Reduction of the infectivity of hepatitis C virus pseudoparticles by incorporation of misfolded glycoproteins induced by glucosidase inhibitors

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INTRODUCTION

Hepatitis C virus (HCV) is a major cause of human illness, with more than 170 million people infected worldwide (Davis et al., 2003; Seeff, 2002). Patients with chronic hepatitis C are at risk of developing liver diseases including cirrhosis and hepatocellular carcinoma (Liang & Heller, 2004). With current therapies, which combine pegylated alpha interferon and ribavirin, around 40% of patients do not show a sustained virological response to treatment (Foster, 2004; Pawlotsky, 2005). Therefore, new therapeutic options are necessary to eradicate HCV infection. With the recent development of easy-to-handle in vitro HCV replication systems (Lohmann et al., 1999), HCV antiviral research has finally taken off and many inhibitors of HCV enzymes are being studied at preclinical up to advanced clinical trial levels (Foster, 2004; Pawlotsky, 2005). Although such highly specific enzyme-targeting antivirals are expected to be beneficial, there may be problems with drug resistance, as has been well documented for human immunodeficiency virus and hepatitis B virus therapies (Johnson et al., 2005; Locarnini et al., 2004).

We are investigating the inhibition of viral assembly and infectivity as a complementary approach. By using bovine viral diarrhea virus (BVDV) as a surrogate model of HCV, we demonstrated previously that glucose analogue-derived iminosugars, i.e. deoxynojirimycin (DNJ) derivatives, inhibit BVDV assembly. We showed that DNJ derivatives, which are inhibitors of endoplasmic reticulum (ER) α-glucosidases I and II, prevent the correct processing of N-linked glycans on nascent polypeptide chains and, consequently,
the calnexin-dependent folding and assembly of viral envelope glycoproteins E1 and E2 (Branza-Nichita et al., 2001). The resulting misfolding/missassembly leads to an accelerated degradation of E1–E2 heterodimers and a decrease in the availability of these complexes for viral budding and morphogenesis. The antiviral activity of a DNJ derivative containing a nine-carbon alkyl chain branched on the ring nitrogen (N-nonyl-DNJ; NN-DNJ) was greater than that of DNJ derivatives containing a shorter alkyl chain (e.g. N-butyl-DNJ; NB-DNJ) (Durantel et al., 2001; Zitzmann et al., 1999).

Recently, two methods, enabling either the complete replication of HCV with production of infectious HCV (JFH1 strain) particles (HCVcc) in Huh7 cells, or the production of pseudotyped particles (HCVpp), which are formed by incorporation of native HCV envelope glycoprotein heterodimers (E1–E2) onto retroviral core particles, have been described (Bartosch et al., 2003; Lindenbach et al., 2005; Pohllmann et al., 2003; Wakita et al., 2005; Zhong et al., 2005). The latter model was validated to study glycoprotein incorporation onto particles, their functionality in terms of cell entry, and neutralization of HCV cell entry (Bartosch et al., 2003; Callens et al., 2005; Goffard et al., 2005; Pohllmann et al., 2003). With such development, it has become possible to measure the antiviral effect directly and to delineate the mechanism of action of glucosidase inhibitors against HCV itself.

In this report, we evaluated DNJ derivatives as inhibitors of HCV assembly and infectivity by using both models and studied the mechanism of action of these inhibitors by using the pseudotyping model. We show that, as in the BVDV surrogate model, DNJ derivatives inhibit ER α-glucosidases in cellulo, resulting in misfolding of HCV glycoproteins. We show that iminosugars reduce the incorporation of E1–E2 complexes into HCVpp and modify their N-glycosylation patterns. Eventually, we demonstrate that the infectivity of HCVpp released under iminosugar treatment is reduced and that this reduction in infectivity is probably due to the incorporation of misfolded HCV envelope glycoproteins in secreted HCVpp.

**METHODS**

**Cells and inhibitors.** Huh7 cells (kindly provided by Dr C. Seeger, Fox Chase Cancer Center, Philadelphia, PA, USA) and Huh7.5 human hepatoma cells (kindly provided by Dr C. Rice, Rockefeller University, New York, USA), as well as HEK-293T human kidney cells (ATCC), were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin at 37°C in a 5% CO2 atmosphere.

NB-DNJ and NN-DNJ were provided by United Therapeutics Corporation. Other inhibitors, including deoxyxymannojirimycin (DManJ), castanospermin and tunicamycin, were purchased from Sigma-Aldrich.

**Antibodies.** Monoclonal antibodies (mAbs) anti-E1 (A4) and anti-E2 (H14, H35, H47, H48 and H53) were described previously (Chouchi et al., 1999; Coczquerel et al., 1998, 2001; Dubuisson et al., 1994). Rat anti-murine leukemia virus (MLV) capsid antibody R187 was purchased from the NIH. Anti-calnexin and anti-calreticulin polyclonal antibodies were purchased from Stressgen Biotechnologies. Anti-actin, as well as anti-mouse and anti-rabbit, horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Sigma.

