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Host cell mTORC1 is required for HCV RNA replication

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ABSTRACT

Objective Chronically HCV-infected orthotopic liver transplantation (OLT) recipients appear to have improved outcomes when their immunosuppressive regimen includes a mammalian target of rapamycin (mTOR) inhibitor. The mechanism underlying this observation is unknown.

Design We used virological assays to investigate mTOR signalling on the HCV replication cycle. Furthermore, we analysed HCV RNA levels of 42 HCV-positive transplanted patients treated with an mTOR inhibitor as part of their immunosuppressive regimen.

Results The mTOR inhibitor rapamycin was found to be a potent inhibitor for HCV RNA replication in Huh-7.5 cells as well as primary human hepatocytes. Half-maximal inhibition was observed at 0.01 $\mu\text{g/mL}$, a concentration that is in the range of serum levels seen in transplant recipients and does not affect cell proliferation. Early replication cycle steps such as cell entry and RNA translation were not affected. Knockdown of raptor, an essential component of mTORC1, but not rictor, an essential component of mTORC2, inhibited viral RNA replication. In addition, overexpression of raptor led to higher viral RNA replication, demonstrating that mTORC1, but not mTORC2, is required for HCV RNA replication. In 42 HCV-infected liver-transplanted or kidney-transplanted patients who were switched to an mTOR inhibitor, we could verify that mTOR inhibition decreased HCV RNA levels in vivo.

Conclusions Our data identify mTORC1 as a novel HCV replication factor. These findings suggest an underlying mechanism for the observed benefits of mTOR inhibition in HCV-positive OLT recipients and potentiate further investigation of mTOR-containing regimens in HCV-positive recipients of solid organ transplants.

INTRODUCTION

Infection with the HCV commonly causes chronic liver disease and thus remains a major indication for orthotopic liver transplantation (OLT) worldwide.¹ Approximately 170 million individuals are chronically infected. Pegylated interferon alpha in combination with ribavirin was the standard of treatment, until 2011, when the first direct acting antiviral (DAA) drugs were approved.² DAA are currently employed in interferon-containing and interferon-free combinations and have led to higher

Significance of this study

What is already known on this subject?

- Immunosuppression based on mammalian target of rapamycin (mTOR) inhibitors results in slower disease progression and reduced rates of advanced fibrosis in HCV-positive recipients after liver transplantation.
- Treatment with the mTOR inhibitor rapamycin significantly suppressed viral replication in HCV-positive renal transplanted patients.
- HCV activates mTOR signalling to inhibit apoptosis and affects mTOR signalling to influence glucose metabolism.

What are the new findings?

- mTOR inhibitors are able to block HCV RNA replication independent from cytotoxic or antiproliferative effects.
- Knockdown of raptor, an essential component of mTOR complex 1, inhibited viral RNA replication.
- mTOR complex 1 is a previously unknown essential host factor for HCV RNA replication.
- Treatment with mTOR inhibitors reduces HCV RNA levels in patients after liver and kidney transplantation.

How might it impact on clinical practice in the foreseeable future?

- Our study provides a possible underlying mechanism for the clinical observation that mTOR inhibition is beneficial in HCV-positive orthotopic liver transplantation recipients. Moreover, this might be a rationale for further evaluating mTOR inhibitor containing regimens in this difficult-to-treat population.

response rates with fewer side effects.^{3,4} However, prevention of HCV reinfection of the graft after OLT is still an unresolved problem in clinical practice and antiviral treatment of HCV after OLT remains challenging.^{1,5,6} Interactions between antiviral and immunosuppressive drugs and their severe side effects in combination with other comorbidities lead to inferior outcomes of HCV-positive OLT recipients compared with those who are transplanted for other indications.⁷ Within 5 years after



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transplantation, up to 30% of patients redevelop cirrhosis and are therefore at risk of decompensation and need for retransplantation.^{8–9} Consequently, survival rates of HCV-infected liver transplanted patients are below those of uninfected ones.¹⁰ The accelerated progression of chronic hepatitis C in OLT recipients is thought to be at least in part due to immunosuppression allowing enhanced viral replication. In addition, we have been able to show that glucocorticoids that are routinely part of immunosuppressive regimens have direct pro-viral effects.¹¹ Optimising immunosuppressant regimens might be a conceivable key factor in improving outcome in HCV-positive OLT recipients, yet clinical data on different agents remain inconclusive.¹ Thus at present, a triple combination of a calcineurin inhibitor (tacrolimus or cyclosporine A (CsA)) in combination with mycophenolate mofetil and steroids remains the standard approach to prevent long-term rejection.¹² Different regimens with more pronounced inherent antiviral activity would be an attractive prospect.

Inhibitors of the serine/threonine kinase mammalian target of rapamycin (mTOR) are an increasingly employed class of immunosuppressive drugs, but their exact impact on the HCV replication cycle is unknown. mTOR is a key component of two structurally and functionally distinct protein complexes that regulate cell signalling pathways by phosphorylating several downstream targets.^{13–14} These regulate host cell energy as well as cell growth and metabolism in response to nutrients and growth factors.¹⁵ The mTOR protein is present in two distinct complexes, the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), that are activated by different classes of stimulators and differ in their upstream and downstream effectors.¹⁴ mTORC1 contains regulatory associated protein of mTOR (raptor) and controls growth-related processes, such as protein synthesis, cellular autophagy, lipid synthesis and progression through the cell cycle.^{16–17} Rapamycin and everolimus bind to the mTOR-FRB (FKBP12/rapamycin-binding domain), which exclusively leads to inhibition of mTORC1. Thus, proliferation, especially of tumour cells and T-cells, is inhibited, resulting in an immunosuppressive effect.¹⁸ mTORC2 activates the proto-oncogene Akt (S473) and is responsible for cell survival, regulation of metabolism and cytoskeletal dynamics.¹⁹ In contrast to mTORC1, mTORC2 comprises the rictor (rapamycin-insensitive companion of mTOR) protein and is insensitive to rapamycin and everolimus inhibition,¹⁹ at least in short-term treatments. Long-term administered rapamycin also affects mTORC2.²⁰

Inhibitors of the mTOR like everolimus and rapamycin are important drugs in tumour therapy and immunosuppression after solid organ transplantation.^{21–22} A retrospective study evaluating the benefit of mTOR inhibitor treatment in >500 HCV-positive liver recipients has shown that mTOR inhibitors slow down disease progression and reduce rates of advanced fibrosis in HCV-positive recipients.²³ In addition, Asthana *et al*²⁴ observed in a retrospective analysis of 141 patients that de novo rapamycin-based immunosuppression leads to slower progression of fibrosis. In an interventional open-label prospective study, renal transplanted patients with HIV and HCV benefited from rapamycin monotherapy in comparison to treatment with CsA.²⁵ Moreover, rapamycin treatment significantly suppressed viral replication in HCV-positive renal transplanted patients compared with patients receiving CsA.²⁵ However, little is known about the relation between mTOR-mediated signalling and HCV RNA replication from a molecular point of view. It is known that HCV activates mTOR signalling to inhibit apoptosis, which may promote chronic infection.²⁶

In addition, HCV affects mTOR signalling to influence glucose metabolism by inhibition of TSC-1/TSC-2 and consequently activation of mTORC1 and S6K1.^{27–28} However, whether mTOR-mediated signalling has direct effects on the HCV replication cycle is unknown. This study was undertaken to clarify this point.

Based on multiple lines of evidence, we show that mTORC1 is a previously unknown essential host factor for HCV RNA replication and new particle production since these key processes of the HCV replication cycle depend on mTORC1-mediated but not mTORC2-mediated signalling. Thus, we provide a possible underlying mechanism for the clinical observation that mTOR inhibition is beneficial in HCV-positive OLT recipients and a rationale for further evaluating mTOR inhibitor containing regimens in this difficult-to-treat population.

MATERIALS AND METHODS

Compounds

Rapamycin (sirolimus, molar mass 914.172 g/mol), everolimus (molar mass 958.224 g/mol), LY-294002 hydrochloride, PP242 hydrate and mitomycin were purchased from Sigma-Aldrich, Seelze, Germany.

HCV replication and infection assay

Huh-7.5 cells were electroporated with 5 µg RNA of the reporter virus Luc-Jc1 (genotype 2a) as described.²⁹ After 4 h, different concentrations of rapamycin or everolimus were added to the cell culture medium. HCV RNA replication was quantified by measuring luciferase activity. After 48 h, supernatants were collected, filtered through 0.45 µm pore size filters and used to infect naive Huh-7.5 target cells. In general, 500 µL containing virus supernatant with a viral titre of 1×10^4 in TCID₅₀ (multiplicity of infection (MOI) 0.05–0.1) was used to inoculate target cells. HCV titres were determined as published recently.³⁰ The 50% tissue culture infectious dose (TCID₅₀) was calculated based on the methods described by Spearman and Kaerber.

Patient characteristics

All kidney-transplanted or liver-transplanted patients with chronic HCV (genotype 1) at Hannover Medical School have been reviewed for a switch of their immunosuppressive regimen to an mTOR inhibitor. HCV RNA levels were compared up to 4 weeks before the introduction of the mTOR inhibitor and 4–12 weeks after the change of the immunosuppressive regimen. Trough levels of the mTOR inhibitor were adjusted to 4–8 ng/mL.

