Inhibitors of Endoplasmic Reticulum α-Glucosidases Potently Suppress Hepatitis C Virus Virion Assembly and Release

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α-Glucosidases I and II are endoplasmic reticulum-resident enzymes that are essential for N-linked glycan processing and subsequent proper folding of glycoproteins. In this report, we first demonstrate that down-regulation of the expression of α-glucosidase I, II, or both in Huh7.5 cells by small hairpin RNA technology inhibited the production of hepatitis C virus (HCV). In agreement with the essential role of α-glucosidases in HCV envelope glycoprotein processing and folding, treatment of HCV-infected cells with a panel of imino sugar derivatives, which are competitive inhibitors of α-glucosidases, did not affect intracellular HCV RNA replication and nonstructural protein expression but resulted in the inhibition of glycan processing and subsequent degradation of HCV E2 glycoprotein. As a consequence, HCV virion assembly and secretion were inhibited. In searching for imino sugars with better antiviral activity, we found that a novel imino sugar, PBDNJ0804, had a superior ability to inhibit HCV virion assembly and secretion. In summary, we demonstrated that glucosidases are important host factor-based antiviral targets for HCV infection. The low likelihood of drug-resistant virus emergence and potent antiviral efficacy of the novel glucosidase inhibitor hold promise for its development as a therapeutic agent for the treatment of chronic hepatitis C.

Hepatitis C virus (HCV) chronically infects more than 170 million people worldwide. Current standard therapy for chronic hepatitis C, the combination of pegylated alpha interferon (IFN-α) and ribavirin, is associated with a less than 50% sustained virological response in patients infected with genotype 1 virus. In the search for more effective therapeutic agents, the development of direct-acting antiviral agents to target viral functions, such as NS3/4A protease and NS5B RNA-dependent RNA polymerase, has been the main focus during the last 2 decades (26). However, an important lesson learned from clinical studies is that although inhibition of the essential viral functions potently inhibited HCV replication and resulted in a rapid drop in viremia, development of drug resistance eventually limited the antiviral efficacy of these drugs (22, 32, 36). Therefore, viral function inhibitors will likely be used not as monotherapeutic agents but rather as part of therapeutic regimens in combination with IFN-α and/or other HCV inhibitors.

Like all other viruses, HCV relies on many host functions to propagate. In addition, the virus needs to counteract or evade the cellular antiviral response to colonize its host cells (23). Therefore, an alternative approach to inhibiting HCV infection is to target host cellular functions required for HCV replication and/or activate the host cellular antiviral response (17, 24, 37). In fact, compared to targeting viral functions, an obvious advantage of targeting host functions is the low likelihood of drug resistance (28). Currently, inhibitors targeting several cellular proteins, including 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (19, 41), cyclophilin (21, 25), phosphatidylinositol 4-kinase alpha (PI4K-α) (2, 6), and heat shock proteins (13), have been shown to inhibit HCV infection in cultured cells and a cyclophilin inhibitor, alisporivir (Debio 025), has been demonstrated to reduce HCV viremia in people (12).

α-Glucosidases I and II catalyze the sequential removal of the three terminal glucose residues from the asparagine-linked (N-linked) oligosaccharides on glycoprotein precursors. These reactions are the first steps of glycan processing and are essential for the proper folding and function of certain cellular and viral glycoproteins (10). We and others have shown previously that treatment of cells with α-glucosidase inhibitors such as imino sugars that are glucose mimics and act as competitive inhibitors of the enzymes inhibited the infection of many enveloped viruses (4, 5, 8, 10, 14). Although the effect of imino sugar on HCV was achieved mainly by using bovine viral diarrheaa virus (BVDV) as a model virus, in one recent study, several imino sugars were being tested and their anti-HCV effects were being confirmed using an HCV tissue culture infection system (35). Moreover, α-glucosidase inhibitors have also been demonstrated to inhibit woodchuck hepatitis virus in chronically infected woodchucks (4), several flaviviruses in mice (33, 38, 40), and HCV in people in a phase II clinical trial (9).

