Antiviral effect of $\alpha$-glucosidase inhibitors on viral morphogenesis and binding properties of hepatitis C virus-like particles

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Hepatitis C virus (HCV) infections are a major public-health concern. New antiviral drugs are needed urgently to complement and improve the efficacy of current chemotherapies. The morphogenesis of HCV represents an interesting, and still unexploited, novel molecular target. $\alpha$-Glucosidase inhibitors derived from the glucose analogue deoxynojirimycin (DNJ) inhibit viral morphogenesis in cellulo via perturbation of the $N$-glycosylation pathway and hence the misfolding of viral glycoproteins that depend on certain $N$-glycans for correct folding. Due to the heavy $N$-glycosylation of HCV glycoproteins, it was hypothesized that such inhibitors would also affect HCV morphogenesis. To study the effect of $\alpha$-glucosidase inhibitors on viral morphogenesis and binding properties, HCV virus-like particles (VLPs) were produced by using baculovirus loaded with HCV structural-protein genes. Here, it is demonstrated that, in the presence of these $\alpha$-glucosidase inhibitors, viral glycoproteins synthesized and retained in the endoplasmic reticulum (ER) (i) contain unprocessed, triglucosylated $N$-glycans, (ii) are impaired in their interaction with calnexin and (iii) are at least partially misfolded. Moreover, it is shown that, although the production of VLPs is not affected by $\alpha$-glucosidase inhibitors, these VLPs contain unprocessed, triglucosylated $N$-glycans and potentially misfolded glycoproteins. Finally, it is demonstrated that VLPs produced in the presence of $\alpha$-glucosidase inhibitors have impaired binding properties to hepatoma cells. The inhibitors of morphogenesis studied here target steps of the HCV viral cycle that may prevent or delay viral resistance. These $\alpha$-glucosidase inhibitors may prove to be useful molecules to fight HCV infection in combination protocols.

INTRODUCTION

Hepatitis C virus (HCV), a member of the family Flaviviridae, is a small, positive-sense, enveloped virus of approximately 9600 nt (Choo et al., 1989, 1991). The viral genome, containing a unique open reading frame, is translated into a single polyprotein precursor of 3000 aa that is processed by host and viral proteases (Bartenschlager et al., 2004; Tellinghuisen & Rice, 2002). The amino-terminal part of the genome encodes structural proteins, including the core protein, two envelope glycoproteins (gpE1 and gpE2) and p7, a small ion-channel protein. These proteins are mainly involved in HCV morphogenesis, although some might have other regulatory functions (Bartenschlager et al., 2004; Tellinghuisen & Rice, 2002). gpE1 and gpE2 can either dimerize in a non-covalent interaction in the endoplasmic reticulum (ER) to form potentially functional pre-budding complexes, or derive disulfide-linked aggregates that contain misfolded, non-functional glycoproteins (Dubuisson, 2000). It has been shown that two subsets of host chaperone proteins participate in the formation of both of these types of complex. Calnexin interacts with glycoproteins engaged in the formation of functional non-covalent complexes, whilst calreticulin and Bip interact with misfolded glycoproteins destined to aggregate (Dubuisson, 2000).

Worldwide, around 170 million people, representing 3 % of the population, have been infected with HCV (Davis et al., 2003; Poynard et al., 2000; Seeff, 2002). More than 70 % of HCV-infected patients become chronic carriers. Long-term exposure to persistent HCV replication predisposes patients to cirrhosis and hepatozellular carcinoma (Liang & Heller, 2000).
2004). In the absence of any prophylactic or therapeutic vaccines, the only therapeutic option consists of the administration of pegylated alpha interferon in combination with ribavirin (Fried et al., 2002; Manns et al., 2001). However, the efficacy of this treatment depends on the viral genotype and is ineffective in 40% of patients (Fried et al., 2002; Manns et al., 2001). Other therapies based on new molecules are clearly required to combat HCV infection more successfully. Molecules specifically targeting viral activities (e.g., protease, helicase and polymerase inhibitors) are the most attractive in terms of drug development and are therefore the most studied (Foster, 2004; Pawlotsky, 2005). However, an antiviral strategy based solely on the use of these types of molecule is expected to face the problem of viral resistance, as already described for human immunodeficiency virus and Hepatitis B virus (Fung & Lok, 2004; Johnson et al., 2005; Locarnini et al., 2004). Therefore, alternative approaches are needed to complement antiviral strategies based on inhibitors of viral enzymes.