Software and statistical analyses

Data were analysed using Excel (Microsoft, Redmond, Washington, USA) or GraphPad Prism 5 (GraphPad Software, La Jolla, California, USA). In vitro experiments were repeated on separate occasions. Each repetition was performed in multiple replicates. The mean ± SD of the replicates from one representative experiment is shown with the number of replicates indicated. Unpaired two-sided Student's *t* test or one-way analysis of variance (ANOVA) with Bonferroni post test were performed accordingly to determine statistical significance. One-way ANOVA was performed to determine statistical significance. Values are represented by **p* < 0.05, ***p* < 0.01 and ****p* < 0.001.

RESULTS

Rapamycin inhibits HCV RNA replication, but has no effect on HCV entry

To ensure that mTOR signalling is active during HCV cell culture-derived particles (HCVcc) infection, Huh-7.5 cells were

transfected with HCVcc and treated with rapamycin for 48 h. Phosphorylation of mTOR and the downstream protein p70S6K was measured using a chemiluminescent assay, which detects cellular proteins only when phosphorylated at the specified residues. In HCVcc-infected Huh-7.5 cells, we observed an

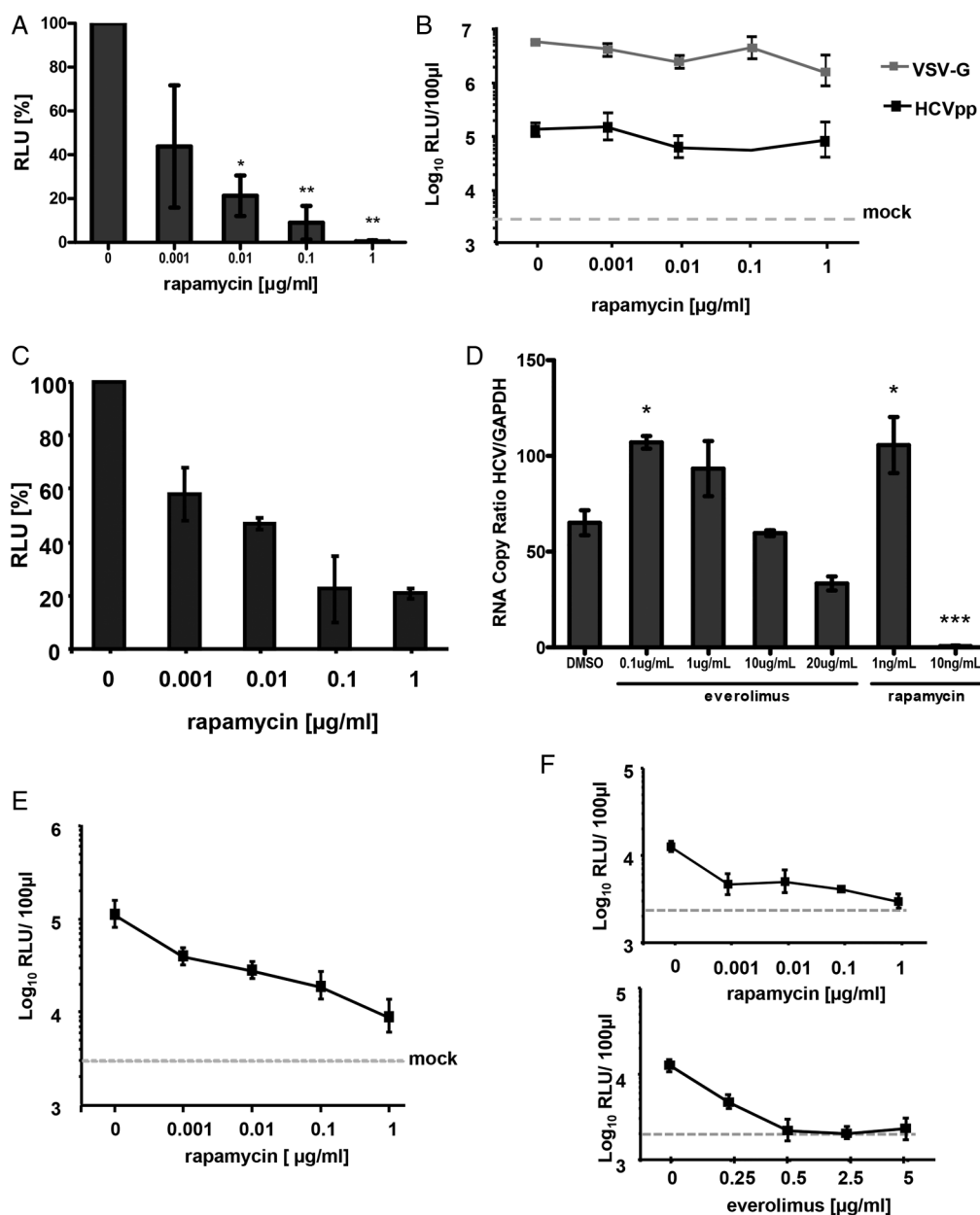


Figure 1 Rapamycin inhibits HCV RNA replication and production of new particles, but had no effect on HCV entry. (A) Huh-7.5 cells were inoculated with Luc-Jc1 reporter viruses in the presence of rapamycin. Infected cells were lysed 3 days later and luciferase activity was determined. The mean of three independent repetitions performed in triplicates is shown. Results were normalised to untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) Huh-7.5 cells were transduced with HCV pseudoparticles (HCVpp) of genotype 1b (Con1) in the presence of rapamycin. The inoculum was removed 4 h later. Infected cells were lysed 3 days later and luciferase activity was determined. A representative experiment of three independent repetitions performed in triplicates is shown. (C) Huh-7.5 cells were electroporated with the reporter virus Luc-Jc1 and 4 h later rapamycin was added. Replication efficiency after 48 h was assessed using a luciferase assay. Mean of three independent experiments performed in duplicates is shown. Results are expressed as per cent of untreated control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. (D) Huh-7.5 cells were infected with Jc1 wildtype virus and 4 h later rapamycin or everolimus was added. Inhibition of HCV RNA replication was measured by quantitative real-time PCR. Results were normalised to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Mean of four independent experiments performed in duplicates is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. (E) 48 h culture fluid of the cells from (C) was used to inoculate naïve Huh-7.5 cells. Infected cells were fixed 3 days later and luciferase activity was determined. A representative experiment performed in triplicates of three independent repetitions with SDs of the means is shown. (F) Differentiated Huh-7.5 cells were produced by treatment with dimethyl sulfoxide (DMSO). Then the cells were inoculated with Luc-Jc1 viruses in the presence of rapamycin or everolimus. 48 h later luciferase signal was measured. Mean values and SDs of four independent experiments are shown. RLU, relative light units; VSV, vesicular stomatitis virus.

increase in mTOR and p70S6K phosphorylation relative to naive Huh-7.5 cells (see online supplementary figure S1A). These experiments were also confirmed in primary human hepatocytes (PHH) as a more relevant *in vitro* system. Importantly, treatment with rapamycin in HCV-infected cells reduces phosphorylation of mTOR and p70S6K, confirming inhibition of mTOR catalytic activity following rapamycin treatment in Huh-7.5 cells and PHH. In addition, we investigated whether HCV infection changes endogenous mTOR levels in Huh-7.5 cells and PHH, but could not observe any significant differences (see online supplementary figure S1B).

Next we evaluated the effects of the commonly used mTOR inhibitor rapamycin on HCV replication and infectivity using firefly luciferase reporter viruses. First, we used full-length HCVcc to infect naive Huh-7.5 cells and added increasing concentrations of rapamycin. As shown in figure 1A, the mTOR antagonist inhibits HCVcc infection in a dose-dependent manner (figure 1A, IC50 0.10 nM). To further investigate which step of the viral replication cycle is affected, we used HCV pseudoparticles (HCVpp) to assess specifically the influence on HCV entry. Viral entry of HCVpps derived from genotype 1a (figure 1B) and genotype 2a (data not shown) was not affected by rapamycin, indicating that HCV cell entry is unaffected by mTOR inhibition.

To further elucidate the effects on HCV RNA replication, Huh-7.5 cells were electroporated with the reporter virus genome and 4 h later increasing doses of rapamycin were added. Replication efficiency was assessed 48 h after transfection using luciferase assay. A dose-dependent inhibition of HCV RNA replication was detectable (figure 1C). In addition, we also used JFH1 subgenomic replicons to study the effect on HCV replication. Inhibition of a JFH1 subgenomic replicon by rapamycin was lower in comparison to a full-length virus or an NS2-5B replicon as well as a Con1 replicon (genotype 1), suggesting that rapamycin sensitivity may be linked to replication fitness of HCV RNAs (see online supplementary figure S2A).

To mimic a chronic HCV infection, we treated Huh-7.5 cells that stably express a neomycin-resistant JFH1 replicon (Luc Ubi Neo JFH1 NS3-3') with rapamycin and measured HCV replication after 48 h. As shown in online supplementary figure S2B, mTOR inhibition also has an antiviral effect on an already established ongoing replication.

