NIM811, a Cyclophilin Inhibitor, Exhibits Potent In Vitro Activity against Hepatitis C Virus Alone or in Combination with Alpha Interferon

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Host factors involved in viral replication are potentially attractive antiviral targets that are complementary to specific inhibitors of viral enzymes, since resistant mutations against the latter are likely to emerge during long-term treatment. It has been reported recently that cyclosporine, which binds to a family of cellular proteins, cyclophilins, inhibits hepatitis C virus (HCV) replication in vitro. Here, the activities of various cyclosporine derivatives were evaluated in the HCV replicon system. There was a strong correlation between the anti-HCV activity and cyclophilin-binding affinity of these compounds. Of these, NIM811 has been selected as a therapeutic candidate for HCV infection, since it binds to cyclophilins with higher affinity than cyclosporine but is devoid of the significant immunosuppressive activity associated with cyclosporine. NIM811 induced a concentration-dependent reduction of HCV RNA in the replicon cells with a 50% inhibitory concentration of 0.66 μM at 48 h. Furthermore, a greater than three-$\log_{10}$ viral RNA reduction was achieved after treating the cells with as little as 1 μM of NIM811 for 9 days. In addition, the combination of NIM811 with alpha interferon significantly enhanced anti-HCV activities without causing any increase of cytotoxicity. Taken together, these promising in vitro data warrant clinical investigation of NIM811, an inhibitor of novel mechanism, for the treatment of hepatitis C.

Chronic hepatitis C continues to be a major global health burden. An estimated 170 million people are infected with hepatitis C virus (HCV) worldwide (22). HCV displays a high degree of genetic variability translated into the classification of six genotypes and many subtypes, of which genotype 1 is the most prevalent genotype in North America, Europe, and Japan. The current standard therapy for chronic hepatitis C is pegylated alpha interferon (IFN-α) in combination with ribavirin for up to 1 year. However, only up to 50% of patients with genotype 1 virus can be successfully treated with this regimen. Moreover, both IFN-α and ribavirin are associated with significant adverse effects. Therefore, more efficacious and better-tolerated drugs for hepatitis C are greatly needed.

HCV, first identified in 1989 (6), is a single-stranded RNA virus with a 9.6-kilobase genome of positive polarity. It encodes a single polyprotein that is cleaved upon translation by cellular and viral proteases into at least 10 individual proteins: C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (14). Current HCV drug discovery efforts focus primarily on two viral enzymes, the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase, both of which are essential for viral replication. However, due to the high heterogeneity and mutation rate of the virus, drug-resistant mutations in the viral genome are likely to emerge during treatment with specific inhibitors of HCV protease and polymerase (7). An alternative and complementary strategy is to target host factors that are also required for viral replication, which may be less prone to

resistance, and such an inhibitor can be used in combination with direct inhibitors of viral proteins. NIM811, a cyclosporine derivative targeting the host protein cyclophilin (Cyp), represents such an approach.

It has been reported recently that cyclosporine inhibited both HCV replicons and infectious virus in vitro (23, 16). K. Watashi et al. further demonstrated that cyclophilin B bound to HCV NS5B polymerase directly and increased its RNA-binding activity, the functions of which were blocked in the presence of cyclosporine (24). Moreover, although the antiviral effect of cyclosporine itself remains to be demonstrated with hepatitis C patients, it was reported that the combination of IFN-α and cyclosporine resulted in significantly higher virological and biochemical response rates than IFN-α monotherapy in one controlled clinical trial (9). However, there are obvious concerns about using cyclosporine, a highly immunosuppressive drug, to treat a chronic viral disease. Cyclosporine primarily exerts its immunosuppressive function by forming a complex with CypA, which subsequently binds to and inhibits calcineurin, a serine/threonine protein phosphatase that controls NF-AT-mediated T-cell activation. NIM811 (MeIle[4]-cyclosporine) is a cyclosporine derivative that has higher Cyp-binding affinity than cyclosporine (1). As shown in Fig. 1, NIM811 is structurally very similar to cyclosporine, with an isobutyl group replaced by a sec-butyl group at position 4. However, this small modification essentially blocks the recognition site of CypA/cyclosporine by calcineurin and thus abolishes the immunosuppressive function associated with cyclosporine. Therefore, NIM811 is a more attractive candidate as an antiviral agent. It has been demonstrated previously that NIM811 displays inhibitory activities against several viruses, including human immunodeficiency virus (HIV), hepatitis B virus, and vesicular sto-