**MTS cell-proliferation assay.** HEK-293T or Huh7.5 cells were grown and incubated in 96-well plates at 37°C in the presence of different concentrations of inhibitors (in triplicate) for 3 days. The number of proliferating cells was determined by using the CellTiter 96 AQuo reactive cell-proliferation assay (Promega) following the manufacturer’s instructions. The cytotoxic concentration 50 (CC50) was determined as the drug concentration at which half of the cells were not proliferating compared with untreated control cells.

**HCVcc production.** Plasmid pLuc-JFH1 was constructed in our laboratory as reported previously (Wakita et al., 2005), starting from the plasmid pJFH1 that was generously provided by Dr T. Wakita, Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Japan. This plasmid enables the in vitro transcription (MEGAscript kit; Ambion) of a bicistronic Luc-JFH1 RNA that contains in the first cistron a luciferase gene fused to the N-terminal part of core driven by the HCV internal ribosomal entry site (IRES), and in the second cistron the sequence encoding the complete HCV polyprotein driven by the encephalomyocarditis virus (EMCV) IRES. In vitro-transcribed Luc-JFH1 RNA was delivered to cells by electroporation as described previously (Wakita et al., 2005). HCV particles (HCVcc) containing the bicistronic Luc-JFH1 genome were harvested 4 days after electroporation, filtered through a 0.45 μm filter and used for infection of fresh Huh7.5 cells.

**Production, purification and infection of hepatoma cells with HCVpp.** HCVpp were produced in HEK-293T cells and purified from cell-culture medium as described previously (Bartosch et al., 2003). Note that a package insert contract containing a luciferase gene under control of the cytomegalovirus (CMV) promoter (pMLVψCMV-Luc) was used as reporter, rather than a GFP (green fluorescent protein) construct. Sixteen hours after transfection (p.t.) with plasmids enabling HCVpp production, the medium was replaced with fresh medium containing DNJ derivatives at the indicated concentrations. Supernatants containing pseudoparticles were harvested 24 h later, i.e. 40 h p.t., filtered through 0.45 μm pore-sized membranes and stored at −80°C before use in infection of Huh7 cells. For some experiments, partially purified particles were obtained by ultracentrifugation of supernatants through a 1.5 ml 20% sucrose cushion in an SW41 Beckman rotor at 30000 r.p.m. for 2 h at 4°C. Viral pellets were suspended overnight at 4°C in PBS (100-fold concentration). In some experiments, transfected HEK-293T cells were pulse-labelled metabolically with 35S. Infection of Huh7 cells (105 cells per well in a 12-well plate) was done with diluted (1:2 up to 1:10) HCVpp in the absence of any entry enhancer (e.g. polybrene). Seventy-two hours post-infection (p.i.), cells were lysed in 1x passive lysis buffer (Promega) and luciferase activity was monitored by using the Renilla luciferase assay system (Promega).

**Protein analysis and immunoprecipitation.** For intracellular analysis, cells were lysed with either TNET [10 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100] or CHSE [2% CHAPS, 50 mM HEPES (pH 7.5), 200 mM NaCl, 2 mM EDTA] buffers for Western blotting or immunoprecipitation, respectively. Proteins were quantified by using Bradford reagent (Sigma). For extracellular analysis, concentrated HCVpp, purified by sucrose-gradient centrifugation, were used. For Western blot analysis, ER- or HCVpp-derived proteins (20 μg) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blots were performed by using
anti-E1 (mAb A4; 1:1000 dilution), anti-E2 (mAb H47; 1:1000 dilution) (Cocquerel et al., 2001; Dubuisson et al., 1994) or anti-actin (1:1000 dilution) as primary antibodies, and an anti-mouse HRP-conjugated (1:10 000 dilution) secondary antibody (Sigma). Bound antibodies were detected by using the ECL system as specified by the manufacturer (Amersham Biosciences). For immunoprecipitation, an equal or equivalent amount of ER- or HCVpp-derived proteins (100 μg) diluted in 500 μl CHSE buffer was preclarified with 20 μl protein A-Sepharose (Amersham Biosciences) for 1 h at 4°C. The cleared lysates were incubated overnight at 4°C with anti-E2 (mAbs H53, H14 or H47) antibodies diluted at 1:100. Fifty microlitres of protein A-Sepharose was then added to the reaction and the incubation was continued for 1 h at 4°C. The slurry was washed three to six times in modified CHSE buffer containing 0.5% CHAPS. Immunoprecipitated complexes were eluted by boiling in SDS-PAGE loading buffer for 10 min, then analysed by SDS-PAGE. For 35S-labelled immunoprecipitated proteins, the gel was dried and exposed to film. For non-labelled immunoprecipitated proteins, Western blot analysis was performed as described above.