To rule out that the antiviral effect is due to an inhibition of luciferase activity and not a direct effect on HCV RNA replication, we used a non-genetically modified virus (Jc1 wildtype) and measured inhibition of HCV RNA by quantitative real time (RT)-PCR. At concentrations, which were in clinical use, we could detect an inhibition of HCV RNA replication. However,

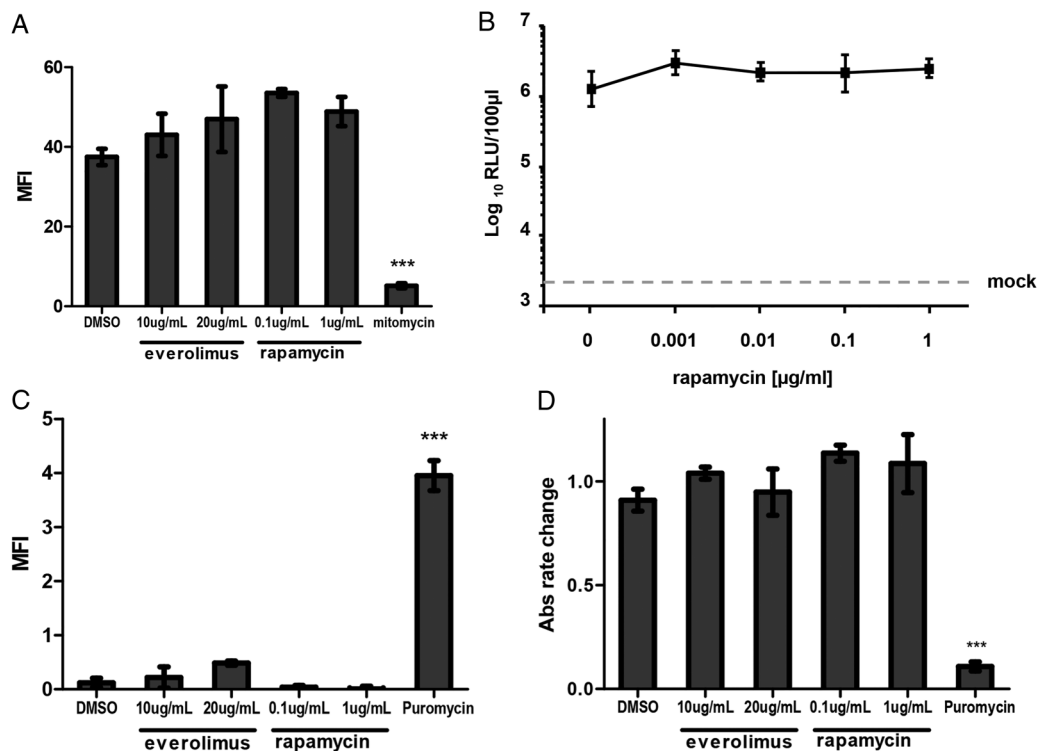


Figure 2 Inhibition of HCV by rapamycin is not due to antiproliferative or cytotoxic effects. (A) Huh-7.5 cells were treated with increasing concentrations of rapamycin in the presence of EdU, a nucleoside analogue to thymidine. 48 h later, EdU incorporation was measured by fluorescence-activated cell sorting (FACS). Mitomycin served as positive control. Mean fluorescence intensity (MFI) was determined using the geometric mean of the gated population. Mean values and SEM of three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) Huh-7.5 stably expressing firefly luciferase from a cytomegalovirus promoter (Huh-7.5/Fluc) were incubated with rapamycin for 72 h. Then cells were fixed and luciferase signal was determined. Mean values and SDs of three independent experiments are shown. (C) Huh-7.5 were incubated for 48 h with increasing concentrations of the drugs. Cells were then stained with propidium iodide. Dye incorporation was measured by FACS. MFI was determined using the geometric mean of the gated population. Mean values and SEM of three independent experiments are shown. Puromycin was used as a positive control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. (D) Huh-7.5 were incubated for 48 h with increasing concentrations of the drugs. Cells were then incubated with 1.2 mM MTT. Absorbance at 540 nm was then read on a plate reader. Mean values and SEM of three independent experiments with five replicates per drug concentration are shown. Puromycin was used as a positive control. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA.

for sub-therapeutic concentrations of rapamycin and everolimus there is a slight increase in viral RNA, as shown in figure 1D.

In parallel, supernatant from electroporated and rapamycin-treated cells from figure 1C was used to inoculate naive cells in order to detect a possible influence of the drug on virus production (figure 1E). As expected and in line with reduced HCV RNA replication, we could observe that production of new viral particles was affected. All experiments (figure 1A–E) were performed with both US Food and Drug Administration-approved mTOR inhibitors (rapamycin and everolimus) leading to similar results. To rule out that the inhibitory effect is limited to a genotype 2 isolate, we also tested the antiviral effect of rapamycin on a genotype 3 chimeric full-length virus in direct comparison with a genotype 2a isolate. As shown in online supplementary figure S3, rapamycin also inhibited replication of a HCV genotype 3 chimeric isolate.

Finally, we generated differentiated Huh-7.5 cells as previously reported by Sainz *et al.*⁸ Upon differentiation Huh-7.5 cells stop dividing, enter G0 state and assume a more ‘differentiated’ hepatocyte-like state characterised by upregulation of liver-specific genes such as albumin, HNF4 α and α 1-antitrypsin. Differentiated Huh-7.5 cells were infected and treated with

different concentrations of rapamycin. As in undifferentiated Huh-7.5 cells, we could observe that rapamycin inhibits HCV RNA replication in a dose-dependent manner (figure 1F), indicating that the effect on HCV RNA replication is independent from cell proliferation.

These data suggest that the mTOR inhibitors potently inhibit HCV RNA replication but not viral entry. Importantly, the effect of rapamycin on HCV RNA replication was similar in dividing and non-dividing cells.

Inhibition of HCV by rapamycin is not caused by antiproliferative or cytotoxic effects

To test whether the inhibitory effect of rapamycin on HCV RNA replication is due to direct antiviral activity or indirectly mediated through non-specific cytotoxic and/or antiproliferative effects of the drug, we used different cytotoxicity and proliferation assays. Since rapamycin is known to be a potent inhibitor of cell proliferation, we performed a standard EdU proliferation assay. In this assay, we could not observe any significant growth arrest after treatment with concentrations of rapamycin we used for our *in vitro* experiments in this study (figure 2A). The proliferation inhibitor mitomycin C served as a positive control. As an aggregate measure of cell proliferation and viability, we used

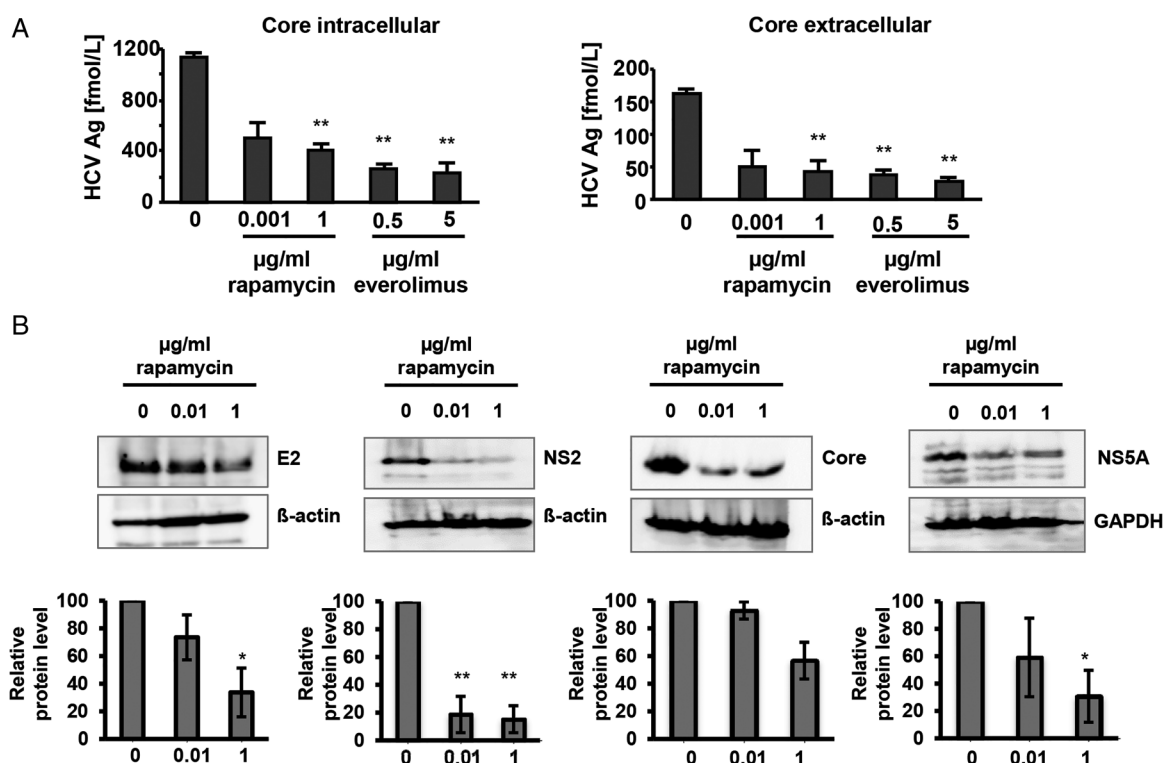


Figure 3 Rapamycin has no influence on new viral particle production and the lipoprotein association of virus particles. (A) Huh-7.5 cells were transfected with Luc-Jc1 and inoculated with mammalian target of rapamycin (mTOR) inhibitors. After 48 h, intracellular and extracellular core levels were measured. Mean values of two independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) Huh-7.5 cells were treated with rapamycin for 48 h in two different concentrations. Levels of HCV proteins E2, NS2, core and NS5A were determined by western blot. Mean of three independent experiments and a representative western blot are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. (C) Huh-7.5 cells were pretreated with increasing concentrations of rapamycin for 24 h. Then cells were washed three times with phosphate buffered saline and inoculated with Luc-Jc1 virus. 48 h after infection luciferase signal was measured. Mean values and SD of three independent experiments are shown. (D) Buoyant density and specific infectivity of Jc1 viruses treated with rapamycin versus untreated. Jc1 was harvested 48 h after transfection of Huh-7.5 cells±rapamycin and were resolved using an iodixanol step gradient. Ten fractions were harvested from the bottom, and HCV core protein, HCV RNA, and infectivity were determined for each fraction. Values are plotted against the density of the respective fraction measured by refractometry. The results are from a representative of two independent experiments. (E) Huh-7.5 cells were transfected with Jc1 wildtype and 48 h post-transfection supernatants were collected. In parallel, virus-producing cells were washed and lysed by repetitive cycles of freeze and thaw. Extracellular (dark grey bars) and intracellular infectivity (light grey bars) were determined by limiting dilution assay. Mean values of three independent experiments and the SD of the means are presented. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