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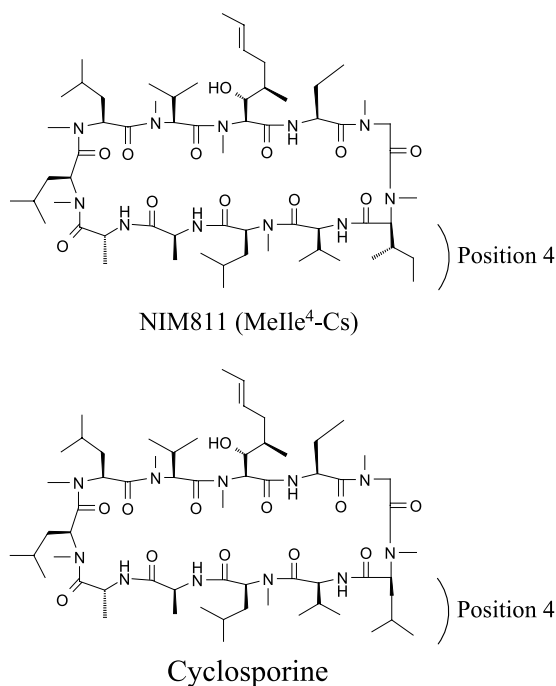
FIG. 1. Chemical structures of NIM811 and cyclosporine.

matitis virus (1, 4, 5, 21). Here, the anti-HCV activities of NIM811 were evaluated in vitro using the HCV replicon system. The effects of the combination of NIM811 and IFN-α were also investigated.

MATERIALS AND METHODS

Compounds. NIM811 and other cyclosporine derivatives were prepared at Novartis (Basel, Switzerland). The compounds were stored at −20°C as 20 mM dimethyl sulfoxide (DMSO) stock solutions until being used in the assay. Recombinant human IFN-α was purchased from Calbiochem (La Jolla, California) and was stored at −80°C.

Cells. The subgenomic genotype 1b (con1) HCV replicon cell line, clone A, was obtained from Charles Rice and Apath LLC (St. Louis, Missouri) (2). The genomic genotype 1b (con1) HCV replicon cell line, Huh 21-5, was obtained from Ralf Bartenschlager and ReBLikon GmbH. The subgenomic and genomic genotype 1a (H77) HCV replicon cells were also obtained from Apath LLC (3). All these replicon cell lines were cultured in Dulbecco's modified Eagle's medium, supplemented with 2 mM L-glutamine, 1× nonessential amino acids, 10% heat-inactivated fetal bovine serum (FBS), and 1 mg/ml G418 (Invitrogen, Carlsbad, CA). Another subgenomic genotype 1b (con1) HCV replicon cell line containing a luciferase reporter gene, Huh-Luc/neo-ET, was kindly provided by Ralf Bartenschlager (11) and was maintained in the same medium described above except that only 0.25 mg/ml G418 was used. The human serum for the protein-binding study was prepared from fresh donated blood and had been prescreened to be HCV negative (Bioreclamation, Hicksville, NY). The serum was stored at −20°C and thawed at 37°C immediately before use.

QRT-PCR based HCV replicon assay. The antiviral activities and cytotoxicities of compounds were determined with various HCV replicon cell lines in a quantitative RT-PCR (QRT-PCR)-based assay. Briefly, 10,000 replicon cells were seeded in each well of 96-well tissue culture plates and were allowed to attach in complete culture medium without G418 overnight. On the next day, the culture medium was replaced with medium containing serially diluted compounds in the presence of 2% FBS and 0.5% DMSO. After the cells were treated for 48 h, total RNA was extracted using an RNeasy 96 Kit (QIAGEN, Valencia, CA). The level of HCV RNA was measured by real-time quantitative RT-PCR (Taqman assay; Applied Biosystems, Foster City, California) using HCV-specific primers (5′-TCT TCA CGC AGA AAG CGT CTA-3′ and 5′-CTG GCA ATT CCG GTG TAC T-3′) and probe (5′-6-FAM-TCC TGG AGG CTG CAC GAC ACT CAT A-TAMRA-3′). The absolute copy numbers of HCV RNA were deter-

mined using a standard curve that was established with known quantities of in vitro-transcribed RNA. The level of HCV RNA was normalized for each sample against the amount of total RNA extracted, which was determined using a Quant-iT RNA assay kit (Molecular Probe; Invitrogen). Each data point represents the average for six replicates in cell culture in a single experiment. The percentage of inhibition was calculated as follows: % inhibition = 1 − (average of compound-treated cells)/(average of control cells). IC_{50} is the concentration of compound at which the HCV RNA level in the replicon cells is reduced by 50%. To monitor the cytotoxic effect of the compounds, the viability of the replicon cells following 48 h of compound treatment was determined using a tetrazolium compound (MTS)-based assay (CellTiter 96 AQueous One Solution cell proliferation assay; Promega, Madison, WI). The percentage of cytotoxicity was calculated as follows: % cytotoxicity = 1 − (average of compound-treated cells)/(average of control cells). Each data point represents the average of three replicates in cell culture.