In this study, in our effort to search for imino sugar derivatives with better antiviral activity against HCV, we first formally demonstrated with small interfering RNA (siRNA) technology that both α-glucosidases I and II are essential host factors in HCV infection. In addition, we demonstrated that known imino sugar glucosidase inhibitors impair HCV infec-
tion at the step of virion assembly and secretion. Interestingly, we found a novel imino sugar derivative, PBDNJ0804, with superior antiviral activity against HCV through a similar mechanism that is consistent with the inhibition of glucosidases.

**MATERIALS AND METHODS**

**Compounds.** Deoxynojirimycin (DNJ) and N-nonyl-DNJ (NNDNJ) were purchased from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). N-butyl-DNJ (NBDNJ) was kindly provided by Raymond Dwek (University of Oxford, Oxford, United Kingdom). All other imino sugar derivatives were as described previously (8).

**Cell culture and virus.** Huh7.5 cells were maintained in Dulbecco’s modified minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (3). A plasmid containing the full-length HCV Jc1 cDNA was cloned from chemically synthesized DNA oligomers (29) (GenScript, Piscataway, NJ). HCV RNA was transcribed in vitro with the MEGAscript kit (Ambion, Austin, TX) and electroporated into Huh7.5 cells (15). Generation of a virus stock and determination of virus titers (50% tissue culture infective doses [TCID50] per milliliter) were done as described previously (20). In general, infection of Huh7.5 cells at a multiplicity of infection (MOI) of 0.015 for 4 days resulted in virus yields of $0.5 \times 10^4$ to $0.5 \times 10^5$ TCID50/ml. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay; Sigma, St. Louis, MO) was performed to measure cytotoxicity following the treatment of Huh7.5 cells with serial doses of each compound for 4 days (Table 1).

**Establishment of stable cell lines expressing shRNA.** Huh7.5 cells were infected with vesicular stomatitis virus G protein-pseudotyped lentivirus encoding (i) a small hairpin RNA (shRNA) targeting human α-glucosidase I (GCS1, SHCLNV-NM_006302), α-glucosidase II (GANAB, SHCLNV-NM_014610), or PI4Kα (SHCLNV-NM_002650) or (ii) a nontargeting shRNA (Sigma, St. Louis, MO) to establish corresponding stable cell lines.

**Indirect immunofluorescence.** HCV-infected cells were fixed with phosphate-buffered saline containing 1% paraformaldehyde and then incubated with cold methanol for 20 min at $-20^\circ$C. Cells were then blocked and incubated with HCV NS3 antibody (clone H23; Abcam, Cambridge, MA). Bound primary antibody was visualized by Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA).

**Western blot analysis.** Cells were lysed with Laemmli buffer and separated on Tris-glycine gel, followed by transfer onto nitrocellulose membrane (Invitrogen). Membranes were blocked and probed with antibodies against HCV NS3 (clone H23; Abcam, Cambridge, MA), HCV E2 (clone AP33; provided by Arvind Patel through Genentech, Inc.), α-glucosidase I (Sigma, St. Louis, MO), α-glucosidase II (Sigma, St. Louis, MO), or glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Sigma, St. Louis, MO). This was followed by incubation with IRDye secondary antibodies and imaging with the LI-COR Odyssey system (LI-COR Biotechnology, Lincoln, NE).