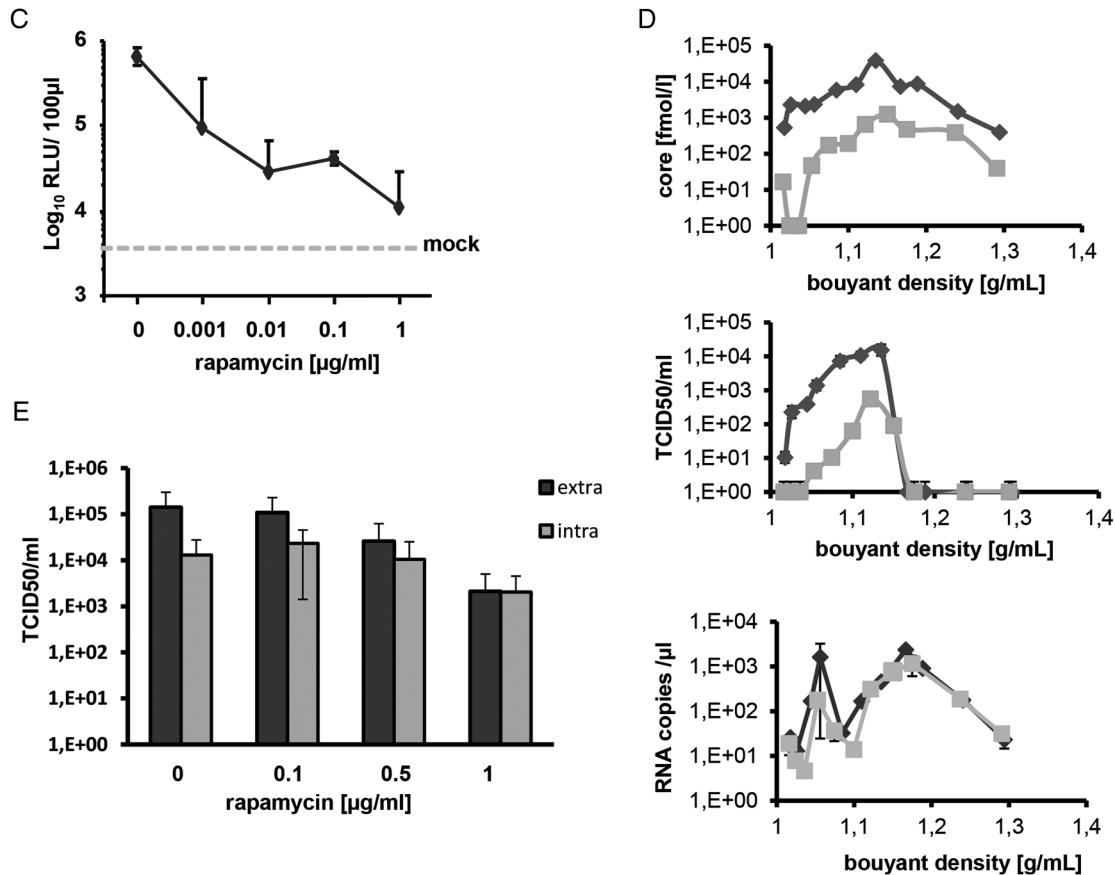


Figure 3 Continued

Huh-7.5/Fluc cells stably expressing firefly luciferase under control of a cytomegalovirus promoter. When these cells were exposed to rapamycin at concentrations of up to 1 $\mu\text{g/ml}$ for 72 h, the luciferase signal was unaffected (figure 2B).

In addition, cytotoxicity was investigated by propidium iodide (PI) staining (figure 2C) and MTT assay (figure 2D), confirming that the concentrations of rapamycin and everolimus that block HCV RNA replication were clearly not cytotoxic.

Taken together, mTOR inhibitors do not show any antiproliferative or cytotoxic effects in our assays at concentrations that were used for inhibition of HCV replication in this study.

Rapamycin has no influence on new viral particle production and the lipoprotein association of virus particles

To further characterise how mTOR inhibitors affect HCV RNA replication, we assessed the amount of different HCV proteins in Jc1-transfected Huh-7.5 cells. Cells were treated with rapamycin for 48 h, then viral protein expression was assessed by western blot (E2, NS2, NS5A) or ELISA (Core). The amount of intracellular and extracellular core protein, a structural component of HCV virions, was markedly reduced after treatment with rapamycin or everolimus (figure 3A), which was confirmed by western blot analysis (figure 3B). Moreover, also production of the viral proteins E2, NS2 and NS5A was reduced in a dose-dependent manner after treatment with rapamycin for 48 h (figure 3B).

To confirm that the inhibitory effect of rapamycin is due to an alteration of the target cell rendering it HCV-resistant, we administered rapamycin 24 h prior to infection. Pretreatment of host cells with rapamycin prior to inoculation with the virus

reduced HCV infection (figure 3C), indicating that rapamycin inhibits HCV by targeting the host cell and not the incoming viral particle.

It is well known that mTOR plays a key role in lipid homeostasis. To investigate whether mTOR inhibitors change lipid association of viral particles, we performed a gradient analysis of viral particles in the presence and absence of rapamycin. As shown in figure 3D, we observed a reduced viral infectivity by treatment of rapamycin independent of the gradient density. The same effects can be noted when we determined viral core amounts by a commercial ELISA and viral RNA by qRT-PCR. These new data demonstrate that rapamycin is not affecting the lipoprotein association and/or biophysical integrity of virus particles.

In addition, we determined the effect of rapamycin on intracellular and extracellular virus particle infectivity and observed a dose-dependent reduction of both particles types. These results therefore indicate that in line with the reduced RNA replication also intracellular and extracellular virus infectivity is reduced and there is no additional inhibition of virus release (figure 3E).

Inhibition of PI3K-PKB inhibits HCV RNA replication

To exclude the possibility that the effect of rapamycin on HCV RNA replication represents an mTOR-independent off-target effect, we next wanted to investigate the effect of LY294002 on HCV. LY294002 is a well-characterised phosphoinositide-3-kinase (PI3K) inhibitor acting upstream of the PI3K/mTOR signalling pathway. When Huh-7.5 cells were treated with non-toxic concentrations (0–10 μM) of LY294002, a reduction of HCV RNA replication was detectable similar to that observed

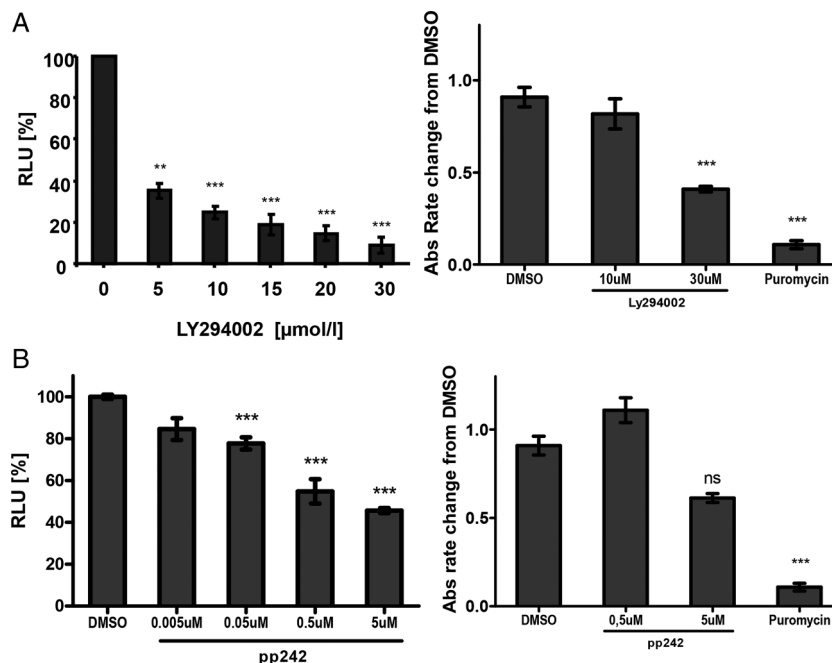


Figure 4 Inhibition of phosphoinositide-3-kinase (PI3K)-PKB inhibits HCV RNA replication. (A) Huh-7.5 cells were transfected with Luc-Jc1 reporter viruses. After 4 h incubation LY294002, an inhibitor of PI3K was added. Viral RNA replication was measured 48 h later by luciferase assay. Mean value and SEM of three independent experiments with two replicate measurements per drug concentration are shown (left panel). In addition, Huh-7.5 were incubated for 48 h with increasing concentrations of the drug. Cells were then incubated with 1.2 mM MTT. Absorbance at 540 nm was then read on a plate reader. Mean values and SEM of three independent experiments with five replicates per drug concentration are shown. Puromycin was used as a positive control (right panel). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) Huh-7.5 cells were transfected with Luc-Jc1 viruses. After 4 h incubation PP242, an inhibitor of mTORC1 and mTORC2, was added and amount of viral RNA replication was measured 48 h later by luciferase assay. Mean values and SEM of three independent experiments with five replicates per drug concentration are shown (left panel). In addition, Huh-7.5 were incubated for 48 h with increasing concentrations of the drug. Cells were then incubated with 1.2 mM MTT. Absorbance at 540 nm was then read on a plate reader. Mean values and SEM of three independent experiments with three replicates per drug concentration are shown. Puromycin was used as a positive control (right panel). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. DMSO, dimethyl sulfoxide.

with rapamycin (figure 4A). This inhibitory effect was independent from cytotoxicity (figure 4A, right panel).

