Antiviral Effects of Amantadine and Iminosugar Derivatives against Hepatitis C Virus

Eike Steinmann,[1] Thomas Whitfield,[2] Stephanie Kallis,[1] Raymond A. Dwek,[2] Nicole Zitzmann,[2] Thomas Pietschmann,[1] and Ralf Bartenschlager[1]

Current therapy of chronic hepatitis C is based on the combination of pegylated interferon-α and ribavirin. In spite of 50% sustained virological response, therapy is still limited by unsatisfying success rates with genotype 1 infections and adverse side effects. One attempt to increase success rates is triple combination therapy of interferon and ribavirin with amantadine, a drug assumed to interfere with HCV p7 ion channel function. However, results from clinical trials indicate limited efficacy and the antiviral activity is unclear. In contrast, NS3 protease inhibitors have shown potent antiviral effects in clinical trials but rapid selection for drug resistance may limit their benefit. Targeting cellular factors required for HCV is therefore an attractive alternative. In this study, employing a system for production of infectious HCV particles in cell culture, we determined the antiviral effects of amantadine and iminosugar derivatives; the second of which primarily target host cell glucosidases required for folding and maturation of HCV envelope glycoproteins. We found that across a spectrum of HCV isolates and genotypes, amantadine affected neither RNA replication nor the release or infectivity of HCV particles. In agreement, p7 ion channel activity was not affected by amantadine, demonstrating that amantadine is not an HCV-selective antiviral. In contrast, a dose-dependent reduction of virus titers was achieved with iminosugars. Furthermore, HCV was rapidly eliminated from cell culture upon passage in the presence of a long alkyl chain deoxynojirimycin (DNJ). *Conclusion:* Iminosugar derivatives are potential drugs for treatment of HCV infections. (HEPATOLOGY 2007;46:330-338.)

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. Worldwide, about 170 million people are chronically HCV-infected and have a high risk of developing cirrhosis and hepatocellular carcinoma.[1] HCV is a positive-strand RNA virus belonging to the family *Flaviviridae*.[2] Its 9.6-kilobase genome is composed of the 5′ nontranslated region, an open reading frame encoding a large polyprotein, and the 3′ nontranslated region.[3] The polyprotein is cleaved into 10 individual components Core, E1, E2 building up the virus particle and most of the nonstructural proteins required for RNA replication. The transmembrane protein p7 is a small hydrophobic polypeptide located at the junction of the structural and nonstructural proteins. p7 is important for productive infection *in vivo*,[4] but dispensable for RNA replication.[5]

At present, neither a selective antiviral therapy nor a vaccine is available, and the current treatment with a combination of pegylated interferon alpha (IFN-α) and ribavirin is only effective in a fraction of patients. Although the first clinical studies with NS3 protease and NS5B polymerase inhibitors demonstrated antiviral effects, at the same time it became apparent that antiviral drug resistance rapidly develops (for review see Tomei et al.[6]). It is therefore important to target different steps in the viral replication cycle, ideally leading to treatment that is successful against all HCV genotypes.

Abbreviations: BVDV, bovine viral diarrhea virus; DGJ, deoxygalactonojirimycin; DNJ, deoxynojirimycin; ER, endoplasmic reticulum; FPV, fowl plaque virus; IC_{50}, median inhibitory concentration; N*B-DNJ,* N*-butyl deoxynojirimycin;* N*N-DGJ,* N*-nonyl deoxygalactonojirimycin;* N*N-DNJ,* N*-nonyl deoxynojirimycin; RLU, relative light units; RT-PCR, reverse-transcription PCR; $TCID_{50}$, 50% tissue culture infectivity dose.*

From the [1]Department of Molecular Virology, University of Heidelberg, Heidelberg, Germany; [2]Glycobiology Institute, Department of Biochemistry, University of Oxford, Oxford, UK.

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Address reprint requests to: Ralf Bartenschlager, Department for Molecular Virology, University of Heidelberg, Im Neuenheimer Feld 345, 69120 Heidelberg, Germany. E-mail: Ralf_Bartenschlager@med.uni-heidelberg.de; fax: (49) 6221 564570.

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Using the recently described HCV cell culture system that allows production of infectious virus in human hepatoma cells,[7-9] in this study we analyzed the antiviral effect of 2 classes of compounds targeting virus egress and entry. The first one is amantadine, a well-studied inhibitor of the influenza A virus M2 ion channel.[10] HCV p7 also exerts ion channel activity in planar lipid bilayers[11-13] that appears to be inhibited by amantadine *in vitro*[11] and in a cell culture surrogate system.[14] However, conflicting results were obtained when treating HCV-infected patients.[15]

The second class of inhibitors studied in this report are iminosugars, which are monosaccharide mimics. Glucose analog-carrying iminosugars inhibit the endoplasmic reticulum (ER)-resident α-glucosidases I and II. As a result, the hyperglucosylated envelope glycoproteins cannot interact with calnexin/calreticulin; they misfold and become substrates for ER-associated protein degradation.[16] Proteins selected for ER-associated protein degradation are retrotranslocated from the ER and degraded by cytosolic proteasomes. The dependence of the HCV envelope glycoproteins on cellular chaperones like calnexin for their folding and the successful inhibition of replication of several viruses by iminosugars argue that these compounds may also be effective against HCV.

Here, we evaluate amantadine and iminosugars as potential anti-HCV agents and present results showing that HCV replication is not affected by amantadine whereas iminosugars have potent antiviral effects.

Materials and Methods

Compounds. Amantadine hydrochloride and *N*-butyl deoxynojirimycin (*N*B-DNJ) were purchased from Sigma-Aldrich. *N*-nonyl deoxynojirimycin (*N*N-DNJ) and *N*-nonyl deoxygalactonojirimycin (*N*N-DGJ) were purchased from Toronto Research Chemicals, Inc., Canada.