Transduction, infection and luciferase assay. HCVcc or HCVpp were used to infect/transduce Huh7 cells (2 × 10^4 cells cm^-2). Cells infected with HCVcc or HCVpp containing the luciferase transgene were lysed 3 days p.t. with 50 μl passive lysis buffer cm^-2 (Promega) and the luciferase activity was measured with 50 μl luciferase assay substrate (Promega) per 10 μl cell lysate, using a luminometer (Luminoskan Ascent; Thermo Labsystems).

Infectivity assay. The number of HCVpp was standardized by quantifying both E1 and E2 after serial dilution by dot-blot using non-conformation-dependent anti-E1 (A4) and anti-E2 (H47) antibodies. The dosing of MLV p30 was irrelevant, as some non-enveloped nucleocapsids are released from producer cells and this release is not sensitive to drug inhibition. The same relative numbers of particles were used to infect Huh7 cells and the infection efficacy was measured as described above by quantifying luciferase activity. Note that infection was performed in the absence of any entry-enhancing substances.

CD81 pull-down assay. CD81 pull-down experiments were performed as described previously (Cocquerel et al., 2003). Recombinant large extracellular loop of human CD81 (hCD81) protein, fused to glutathione S-transferase (GST), was pepsin-digested on glutathione-Sepharose AB beads according to the manufacturer's recommendations (Pharmacia Biotech). Standardized numbers of concentrated HCVpp, produced in the absence or presence of 500 μM NB-DNJ or 100 μM NN-DNJ, were incubated with immobilized hCD81–GST at 4°C for 1 h. After three washes with PBS, precipitates were separated by SDS-PAGE (10% gel) and analysed by Western blotting with anti-E1 (A4) and anti-E2 (H47) mAbs.

Immunogold labelling of purified HCVpp. HCVpp, concentrated through a 20% sucrose cushion as described above, were further purified on a 20–60% sucrose gradient by using an SW41 Beckman rotor at 30 000 r.p.m. for 16 h at 4°C. HCVpp in the positive fraction were then reconstituted by ultracentrifugation at 30 000 r.p.m. for 2 h at 4°C. Ten microlitres of concentrated HCVpp was loaded onto carbon-coated grids for 1 min. Then, grids were negatively stained with 2% uranyl acetate before complete drying and examination with a JEOL 1010 electron microscope. For immunogold labelling, HCVpp were first incubated overnight at 4°C with either anti-E1 (A4) or anti-E2 (H48) antibodies, diluted 1:20 in PBS. HCVpp were further incubated for 3 h at room temperature with protein A–colloidal gold (15 nm in diameter; British Biocell), diluted 1:20 in PBS. Negative staining of the HCVpp was then performed as described above.

RESULTS

Anti-HCV properties of α-glucosidase inhibitors derived from DNJ

Previously, we reported that the iminosugars NB-DNJ and NN-DNJ, which are ER α-glucosidase inhibitors, have in vitro antiviral properties against BVDV, to date the sole HCV surrogate virus used to study the inhibition of viral morphogenesis and infectivity. Very recently, a system based on the utilization of a strong, replication-competent HCV strain (i.e. JFH1 genotype 2a strain), enabling the complete replication of HCV including the in vitro production and secretion of HCVcc, was developed. By using a bicistronic construct containing a luciferase gene placed under control of the HCV IRES and the entire HCV JFH1 open reading frame placed under the control of the EMCV IRES for translation, we evaluated the overall anti-HCV activity of NB-DNJ and NN-DNJ. In vitro-transcribed Luc-JFH1 RNA was transfected into Huh7.5 cells, then cells were treated immediately with an increasing concentration of iminosugars for 72 h. At the concentrations used, no toxicity was observed in Huh7.5 cells, as determined by MTS assay. The CC50 of NN-DNJ and NB-DNJ was determined to be 235 ± 5.25 μM and >1 mM, respectively. At the concentration used, no change in luciferase activity was observed (data not shown). As the luciferase activity measured in these transfected cells correlates directly with the replication of the genome, the absence of reduction of this activity with increasing drug concentrations indicates that glucosidase inhibitors do not inhibit genome replication. HCVcc produced were subsequently used to infect new Huh7.5 cells in the absence of drugs. Seventy-two hours p.i., luciferase activity was measured on cell lysates (Fig. 1). The long-alkyl chain-containing iminosugar NN-DNJ was more potent than NB-DNJ, with an IC50 i.e. the concentration inhibiting 50% of the luciferase activity in Huh7 cells infected with virus produced in the presence of drugs compared with untreated infected cells, of <10 μM (IC50 of approx. 6.25 μM), whilst the IC50 of NB-DNJ was around 50 μM (Fig. 1). Although it is very interesting to study many aspects of HCV biology, the system based on the use of HCV JFH1 strain remains difficult to use to study the inhibition of morphogenesis and entry by iminosugars, as HCVcc production rate is limited, the nature of the particles produced is poorly known and many reagents, including conformational antibodies recognizing envelope glycoproteins, are still lacking.