### TABLE 1. Structures, antiviral properties, and α-glucosidase I-inhibitory activities of imino sugar derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>R in structure</th>
<th>EC50 (μM)a,b</th>
<th>CC50 (μM)c</th>
<th>α-Glucosidase IC50 (μM)d</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNJ</td>
<td></td>
<td>&gt;100</td>
<td>&gt;500</td>
<td>1.44</td>
</tr>
<tr>
<td>NBDNJ</td>
<td></td>
<td>&gt;100</td>
<td>&gt;500</td>
<td>0.68 ± 0.05</td>
</tr>
<tr>
<td>NNDNJ</td>
<td></td>
<td>4.0 ± 0.5</td>
<td>87 ± 8.7</td>
<td>0.54 ± 0.08</td>
</tr>
<tr>
<td>OS-95II</td>
<td></td>
<td>28.8 ± 12.3</td>
<td>&gt;500</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>CM-9-78</td>
<td></td>
<td>52.5 ± 14.2</td>
<td>&gt;500</td>
<td>0.42 ± 0.09</td>
</tr>
<tr>
<td>CM-10-18</td>
<td></td>
<td>27.2 ± 5.4</td>
<td>&gt;500</td>
<td>0.46 ± 0.11</td>
</tr>
<tr>
<td>PBDNJ0802</td>
<td></td>
<td>3.5 ± 1.0</td>
<td>&gt;500</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>PBDNJ0803</td>
<td></td>
<td>1.7 ± 0.8</td>
<td>208 ± 17.6</td>
<td>0.51 ± 0.09</td>
</tr>
<tr>
<td>PBDNJ0804</td>
<td></td>
<td>5.4 ± 2.6</td>
<td>&gt;500</td>
<td>0.50 ± 0.16</td>
</tr>
</tbody>
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*a Results represent mean values and standard deviations from three independent experiments. 
*b EC50 was determined by in-cell Western immunoassay as described in the legend to Fig. 4. 
*c CC50 was determined by MTT assay. 
*d Determined by using the substrate Glc3Man3GlcNAc2 as previously described (1).
RNA quantification by qRT-PCR. Total cellular RNA or RNA in culture medium was extracted using TRizol reagent (Invitrogen) or a QiAamp viral RNA minikit (Qiagen) and reverse transcribed using SuperScript III (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) was performed on an Applied Biosystems 7500 thermocycler using the probe 5′-6-carboxyfluorescein-CCT TGT GGT ACT GCC TGA-molecular-groove binding nonfluorescent quencher-3′ (Applied Biosystems) and forward and reverse primers 5′-AGCGTTGGGTGCGAAAG-3′ and 5′-CAGCGCAAGGCCCT-3′, respectively. The standard curve was generated using serial 10-fold dilutions of in vitro-transcribed full-length HCV RNA.

In-cell Western immunoassay. The in-cell Western immunoassay was performed largely as previously described for the detection of dengue virus (DENV) (18), except that an HCV NS3 monoclonal antibody (H23) was used. Briefly, cells were fixed with 3.7% formaldehyde and permeabilized with 0.25% Triton X-100. Intracellular levels of HCV NS3 protein were revealed by sequential incubation with an HCV NS3-specific monoclonal antibody (clone H23; Abcam, Cambridge, MA) and IRDye 800 goat anti-mouse IgG (LI-COR, Lincoln, NE). Cell viability was determined by DRAQ5 (Biostatus Limited, Leicestershire, United Kingdom) and Sapphire 700 (LI-COR, Lincoln, NE) staining. Fluorescence signal intensity was quantified with LI-COR Odyssey.

In vitro α-glucosidase I enzymatic assay. Free oligosaccharide Glc3Man5GlcNAc2, a substrate for glucosidase I, was incubated with purified α-glucosidase I (from rat liver) together with various concentrations of imino sugar derivatives as previously described (1). Separation of the hydrolysis products was performed using normal-phase high-performance liquid chromatography (Waters). Dose-response curves were generated to calculate the concentration required to inhibit enzyme activity by 50% (IC50).