Plasmids. Constructs JFH1 and JFH1/ΔE1E2 have been described recently.[7] All other HCV genomes used in this study have been described elsewhere.[17,18]

Peptide Synthesis. p7 (GT 1a, isolate H77) was synthesized by standard butyloxycarbonyl (Boc) methodology,[19] purified by preparative HPLC, and confirmed by mass spectrometry.

Channel Recordings. The artificial membrane used consists of a 4:1:1 mixture of 1,2-dioleoyl-*sn*-glycero-3 phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS) (Avanti Polar Lipids, Alabaster, AL), which were dissolved in chloroform, dried under N_2 gas, and resuspended in *n*-decane at 25 mg/ml. For channel recordings, a Delrin cup chamber system was used with an aperture diameter of 150 μm. The chambers separated by the aperture were filled with 1 mL buffer (300 mM KCl, 5 mM K^+-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.0). Formation of the bilayer and induction of channel activity was achieved using standard techniques described elsewhere.[12]

Cell Culture and Fowl Plaque Virus Inhibition Assay. Two cured Huh7 replicon cell lines designated Huh7-Lunet[20] and Huh7.5[21] were used and cultured as recently described.[18] Cytotoxicity was determined by using a water-soluble tetrazolium salt (WST) assay according to the manufacturer's instructions (Roche, Mannheim, Germany). For fowl plaque virus (FPV) inhibition assay, we preincubated Huh7-Lunet cells for 30 minutes with amantadine at different concentrations, washed them with medium, and infected them for 1 hour with serial dilutions of FPV in the presence of different concentrations of amantadine. We cultured cells in the appropriate drug dilution and stained them 3 days later with a solution of 1% crystal violet, 3.6% formaldehyde, 1% methanol, and 20% ethanol.

HCV Replication and Infection Assay. We assessed possible effects of amantadine and iminosugar derivatives on HCV RNA replication using Huh7-Lunet and Huh7.5 replicon cells, which carry a JFH1-based selectable subgenomic luciferase replicon (V. Lohmann, unpublished data), analogous to the design described recently.[22] Infection studies based on luciferase reporter viruses have been described elsewhere.[18]

Immunohistochemical Staining, Virus Titration, and Indirect Immunofluorescence. We determined the titers of infectious virus by using a limiting dilution assay on Huh7.5 cells with a few minor modifications; and we determined the 50% tissue culture infectivity dose ($TCID_{50}$), as described.[8] The indirect immunofluorescence method has been described elsewhere.[18]

Metabolic Radiolabeling of Proteins, Immunoprecipitation, and Deglycosylation. We transfected Huh7.5 cells with Jc1 or a subgenomic replicon and after 4 hours we added iminosugar derivatives to the cell culture medium. We washed the cells 24 hours later, then starved them in methionine-free medium for 60 minutes, and incubated them overnight with [^{35}S]methionine/cysteine–containing medium in the presence of the drug. We used cell lysates for immunoprecipitation as described elsewhere[23] by using the E2-specific antibody AP33.[24] For deglycosylation studies, we treated the immunocomplexes with 2000 U endoglycosidase H (New England Biolabs) as recommended by the manufacturer. We stopped the deglycosylation by adding an equal volume of protein sample buffer. We analyzed the immunocom-

plexes by SDS-PAGE and we detected the separated proteins by autoradiography.

Quantitative Detection of HCV Core Protein and RNA. We detected the Core protein using the Trak-C-Core enzyme-linked immunosorbent assay (ELISA) as recently described.[25] We quantified the HCV RNA using reverse-transcription PCR (RT-PCR) employing an ABI Prism 7000 Sequence Detector (Applied Biosystems, Darmstadt, Germany). Reactions were carried out in 3 stages exactly as described.[26]

Results

No Effect of Amantadine on HCV RNA Replication, Virus Release, or Infectivity of Virus Particles. Before addressing the impact of amantadine on HCV replication, we set up an assay for FPV as a positive control for virus inhibition by amantadine in Huh7 cells as described.[27] We found that FPV was inhibited by amantadine in a dose-dependent manner both in Huh7-Lunet and in Huh7.5 cells (Fig. 1A; and data not shown for Huh7.5 cells). Due to their very high permissiveness for HCV RNA replication, Huh7-Lunet cells were primarily used for transfections, whereas Huh7.5 cells, which are highly infectible and support rapid virus spread,[28] were used for infection experiments with authentic wild-type genomes. To determine whether amantadine has an effect on HCV RNA replication, we transfected Huh7-Lunet cells with the Luc-JFH1 genome and measured HCV RNA replication by luciferase assays.[18] The addition of 5 or 10 μg/ml amantadine to the cell culture medium had no effect on Luc-JFH1 replication, demonstrating that amantadine did not affect HCV RNA replication or cell growth (Fig. 1B). Cytostatic and cytotoxic effects were only observed at amantadine concentrations $\geq$20 μg/ml by using the WST cell proliferation assay (data not shown). The release of core protein into the cell culture supernatant, a surrogate marker for virus particle formation, was also not affected by amantadine (Fig. 1C). Moreover, upon inoculation of naive cells with the reporter virus prepared in the presence of increasing amounts of amantadine, the compound did not interfere with HCV infection (Fig. 1D), indicating that amantadine does not affect replication, assembly, release, or infectivity of virus particles.

Recent studies have suggested that amantadine affects the function of genotype 1b p7.[11] Since JFH1 is a genotype 2a isolate, the failure of amantadine's antiviral action could have been due to genotypic differences. To address this possibility, we repeated the virus release and infectivity assay by using chimeric genomes in which the region encoding core to the N-terminus of NS2 of JFH1 is replaced by the analogous region of H77 (genotype 1a), Con1 (genotype 1b), or J6 (genotype 2a) (Fig. 2).[17] Four hours after transfection, we added amantadine at 2 concentrations to the culture medium and we quantified infectivity released 48 hours posttransfection using a limiting dilution assay performed in the presence of the drug (Fig. 2A). Although the different viruses grew to varying peak titers,[17] we observed no adverse effect of amantadine on the production of infectious genotype 1a, 1b, or 2a virus particles.