Luciferase-based HCV replicon assay. The antiviral activity and cytotoxicity of compounds were determined using an HCV replicon cell line (Huh-Luc/neo-ET) containing a luciferase reporter gene, the expression of which is under the control of HCV RNA replication and translation. Briefly, 5,000 replicon cells were seeded in each well of 96-well tissue culture plates and were allowed to attach in complete culture medium without G418 overnight. On the next day, the culture medium was replaced with medium containing serially diluted compounds in the presence of 10% FBS and 0.5% DMSO. After a 48-h compound treatment, the remaining luciferase activities in the cells were determined using the BriteLite reagent (Perkin Elmer, Wellesley, Massachusetts) with an LMaxII plate reader (Molecular Probe; Invitrogen). Each data point represents the average for four replicates in cell culture in a single experiment. IC_{50} is the concentration of compound at which the luciferase activity in the replicon cells is reduced by 50%. The cytotoxicity of the compound is evaluated using the same MTS-based assay described above.

Nine-day HCV RNA reduction assay. HCV replicon cells, clone A, were seeded at a low density of 500 cells per well in 96-well plates so that the cells would not become overconfluent after nine continuous days in culture. The cells were treated with compounds serially diluted in Dulbecco's modified Eagle's medium containing 10% FBS and 0.2% DMSO but no G418. The culture medium was replaced every 3 days with fresh medium containing compounds or 0.2% DMSO control. After the cells were treated with compounds for 3, 6, or 9 days, total RNA was extracted, and the level of HCV RNA was determined as described above and was normalized against the amount of total RNA extracted for each sample. Each data point represents the average for five replicates in cell culture in a single experiment. The HCV RNA reduction after each period of treatment was calculated by comparing the remaining level of HCV RNA in compound-treated cells to that of control cells treated with 0.2% DMSO for the same duration.

Synergy analysis. To determine whether the effect of the combination of NIM811 and IFN-α was synergistic, additive, or antagonistic, MacSynergy (kindly provided by Mark Prichard), a mathematical model based on Bliss Independence theory, was used to analyze the data from the 48-h luciferase-based HCV replicon assay (20). In this model, a theoretical additive effect with any given concentration of NIM811 and IFN-α can be calculated using the equation: $Z = X + Y(1 − X)$, where X and Y represent the inhibition produced by NIM811 or IFN-α alone, respectively, and Z represents the effect produced by the combination of two compounds if they were additive. The theoretical additive effects were compared to (subtracted from) the actual experimental effects at various concentrations of the two compounds and were plotted as a three-dimensional differential surface that would appear as a horizontal plane at 0% if the combination were additive. Any peak above this plane (positive values) would indicate synergy, whereas any depression below it (negative values) would indicate antagonism. The 95% confidence interval of the experimental dose-response was considered to reveal only effects that were statistically significant.

RESULTS

NIM811 is a potent inhibitor of HCV RNA replication in the replicon cells. The anti-HCV activity of NIM811 was first examined using a subgenomic, genotype 1b (con1) HCV replicon (15). Replicon cells were treated with various concentrations of NIM811 for 48 h. The remaining levels of HCV RNA in the cells were determined by quantitative RT-PCR using HCV sequence-specific primers and probes. The HCV copy numbers were normalized against the total amount of RNA extracted
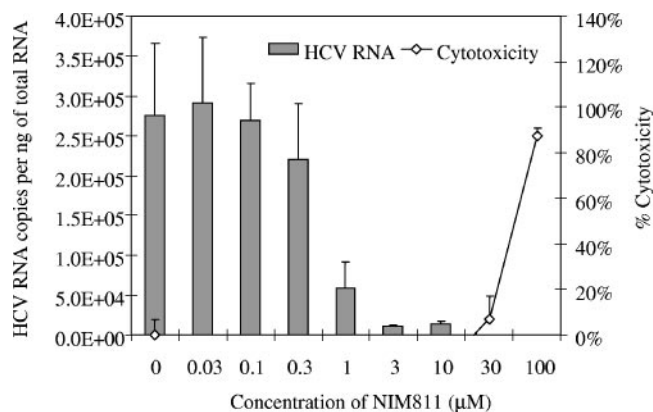
FIG. 2. Concentration-dependent reduction of HCV RNA following treatment of replicon cells with NIM811 for 48 h. HCV replicon cells (clone A) were incubated for 48 h with various concentrations of NIM811 (0.03 to 10 μM for anti-HCV activity and 0.3 to 100 μM for cytotoxicity). To determine antiviral activity, the level of remaining HCV RNA was measured by QRT-PCR and was normalized against the amount of total RNA extracted for each sample. Each data point represents the average for six replicates in cell culture in a single experiment. To monitor cytotoxic effect, the viability of the replicon cells following compound treatment was determined using a tetrazolium compound (MTS)-based assay and compared to that of untreated control cells. Each data point represents the average for three replicates in cell culture.