RESULTS

α-Glucosidases I and II are essential host factors for HCV infection. Although it has been speculated and experimentally demonstrated with small molecular inhibitors that endoplasmic reticulum (ER)-resident α-glucosidases I and II are essential for the processing of N-linked glycans of viral envelope glycoproteins and thus required for viral particle assembly and the secretion of many enveloped viruses, the function of α-glucosidase II was validated only recently in a genome-wide siRNA screening study for DENV infection (34). To provide a solid basis for the development of α-glucosidase inhibitors as antiviral targets against HCV and determine the role of both α-glucosidases I and II in HCV infection, we first established stable Huh7.5 cell lines expressing shRNA molecules targeting α-glucosidase I, II, or both. As shown in Fig. 1A, compared with the Huh7.5 cell line expressing a nontargeting shRNA (sh-control) or shRNA targeting glucosidase I (sh-glucosidase I), glucosidase II (sh-glucosidase II), or both (sh-glucosidase I&II), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B), reduced the HCV yield by 14-fold (Fig. 1B). However, downregulation of either glucosidase I or II expression in Huh7.5 cells resulted in an approximately 5-fold reduction of progeny HCV production. Not surprisingly, knocking down both of the enzymes led to a slightly but significantly more profound reduction of HCV yield than that observed in cells with either one of the enzymes targeted by the shRNA. As a positive control, ablation of a well-known HCV host factor, PI4K-α (2, 6), reduced the HCV yield by 14-fold (Fig. 1C). These results thus demonstrate that both α-glucosidases I and II are essential HCV host factors.

Inhibition of α-glucosidases impairs HCV E2 protein glycan processing and reduces HCV release. To obtain detailed knowledge of the biological function of α-glucosidases in HCV replication and explore the therapeutic potential of glucosidase inhibitors, we first tested the effects of a panel of three imino sugar derivatives, OSL-95II, CM-10-18, and CM-9-78, on HCV infection. These derivatives had been demonstrated to inhibit α-glucosidase I in in vitro biochemical assays (Table 1). IFN-α was used as a positive control. As shown in Fig. 2, treatment of cells immediately after HCV infection (at an MOI of 0.015) with either IFN-α or any one of the three imino...
sugars efficiently inhibited virus spread (Fig. 2A) and accumulation of intracellular viral RNA (Fig. 2B) and reduced the yields of progeny HCV by more than 1 log (Fig. 2C). While these results, together with the results presented in Fig. 1, convincingly demonstrate that inhibition of host cellular α-glucosidases impairs HCV infection, the viral replication steps requiring the host enzymes could not be deduced.

As discussed previously, the ER-resident α-glucosidases are presumably involved in the glycan processing of HCV envelope glycoproteins E1 and E2 and thus required for virion assembly and secretion. To test this hypothesis, Huh7.5 cells were infected with HCV and cultured for 4 days to allow the virus to spread and infect almost 100% of the cells in the culture, as judged by immunofluorescent staining with an antibody against HCV NS3 protein (Fig. 3A). At this time, cells persistently infected with HCV were left untreated or treated with IFN-α (positive control) or imino sugar derivatives. As expected, treatment of HCV-infected cells with IFN-α for 4 days dramatically reduced the levels of intracellular HCV NS3 proteins and HCV RNA (Fig. 3A, B, and C); as a consequence, the HCV yield in the culture medium was reduced by more than 1,000-fold. However, treatment of HCV-infected cells with any one of the three imino sugars did not apparently reduce the levels of intracellular HCV NS3 protein (Fig. 3A and C) and HCV RNA (Fig. 3B), suggesting that the α-glucosidases are not involved in HCV protein translation and RNA replication. This notion was further confirmed by experiments demonstrating that treatment of Huh7.5 cells containing HCV subgenomic replicons (genotype 1b, Con1 strain) with the imino sugars did not inhibit the replication of the replicon (data...
On the contrary, in supporting the notion that HCV \(-\)glucosidases are required for glycan processing of HCV envelope glycoproteins, imino sugar treatment resulted in an electrophoresis mobility shift and a subsequent decay of HCV E2 and E2-p7 proteins (an unprocessed product at E2/p7 site [16]), the hallmark of a failure to properly process the terminal glucose residues of the N-linked oligosaccharide(s) of glycoproteins in the ER (Fig. 3C). In agreement with the observed inhibition of E2 glycoprotein processing and degradation, production of progeny HCV was reduced approximately 5\(\times\)fold by imino sugar treatment (Fig. 3B).