We also analyzed infectivity of the genotype 2a particles produced in the presence of amantadine in immunofluorescence studies and found no impairment (Fig. 2B). About 90% of cells could be infected with Jc1 irrespective

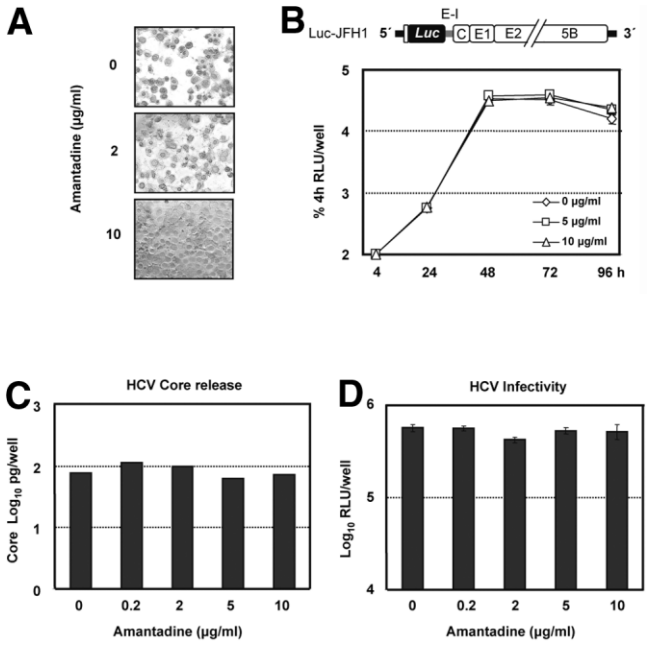

Fig. 1. Effect of amantadine on HCV RNA replication, core release, and virus infectivity. (A) We infected Huh7-Lunet cells with FPV in the presence of given concentrations of amantadine. We further incubated the cells 72 hours under given drug concentration, fixed and stained. (B) A schematic representation of the Luc-JFH1 genome with a luciferase reporter gene is shown at the top. Viral proteins are expressed via an internal encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES) (E-I; light gray bar). The luciferase gene is fused in frame to the 16 N-terminal residues of HCV core. We incubated Huh7-Lunet cells transfected with the Luc-JFH1 RNA in medium containing given amantadine concentrations, lysed them at the times specified at the bottom, and determined the luciferase activity. The percentages of relative light units (RLU) per well after normalization for the 4-hour value are given. Each point is the average value of duplicate wells; the error bars show SDs of the means. (C) We quantified Core released into the supernatant of Luc-JFH1 transfected cells in the presence or absence of amantadine, employing a core-specific immunoassay. (D) We determined the infectivity present in the culture supernatant of Luc-JFH1 transfected cells by inoculation of naive Huh7.5 cells cultured in the presence of given amantadine concentrations. We determined luciferase activity 72 hours postinfection. Mean values of 2 independent wells are given. Error bars show SDs of the means.

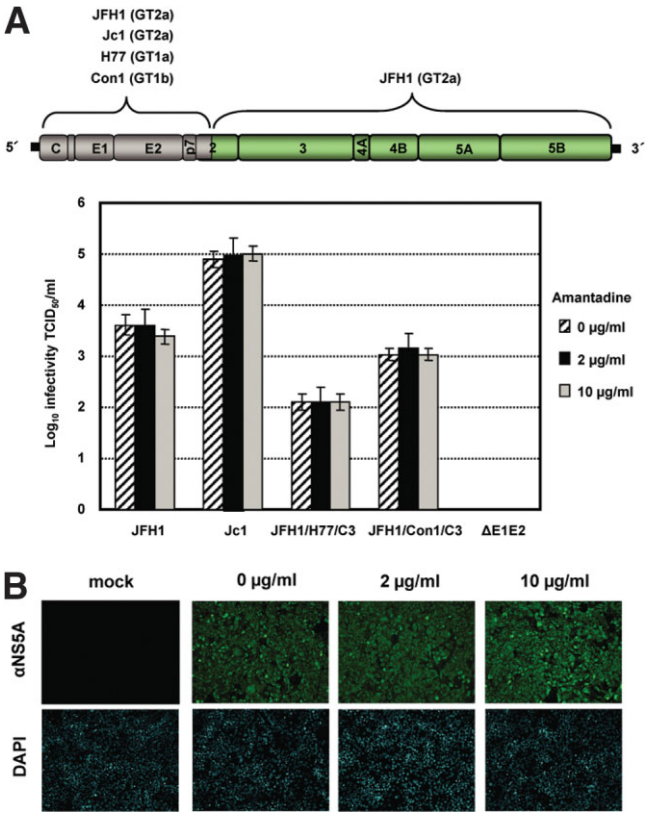

Fig. 2. Effect of amantadine on the production of infectious HCV chimeras. (A) We transfected the chimeric genomes shown at the top into Huh7-Lunet cells, and cultured them in medium supplemented with the given amantadine concentrations. We determined the infectivity released after 48 hours by $TCID_{50}$ assay in the presence of the drug. (B) We grew Huh7.5 cells on cover slips and infected them at a multiplicity of infection of 1 $TCID_{50}$/cell with Jc1 virus produced in the presence of amantadine. After 72 hours incubation in medium containing the given amantadine concentrations, we visualized the infected cells by NS5A-specific immunofluorescence (green). We counterstained the nuclear DNA with 4′,6′-diamino-2-phenylindole (DAPI) (blue).

of whether virus production and infection was conducted in the presence or absence of amantadine. These findings demonstrate that amantadine does not affect assembly, release, or infectivity of HCV particles derived from genotype 1 and 2 isolates.