Therefore, to characterize further the inhibitory activity of glucosidase inhibitors against HCV, in particular the effect on viral infectivity, we used the system enabling the production and secretion of infectious retroviral particles pseudotyped with HCV envelope glycoprotein (HCVpp). HEK-293T cells transfected with plasmid enabling the production of HCVpp were treated with increasing concentrations of iminosugars. HCVpp produced over a period of 24 h in the presence of drug were used to infect
Huh7 cells. With both DNJ derivatives tested, a significant and dose-dependent inhibition of the production and infectivity of HCVpp, as measured by luciferase activity in the lysate of infected Huh7 cells, was observed (Fig. 2). At the concentrations used, no toxicity was observed on HEK-293T cells with DNJ derivatives, as determined by MTS assay. The CC50 of NN-DNJ and NB-DNJ was 240 ± 12.5 μM and 1 mM, respectively. Consistent with results obtained with the HCVcc system, the long-alkyl chain-containing iminosugar NN-DNJ was more potent than NB-DNJ, with an IC50 of <10 μM, whilst the IC50 of NB-DNJ was around 50 μM (Fig. 2). Two types of control experiment were performed. We either omitted (no-envelope control) or replaced the plasmid encoding HCV glycoproteins with a plasmid encoding glycoprotein G of vesicular stomatitis virus (VSV). The morphogenesis of VSV was shown previously to be insensitive to glucosidase inhibitors, as its G protein is not exclusively dependent on the chaperone activity of calnexin to fold correctly (Hammond & Helenius, 1994). Whilst neither iminosugar had an effect against the production and secretion of bald ‘gag particles’ (no-envelope control), surprisingly, we observed that the production and/or infectivity of VSV-G pseudoparticles were increased by treatment with DNJ derivatives (Fig. 2). Although this result remains unexplained, it confirms that, at the concentrations used, these molecules are not toxic (i.e. the same number of producing cells are present) and therefore strengthens the demonstration of the inhibitory activity of DNJ derivatives on HCVpp production and/or infectivity.

Collectively, the results obtained with both systems (HCVcc and HCVpp) demonstrate the anti-HCV activity of glucosidase inhibitors derived from DNJ. Due to the lack of reagents to work with the HCVcc system and because the anti-HCV activity was similar with both systems, the rest of the study was performed with infectious HCVpp.

Effect of α-glucosidase inhibitors on viral envelope glycoprotein accumulation, N-glycosylation and folding in transfected HEK-293T cells

Western blotting was used to analyse the effect of iminosugars on the accumulation of E1 and E2 in the ER and their N-glycosylation. Whilst no change in the

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**Fig. 1.** Effect of DNJ derivatives on HCVcc secretion and/or infectivity. Huh7.5 cells (8×10^5 cells per 35 mm well) were transfected with in vitro-transcribed RNA obtained from pLuc-JFH1 plasmid. Six hours p.t., the medium was replaced with fresh medium containing increasing concentrations of NB-DNJ (a) or NN-DNJ (b) as shown. Three days p.t., HCVcc were harvested and used as inoculum to infect Huh7.5 cells (1×10^5 cells). Seventy-two hours p.i., cells were lysed and luciferase activity was determined by using a luminometer. Luciferase activity is not reported as an absolute value, but is calculated relative to the ‘no drug’ condition and reported on the y-axis as a percentage. The data shown represent the means of two independent experiments.

**Fig. 2.** Effect of DNJ derivatives on VSV-G and HCV pseudoparticle secretion and/or infectivity. HEK-293T cells (3×10^5 cells per 35 mm well) were transfected with a plasmid enabling the production and secretion of either VSV-G or HCV (E1–E2) pseudoparticles in the culture medium. For negative-control experiments (No env), the plasmid encoding envelope glycoproteins was omitted from the transfection. Sixteen hours p.t., the medium was replaced with fresh medium containing increasing concentrations of NB-DNJ (a) or NN-DNJ (b). Forty hours p.t., NoENVpp, VSG-Gpp and HCVpp were harvested and filtered through 0.45 μm membranes. Pseudoparticles (125 μl; one-sixteenth of the production) were inoculated on Huh7 cells (4×10^4 cells) seeded the day before. Seventy-two hours p.i., cells were lysed and luciferase activity was determined by using a luminometer. Please note that luciferase activity is not reported as an absolute value, but is calculated relative to the ‘no drug’ condition and reported on the y-axis as a percentage. The data shown represent the means of three independent experiments.
accumulation of E1 and E2 was noticed upon iminosugar treatment, the electrophoretic mobility of E1 and E2 was reduced by treatment with DNJ (Fig. 3). This reduction of mobility in PAGE is due to the retention of hyperglycosylated N-glycans (Glc3Man9GlcNAc6) in the viral envelope proteins and is a direct consequence of the inhibition in cellulo of ER α-glucosidases. Such an upwards shift was also observed in the BVDV model used previously to analyse the antiviral effect of α-glucosidase inhibitors (Branza-Nichita et al., 2001; Durantel et al., 2001).