HCV morphogenesis is a potential target for antiviral strategies. However, studies of the HCV life cycle, including HCV morphogenesis, and new antiviral targets have long been hampered by the lack of an efficient cell-culture system able to support HCV replication. Such a system has recently been described and promises to benefit HCV research greatly (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). Until this development, various HCV surrogate models have been used to address some aspects of HCV biology. By using Bovine viral diarrhea virus (BVDV) as an HCV surrogate model, we have previously studied the antiviral properties and determined the mechanism of action of a new class of antiviral compounds, generically called iminosugars, that inhibit morphogenesis in cellulo (Branza-Nichita et al., 2001; Durantel et al., 2001, 2004; Zitzmann et al., 1999). Iminosugars act as charge/transition-state analogues of sugars and are inhibitors, in vitro and in vivo, of many glycosidases (Dwek et al., 2002). Two \( \alpha \)-glucosidases play an important role in the processing of \( N \)-linked glycans within the ER by removing in a step-wise fashion the terminal glucose residues from the initial \( N \)-glycan structure added to nascent polypeptides. This glucose removal is crucial, as unfolded glycoproteins carrying monoglucosylated \( N \)-glycans can interact with the lectin-like ER chaperones calnexin and calreticulin until they are folded correctly (Helenius & Aebi, 2004). HCV glycoproteins are highly \( N \)-glycosylated: gpE1 and gpE2 contain five to six and 11 \( N \)-glycosylation sites, respectively, and are strictly dependent on calnexin interaction to fold and assemble properly (Dubuisson, 2000).

Based on data that we obtained by using the BVDV system and molecular data concerning the folding and assembly of HCV glycoproteins, we hypothesized that iminosugars derived from deoxynojirimycin (DNJ), which are inhibitors of ER \( \alpha \)-glucosidases, could have a potent effect on the folding and assembly of HCV glycoproteins and could therefore be evaluated as HCV antivirals. The aim of this work was to study the antiviral effect and the mechanism of action of \( \alpha \)-glucosidase inhibitors on HCV morphogenesis and infectivity (i.e., binding properties). We produced HCV ‘virus-like particles’ (VLPs) in insect cells by using a baculovirus system, as described by Baumert et al. (1998). These VLPs share some morphological, biophysical and antigenic properties with natural HCV virions (Baumert et al., 1998; Saunier et al., 2003; Triyatni et al., 2002). This model, until the very recent publication of a full replication system, was one of the best models to study HCV morphogenesis. The model allows the budding into the lumen of an ER-derived compartment of a ‘capsid-like’ structure in a phospholipidic vesicle containing viral glycoproteins. By using this model, we show here that glucosidase inhibitors (i) do not prevent the formation of VLPs in insect cells, although they inhibit \( \alpha \)-glucosidases in cellulo and subsequently impair the folding of gpE2 to a certain extent, (ii) do not modify the composition of VLPs, but (iii) do reduce the binding of VLPs to human hepatoma cells.

**METHODS**

**Iminosugars.** Two \( \alpha \)-glucosidase inhibitors were used in this study. N-Butyl-deoxyojirimycin (NB-DNJ) was purchased from Sigma-Aldrich. N-Nonyl-DNJ (NN-DNJ) was provided by United Therapeutics Inc. N-Nonyl-deoxygalactonojirimycin (NN-DGJ), an iminosugar that has no \( \alpha \)-glucosidase-inhibitory activity and was used here as a control in some experiments, was also provided by United Therapeutics Inc.

**Antibodies.** Anti-E1 (A4) and anti-E2 (H14, H35, H47, H48 and H53) monoclonal antibodies (mAbs) were described previously (Cocquerel et al., 1998, 2001; Dubuisson et al., 1994). The anti-core mAb was a gift from C. Jolivet (Biomerieux, Lyon, France). Anti-calnexin and anti-calreticulin polyclonal antibodies were purchased from Stressgen Biotechnologies. Anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were from Sigma.

**Insect-cell cultures and baculovirus construct.** Spodoptera frugiperda Sf9 insect cells were cultured as described by King & Possec (1992), either in suspension or as a monolayer, at 28 °C in SF-900 II medium (Invitrogen) containing either 2.5% (v/v) (maintenance) or 10% (v/v) (infection) fetal calf serum (FCS) (Perbio). Baculoviral stock production and plaque assays for virus titration were performed as described by King & Possec (1992).

A baculovirus carrying an H77 (genotype 1a)-derived HCV sequence encoding capsid, gpE1, gpE2 and p7 proteins was constructed. First, the HCV sequence was cloned into the triple expression vector pTriEx-1.1 (Novagen) to derive the transfer vector, pTriEx-Struct-HCV-H77. The E2p7 sequence was subcloned directly from the pTM1-HCV plasmid (Dubuisson et al., 1994) into the NcoI/Xhol sites of pTriEx-1.1 to generate pTriEx-E2p7. The CE1 sequence was amplified by PCR using the pTM1-HCV plasmid as template and the following primers: 5’-AGACCGTGATCATGAGCAGCATCCTCC-3’ (sequence of H77 strain; BspHI site underlined) and 5’-GTTCCTCAGGGCGCTCTCGGTGGATGAG-3’ (sequence of H77 strain; Xhol site underlined). The PCR product was double-digested with BspHI/NcoI and cloned into the unique NcoI site of pTriEx-E2p7 to obtain pTriEx-Struct-HCV-H77. The recombinant baculovirus, Bac-CE1E2p7, was generated by co-transfection of Sf9 cells with pTriEx-Struct-HCV-H77 and linearized baculovirus DNA as recommended by the manufacturer (BacVector 3000; Novagen). Amongst several clones picked by plaque
Effect of glucosidase inhibitors on HCV morphogenesis