Effect of Iminosugar Derivatives on HCV RNA Replication. Given the recent demonstration that antiviral compounds targeting the NS5B polymerase and the NS3/4A protease lead to rapid selection for resistance both in cell culture and in vivo, we sought to validate iminosugars primarily targeting cellular enzymes and thus reducing the risk for resistance development, as a novel approach to interrupt the HCV replication cycle. Using the HCV surrogate model bovine viral diarrhea virus (BVDV), we previously showed that deoxynojirimycin (DNJ)-containing iminosugars such as *N*B-DNJ and *N*N-DNJ (Fig. 3A) are potent antivirals inhibiting ER α-glucosidases I and II.[29] In addition, long alkyl chain derivatives (*N*N-DNJ and *N*N-DGJ; Fig. 3A) also inhibit HCV p7 ion channel activity in vitro, whereas the short alkyl chain derivatives (*N*B-DGJ, *N*B-DNJ) are inactive in this assay.[12]

As DNJ-derived iminosugar derivatives target cellular enzymes, we first wanted to rule out any effect of these compounds on host cell growth and HCV RNA replication, which are tightly coupled.[30] We treated Huh7.5 and Huh7-Lunet cells harboring an autonomously replicating JFH1 luciferase reporter construct (Fig. 3B) with differ-

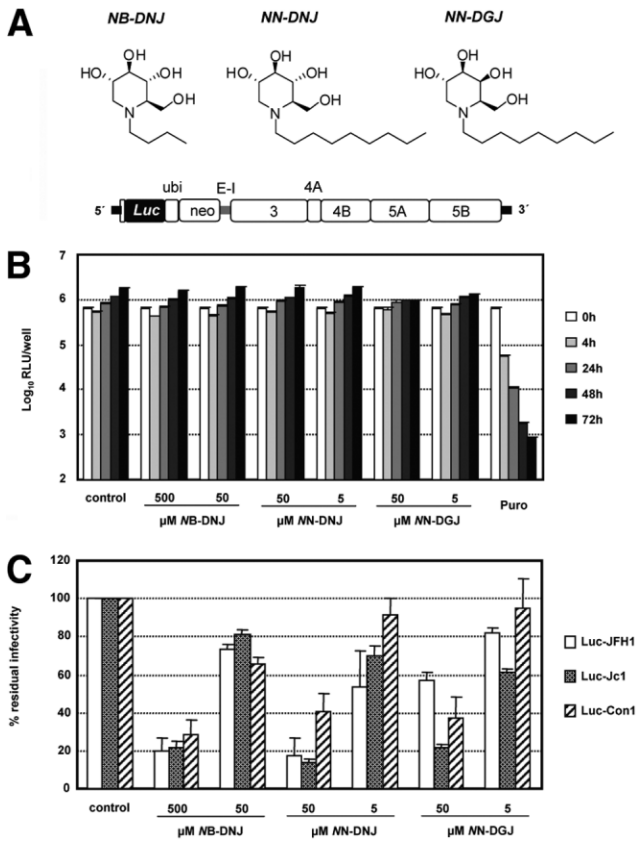

Fig. 3. Effect of iminosugar derivatives on HCV RNA replication and infectious virus production. (A) Chemical structures of iminosugar derivatives used in this study. (B) Schematic representation of a subgenomic selectable JFH1 replicon directing the expression of the firefly luciferase fused in-frame to ubiquitin (ubi) and the selectable marker (*neo*). We incubated Huh7.5 cells carrying this replicon with iminosugar derivatives at given concentrations and monitored the HCV RNA replication by measuring reporter activity at the times specified on the right. As a control for cytotoxicity, we grew replicon cells in the presence of puromycin (4 μg/mL). Mean values of duplicate wells and the SEMs are given. (C) We used Luc-JFH1, Luc-Jc1, or Luc-Con1 viruses produced in the presence of given iminosugar concentrations to infect naive Huh7-Lunet cells. After incubation for 72 hours in the presence of the same inhibitor concentrations, we harvested the cells and quantified the infection by luciferase assay. We normalized the infection data using the intracellular luciferase expression of the producer cells; the data are shown as the percentage of RLU relative to RLU obtained with the inhibitor-free control. Mean values of 3 independent experiments are given. Error bars represent the SDs of the means.

ent iminosugar derivatives for 72 hours. We selected the concentrations according to a recent report on BVDV.[29] Neither compound affected luciferase expression, ruling out an interference of the iminosugars with HCV RNA replication and host cell growth (Fig. 3B; and data not shown for Huh7-Lunet cells).

Iminosugar Derivatives Prevent the Production of Infectious Particles of Different Genotypes. We assessed the impact of iminosugar derivatives on production of infectious HCV particles using JFH1 and chimeric reporter viruses. After transfection of Huh7-Lunet cells and incubation with 2 different iminosugar concentrations, we harvested supernatants and used them to inoculate naive Huh7-Lunet cells. After 72 hours inoculation with the corresponding inhibitor, we determined the luciferase expression. Treatment of Luc-JFH1 transfected cells with *N*B-DNJ and *N*N-DNJ resulted in a dose-dependent reduction of virus amounts down to 20% of the control value at 500 and 50 μM, respectively (Fig. 3C). In contrast, the inhibitory effect of *N*N-DGJ was only moderate (60% reduction). We observed comparable reductions with the genotype 2a chimeras Luc-Jc1 (Fig. 3C) and a genotype 1a chimera (not shown), whereas the *N*N-compounds were slightly less effective with the genotype 1b chimera (Fig. 3C). These results demonstrate that different HCV genotypes are susceptible to iminosugar derivative treatment.

To investigate which step in the viral life cycle is affected by these compounds, we incubated Luc-Jc1 transfected cells with iminosugars and determined the release of core protein and the infectivity of released particles. As shown in Fig. 4A, the reduced amounts of extracellular core correlated well with the reduction of infectious virus titers, indicating that iminosugar derivatives primarily impair virus assembly or release.