The retention of hyperglycosylated N-glycans on a polypeptide being folded in the ER affects its interaction with calnexin (Helenius & Aebi, 2004). When the interaction with calnexin is crucial for a given protein to fold correctly, the inhibition of this interaction has a consequence on the quality of the folding. To determine whether treatment with DNJ derivatives resulted in the misfolding of HCV glycoproteins, an immunoprecipitation of E2 using the conformation-dependent mAb H53 was performed on pulse-labelled proteins from transfected HEK-293T cells treated with various concentrations of NB-DNJ and NN-DNJ. A dose-dependent reduction in the amount of E2 immunoprecipitated was observed with both drugs, NN-DNJ being once again more potent than NB-DNJ, suggesting that the folding of the H53 epitope is somehow affected by these drugs (Fig. 4a). To confirm these data, experiments were performed using non-radioactively labelled proteins. Various concentrations of NB-DNJ and NN-DNJ were used and added from 16 h p.t. until lysis of cells. Proteins were extracted 40 h p.t., then immunoprecipitated with either non-conformation-dependent H47 or conformation-dependent H53 anti-E2 mAbs to demonstrate that the reduction of the amount of E2 immunoprecipitated with H53 was indeed due to misfolding and not to a decrease in the overall amount of E2 in cell lysates.

Fig. 3. Effect of DNJ derivatives on protein accumulation and N-glycosylation. HEK-293T cells (3 × 10⁶ cells per 35 mm well) were transfected with a plasmid enabling the production of HCVpp. Sixteen hours p.t., the medium was replaced with fresh medium containing increasing concentrations of NB-DNJ or NN-DNJ. Forty hours p.t., cells were lysed and 20 μg protein was analysed by Western blot with anti-E1, anti-E2 and anti-actin antibodies (diluted 1:1000). Although the hybridization was performed sequentially, the blot shows all three proteins.

immunoprecipitated material was analysed by Western blotting using mAb H47. To demonstrate that equal amounts of cell lysate were used for the immunoprecipitation, Western blot analysis was also performed with both anti-E1 and anti-E2 antibodies. A dose-dependent reduction in the amount of E2 immunoprecipitated was observed with H53, but not with H47, confirming that the folding of the H53 conformational epitope was impaired for some E2 proteins (Fig. 4b). In the same time, no significant modification of the amount of E1 and E2 was observed (Fig. 4c), thus showing that the same amount of material was used to perform the immunoprecipitation. It is worth noting that the amount of E1 co-immunoprecipitated with E2 by a conformation-dependent anti-E2 antibody was also reduced (Fig. 4d). This result was expected, due to the interaction between E1 and E2. Together, these results suggest that the H53 epitope is misfolded in some E2 proteins as the result of α-glucosidase inhibition by the iminosugar derivatives and consequent impairment of the interaction with calnexin. Similar results were obtained with other conformation-dependent anti-E2 antibodies (H35 and H48) (data not shown), suggesting that the misfolding of E2 is not restricted to a specific epitope, but extends to the whole protein.

**Glucosidase inhibitors lead to a reduction in the incorporation of E1–E2 into HCVpp and modify the pattern of N-glycosylation of residually secreted HCV pseudoparticles**

The supernatants of transfected HEK-293T cells treated or not with increasing amounts of NB-DNJ and NN-DNJ were concentrated (100 ×) and analysed by Western blot with both anti-E1 and anti-E2 antibodies, as well as with an anti-p30 antibody that recognizes the core protein of MLV. Ten microlitres of concentrated HCVpp, equivalent to 1 ml cell-culture medium, was analysed (Fig. 5). A reduction in the amount of E1 and E2 detected was observed with both DNJ derivatives, suggesting that the reduction of the amount of E1–E2 complexes in the ER of HCVpp-producing HEK-293T cells after glucosidase inhibition has consequences on the incorporation of E1–E2 into HCVpp. In parallel, no significant reduction of the amount of MLV ‘bald’ particles, as measured by detection of the capsid protein (p30), was observed. This result was anticipated, as the MLV core has been described previously to be secreted as ‘bald’ particles in the absence of any expressed envelope glycoproteins (Bartsch et al., 2003).

