL * vs †, $P < 0.0001$, * vs ‡, $P < 0.0001$; Triglycerides * vs ‡, $P = 0.006$;