To determine whether iminosugar derivatives inhibit ER-α-glucosidases in cellulo, we analyzed the biosynthesis and processing of HCV envelope glycoproteins. Electrophoretic mobility of E1 and E2 isolated from cells after treatment with *N*B-DNJ and *N*N-DNJ was slightly reduced, arguing for an inhibition of *N*-linked glycan trimming and hyperglucosylation of the proteins (Fig. 4B). As expected, DGJ derivatives that do not inhibit ER-α-glucosidases did not lead to an altered electrophoretic mobility of E proteins. None of the inhibitors affected viral protein synthesis and processing of the polyprotein precursor, corroborating the lack of cytotoxicity at the chosen concentrations.

Next, we analyzed whether iminosugar treatment affects the HCV infection process; therefore, we used Huh7-Lunet cells that were treated with iminosugar derivatives only during the 4 hour period of inoculation with Luc-Jc1 viruses that had been produced in the absence of the drug. After a washing step, we determined virus infectivity by measuring luciferase expression 72 hours after infection. Under these conditions, *N*B-DNJ did not affect infection, whereas *N*N-DNJ and *N*N-DGJ reduced HCV entry to 40% and 20% of the control virus, respectively (Fig. 4C). These results indicate that *N*N-DNJ and *N*N-DGJ also interfere with HCV entry into host cells, similar to what was observed with BVDV.[29]

As an additional assay to visualize the inhibition of HCV spread by iminosugars, we infected Huh7.5 cells with Jc1 virus lacking a reporter gene, and after culturing the cells for 72 hours in iminosugar-containing medium, we analyzed the cells using immunofluorescence. As shown in Fig. 5, more than 90% of the cells were positive for NS5A when the cells were cultured in the absence of iminosugar, whereas this number was greatly reduced in case of viruses grown in the presence of *N*B-DNJ, *N*N-DNJ, and *N*N-DGJ. These results demonstrate that iminosugar derivatives efficiently suppress spread of HCV.

Clearance of Infectious HCV from Cell Culture by Iminosugars. To determine if these compounds are capable of clearing HCV from cell culture, we performed 5 passages of Jc1 in the presence of iminosugar derivatives. After each passage, we quantified residual extracellular viruses by inoculation of naive Huh7.5 cells and we determined cell-associated HCV RNA copies by quantitative RT-PCR. Passaging of Jc1 viruses in the absence of iminosugar derivatives was efficient and virus titers were constantly high, yielding between 10^8 and 10^9 RNA copies per well (Fig. 6). Virus passage at 500 μM *N*B-DNJ led to a strong reduction of virus titers, reaching almost background levels after the third passage, and after 5 passages no infectivity was detectable in the respective supernatant (Fig. 6A). An even higher potency was found in the case of high-dose *N*N-DNJ treatment, and even at the low dose, virus titer dropped 10-fold after 5 passages (Fig. 6B). Virus passage in the presence of 50 μM *N*N-DGJ reduced HCV RNA levels to background and no virus infectivity was found. An almost 50-fold reduction in HCV RNA copy numbers was achieved after 5 passages in medium containing only 5 μM *N*N-DGJ (Fig. 6C). These data demonstrate the antiviral potency of certain iminosugars.

No Inhibition of p7 Ion Channel Activity* In Vitro *at Noncytotoxic Concentrations of Amantadine. Having evaluated the antiviral activity of amantadine and iminosugars on HCV replication in cell culture, we characterized these compounds for p7 inhibition by using an *in vitro* assay for 2 reasons. First, it has been described that amantadine blocks p7 function *in vitro*, which contrasts our data obtained in cell culture; second, for some of

the iminosugars an inhibition of p7 function has been described, raising the question of whether part of the inhibition we observed is due to blocking p7 function. We therefore reconstituted chemically synthesized HCV p7 in artificial lipid membranes and measured its ion channel activity at increasing concentrations of amantadine or one of the iminosugar derivatives.

p7 ion channel activity was not affected up to a concentration of 10.3 μg/ml amantadine (Fig. 7A and B), confirming the results obtained in cell culture (Fig. 2B). *In vitro* testing of higher amantadine concentrations, which affected Huh7 cell viability and therefore could not be analyzed in cell culture, gave rise to a sigmoidal dose-response curve, which allowed an estimate of 2 approximate IC_{50} values, at 12.9 μg/ml (69 μM) and 47.5 μg/mL (253 μM) (Fig. 3C). Although these supraphysiological amantadine concentrations affect ion channel activity, even at the highest concentrations used (188 μg/ml, corresponding to about 1 mM), approximately 5% of the initially recorded average bilayer current could still be observed (Fig. 7A). Thus, total inhibition of p7 was not achieved. Similarly, the short alkyl chain iminosugar *N*B-DNJ did not significantly inhibit p7 channel activity, consistent with *N*B-DNJ exerting its antiviral effect by inhibiting ER α-glucosidases. In contrast, the long alkyl chain iminosugars *N*N-DNJ and *N*N-DGJ were much more potent, with IC_{50} values of 5.8 μg/ml (31 μM) and 6.3 μg/ml (34 μM), respectively (Fig. 7B). Unlike amantadine, these iminosugar derivatives also achieve complete inhibition of p7 ion channels in the artificial membrane, at 15 μg/ml (80 μM) and 18.8 μg/ml (100 μM), respectively.