Next, the pattern of N-glycosylation of HCVpp produced in the presence or absence of drug was analysed. Concentrated HCVpp were submitted to mock, endoglycosidase H (Endo H) or peptide: N-glycosidase F (PNGase F) treatment and subjected to Western blot analysis using both anti-E1 and anti-E2 antibodies. As described previously (Flint et al., 2004; Op De Beeck et al., 2004), HCVpp produced in the absence of drug contained E2 carrying mainly Endo H-resistant complex N-glycans, whereas the
E1 produced carries both Endo H-sensitive high-mannose and Endo H-resistant complex N-glycans (data not shown). In contrast, in the presence of DNJ derivatives, the pattern of N-glycosylation was modified (data not shown). Upon NB-DNJ treatment, HCVpp contained E1 and E2 carrying more Endo H-sensitive high-mannose N-glycans than HCVpp produced without drug. With NN-DNJ, the effect was more pronounced, as both E1 and E2 contained mainly Endo H-sensitive high-mannose N-glycans. The retention of high-mannose N-glycans, which are probably hyperglucosylated N-glycans, in HCVpp is the consequence of glucosidase inhibition.

HCVpp produced under α-glucosidase inhibitor treatment are less infectious

Having established that iminosugars induce a reduction of the incorporation of E1–E2 complexes into HCVpp as a consequence of glucosidase inhibition, we sought to demonstrate whether they would also affect the infectivity of these HCVpp, which are formed and secreted residually under an incomplete drug-induced block. An infectivity assay was performed by using standardized numbers of HCVpp produced in the presence or absence of drug. The standardization was performed by dosing both E1 and E2 after serial dilution by Western blot using non-conformation-dependent anti-E1 (A4) and anti-E2 (H47) antibodies (Fig. 6a). The dosing of MLV p30 was of no interest here, as some non-enveloped nucleocapsids are released from producer cells and this release is not sensitive to drug inhibition, as shown in Fig. 5. The same relative numbers of particles produced either with or without drug were used to infect Huh7 cells. Seventy-two hours p.i., intracellular luciferase activity, which is proportional to viral infectivity, was assayed.

Fig. 5. Secretion of HCVpp is inhibited by DNJ derivatives. HEK-293T cells (2×10^6 cells per 10 cm plate) were transfected with a plasmid enabling the production of HCVpp. Sixteen hours p.t., the transfection mixture was replaced with fresh medium containing increasing amounts of NB-DNJ and NN-DNJ. Forty hours p.t., secreted HCVpp were harvested from the medium and filtered through 0.45 μm membranes. Ten microlitres of 100-fold-concentrated HCVpp was subjected to SDS-PAGE and Western blot analysis using anti-E2 (H47) and anti-E1 (A4) antibodies, as well as an anti-MLV capsid (p30) antibody.
entry, was quantified by using a luminometer (Fig. 6b). A reduction in activity was observed with both DNJ derivatives. With NB-DNJ, the luciferase activity was reduced to 30% of that of the control, whilst it was reduced to 10% with NN-DNJ, suggesting a greater effect of the long-alkyl chain DNJ derivative on the infectivity of HCVpp.

The reduced infectivity of HCVpp produced under iminosugar treatment is due to the incorporation of misfolded envelope glycoprotein rather than to modification of N-glycosylation

Two hypotheses were tested to determine why HCVpp produced under glucosidase inhibitors treatment were less infectious. First, we sought to show whether the reduced infectivity was due to the presence of high-mannose glycans in HCVpp, as shown above. To this end, we produced HCVpp in HEK-293T cells in the presence of DMJ, a mannosidase inhibitor that is known to block the trimming of mannos from N-glycans (Bischoff et al., 1986). HCVpp secreted in the presence of DMJ contained, as reported previously (Lozach et al., 2004), high-mannose structures on E1 and E2 (data not shown), but remained as infectious as HCVpp produced without DMJ (Fig. 6). Therefore, the high-mannose status of N-glycans is not the cause of the reduced infectivity of HCVpp produced in the presence of iminosugars.

We then examined whether the lack of infectivity could be due to the incorporation of misfolded HCV envelope glycoprotein in HCVpp. Immunoprecipitation with either non-conformation-dependent (H47) or conformation-dependent (H53) anti-E2 antibodies was performed by using the same standardized relative number of secreted HCVpp produced in the absence or presence of NN-DNJ. The number of HCVpp was standardized as described above. The immunoprecipitated material was analysed by Western blot using anti-E2 (H47). A reduction in the amount of E2 immunoprecipitated was observed with H53, but not with H47. The quantification of immunoprecipitated E2, done by measurement of luminescence using the Chemidoc XRS system from Bio-Rad and Quantity One analysis software, enabled us to determine the E2 folding status, defined as amount of E2 immunoprecipitated with H53 divided by the amount of E2 immunoprecipitated with H47. As presented in Fig. 7(a), this E2 folding ratio was reduced for HCVpp produced under NN-DNJ treatment compared with HCVpp produced without treatment. To confirm this result, we used the previously described CD81 pull-down assay (Cocquerel et al., 2003). CD81, a potential co-receptor for HCV (Bartosch & Cosset, 2006), is able to interact with E2 and it was shown that this interaction is dependent on the conformation of E2 (Flint et al., 2000). We therefore used hCD81–GST fusion protein to probe further the conformation status of E2 within secreted HCVpp produced in the absence or presence of glucosidase inhibitors. The number of HCVpp was standardized as described above and was verified by direct Western blotting using anti-E1 (A4) and anti-E2 (H47) mAbs (Fig. 7b, left panels). A standardized number of HCVpp were incubated with hCD81–GST immobilized on glutathione–Sepharose beads. The interaction between hCD81–GST and HCVpp was revealed by Western blot analysis of Sepharose-associated proteins with anti-E1 (A4) and anti-E2 (H47) mAbs. As shown in Fig. 7(b) (right panels), a significant reduction of the amount of E1 and E2 pulled down was observed with treated samples, suggesting a misfolding of the CD81-binding domain of E2 induced by glucosidase inhibitors. Eventually, to strengthen the demonstration, secreted HCVpp produced in the absence or presence of NN-DNJ were purified on a sucrose gradient, reconcentrated and then loaded onto carbon-coated grids. Immunogold labelling was performed by using either a conformation-dependent anti-E2 mAb (H48) or
a conformation-independent anti-E1 mAb (A4). HCVpp produced without drug were stained by both conformation-dependent and -independent antibodies, suggesting the presence of HCV glycoproteins in HCVpp and the correct folding of E2 in these particles (Fig. 7c). In contrast, HCVpp produced under NN-DNJ treatment were not recognized by the conformation-dependent antibody, thus suggesting the presence of misfolded E2 incorporated in the particles.