Imino sugar derivative PBDNJ0804 is a potent HCV inhibitor with low cytotoxicity. During the last decade, we have been making consistent efforts to improve the antiviral efficacy of imino sugars by generating several categories of DNJ derivatives. One of the important findings from these studies is that a DNJ derivative with a hydroxylated cyclohexyl side chain (OSL-95II) has an improved antiviral efficacy and lower cytotoxicity (14). Accordingly, a family of imino sugar derivatives containing oxygenated side chains and terminally restricted ring structures were synthesized (as depicted in Table 1) and demonstrated to have low cytotoxicity and superior antiviral

FIG. 3. Effects of three imino sugars on viral replication, protein expression, and virion production in persistently HCV-infected Huh7.5 cells. Huh7.5 cells were infected with HCV at an MOI of 0.015. Four days postinfection (when approximately 100\% of the cells were infected, as judged by positive staining of HCV NS3 by indirect immunofluorescence assay), cells were seeded into the wells of 24\-well plates at a density of 8 \(\times\) 10\(^4\)/well. Cells were left untreated (Control) or treated at 24 h postseeding with 100 IU/ml IFN-\(\alpha\) (positive control) or 100 \(\mu\)M OSL-95II, CM-10-18, or CM-9-78 for 4 days. (A) At day 4 posttreatment, HCV NS3 protein was detected with an indirect immunofluorescence assay. Cell nuclei were stained with DAPI. (B) Intracellular HCV RNA levels (black bar) and HCV yields in culture media (open bar) at day 4 posttreatment were determined with qRT-PCR and titration assays, respectively. The results are expressed as percentages of the levels in control cells. Values represent averages and standard deviations of results obtained from four independent experiments. (C) Cells were harvested at 3 and 4 days postinfection, and intracellular levels of HCV E2 (arrowheads) and E2/E2p7 (asterisks) or NS3 proteins were determined by Western immunoblot assay. GAPDH served as a loading control.
activity against several members of the Flaviviridae family, including BVDV, DENV, and West Nile virus (WNV) (8).

Figure 4 shows the results obtained in a study that systematically compared the anti-HCV activities of representative compounds from the different generations of imino sugar derivatives with an in-cell Western assay. Figure 4A shows a typical in-cell Western assay result. The amount of intracellular HCV NS3 protein was visualized by immunostaining with a specific antibody (green color). Total cellular DNAs were stained with DRAQ5 and Sapphire 700 (red color). Hence, the reduced intensity of the red signal indicates the cytotoxicity of the compounds. The accuracy of the assay was evaluated with IFN-α as a positive control. In agreement with previous reports, IFN-α inhibited HCV infection with an EC50 of 1 IU/ml (Fig. 4B). Consistent with what we observed in previous studies with other flaviviruses, DNJ and DNJ with a short alkyl side chain (NBBDNJ) showed little antiviral activity against HCV at a 100 μM concentration. DNJ with a nine-carbon alkyl side chain (NNDNJ) showed strong antiviral activity, with an EC50 of 4 μM, but also was relatively toxic to cells (concentration inhibiting cell growth by 50% [CC50], 87 μM) (Fig. 4B and Table 1).

As demonstrated in the previous two sections and a more complete dose-response study presented in Fig. 4B, three DNJ derivatives, with either a hydroxylated or an oxygenated side chain and a terminal ring structure (OSL-95II, CM-10-18, and CM-9-78) showed antiviral activity with EC50 ranging from 27 to 52 μM and lower cytotoxicity (CC50 of >500 μM). Interestingly, PBBDNJ0802, a stereoisomer of CM-9-78, and two additional compounds (PBBDNJ0803 and PBBDNJ0804) with modifications of the terminal ring structure demonstrated much-improved antiviral activity against HCV, with EC50 ranging from 1.7 to 5.4 μM (Fig. 4B and Table 1). This represents a 10- to 30-fold improvement of antiviral efficacy over that of the parental compound, CM-9-78. More importantly, our results imply that the terminal ring structure is a key moiety for antiviral activity.