Production of HCV VLPs in insect cells. HCV VLPs were produced by infection of 2 × 10^6 SF9 cells at an m.o.i. of 10–20. To study the effect of z-glucosidase inhibitors on glycoprotein accumulation, NB-DNJ and NN-DNJ were added at the same time as the virus at the concentrations indicated in the text and maintained in the culture throughout 72 h production. For some experiments, infected cells were treated with 5 mM tunicamycin (Sigma), an inhibitor that prevents the N-glycosylation of proteins. Cells were lysed in TNC buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM CaCl_2] containing 0-25% (w/v) digitonin and VLPs were purified as described by Baumert et al. (1998). Following purification by sucrose-gradient centrifugation (100 000 g, 16 h), fractions where gPE1 and gPE2 cosedimented were detected by Western blotting. These fractions were pooled, diluted in TNC buffer and VLPs were reconcentrated by ultracentrifugation (100 000 g, 3 h). Concentrated VLPs were resuspended in TNC buffer and stored at −20°C until further use. VLPs observed by transmission electron microscopy after negative staining. Concentrated VLPs (10 μl) were loaded onto carbon-coated grids, then the grids were stained with 2% uranyl acetate before examination with a JEOL 1010 XC electron microscope.

Analysis of N-glycosylation of ER-retained and VLP-associated glycoproteins. Glycoproteins retained in the ER of infected SF9 cells were extracted in TNET buffer [10 mM Tris (pH 7-5), 150 mM NaCl, 2 mM EDTA, 0-5% Triton X-100] for 30 min on ice and quantified by the Bradford method (Sigma). Concentrated VLPs resuspended in TNC buffer were also used for N-glycosylation analysis. ER-retained or VLP-associated glycoproteins (20 μg) were digested with endoglycosidase H (Endo-H; New England Biolabs) according to the supplier’s recommendations. Digestion products were separated by SDS-PAGE and analysed by Western blotting as described below.

Western blot analysis. Infected SF9 cells were lysed in TNET buffer containing a mixture of protease inhibitors (Roche) for 30 min on ice. Cell lysates were clarified by centrifugation at 16 000 g for 2 min. VLPs were lysed as described above in TNC buffer containing protease inhibitors and 0-25% (w/v) digitonin on ice for 4 h. Protein concentration was determined by using Bradford reagent (Sigma). ER-retained VLPs or glycosidase-digestion products were separated by SDS-PAGE. They were transferred to a nitrocellulose membrane by using a semi-dry electroblotter (Bio-Rad) and detected with primary mAbs anti-E1 (A4; 1 : 1000 dilution), anti-E2 (H47 and H53; 1 : 800 dilution), anti-core (1 : 1000 dilution) or anti-core (1 : 1000 dilution) antibodies. Immunoprecipitation was performed as described above, with anti-chaperone (diluted 1 : 100) or anti-E2 (H47 or H53; diluted 1 : 100) antibodies. Immunoprecipitated proteins were separated by SDS-PAGE and analysed by Western blotting with anti-E1 or anti-E2 antibodies.

Binding assay on Huh-7 cells. Standardized relative amounts of VLPs produced with or without iminosugars and concentrated as described before were incubated with Huh-7 cells (0-5 × 10^5) for 1 h at 4°C. Cells were washed twice with flow-cytometry (FC) buffer (0-1% BSA in PBS) and incubated with anti-E1 (A4, 1 : 100) at 4°C for 45 min. Cells and antibodies were washed twice with FC buffer and incubated with fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Sigma; 1 : 200) for 30 min at 4°C. After two washes in FC buffer, cell-bound VLPs were diluted in 1 ml FC buffer and analysed by flow cytometry (Dako Galaxy). The mean fluorescence intensity of bound VLPs was determined after subtraction of the non-specific fluorescence value.

RESULTS

Effect of glucosidase inhibitors on HCV glycoprotein accumulation and N-glycosylation

To determine whether HCV structural proteins were expressed from our baculovirus system, SF9 cells were infected at high m.o.i. with Bac-CE1E2p7. Proteins were extracted at the indicated time and analysed by Western blotting with either anti-core, anti-E1 or anti-E2 antibodies (Fig. 1a). Core, gPE1 and gPE2 proteins were detected 48 h p.i. at their expected sizes (16–17, 25–27 and 66–68 kDa, respectively), thus confirming the expression and processing of these HCV proteins. A kinetic study revealed a maximal accumulation of gpE1 and gPE2 at 48 h p.i. (Fig. 1b). The expression of these proteins from baculovirus is consistent with previously published studies (Baumert et al., 1998).