Collectively, the results presented in Figs 6 and 7 suggest that the reduced infectivity of HCVpp produced under iminosugar treatment may be due to the incorporation of misfolded HCV glycoprotein in particles.

DISCUSSION

The assembly and infectivity of HCV represent interesting, but as yet unexploited, targets for an antiviral strategy. We have previously reported the antiviral effects of various iminosugars using the BVDV model, a surrogate virus of HCV, and demonstrated that these molecules inhibit viral morphogenesis (Branza-Nichita et al., 2001; Durantel et al.,
2001; Zitzmann et al., 1999). Amongst iminosugars, DNJ derivatives, which are inhibitors of ER \( \alpha \)-glucosidases, were the most active. ER \( \alpha \)-glucosidases catalyse the stepwise removal of three glucose residues from triglcosylated N-linked glycans (Glc\(_3\)Man\(_9\)NAcGln\(_2\)), which are initially attached to some nascent glycoproteins transiting via the ER. This glucose removal leads to the formation of monoglcosylated N-linked glycans (Glc\(_2\)Man\(_9\)NAcGln\(_2\)) that play a crucial role in mediating the interaction between the glycoproteins to be folded and the lectin-like ER chaperone calnexin (Braakman & van Anken, 2000; Helenius & Aebi, 2004).

The anti-BVDV action of these molecules is due in part to the disruption of the folding and assembly of viral glycoprotein pre-budding complexes that consequently cause the inhibition of viral morphogenesis and secretion (Branza-Nichita et al., 2001). Although BVDV is, in many respects, a valid surrogate model of HCV due to their close genetic organization and related biochemical functions of their proteins, it nevertheless remains a distinct virus. One important difference is the presence of an additional structural gene, E\(^{TTS}\), encoding a protein that may be involved in the entry of BVDV (Iqbal et al., 2000). Moreover, it has been shown recently that \( \alpha \)-glucosidase inhibitors affect the biological function of E\(^{TTS}\) (Branza-Nichita et al., 2004). The results of the BVDV study prompted us to study the antiviral properties of \( \alpha \)-glucosidase inhibitors by using other models of HCV assembly and entry.

In this study, we used the two most relevant HCV models to provide further evidence of the antiviral action properties of \( \alpha \)-glucosidase inhibitors derived from DNJ derivatives and to decipher their mechanism of action further. The first model used consisted of the production in Huh7.5 cells of HCV particles (HCVcc) that have encapsidated a bicistronic genome containing (i) a luciferase gene in the first cistron, translation of which is mediated by the HCV IRES, and (ii) the sequence encoding the JFH1 polyprotein in the second cistron, translation of which is mediated by the EMCV IRES. Moreover, it was shown that these HCVcc produced in Huh7.5 cells were infectious \textit{in vitro} and \textit{in vivo} (Wakita et al., 2005). The second model consists of the production of pseudotyped particles (HCVpp) that are formed by incorporation of native HCV envelope glycoprotein heterodimers (E1–E2) onto MLV retroviral core particles (Bartosch et al., 2003; Lindenbach et al., 2005; Pohlmann et al., 2003; Wakita et al., 2005; Zhong et al., 2005).

In both models, we find that \( \alpha \)-glucosidase inhibitors derived from DNJ have an overall inhibitory activity, as measured by the reduction of luciferase expression in Huh7/Huh7.5 cells infected by HCVcc or HCVpp produced in the presence of drugs. Confirming results obtained with BVDV, a DNJ derivative bearing a nonyl chain on the nitrogen of the cycle (i.e. NN-DNJ) is found to be more active than its butyl counterpart (i.e. NB-DNJ). In the BVDV model, the higher antiviral activity of NN-DNJ was due neither to better penetration of the compound nor to better inhibition of ER \( \alpha \)-glucosidases, but rather to another, as yet not fully characterized, mechanism of action (Durantel et al., 2001; Zitzmann et al., 1999).