Discussion

In this study, we characterized the potential antiviral effects of amantadine and iminosugar derivatives on HCV by using the recently developed virus production system,[7-9] and we found that amantadine does not affect replication, particle production, or infectivity of JFH1 and several JFH1-derived chimeras. Amantadine is well known to inhibit influenza A virus cell entry by interacting with the viroporin M2 and it has proven efficacy in preventing influenza A virus infections. In 1997, Smith[31] reported that amantadine treatment was effective in inducing a sustained biochemical and virological response in patients with chronic hepatitis C who had previously

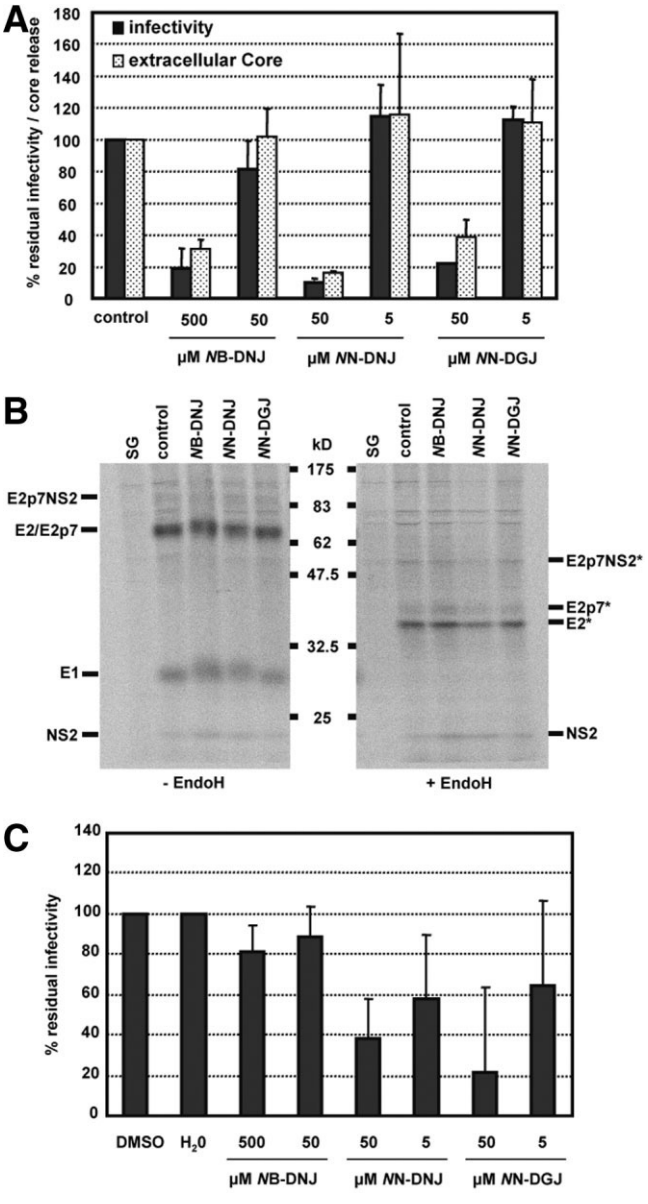

Fig. 4. Effect of iminosugar derivatives on virus particle production. (A) Four hours after transfection of Luc-Jc1 into Huh7-Lunet cells, we added media containing iminosugar derivatives at given concentrations. After 48 hours, we determined the release of core protein by core-specific enzyme-linked immunosorbent assay (ELISA) (dotted bars). In addition, we determined infectivity in supernatants (black bars). We normalized the data using the intracellular luciferase expression at 48 hours posttransfection; the data are shown as percentages relative to the yield achieved in the absence of inhibitor. Mean values of 4 independent experiments are given. Error bars show SDs of the means. (B) We transfected Huh7.5 cells with Jc1 or a subgenomic replicon (SG) and after 4 hours we mock-treated the cells (control) or treated them with 500 μM *N*B-DNJ, 50 μM *N*N-DNJ, 50 μM *N*N-DGJ. At 24 hours posttransfection, we radiolabeled the proteins in the presence of the drug and we isolated the E proteins by immunoprecipitation under nondenaturing conditions. We either mock-treated the immunocomplexes (− EndoH) or deglycosylated them by endoglycosidase H treatment (+ EndoH). After SDS-PAGE, we detected the proteins by autoradiography. The deglycosylated forms of the proteins are labeled with an asterisk (right panel). (C) We used Luc-Jc1 to inoculate naive Huh7-Lunet cells in the presence of indicated inhibitors for 4 hours. We then added inhibitor-free medium and we quantified residual infectivity by assessing the amount of reporter activity expressed in target cells 72 hours after inoculation. Mean values of 3 independent experiments are given. Error bars represent the SDs of the means.