Next, the effect of two glucosidase inhibitors, NB-DNJ and NN-DNJ, on glycoprotein accumulation and N-glycosylation was studied. Insect cells were infected with Bac-CE1E2p7 and treated with increasing concentrations (0–1000 μM) of immediately for 30 min on ice in CHAPS–HSE buffer [2% CHAPS (3-[3-chloroamidopropyl]-dimethylammoniopropyl)-1-propanesulfonate] in 50 mM HEPES (pH 7-5), 200 mM NaCl, 2 mM EDTA] with a mixture of protease inhibitors (Roche) and iodoacetamide (20 mM) to alkylate free thiol groups and prevent non-specific aggregation. Labelled cell lysates were pre-cleaned by incubation with 50 μl protein A-Sepharose overnight at 4°C. Lysates were then centrifuged twice (3000 g, 5 min) and incubated with anti-chaperone (diluted 1 : 100) or anti-E2 conformation-dependent (H53; diluted 1 : 100) antibodies for 1 h at 4°C. Protein A–Sepharose (50 μl) was then added to supernatants for 2 h at 4°C. The slurry was washed three times with 0-5% CHAPS–HSE buffer. Bound proteins were eluted by boiling samples in SDS-PAGE sample buffer in the presence (reducing conditions) or absence (non-reducing conditions) of 5% (v/v) 2-mercaptoethanol. Samples were separated by SDS-PAGE. The gels were treated with enhancing reagent (Amplexr; Amersham Biosciences), dried and exposed at −70°C to Hyperfilm-M (Amer- sham Biosciences). Alternately, immunoprecipitation was performed under non-radioactive conditions. In this case, infection of SF9 cells was performed as described above and the drug was added 1 h post-infection (p.i.) and kept until cell lysis 30 h after infection. The immunoprecipitation was performed as described above, with anti-chaperone (diluted 1 : 100) or anti-E2 (H47 or H53; diluted 1 : 100) antibodies. Immunoprecipitated proteins were separated by SDS-PAGE and analysed by Western blotting with anti-E1 or anti-E2 antibodies.

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NB-DNJ or NN-DNJ. ER-retained glycoproteins were extracted 24 h p.i. and analysed by Western blotting with both anti-E1 and anti-E2 antibodies (Fig. 2). Two main dose-dependent effects were observed with increasing concentration of DNJ derivatives. First, an overall increase in the accumulation of gpE1 and gpE2 was observed with both NB-DNJ and NN-DNJ. The reduction in gpE1 and gpE2 produced in the presence of 1 mM NN-DNJ was due to toxic effects of this drug, which has an IC₅₀ in this system of approximately 200 μM. Second, a reduction of HCV glycoprotein mobilities, obvious for gpE1 and less marked for gpE2, was also observed with both drugs. This upward shift is a direct consequence of the inhibition of ER α-glucosidases in cellulo and is due to the retention of bi- and triglucosylated N-glycans (Glc2–Man9–NAcGln2 and Glc3–Man9–NAcGln2) on glycoproteins. No upward shift was observed with 1 mM NN-DGJ, an iminosugar that has no α-glucosidase-inhibitory activity (Durantel et al., 2001). Together, these results demonstrate that glucosidase inhibitors derived from DNJ are active and induce a retention of hyperglycosylated N-glycan structures on ER-retained proteins in insect cells.

Glucosidase inhibitor disrupts the interaction of viral glycoproteins with calnexin

Many viral glycoproteins depend on interactions with calnexin to fold and, more importantly, to assemble properly (Braakman & van Anken, 2000; Helenius & Aebi, 2004). It has been shown previously that calnexin assists the folding of HCV glycoproteins (Dubuisson, 2000). The interaction between glycoproteins and calnexin is based on the lectin-like affinity of calnexin for monoglucosylated N-linked glycans attached to the nascent polypeptide (Helenius & Aebi, 2004). In order to interact with calnexin, the triglucosylated N-linked glycan that is initially added to the nascent polypeptide in the ER needs to be processed by α-glucosidases I and II. Having demonstrated that α-glucosidase inhibitors prevent this processing, we set out to determine whether the interaction of the glycoprotein with calnexin was affected. A radioactive pulse–chase experiment was performed 24 h p.i. on either mock- (uninfected) or Bac-CE1E2p7-infected Sf9 cells in the presence or absence of 2 mM NB-DNJ, followed by an immunoprecipitation with an anti-calnexin antibody. Immunoprecipitated complexes were subjected to SDS-PAGE under reducing conditions (Fig. 3a). A strong decrease (>60%) in the amount of gpE1 and gpE2 co-immunoprecipitated was observed in the presence of NB-DNJ. Another immunoprecipitation experiment with the anti-calnexin antibody was performed to confirm the previous result, but using non-radioactive conditions. Immunoprecipitation was done on lysates of infected Sf9 cells in the absence or presence of 1 mM NB-DNJ. Immunoprecipitated complexes were subjected to SDS-PAGE under reducing conditions and analysed by Western blotting with both anti-E1 and anti-E2 antibodies.