To determine whether these molecules could, in addition to their already identified activity on glycoprotein folding and assembly that results in inhibition of BVDV morphogenesis, have another mode of action, we also studied their effect on viral infectivity and entry. The HCVpp model may not be the most valid model to study HCV morphogenesis, as it is based on pseudotyping, but represents a validated model to study HCV entry and therefore, by extension, a suitable model to study the infectivity properties of particles produced. For instance, this model was used successfully to study the importance of \( \alpha \)-glucosylation for the incorporation of functional E1–E2 complexes in particles (Goffard et al., 2005), as well as to study viral entry and its inhibition by neutralizing antibodies (Bartosch et al., 2003; Callens et al., 2005; Flint et al., 2004; Goffard et al., 2005; Lozach et al., 2004; Op De Beeck et al., 2004; Pohlmann et al., 2003; Voisset et al., 2005). To study in depth the mechanism of action of glucosidase inhibitors, this model was preferred to the model based on replication of the HCV JFH1 strain, for several reasons. First, antibodies able to recognize conformational epitopes in JFH1 glycoproteins, which are important for an exhaustive demonstration of the incorporation of misfolded complexes in particles, are currently lacking. Second, presently one cannot be sure that the production of viral particles will be sufficient to perform biochemical studies, including immunogold study with conformation-dependent antibodies. Hence, to date, no publication has shown high secretion of particles by electron microscopy studies. Furthermore, the nature and composition of the particles produced in Huh7 cells upon JFH1 (or chimera) infection is still a matter of discussion. All of these points need clarification before one can address with this new model specific issues on the mechanism of action of molecules probably targeting the morphogenesis of proper virions.

By using the HCVpp model, we confirm that DNJ derivatives inhibit ER \( \alpha \)-glucosidases \textit{in cellulo}. This inhibition leads to the retention of hyperglcosylated \( N \)-glycans on viral glycoproteins, which in turn prevents their interaction with the lectin-like chaperone calnexin. This lack of interaction has a consequence on the folding and assembly of viral glycoproteins (Branza-Nichita et al., 2001). Here, we demonstrate, by immunoprecipitation with antibodies recognizing correctly folded E2, that DNJ derivatives impair the folding of HCV glycoproteins and reduce the amount of correctly folded and assembled E1–E2 complexes. The diminution of the amount of correctly folded and assembled E1–E2 pre-budding complexes probably accounts for the observed inhibition of E1–E2 complex incorporation into HCVpp. Furthermore, we demonstrate for the first time that \( \alpha \)-glucosidase inhibitors reduce the infectivity of viral particles that are released under incomplete drug-induced inhibition of secretion. We give...
evidence suggesting that the reduced infectivity of HCVpp produced in the presence of DNJ derivatives is not due to the retention of high-mannose N-glycans on secreted HCVpp. Instead, we demonstrate by using three different experimental approaches that the reduction of the infectivity of HCVpp is due to the incorporation of the misfolded/misassembled envelope glycoproteins in HCVpp. The fact that misfolded E1–E2 complexes are found in HCVpp implies that the incorporation of E1–E2 into HCVpp could occur with misfolded/misassembled E1–E2 complexes. This result would confirm the crucial predominant role of glycoprotein transmembrane domains over luminal ectodomains for E1–E2 assembly and incorporation into particles (Dubuisson, 2000). Further studies, using in particular the complete HCV replication system, are required to confirm whether misfolded HCV glycoprotein can be incorporated into virions and to understand better how the infectivity is decreased. It also remains to be determined whether the inhibition by the drugs is due to inhibition of binding, internalization or fusion.

The sum of our results demonstrates that the antiviral effect of z-glucosidase inhibitors, such as DNJ derivatives, is due to both an inhibition of E1–E2 assembly and incorporation into HCVpp, as well as a reduction in viral infectivity due to the incorporation of misfolded glycoprotein complexes in virions. These properties further suggest the potential usefulness of z-glucosidase inhibitors in combating HCV infection.

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Braakman, I. & van Anken, E. (2000). Potential usefulness of complexes in virions. These properties further suggest the infectivity is decreased. It also remains to be determined whether the inhibition by the drugs is due to inhibition of binding, internalization or fusion. The sum of our results demonstrates that the antiviral effect of z-glucosidase inhibitors, such as DNJ derivatives, is due to both an inhibition of E1–E2 assembly and incorporation into HCVpp, as well as a reduction in viral infectivity due to the incorporation of misfolded glycoprotein complexes in virions. These properties further suggest the potential usefulness of z-glucosidase inhibitors in combating HCV infection.

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