PBBDNJ0804 more efficiently disrupts HCV E2 glycan processing and potently inhibits HCV release. Compared with CM-10-18 and other structurally similar DNJ derivatives, PBBDNJs are not actually better glucosidase I inhibitors, as indicated by the IC50 obtained in an in vitro enzymatic assay (Table 1). Therefore, one possible explanation for the superior antiviral activity of PBBDNJs is that the compounds are more efficiently accumulated in the ER, which results in better inhibition of the cellular enzymes and stronger antiviral activity. Alternatively, besides inhibition of the ER α-glucosidases, the compounds may disrupt an additional replication step(s) of the HCV life cycle. To distinguish these two possibilities, we first compared the effects of PBBDNJ0804 and CM-10-18 on their abilities to inhibit HCV by adding the compounds to culture media immediately after infection. The results showed that PBBDNJ0804 dose dependently reduced intracellular and extracellular levels of HCV RNA and infectious viral yields (a 3-log reduction at 100 μM) (Fig. 5A). Moreover, the intracellular levels of the NS3 and E2 proteins were also reduced by PBBDNJ0804 (Fig. 5B). Interestingly, consistent with the results obtained with the in-cell Western immunoassay (Fig. 4C), treatment of cells with 10 μM PBBDNJ0804 reduced HCV RNA levels and virus production to extents similar to those observed in cells treated with 100 μM CM-10-18.

To further determine if PBBDNJ0804 had a stronger ability to suppress E2 protein glycan processing and virion secretion, Huh7.5 cells 100% persistently infected with HCV were either left untreated or treated with 100 IU/ml IFN-α, 100 μM CM-10-18 (positive controls), and the indicated concentrations of...
PBDNJ0804 for 4 days. The results indicated that, as expected, PBDNJ0804 did not reduce the intracellular levels of viral RNA (Fig. 5C) and NS3 proteins (Fig. 5D, middle) at all of the concentrations tested. However, PBDNJ0804 treatment resulted in an electrophoretic mobility shift and degradation of the E2 and E2-p7 proteins (Fig. 5D, top), as well as a reduction of viral yields (Fig. 5C) in a dose-dependent manner. Similar to the results obtained in the de novo infection assay presented above (Fig. 5A and B), it appeared that 10 μM PBDNJ0804 demonstrated a potency in the inhibition of HCV E2 glycoprotein processing and a viral yield reduction similar to those of 100 μM CM-10-18. Our results thus imply that the novel DNJ derivative PBDNJ0804 more potently inhibited HCV glycoprotein processing and virion secretion but did not disrupt additional steps of HCV replication. This is consistent with the proposed mechanism of action of glucosidase inhibition.

**DISCUSSION**

We show in this study that ER-resident α-glucosidases I and II are essential host factors for HCV infection by using either (i) RNA interference (RNAi) technology to knock down the cellular protein expression of these enzymes or (ii) imino sugar derivatives known to inhibit their enzymatic activity. Mechanistically, we demonstrate that the ER α-glucosidases play an essential role in glycan processing and the function of HCV envelope glycoproteins.

Despite the great potential of imino sugars as broad-spectrum antiviral drugs, their clinical development has been hampered by their relatively low efficacy. For example, NBDNI, an imino sugar that has been approved by the U.S. and European FDAs for use in the treatment of Gaucher's disease, had been terminated for development as an antiviral agent against HIV due to the failure to achieve a therapeutic concentration in
vivo (11). Similarly, another glucosidase inhibitor, Celgosivir (a prodrug of the natural product castanospermine), showed only a modest antiviral effect in chronically HCV-infected patients (5% of the patients tested experienced a >1-log_{10} reduction in viremia) as a mono- or combination therapy in a phase II clinical trial, mostly likely due to the relatively low plasma drug concentration and poor antiviral activity achieved (9).