To investigate whether both mTOR complexes play a role in HCV replication cycle and simultaneous inhibition of both might have synergistic effects, the inhibitor PP242 was tested. PP242 is an ATP-competitive mTOR inhibitor affecting both mTORC1 and mTORC2 in contrast to rapamycin, which exclusively inhibits mTORC1. As shown in figure 4B, PP242 inhibits HCV similarly to rapamycin, but we could not detect any additive effect of inhibition of both mTOR complexes (figure 4B), indicating that mTORC1 is mainly responsible for HCV inhibition.

Gene silencing of raptor by RNAi reduces HCV RNA replication

The two mTOR complexes present in cells differ in that mTORC1 contains raptor while mTORC2 contains rictor. To evaluate the direct effects of raptor and rictor on HCV, Huh-7.5 cells were transduced with integrating lentiviral vectors expressing shRNA specifically targeting raptor, rictor or an irrelevant shRNA as a control (sh irrelevant). As shown in figure 5A, shRNA vectors targeting raptor and rictor achieved marked downregulation of the respective proteins. However, only raptor silencing resulted in a significant decrease of HCV RNA replication and production of new viral particles ($p < 0.001$) while silencing of rictor had no significant effect on HCV. These data support that mTORC1, but not mTORC2, plays an important role in the HCV replication cycle.

To further elucidate mTORC1 involvement in the viral replication cycle, we examined the impact of mTOR-inhibitor treatment on expression of the mTORC1 proteins mTOR and raptor. Naive Huh-7.5 cells were treated with increasing concentrations of rapamycin or everolimus and incubated for 4, 24 or 48 h. As shown in figure 5B, mTOR-inhibitor treatment leads to reduction of mTOR and raptor protein.

To show that raptor indeed plays a role during HCV replication, a cell line overexpressing raptor protein (pLJM1 Flag Raptor) was created. Overexpression was confirmed by western blot (figure 5C). Infection assays with raptor overexpressing cell lines revealed 1.5-fold higher HCV RNA replication levels compared with Huh-7.5 cells ($p < 0.01$, figure 5D).

These data show that mTORC1 plays an important role for HCV RNA replication. While downregulation of the mTORC1 protein raptor decreases RNA replication, overexpression of raptor enhances RNA replication. Since silencing of rictor has no effect on HCV, mTORC2 is likely not required for the HCV replication cycle.

Furthermore, these data indicate that rapamycin indeed targets mTORC1 and has no unforeseen direct off-target effect on a viral protein.

Treatment with mTOR antagonists inhibits HCV RNA replication in PHH

Although Huh-7.5 cells sustain the complete HCV replication cycle, these carcinoma-derived cells functionally differ from PHH. Therefore, PHH were inoculated with HCVcc in the

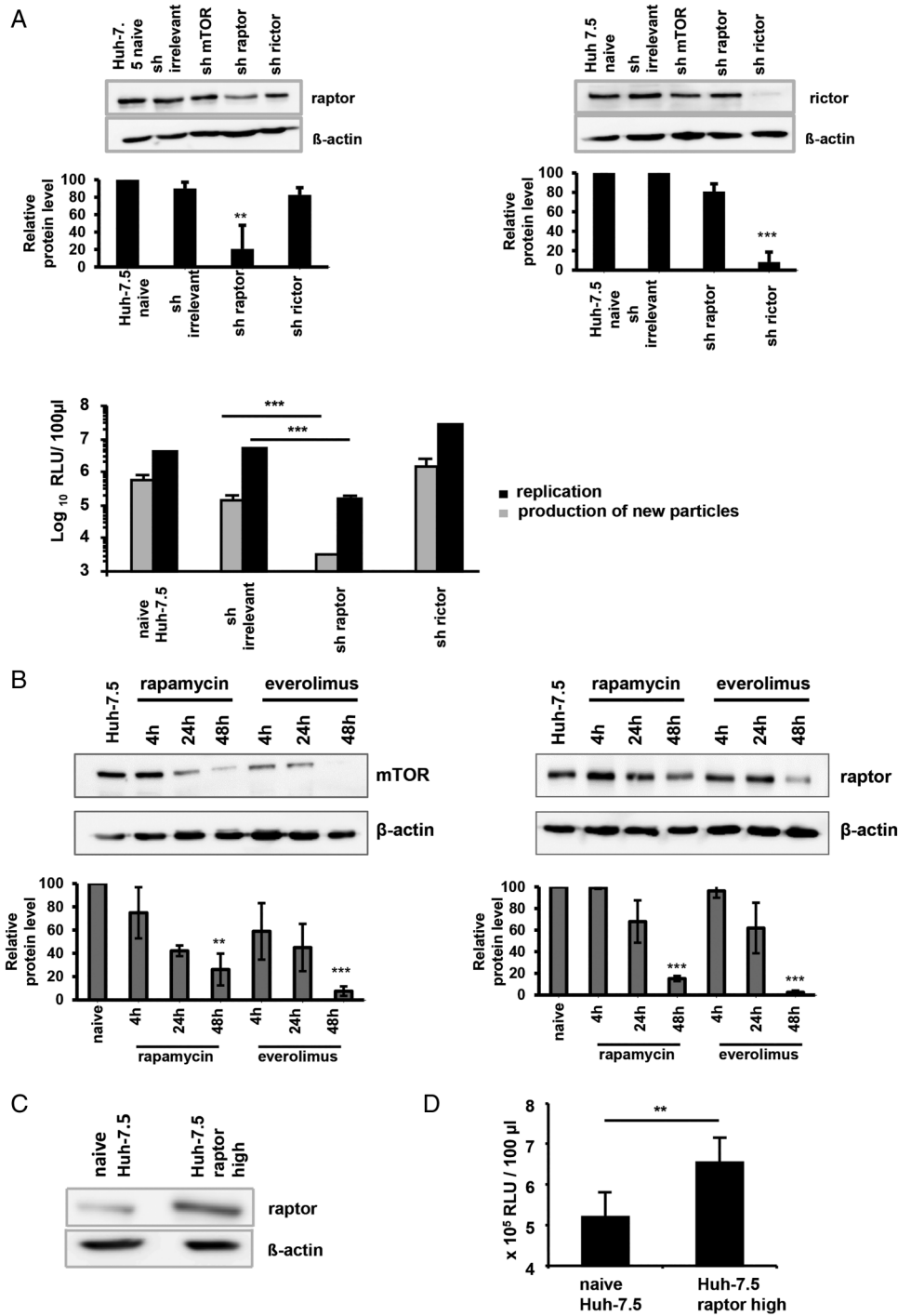


Figure 5 Gene silencing of raptor by RNAi reduces HCV RNA replication. (A). Huh-7.5 cells stably expressing shRNA's targeting raptor or rictor were characterised by western blot (upper panel) and transfected with Luc-Jc1 virus. Viral replication was measured 48 h later by luciferase activity (lower panel). Naive Huh-7.5 cells and a control cell line harbouring an irrelevant shRNA were used as positive controls. For production of new viral particles, culture fluid of the same cells was used to inoculate naive Huh7.5 cells. Infected cells were fixed 3 days later and luciferase activity was determined. A representative western blot and mean values of three independent experiments with SDs of the means is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) Naive Huh-7.5 cells were treated with 1 µg/ml rapamycin or 5 µg/mL everolimus. Expression of mammalian target of rapamycin (mTOR) and raptor protein was determined by western blot. A representative western blot and mean values of three independent experiments with SDs of the means is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. (C) A raptor overexpressing Huh-7.5 cell line (Huh-7.5 raptor high, pJM1 Flag Raptor) was created and raptor protein overexpression was confirmed by western blot. (D) Huh-7.5 raptor high and naive Huh-7.5 cells were transfected with Luc-Jc1 virus and HCV replication was measured by luciferase assay 48 h later. Mean values and SD of three independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA.

presence or absence of rapamycin or everolimus and infectivity was determined through limiting dilution assay. Importantly, both drugs reduce HCV infectivity while rapamycin had overall

a stronger inhibitory effect on PHH than everolimus (figure 6A). In addition, PI staining and MTT assay was performed on PHH, confirming that the concentrations of