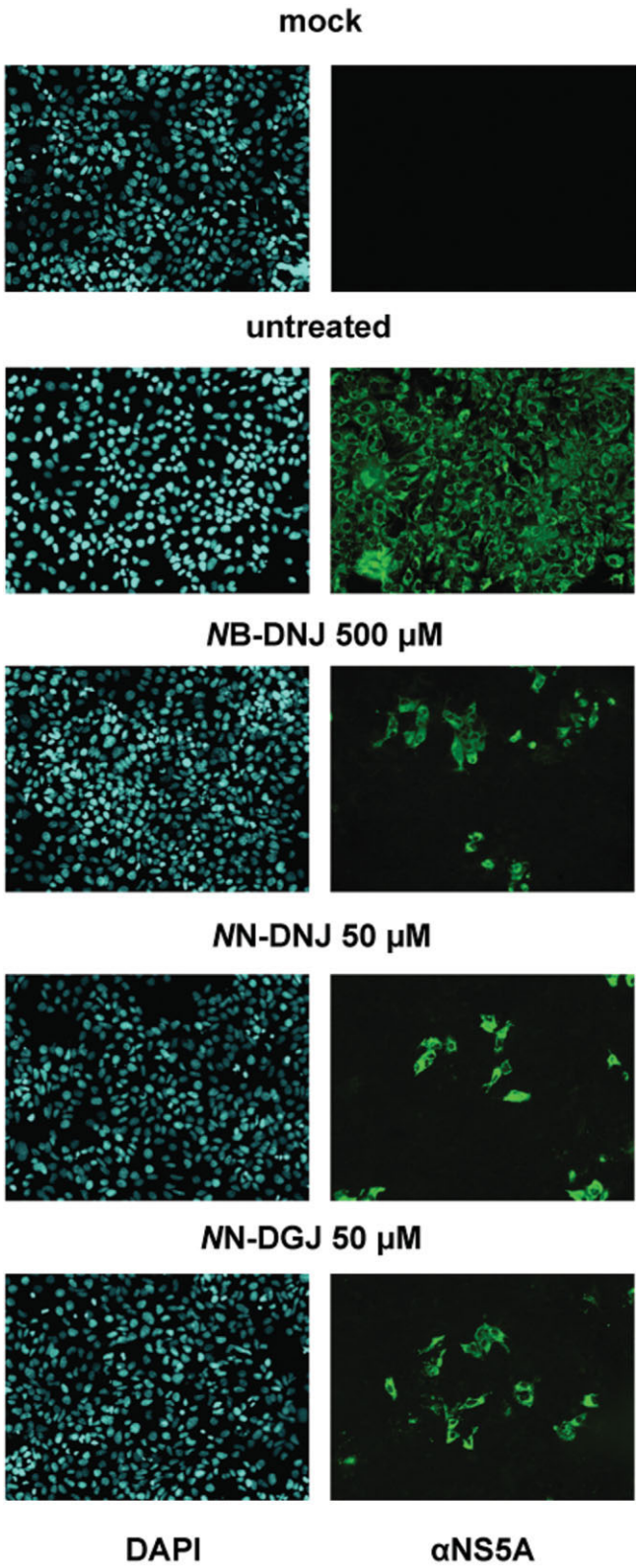

Fig. 5. Impact of iminosugar derivatives on Jc1 spread. We infected Huh7.5 cells with Jc1 at a multiplicity of infection of 1 $TCID_{50}$/ml. After 4 hours, we replaced the medium with fresh medium containing the indicated iminosugar derivatives. Three days later, we visualized the infected cells by NS5A-specific immunofluorescence (green). We counterstained the nuclear DNA with 4′,6′-diamino-2-phenylindole (DAPI) (blue).

failed to respond to interferon monotherapy. However, the possible benefit of amantadine monotherapy or in combination with IFN-α/ribavirin therapy could not be confirmed in many other studies.

Further evidence for an inhibition of HCV by amantadine came from studies showing an inhibition of p7 by amantadine in artificial membranes[11] and in a cell-based hemadsorption surrogate assay.[14] However, we could not confirm this inhibition in an authentic cell culture system using genotype 1a, 1b, and 2a virus particles. When using a p7 *in vitro* assay, p7 ion channel activity was not inhibited by amantadine concentrations up to 10.3 µg/ml. Channel inhibition was only observed at higher concentrations (we tested up to 1 mM) that could not be applied in cell culture due to the cytotoxic effects of the drug. At

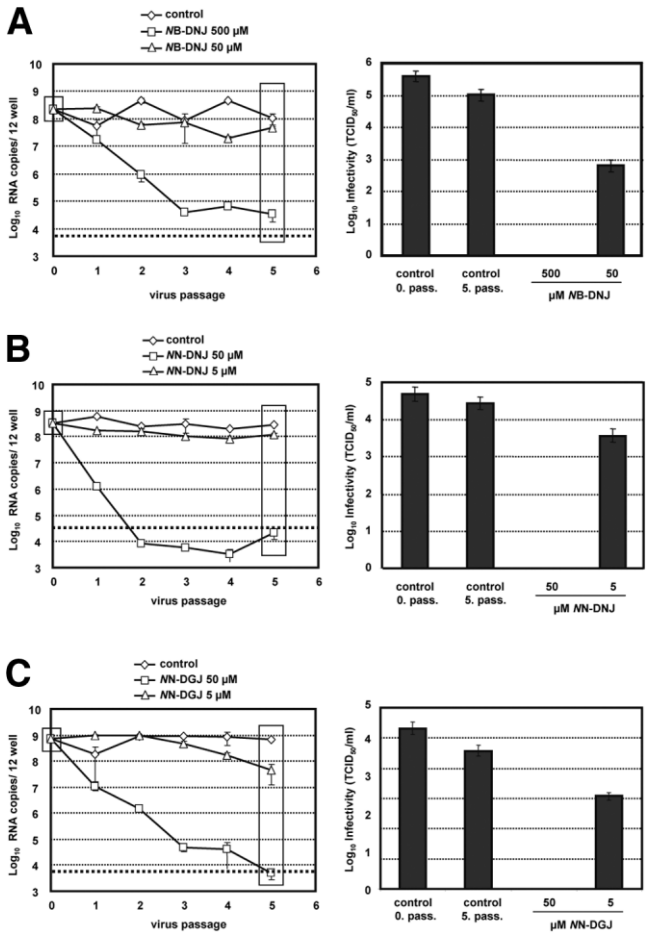

Fig. 6. Elimination of Jc1 from cell culture by iminosugar derivatives. We infected Huh7.5 cells with Jc1 for 48 hours in the presence of given concentrations of *N*B-DNJ (A), *N*N-DNJ (B), and *N*N-DGJ (C). We passaged supernatants from these cells on naive Huh7.5 cells in 48-hour intervals in the presence of inhibitors. We used aliquots of each supernatant to infect Huh7.5 cells in parallel, and 72 hours later we determined the amounts of intracellular HCV RNA by quantitative RT-PCR (left panels). In addition, we determined the infectivity titers of the original virus stock (control, 0. passage) and of supernatants of the fifth passage (5. pass.) (right panels).