Our studies reported herein discovered three novel DNJ derivatives with a six-carbon side chain, represented by PBDNJ0804, with more potent antiviral activity against HCV. Mechanistic analyses revealed that although PBDNJ0804 is not a more potent α-glucosidase I inhibitor, the compound appears to more efficiently inhibit the glycan processing of E2 glycoprotein and thus the assembly and secretion of virions. Although the possibility cannot be ruled out that PBDNJ0804 inhibits additional viral functions, such as the p7 ion channel, as suggested by others for NNDNJ (35), or other, unknown, host functions to achieve improved antiviral activity, the fact that the compound is also a better inhibitor of DENV and WNV (8), which do not encode a p7-like protein, favors the hypothesis that the improved antiviral effect of PBDNJ0804 is caused by enhanced cellular uptake and accumulation of compounds to a higher concentration in the ER. Indeed, it was recently shown that encapsulation of imino sugar into ER-targeting liposomes allowed enhanced delivery, as well as dramatically improved antiviral efficacy against HIV (30). Therefore, in future efforts to improve the antiviral activity of imino sugars, we should take into consideration that the cell-permeating ability and subcellular distribution of the compounds are important factors in determining their biological activity.

Interestingly, we showed that reducing α-glucosidase I, II, or both by approximately 50% using RNAi technology significantly reduced HCV production but did not apparently interfere with host cell growth. These results suggest that HCV and possibly other viruses are more dependent on these host enzymes than the host cell itself and thus provide a solid basis for inhibition of α-glucosidases as a practical antiviral strategy. However, prolonged treatment of cells with α-glucosidase inhibitors may also disrupt the glycan processing and maturation of certain host cellular glycoproteins, including viral receptors, which could, in turn, inhibit virus entry. In fact, it was reported previously that treatment of cells with NNDNJ could modestly inhibit HCV entry (35). In agreement with this observation, we have also demonstrated that pretreatment of MDBK cells with imino sugars for 24 h reduced the permissiveness of the cells to BVDV infection by 50% (data not shown). Moreover, we consistently observed a stronger antiviral activity of the imino sugars in de novo infection assays than in persistently infected cells (Fig. 2, 3, and 5). Hence, it is possible that inhibition of HCV entry contributes, at least in part, to the antiviral activity of imino sugars against HCV.

Experiences in antiviral therapies of HIV and hepatitis B virus infections and more recently in clinical trials of antiviral drugs against chronic HCV infection have shown that although drugs targeting viral functions initially induce a profound drop in viremia, development of drug resistance eventually limits their antiviral efficacy. A strategy of combination therapy with antivirals targeting multiple steps of viral replication has been proved to induce sustained suppression of HIV replication and prevent the emergence of drug resistance (22, 32, 36). It has also been demonstrated in recent clinical trials that a triple combination of PEGylated IFN-α2b, ribavirin, and an NS3/4A protease inhibitor (telaprevir or boceprevir) significantly improved the sustained virological response rate in treatment-naïve HCV patients (27, 31). Moreover, it was demonstrated previously that NNDNJ treatment of HCV-infected HuH7 cells was able to eliminate the virus after only five passages without emergence of drug-resistant viruses (35). The absence of selection of α-glucosidase inhibitor-resistant mutants was also observed in a BVDV infection model (39). These results strongly argue that, unlike drugs targeting viral functions, inhibition of host cellular functions essential for HCV replication, such as α-glucosidases, has a low likelihood of developing resistant mutants. This unique property, in combination with much-improved antiviral efficacy, makes PBDNJ0804 a promising antiviral candidate for combination therapy with IFN-α or drugs that inhibit viral functions (7).

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