Fig. 1. Expression of HCV structural proteins in insect cells. Sf9 cells were infected at an m.o.i. of 20 with Bac-CE1E2p7. (a) At 48 h p.i., the cells were lysed and lysates were subjected to SDS-PAGE under reducing conditions and immunoblotting with anti-core (left panel), anti-E1 (middle panel) or anti-E2 (right panel) antibodies. (b) Kinetic analysis of HCV gpE1 and gpE2 expression.

Fig. 2. Effect of α-glucosidase inhibitors on gpE1 and gpE2 accumulation and N-glycosylation in the ER of infected Sf9 cells. Sf9 cells were infected at an m.o.i. of 20 with Bac-CE1E2p7 in the presence of increasing concentrations of NB-DNJ and NN-DNJ, two α-glucosidase inhibitors derived from DNJ. Twenty-four hours p.i., proteins were extracted, separated by SDS-PAGE under reducing conditions and analysed by Western blotting with anti-E1 and anti-E2 antibodies.
Proteins were pulse-labelled for 15 min with 100 \( \mu \text{Ci} \) [\( ^{35} \text{S} \)]methionine/\( ^{35} \text{S} \)cysteine. Cells were lysed immediately with 2 % CHAPS buffer and proteins were immunoprecipitated with an anti-calnexin antibody. Immunoprecipitated complexes were separated by SDS-PAGE and the gel was exposed to autoradiography confirming that equal amounts of lysates were used (Fig. 3b). It is worth noting that no shift of HCV glycoproteins immunoprecipitated by anti-calnexin was observed, thus confirming that only glycoproteins carrying monoglucosylated N-glycan can interact with calnexin. The fact that some gpE2 and gpE1 still interacts with calnexin despite the treatment with NB-DNJ indicates that, at the concentration used, \( \alpha \)-glucosidase inhibition is only partial. Altogether, the results obtained confirm that the retention of hyperglucosylated N-linked glycans induced by \( \alpha \)-glucosidase inhibition prevents the interaction of viral glycoproteins with calnexin in insect cells.

**Glucosidase inhibitor induces the misfolding of gpE2 and increases its interaction with calreticulin**

Having established that the association of HCV glycoproteins with calnexin is inhibited in the presence of NB-DNJ, we wanted to determine whether this inhibition would impair the folding of HCV glycoproteins. We assessed the folding of gpE2 by using the conformation-dependent anti-E2 H53 antibody (Cocquerel et al., 1998). A pulse–chase experiment was performed 24 h p.i. on either mock- or Bac-CE1E2p7-infected SF9 cells in the absence or presence of 2 mM NB-DNJ, followed by immunoprecipitation with the H53 antibody. The immunoprecipitated complexes were subjected to SDS-PAGE under reducing conditions (Fig. 4a). A small decrease (<25 %), as quantified by scan densitometry, in the amount of immunoprecipitated gpE2 was observed in the presence of 2 mM NB-DNJ, suggesting that the folding of the H53 epitope is somehow affected by NB-DNJ. No significant difference (<10 % by scan densitometry) was observed on co-immunoprecipitated gpE1. Altogether, the effect was weak and incorporation of radioactivity with a 15 min pulse was not strong enough to determine whether the misfolding of gpE2 could affect its interaction with gpE1. Therefore, other immunoprecipitations were performed under non-radioactive conditions in order to analyse the cumulative effect in longer drug exposure. Bac-CE1E2P7-infected cells were left untreated or treated with NB-DNJ (1 mM) from 1 h p.i. until cell lysis. Immunoprecipitation was done with either non-conformation-dependent (H47) or conformation-dependent (H53) anti-E2 antibodies to demonstrate clearly that the reduction of the amount of E2 immunoprecipitated with H53 was indeed due to misfolding and not to a diminution of the overall amount of E2 in cell lysates. Immunoprecipitated complexes were subjected to SDS-PAGE under reducing conditions and analysed by Western blotting with both anti-E1 and anti-E2 antibodies. Moreover, direct Western blotting with either anti-E1 or anti-E2 antibodies was performed on cell lysates to demonstrate that a similar amount of lysate was used for the immunoprecipitation (Fig. 4b). Strengthening the result obtained under radioactive conditions, a decrease (>40%) in the amount of gpE2 immunoprecipitated with anti-E2 antibody (H53) was also observed in the presence of NB-DNJ. In this case, the amount of co-immunoprecipitated gpE1 was reduced significantly. Moreover, the amount of gpE2 immunoprecipitated with a non-conformation-dependent anti-E2 antibody, as well as the amount of calnexin detected by Western blotting, was similar in both treated and untreated samples, thus confirming that equal amounts of lysate were used in the experiment. These results confirm that...
NB-DNJ does not induce a decrease in the overall amount of HCV glycoprotein synthesized, as also shown in Fig. 2, but indeed induces the misfolding of the gpE2 H53 epitope. Equivalent results were also obtained with other conformation-dependent anti-E2 antibodies (H35 and H48) (data not shown), thus suggesting that the misfolding of gpE2 is not restricted to one particular epitope. As no conformation-dependent anti-E1 antibody has been described to date, the folding status of gpE1 could not be checked.