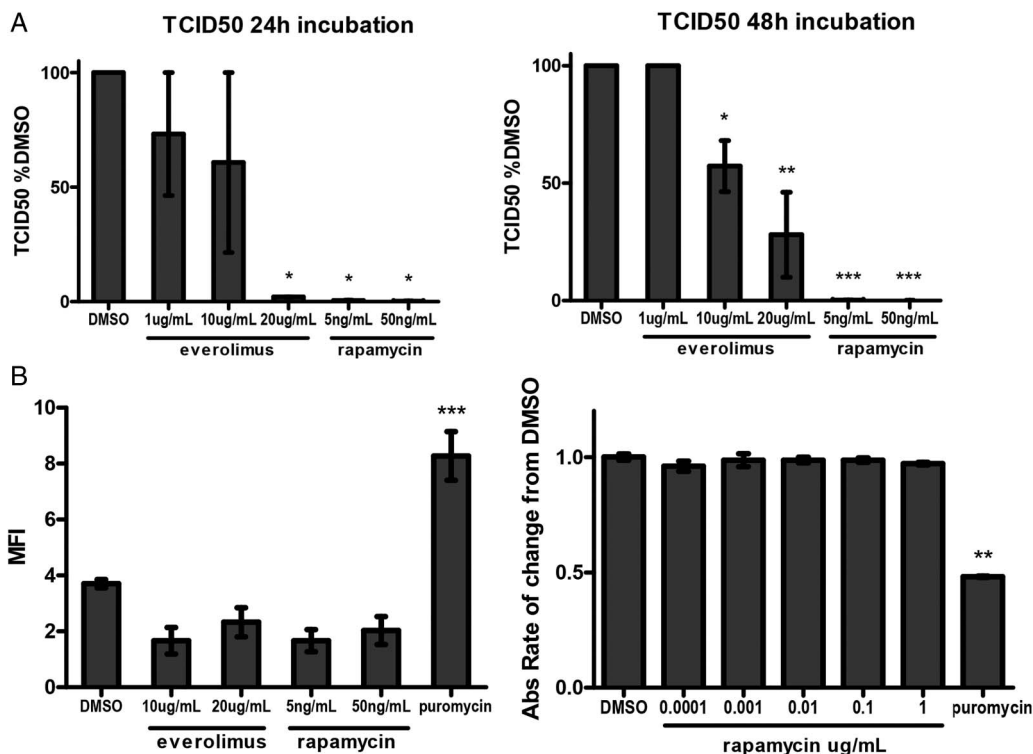


Figure 6 Mammalian target of rapamycin (mTOR) inhibition also decreases HCV RNA replication in primary human hepatocytes (PHH). (A) PHH were infected with Jc1 wildtype virus and treated with increasing concentrations of rapamycin or everolimus. Supernatants were collected after 24 and 48 h and infectivity was determined through limiting dilution assay. Mean values and SEM of two independent experiments are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way analysis of variance (ANOVA). (B) PHH were incubated for 48 h in the presence of rapamycin or everolimus. Cells were then stained with propidium iodide. Dye Incorporation was measured by fluorescence-activated cell sorting. Mean fluorescence intensity (MFI) was determined using the geometric mean of the gated population. Mean values and SEM of three independent experiments are shown. Puromycin was used as a positive control (left panel). Cells were incubated with 1.2 mM MTT. Absorbance at 540 nm was then read on a plate reader. Mean values and SEM of three independent experiments with five replicates per drug concentration are shown. Puromycin was used as a positive control (right panel). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by one-way ANOVA. DMSO, dimethyl sulfoxide.

rapamycin and everolimus used for this experiments were not toxic for PHH (figure 6B).

These data indicate that mTOR antagonists do not only block HCV infection of hepatoma cell lines but also inhibit replication in PHH.

Knockdown of mTORC1 does not alter viral translation

Inhibition of mTORC1 signalling is known to block translation of cellular RNAs. This led us to investigate whether mTORC1 is involved in viral RNA replication or, in addition, mainly viral RNA translation. Therefore, Huh-7.5 cells and Huh-7.5 cells

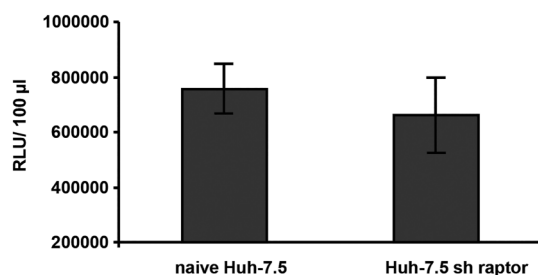


Figure 7 Knockdown of mTORC1 has no significant effect on viral translation. Huh-7.5 and Huh-7.5 sh raptor cells were transfected with a replication defective Luc-Jc1 Δ GDD mutant and viral translation was measured 4 h later by luciferase activity. Mean values and SDs of four independent experiments are shown.

expressing shRNA against raptor were transfected with the replication-defective Luc-Jc1 Δ GDD mutant. Luciferase signal was measured 4 h after electroporation. As shown in figure 7, we could not observe any significant differences in translation efficacy in naive Huh-7.5 vs Huh-7.5 sh raptor cells

These data indicate that inhibition of mTORC1 rather effects RNA replication than translation of viral proteins.

mTOR inhibitors reduce HCV RNA levels in patients after liver and kidney transplantation

Between 2005 and 2014, 386 patients after kidney and liver transplantation at Hannover Medical School were switched to an immunosuppressive regimen including an mTOR inhibitor. Out of these, 67 patients were chronically infected with HCV genotype 1. We retrieved available HCV RNA levels up to 4 weeks before and 4–12 weeks after addition of the mTOR inhibitor in this patient cohort from the patient files. We were thus able to follow-up the course of HCV RNA levels in 42 patients (figure 8A). In liver-transplanted as well as kidney-transplanted patients, HCV RNA levels were significantly reduced after introduction of the mTOR inhibitor, indicating that inhibition of mTOR also has an antiviral effect in vivo. Both mTOR inhibitors rapamycin and everolimus had a similar antiviral effect. Interestingly, decline of HCV RNA was more pronounced in patients after liver transplantation in comparison to kidney-transplanted individuals (figure 8B).

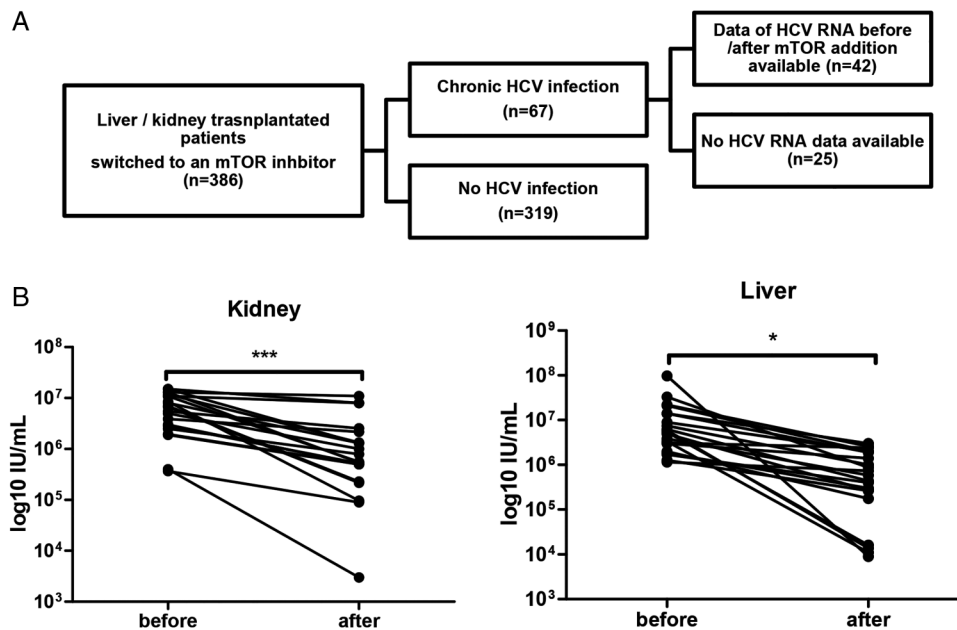


Figure 8 Mammalian target of rapamycin (mTOR) inhibition reduces HCV RNA levels in vivo. (A) Flow chart showing the selection process of participants enrolled in this analysis. (B) HCV RNA levels up to 4 weeks before and 4–12 weeks after changing the immunosuppressive regimen to an mTOR inhibitor were analysed by patient chart review in 42 patients after liver or kidney transplantation. There was no difference in type of mTOR inhibitor (everolimus or rapamycin). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ by two-tailed t test.

DISCUSSION

In this study, we identified the mTOR inhibitors rapamycin and everolimus as potent inhibitors for HCV RNA replication. Inhibition of HCV by mTOR inhibitors (rapamycin and everolimus) was also detectable in differentiated Huh-7.5 cells and in PHH, indicating that this effect is not related to their potential to inhibit cell proliferation. Treatment with rapamycin led to a reduction of HCV proteins E2, Core and non-structural proteins NS2 and NS5A, and thus the downregulation of several important viral proteins. The antiviral effect was not limited to a genotype 2a strain and was confirmed against an HCV genotype 3 chimeric virus. Furthermore, we determined that efficient HCV RNA replication is dependent on the presence of the mTORC1 signalling component raptor and that viral translation was not affected. In a single-centre cohort including 42 HCV genotype 1-infected patients after liver or kidney transplantation, we were able to verify that switch of the immunosuppressive regimen to an mTOR inhibitor is associated with a significant decline of HCV RNA levels. These findings have two important implications.

First, better outcomes for chronically HCV-infected patients after OLT are likely to depend on delivering antiviral and immunosuppressive agents simultaneously. In this study, inhibition of HCV replication was detected at concentrations that are achievable in the serum of transplant recipients. To this end, rapamycin and everolimus may be attractive agents as part of an immunosuppressive regimen for this special patient group. Rapamycin has been seen as an attractive immunosuppressant as it reduces the risk of significant hepatic fibrosis after OLT in HCV-positive patients³¹ and suppresses viral replication in vivo after solid organ transplantation,²⁵ but the molecular reason for these observations had not been identified yet. Our data show for the first time that mTOR inhibitors directly inhibit important steps in the HCV replication cycle. Thus, mTOR inhibitors should be strongly considered as part of immunosuppressive regimens in HCV-positive patients as they may help to limit the

extent of reinfection of the graft. Moreover, treatment of HCV recurrence after OLT is challenging due to drug interactions and contraindications for some antiviral agents. It will be interesting to see whether antiviral treatment will be more successful in patients receiving immunosuppressive regimens containing an mTOR inhibitor. Clinical studies will be necessary to clarify this point. Interestingly, HCV RNA reduction under mTOR-based immunosuppression was more pronounced in patients after liver transplantation than in kidney-transplanted patients. One possible reason for this might be that in our centre patients after kidney transplantation receive glucocorticosteroids while steroids were avoided in HCV-positive liver-transplanted patients.