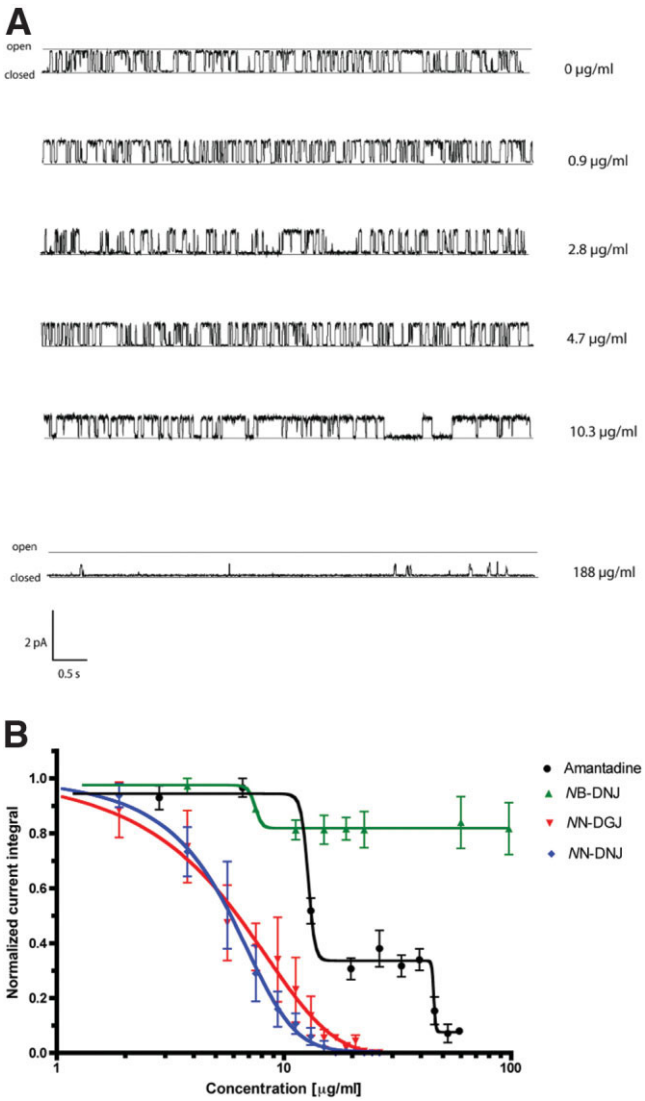

Fig. 7. Inhibition of HCV p7 by amantadine in vitro. (A) Ion channel activity of p7 at +40 mV in a 1,2-dioleoyl-*sn*-glycero-3 phosphocholine (DOPC):1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine] (POPS):1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) (4:1:1) bilayer. We added amantadine from a stock solution to reach the final concentrations indicated on the right-hand side. Channel opening and closing states are indicated by gray lines in the top and bottom traces. The current amplitude is shown in picoamperes (pA). We recorded ion channel traces for each drug concentration for 5 to 10 minutes, 7 seconds of which is shown here. (B) For each data point, ion channel activity is represented as normalized current integral (*y* axis) measured over 5 minutes, which comprises up to 11,000 single opening events. We analyzed the amantadine and iminosugar derivatives were analyzed at the indicated concentrations. The mean of 3 independent experiments and SDs are shown. The *y* axis has been shifted to clearly show the first data point.

concentrations higher than 10.3 µg/ml, inhibition followed a 2-site competition behavior with 2 approximate IC_{50} values of 69 µM and 253 µM, respectively. The same pattern was obtained with synthetic p7 of a different HCV isolate, arguing that the biphasic inhibition is a general property of HCV p7 (N. Zitzmann, unpublished observation).

In contrast to our results, a complete block of the p7 ion channel by 1 µM (0.188 µg/ml) amantadine has been described by Griffin et al.[11] This discrepancy may be due to the different p7 proteins used. Although we used an authentic p7 peptide synthesized *in vitro*, Griffin et al.[11] use GST-p7 and GST-HIS-p7 fusion proteins that may have different properties.

In addition to amantadine, in this study we analyzed the antiviral potency of iminosugars on HCV. Similar to what has been described with BVDV,[29] we did not observe an effect of these compounds on HCV RNA replication. Furthermore, a dose-dependent reduction in the amount of chimeric infectious viruses of genotype 2a and 1b could be demonstrated, similar to what has been described for HBV, dengue virus, yellow fever virus, and Japanese encephalitis virus.[32,33] Thus iminosugars have a broad antiviral spectrum supporting their primary action on a cellular rather than a viral target. Moreover, we found that *N*N-DNJ and *N*N-DGJ have an effect on HCV entry, although this inhibition is moderate. In case of HIV, it has been shown that *N*B-DNJ altered the glycosylation of gp120, resulting in an impairment of virus entry at a step after CD4 binding.[34] It remains to be determined how HCV infectivity is decreased. We also found a reproducible effect of certain iminosugars on HCV E-protein glycosylation. Although this effect was rather moderate, it should be kept in mind that in addition to altering E-protein glycosylation to an extent that is visible on SDS-PAGE, slight changes in glycosylation also affect E1-E2 complex assembly. Formation of this complex, however, is required for full infectivity.

By using the intragenotypic Jc1 chimera,[17] we demonstrated that iminosugars interfere with virus spread and they can be used to clear HCV from cell cultures, most efficiently with *N*N-DNJ. These results confirm data obtained with BVDV, corroborating that *N*N-DNJ is more active than the butyl *N*B-DNJ derivative.[29] This stronger antiviral effect might be explained by the presence of a long alkyl side chain that may confer an HCV p7 inhibitory effect.[12] Thus compounds combining a DNJ headgroup with a long alkyl chain side chain, such as *N*N-DNJ, may have 2 mechanisms of action: inhibition of ER α-glucosidases and blocking p7 ion channel activity.

In summary, our results demonstrate that amantadine does not interfere with the HCV replication cycle. In contrast, by focusing on viral morphogenesis and infectivity as novel targets for HCV therapy, we show that iminosugar derivatives efficiently inhibit virus propaga-

tion in cell culture and thus may be a useful strategy for future therapeutic intervention.

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