It has been shown previously that aggregates containing misfolded gpE1–gpE2, resulting from infection of HepG2 cells with a recombinant vaccinia virus overexpressing HCV glycoproteins, interact strongly and stably with calreticulin rather than calnexin (Dubuisson, 2000). To assay for calreticulin binding, we repeated the experiment described above and used an anti-calreticulin antibody for the immunoprecipitation (Fig. 5). Under radioactive conditions, a weak increase (< 25 %), as quantified by scan densitometry, in the amount of immunoprecipitated gpE2 was observed in the presence of 2 mM NB-DNJ (Fig. 5a). Under non-radioactive conditions, the increase in the amount of immunoprecipitated gpE2 was confirmed for samples treated with 1 mM NB-DNJ compared with the control lane (Fig. 5b). Two other observations were made in this experiment. First, the amount of gpE1 co-immunoprecipitated with gpE2 by anti-calreticulin was reduced rather than increased compared...
with the control, thus suggesting that misfolded gpE2 is less able to interact with gpE1. Second, HCV glycoproteins immunoprecipitated from the sample treated with anti-calreticulin contained hyperglucosylated N-glycans, as demonstrated by their reduced mobility in gel.

Taken together, the results presented in Figs 4 and 5 suggest that NB-DNJ induces the misfolding of gpE2 and increases the interaction of gpE2 carrying triglucosylated N-glycans with calreticulin. Experimentation performed either with an anti-E2 antibody (H14), described previously as recognizing aggregates in mammalian cells, or under non-reducing SDS-PAGE conditions did not allow clear demonstration that glucosidase inhibitors increase the amount of gpE1–gpE2 aggregates dramatically (data not shown). Indeed, with this baculovirus overexpressing system, the basal level of aggregation is fairly high (i.e. without any drug treatment) and non-quantifiable smears are obtained under such conditions. However, as calreticulin was shown previously to interact with gpE1–gpE2 aggregates (Dubuisson, 2000), we can conclude that \( \alpha \)-glucosidase inhibitors induce misfolding and probably somehow increase aggregation (i.e. misassembly) of HCV glycoproteins in insect cells.

### Effect of glucosidase inhibitors on VLP formation and N-glycosylation

Next, we sought to determine the effect of these drugs on VLP formation. VLPs were produced in \( S^9 \) cells in the absence or presence of NN-DNJ (100 \( \mu \)M), which was preferred to NB-DNJ as it is more potent and can be used at a lower concentration. An additional control was performed by producing VLPs in the presence of tunicamycin (5 \( \mu \)M), a potent inhibitor of N-glycosylation. VLPs were purified by sucrose-gradient centrifugation and fractions were analysed for the presence of both gpE1 and gpE2 by Western blotting (Fig. 6a). In the absence of any drug treatment, both gpE1 and gpE2 were detected in the same fraction, confirming the presence of gpE1 and gpE2 in VLPs. In addition, fractions containing gpE1 and gpE2 had the expected densities (1·16–1·22 g cm\(^{-3}\)), consistent with previous studies (Baumert et al., 1998; Saunier et al., 2003; Triyatni et al., 2002). Whilst treatment with NN-DNJ did not alter the amount or the density of VLPs produced significantly, treatment with tunicamycin was rather toxic, although it did not abrogate the signal obtained by Western blotting (Fig. 6a). The presence of glycoproteins at the expected densities was confirmed by negative staining electron microscopy (Fig. 6b).

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**Fig. 6.** Comparison of VLPs produced in the presence or absence of NN-DNJ. (a) \( S^9 \) cells were infected with Bac-CE1E2p7 at an m.o.i. of 20 in the absence or presence of 100 \( \mu \)M NN-DNJ or 5 \( \mu \)M tunicamycin. VLPs were harvested 72 h later and purified as described in Methods. Fractions from sucrose-gradient centrifugation (density indicated in g cm\(^{-3}\)) were analysed by Western blotting analysis with either anti-E2 (H47) or anti-E1 (A4) antibodies. (b) Negatively stained electron micrograph of HCV VLPs produced in the absence or presence of the indicated drug. Bars, 200 nm.
density does not guarantee the quality of VLPs produced. To determine whether treatment with NN-DNJ and tunica-mycin affected the amount and ultrastructure of VLPs, observation of negatively stained particles was made by electron microscopy. Whilst the number and ultrastructure of VLPs produced under NN-DNJ were not significantly different from those of the control, almost no particles could be detected in tunicamycin-treated samples (Fig. 6b). Only protein aggregates could be detected in the latter samples.