We also have to keep in mind that mTOR is a key player in many cellular processes. One must consider that reduced liver damage after OLT upon rapamycin treatment might therefore be an indirect effect of direct inhibition of HCV replication. At this point, this hypothesis cannot be ruled out.

Second, our findings have implications for our understanding of HCV biology: mTOR inhibition leads to a reduction in HCV RNA replication and knockdown of raptor has a similar effect. In addition, overexpression of raptor enhances replication of HCV. Other steps of the viral lifecycle like entry, translation and production of novel viral particles were not affected. Thus, raptor as part of mTORC1 is a novel host factor that promotes HCV RNA replication in infected cells. Recently, McNulty *et al*³² could show that replication of Andes virus (ANDV), a member of the Hantavirus genus, also depends on mTORC1 signalling. However, mTOR primarily regulated ANDV protein expression and not ANDV RNA production. Therefore, ANDV and HCV likely depend on mTORC1 for different reasons. Moreover, our data suggest that mTORC1 is involved in HCV RNA replication rather than its translation. However, we were not able to show a direct co-localisation of mTOR and HCV viral proteins by immunofluorescence (data not shown) although both are localised in close proximity in the ER. This suggests that mTORC1 signalling promotes HCV replication indirectly.

It has been shown by George *et al*³³ that HCV NS5A itself is able to activate mTORC1, which upregulates cap-dependent host protein translation and development of HCV-associated hepatocellular carcinoma. However, a direct link to viral replication and translation was not part of this study. In contrast, Huang *et al*³⁴ published that HCV inhibits mTORC1 via ER stress and enhanced autophagy. In their experiments, HCV core protein level was not changed after activation of the mTORC1 pathway consistent with the hypothesis that inhibition of mTORC1 blocks RNA replication and not viral translation.

One possible explanation might be that mTORC1 is a key regulator in lipid homeostasis: it has been shown before that mTORC1 stimulates lipid synthesis via SREBP1 and influences lipolysis within lipid droplets.³⁵ In addition, it is well known that lipid droplets play an essential role in multiple steps of the viral lifecycle of HCV such as infection, replication and assembly and release of new viral particles.^{36,37} In line with this hypothesis, we also observed an enlargement of lipid droplets after treatment with rapamycin (data not shown) in HCV-infected cells similar to which was found by Andersson *et al*³⁸ after treatment with the cyclophilin inhibitor NIM811. However, we could not detect any differences in lipid composition of viral particles produced in cells treated with an mTOR inhibitor. Further detailed studies are necessary to evaluate the effects of rapamycin on SREBP1 and their influence on lipid droplet content.

Another possible explanation for the antiviral effect can be found downstream of mTOR signalling: the substrate p70 S6 kinase has been shown to phosphorylate PAK1 whose activity was reported to inhibit viral replication in hepatoma cells. In the same study, siRNA knockdown of p70 S6K abrogated PAK1 phosphorylation and enhanced viral replication, suggesting that this mechanism may be related to the antiviral effects of rapamycin and its analogues in the current study.³⁹

In contrast to our data and the above-mentioned work, Beretta *et al* reported a promoting effect arising from Pi3K inhibition on HCV replication.⁴⁰ They showed that the N-Ras-Pi3K-Akt-mTOR is important in regulating viral replication rates in retinoic acid inducible gene 1 (RIG-I) competent cell lines by modulating the phosphorylation state of the viral protein NS5A. Curiously, we also could observe a slight increase of HCV RNA replication at subtherapeutic concentrations of both mTOR inhibitors, which is in line with Beretta *et al* who used Pi3K inhibitors although at much lower concentrations. We cannot rule out that differences between HCV genotypes may play a role in the observed effects as Beretta *et al* reported on a genotype 1b strain. Furthermore, a major difference in comparison to our work is that Beretta *et al* used the RIG-I competent Huh 7 cell line while we were using Huh-7.5 cells, which are known to have a mutated RIG-I. Nonetheless, this observation raises an interesting question about mTOR modulation by viral activity. Not only response to Pi3K inhibition from the virus appears to be different in Huh7 and Huh-7.5 cell lines but also viral replication exhibits an interesting dichotomy of responses when the cells are stimulated with substances that alter mTOR signalling.⁴⁰

In summary, we have discovered that mTORC1 is required for efficient HCV RNA replication and that mTOR inhibitors may be useful as part of immunosuppressive regimens for HCV-positive individuals after OLT or transplantation of other solid organs. We can thus explain the clinical observation made in several studies that rapamycin-containing regimens are associated with better patient outcome in HCV-positive individuals after solid organ transplantation. Moreover, we provide data

suggesting that raptor as part of mTORC1 is a novel host factor for HCV RNA replication.

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Ethics approval Ethics Committee of Hannover Medical School.

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Materials and Methods

Plasmids and viruses:

Development of the reporter virus genomes Luc-Jc1, Luc-JFH1, Luc-JFH1/ NS3-5B, Luc-JFH1 /NS2-5B have been described recently [1]. The replication-defective mutant Luc-Jc1 Δ GDD has an in-frame deletion of 10 amino acids encompassing the GDD motif of NS5B [2]. Short hairpin RNA's (shRNA) Raptor_1 shRNA (Addgene plasmid 1857), rictor_1 shRNA (Addgene plasmid 1853), mTOR1 shRNA (Addgene plasmid 1855) scramble shRNA (Addgene plasmid 1864) and pLJM1 Flag raptor (Addgene plasmid 26633) were all purchased from Addgene, Cambridge, USA [3, 4]. Plasmid pFK-Jc1 [5] and monocistronic *Renilla* luciferase reporter virus genomes designated JcR2a and SA13/5a/R2a were described recently [6]. Luc Ubi Neo JFH1 NS3-3` has been described before [7].

Cell culture:

Huh-7.5 cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% fetal calf serum, non-essential amino acids, L-glutamine and Penicillin/Streptomycin. Cells harboring small hairpin RNA (shRNA) constructs were kept in the presence of neomycin or puromycin. For differentiation, Huh-7.5 cells were seeded in 12 well plates and treated with DMEM + 1% DMSO for at least two weeks as described before [8].

Primary human hepatocytes (PHH) were isolated from patients undergoing partial hepatectomy, plated at a density of 1.3×10^6 on Collagen in P6 dishes, and kept in William's E medium (Invitrogen, Karlsruhe, Germany).

Preparation of lentiviral pseudo-particles and HCVpp infection:

Lentiviral-based pseudotypes bearing vesicular stomatitis virus glycoproteins (VSV-G) or HCV E1-E2 proteins of the Con1 or JFH isolate were generated as described recently [2]. Huh-7.5 target cells were seeded on a 12 well plate and infected with pseudoparticles and

mTOR inhibitors in different concentrations for 5h. Luciferase was measured 72h post transduction.

HCV infection assay on primary human hepatocytes

In general, 1000 µl containing virus supernatant with a viral titer of 2×10^5 TCID₅₀ (MOI 0.5-1) was used to inoculate target cells. After 6 hours, cell culture medium supplemented with different concentrations of rapamycin or everolimus was added. After 24 and 48 hours, supernatant was harvested, filtered and then used to infect naïve Huh-7.5 cells. HCV titers were determined as published recently [9].

Luciferase reporter cell line

To test for anti-proliferative and/or cytotoxic effects, lentiviral VSV-G pseudoparticles were assembled as described above and used to transduce Huh-7.5 cells to stably express firefly luciferase driven by a CMV promoter (Huh-7.5/Fluc). Drugs were added to Huh-7.5/Fluc cells at different concentrations for 48 hours. After 48h luciferase signal was measured.

EdU Proliferation Assay

Huh-7.5 cells +/- rapamycin were stained with EdU (5-ethynyl-2'-deoxyuridine) Flow Cytometry Kit (Base Click GmbH, Tuzing, Germany) following manufacturer's instruction. As a positive control we used mitomycin. A BD FACS Canto flow cytometer (Becton Dickinson, Heidelberg, Germany) was used for acquisition and FlowJo software (Tree Star; Ashland, OR) for data analysis.

Core ELISA

Core protein was measured using HCV core antigen kit received from Wako Chemicals (Neuss, Germany).

Western Blot Analysis

Proteins, resolved by electrophoresis and blotted onto a polyvinylidene difluoride membrane, were detected using anti-E2 (AP33 from Arvind Patel), anti-NS2 (6H6 provided by Charles Rice), anti-NS5A (9E10 provided by Charles Rice) and anti-Core (C7-50, provided by Darius Moradpour). Anti- β -actin (Sigma-Aldrich, Germany) was used as described recently.

Anti-raptor antibody (EP539Y, anti-rabbit monoclonal) and Anti-ricor antibody (7B3, anti-mouse monoclonal) were bought from Abcam, Cambridge, USA. Anti-mTOR antibody (anti-rabbit) was purchased from Cell Signaling Technology, USA. For raptor, ricor and mTOR western blots, sample buffer was used without 2% mercaptoethanol. The ECL Plus Western Blotting Detection System (GE Healthcare Europe, Freiburg, Germany) was used for final protein detection.