Having established that α-glucosidase inhibitors did not affect the quantity of VLPs produced, we sought to determine whether any change in the pattern of N-glycosylation could be observed on VLPs produced in the presence of NB-DNJ (500 μM) or NN-DNJ (100 μM). VLP-associated glycoproteins were submitted to Endo-H digestion. Endo-H is a glycosidase that specifically cleaves the chitobiose core of high-mannose-containing N-linked glycans. Digested proteins were analysed by Western blotting using both anti-E1 and anti-E2 antibodies (Fig. 7). Glycoproteins incorporated in VLPs produced either in the absence or presence of α-glucosidase inhibitors were sensitive to Endo-H digestion, indicating the presence of high-mannose-containing N-linked glycans on these glycoproteins. However, glycoproteins incorporated in VLPs produced in the presence of NB-DNJ or NN-DNJ showed reduced mobility on SDS-PAGE gels compared with those produced in the absence of drug, as observed for the intracellular, ER-retained HCV glycoprotein complexes. This reduced mobility indicates the presence of hyperglycosylated N-linked glycans on these glycoproteins. As glycoproteins bearing hyperglycosylated N-linked glycans do not interact with calnexin and do not benefit from the chaperone activity of this protein to fold, it is likely that HCV glycoproteins incorporated in VLPs produced in the presence of drugs may be misfolded.

Production of VLPs in the presence of α-glucosidase inhibitors leads to impaired binding properties

Comparative binding assays on Huh-7 cells were performed with VLPs produced either in the absence or presence of NN-DNJ (100 μM). To quantify the number of VLPs and to ensure the same relative number in each experiment, precise serial dilutions (1:2 dilutions) of VLPs were dotted on nitrocellulose and analysed by Western blotting with conformation-independent antibodies anti-E1 (A4) (Fig. 8a) or anti-E2 (H47) (data not shown). The luminescent signal was quantified by using the Chemidoc XRS system and Quantity One analysis software (Bio-Rad). The intensity and number of binding events to cells were analysed by flow cytometry after hybridization with anti-E1 and FITC-conjugated secondary antibodies. As shown in Fig. 8(b), binding of VLPs produced in the presence of NN-DNJ to Huh-7 cells was reduced by 75% compared with VLPs produced in the absence of the inhibitor. The same results were obtained when the analysis was performed with anti-E2 antibodies (H47 and H53; data not shown). This strong reduction in binding suggests that α-glucosidase inhibitors derived from DNJ impair the binding properties of VLPs.

DISCUSSION

HCV morphogenesis represents an interesting target for the development of a new generation of antiviral compounds. By using an HCV study model, BVDV, we have previously studied in some detail the antiviral properties and mechanisms of action of a new class of molecules generally called iminosugars (Branza-Nichita et al., 2001; Durantel et al., 2001, 2004; Zitzmann et al., 1999). Amongst the iminosugars, DNJ and N-alkylated derivatives thereof, which are α-glucosidase inhibitors, were the most efficient. DNJ derivatives mediate their anti-BVDV activity both by preventing viral secretion, due to the disruption of folding and assembly of viral glycoprotein pre-budding complexes, and by reducing viral infectivity as the result of the incorporation of non-functional glycoprotein complexes into
Our results demonstrate that DNJ derivatives that are inhibitors of HCV morphogenesis as an alternative antiviral option, the efficacy of iminosugars, in particular DNJ derivatives, against HCV had not been demonstrated. Here we analysed, to some extent, the effect of DNJ derivatives on the assembly of HCV glycoprotein complexes and on the formation and binding properties of HCV pseudoparticles. We used a baculovirus loaded with an HCV sequence (genotype 1a; strain H77) to enable expression of the structural proteins that subsequently assemble into VLPs in insect cells, as described by Baumert et al. (1998). This model, until the recent development of a proper HCV replication system, was one of the best models to investigate some aspects of HCV morphogenesis and therefore to study potential morphogenesis inhibitors.

Our results demonstrate that DNJ derivatives that are α-glucosidase inhibitors inhibit insect ER α-glucosidases and cause the retention of hyperglucosylated N-linked glycans on HCV glycoproteins in a dose-dependent manner, as well as the accumulation of these glycoproteins in the ER of infected insect cells. Moreover, we have confirmed in this model that the interaction of HCV glycoproteins carrying unprocessed, hyperglucosylated N-linked glycans with calnexin is impaired. As expected from previous data (Branza-Nichita et al., 2001, 2002), the consequence of this impaired interaction with the chaperone protein was a partial misfolding of gpE2, as suggested by immunoprecipitation studies with a conformation-dependent antibody (anti-E2 H53). Furthermore, an increased association of gpE2 with calreticulin, another chaperone protein that has previously been shown to interact with misfolded HCV glycoproteins (Dubuisson, 2000), was observed. It is not clear why potentially misfolded HCV glycoproteins accumulate in the ER of infected insect cells. This result differs from those obtained with BVDV, where it was shown that misfolded glycoproteins were degraded by the proteasome and did not accumulate in the ER of BVDV-infected Madin–Darby bovine kidney cells (Branza-Nichita et al., 2002). This accelerated degradation of misfolded BVDV glycoproteins caused a reduction in the amount of pre-budding complexes, which was proposed tentatively as one possible explanation for the inhibition of viral secretion. Two hypotheses can be given to explain this difference. Firstly, the baculovirus overexpresses HCV proteins and may therefore saturate the proteasome. Secondly, baculovirus is associated during its replication cycle in cells with many changes in host physiology, including the shut-off of host protein synthesis and the reorganization of the cellular ultrastructure (Blissard, 1996). Therefore, we cannot exclude the possibility that the function of the proteasome might be affected by baculovirus infection.

In the BVDV model, α-glucosidase inhibitors were shown to prevent viral secretion due to their intracellular inhibitory activity on host ER α-glucosidases (Branza-Nichita et al., 2001; Durantel et al., 2001). However, it was not demonstrated in these studies whether the blockage of viral secretion was a consequence of impaired viral budding from the lumen of the ER or whether it prevented the release of virions from the secretory pathway. VLPs produced after infection of S9 cells by recombinant baculoviruses bud into ER-derived vesicles, but are not released/secreted from cells (Baumert et al., 1998); a mild lysis with 0–25% (w/v) digitonin is necessary to recover them from the intracellular compartment. Extraction with digitonin discriminates between ER membrane-associated glycoproteins and those associated with already budded VLPs. The capacity of DNJ derivatives to prevent the formation of VLPs was analysed in this study. Interestingly, we have shown that DNJ derivatives do not inhibit the formation of VLPs in infected insect cells and/or antibodies were omitted as indicated. The graph presented is the result of four experiments done with two different batches of VLPs.

Fig. 8. Analysis of binding of VLPs produced in the absence or presence of NN-DNJ to Huh-7 cells. (a) Dilutions (1:2) of concentrated VLPs were dotted serially on nitrocellulose and analysed by immunoblotting with anti-E1 (mAb A4) antibody. (b) VLPs produced ± 100 μM NN-DNJ were incubated with Huh-7 cells at 4°C. Bound VLPs were detected by hybridization with primary anti-E1 (mAb A4) and secondary anti-mouse IgG FITC-conjugated antibodies. Binding analysis was performed by using a flow cytometer. The percentage of cells firing above the fixed threshold [10 relative fluorescence units of FL1 (green fluorescence)], as well as the mean value of FL1, was taken into account to calculate the percentage binding. As controls, VLPs and/or antibodies were omitted as indicated. The graph presented is the result of four experiments done with two different batches of VLPs.

virions (Branza-Nichita et al., 2001; Durantel et al., 2001; Zitzmann et al., 1999).
of interaction with Huh-7 cells (Fig. 8). This reduced interaction provides an insight into the ability of DNJ derivatives to reduce viral infectivity. In the BVDV model, the reduction of viral infectivity induced by DNJ derivatives was hypothesized, but not demonstrated (Durantel et al., 2001). Results obtained here represent the first evidence for the inhibition of viral infectivity by inosamogars. The reduced ability of VLPs produced in the presence of $\alpha$-glucosidase inhibitors to bind to hepatoma cells may correlate with the retention of unprocessed, hyperglucosylated N-linked glycans on HCV glycoproteins incorporated in these VLPs (Fig. 7). Indeed, the presence of unprocessed, hyperglucosylated N-linked glycans on HCV glycoproteins represents the first indirect line of evidence for the presence of misfolded HCV glycoproteins in those VLPs. For technical reasons, to date we have not been able to demonstrate the presence of misfolded HCV glycoproteins in VLPs produced in the presence of the DNJ derivatives. Further studies are in progress to address this important issue.

New antivirals are urgently needed to better combat HCV infection, in particular in patients who are resistant to current therapies. In addition to HCV enzyme inhibitors that are being developed and tested in clinical trials (Foster, 2004; Pawlotsky, 2005), it would be of value to develop novel types of antivirals targeting other steps of the HCV viral cycle to prevent or delay viral resistance. The inhibitors of morphogenesis studied here belong to this category of molecules and could play an important role, in particular in combination protocols. To investigate this further, DNJ derivatives will be evaluated in the newly developed cellular system that enables complete replication, including viral morphogenesis and entry (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). These data, in combination with other antivirals tested, will be used to design in vitro new therapies that could be beneficial therapeutically.

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