Freeze and thaw lysates of HCV transfected cells

Huh7.5 cells were transfected with Jc1 RNA, 48 h post transfection cell culture supernatants were harvested and virus titers determined by TCID₅₀. Cell-associated infectivity was prepared essentially as described [10]. Briefly, cells were extensively washed with PBS, scraped and centrifuged for 5 minutes at 400 x g. Cell pellets were resuspended in 1 ml of DMEM containing 5% FCS and subjected to three cycles of freeze thaw using liquid nitrogen and a thermoblock set to 37°C. Samples were then centrifuged at 10,000 x g for 10 minutes at 4°C to remove cell debris, and cell-associated infectivity was determined by TCID₅₀ assay.

Iodixanol density-gradient

Density Gradient centrifugation was performed as described recently [11]. Briefly, viruses were separated by an overnight centrifugation through a 0% - 40% iodixanol step gradient at 154,000 x g in a TH-641 swing-out rotor at 4°C using a Sorvall Ultra WX80 centrifuge. Ten fractions of 1 ml were collected from the bottom and analysed for virus infectivity, core protein levels and viral RNA copies, respectively.

RNA quantification by reverse-transcription PCR (RT-PCR)

RNA was isolated from cells by RNAeasy Kit (Qiagen) as recommended by the manufacturer. HCV-specific RNA and GAPDH (glyceraldehyde-3-phosphate dehydrogenase)-specific mRNA were determined as described previously [6]. HCV RNA and GAPDH were quantified using a Light Cycler 480 II (Roche, Mannheim, Germany) .

MTT viability assay

Cell viability was measured using an MTT assay according to the manufacturer instructions. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was purchased from Life Technologies (Life Technologies GmbH, Darmstadt, Germany).

Propidium Iodide (PI) staining

Cytotoxicity was measured using propidium iodide (PI) staining. Briefly, cells were incubated with the desired drug concentrations for 48h. Medium was collected and adherent cells were trypsinized for 5 minutes at 37°C and centrifuged, together with the culture medium, at 4°C 300g for 5 minutes. The pellet was washed with cold PBS. Cells were resuspended in 350µL of fresh media and conserved on ice. 600ng of PI (Life Technologies GmbH, Darmstadt, Germany) were added to the cell suspension before measuring and the mixture was incubated on ice for 2 minutes and transferred to 7.5mL tubes for immediate FACS analysis. 488nm laser was used for excitation.

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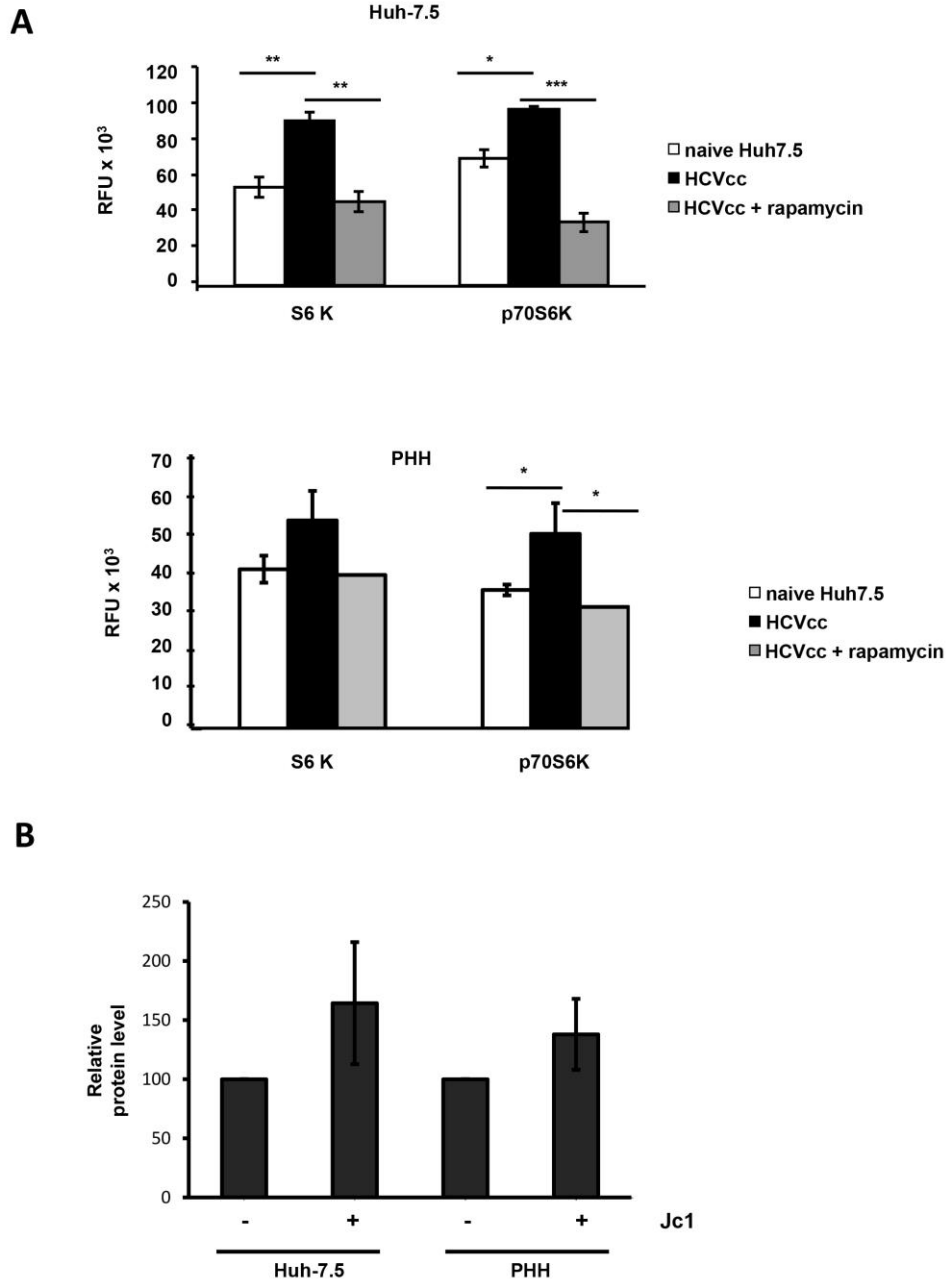
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Supplementary Figures :

Figure S1 mTOR signaling is active during HCVcc infection

A) Huh-7.5 cells (upper panel) or PHH (lower panel) were transfected with HCVcc and treated with 0.1 µg/ml rapamycin for 48h. Phosphorylation of mTOR and the downstream protein S6K was detected by a chemiluminescent assay. Phosphorylation was measured in relative fluorescence units (RFU). Mean values of 3 independent experiments were shown. (B) Huh-7.5 cells and PHH were infected with HCVcc. After 48 hours, mTOR protein expression was measured by Western blot. Mean values of three independent experiments are shown.



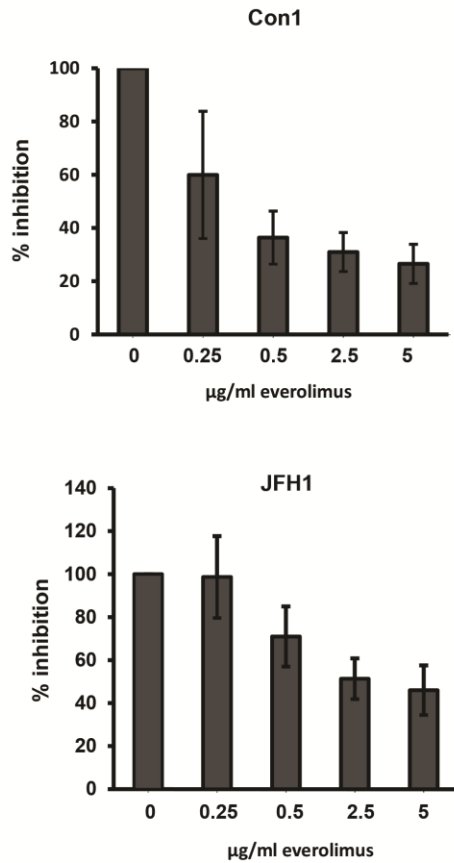
Suppl. Figure 1

Figure S2

(A) Huh7.5 cells were electroporated with subgenomic RNA of Con1 or JFH1, seeded and after 4 hours everolimus was added in increasing concentrations. After 48 hours replication was measured by luciferase assay. Mean values of three independent experiments performed in duplicates are shown.

(B) A Huh-7.5 cell line stably expressing Luc Ubi Neo JFH1 NS3-3' was treated with 0.1 $\mu\text{g/ml}$ rapamycin. After 48 hours cells were lysed and replication was measured by luciferase assay. Mean values of three independent experiments are shown.

A



B

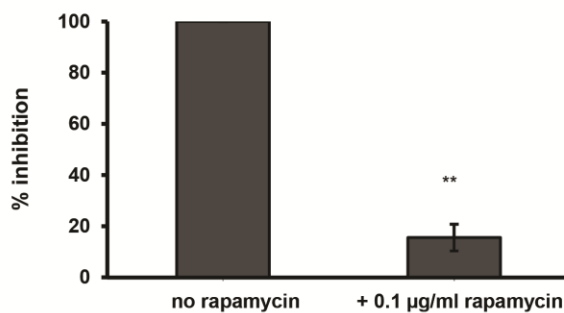


Figure S3

Huh-7.5 cells were electroporated with JcR2a (genotype 2a) or SA13/5a/R2a (genotype 3) RNA and 4 h later rapamycin was added. Replication efficiency after 48 hours was assessed using a luciferase assay. Mean of three independent experiments performed in duplicates is shown. Results are displayed in means of 100% untreated control.