from the cells to offset any potential cytotoxic effect associated with compound treatments or any variation derived from RNA extraction. As shown in Fig. 2, NIM811 resulted in a concentration-dependent reduction of HCV RNA in the replicon cells. The mean IC_{50} of NIM811 after 48 h from three independent experiments was determined to be 0.66 μM. No significant cytotoxicity was observed with NIM811 at concentrations below 30 μM, and no significant inhibition of the expression of the glyceraldehyde-3-phosphate dehydrogenase housekeeping gene was observed (not shown), indicating that the inhibition of HCV RNA replication was specific.

The activities of NIM811 were further evaluated using other HCV replicon systems, the results of which are summarized in Table 1. NIM811 exhibited similar activities ($IC_{50} = 0.64$ μM) with a genomic replicon of genotype 1b (con1) HCV, Huh 21-5, which expresses all the viral structural protein in the cells as well as nonstructural proteins. In addition, NIM811 was tested against both subgenomic and genomic genotype 1a (H77) HCV replicons, for which it appeared to be slightly more potent, with IC_{50}s of 0.42 μM and 0.35 μM, respectively. There was no significant cytotoxicity observed in these cells with NIM811 at concentrations below 30 μM.

Anti-HCV activity of NIM811/cyclosporine derivatives correlates with their ability to bind cyclophilin. It has been shown previously that the cyclophilin-binding activity of cyclosporine appears to be required for its antiviral activity (17, 24). To further establish a quantitative correlation between the two functions, a number of cyclosporine derivatives with different cyclophilin-binding affinities were selected and tested in the HCV replicon assay. A replicon cell line (Huh-Luc/neo-ET) expressing a luciferase reporter under the control of HCV RNA replication was used to determine antiviral activity by measuring the reduction of luciferase activity in the cells. Cy-

clophilin-binding activity and immunosuppressive activity of these compounds have been determined and described in a previous report (1). All three activities of these compounds, described as IC_{50}s, were compared to those of cyclosporine and shown as *n*-fold increase over cyclosporine in Table 2. There was a very good correlation between the anti-HCV activity of these compounds and their cyclophilin-binding affinity but not with their immunosuppressive activity.

Serum protein-binding effect on NIM811. Human serum contains a high level of proteins, which may potentially reduce the in vivo efficacy of drugs that are highly protein bound. The effect of serum protein binding on the anti-HCV activity of NIM811 was examined in the luciferase-based HCV replicon assay by adding 10%, 20%, or 40% human serum to the compound treatment. BILN2061, a specific inhibitor of HCV NS3-4A serine protease (12), was included in this study for comparison. The IC_{50}s of the two compounds in the absence or presence of human serum are summarized in Table 3. As expected, there was a concentration-dependent increase of IC_{50} in the presence of human serum for both compounds. However, the increase of IC_{50} was modest with NIM811 (5.3-fold in the presence of 40% serum) compared to that of BILN2061 (54.5-fold in the presence of 40% serum) in the same experiment. There was no significant cytotoxicity observed with these compounds at the concentrations tested regardless of whether human serum was present.

TABLE 1. Anti-HCV activities and cytotoxicities of NIM811 in replicons[a]

HCV replicon	IC_{50} (μM)	CC_{50} (μM)
Genotype 1b, subgenomic	0.66	>30
Genotype 1b, genomic	0.64	>30
Genotype 1a, subgenomic	0.42	>30
Genotype 1a, genomic	0.35	>30

[a] HCV replicon cells were treated with six different concentrations of NIM811 for 48 h. Anti-HCV activity was determined by measuring the reduction of HCV RNA in the replicon cells using QRT-PCR. IC_{50} is the concentration of compound at which the level of HCV RNA is reduced by 50%. Cytotoxicity was determined using a tetrazolium compound (MTS)-based cell viability assay. CC_{50} is the concentration of compound at which the cell viability is reduced by 50%.

TABLE 2. HCV replicon inhibition, cyclophilin (Cyp) binding, and immunosuppressive activities of cyclosporine derivatives[a]

Compound	HCV replicon IC_{50} (μM)	Fold increase of IC_{50}		
		Replicon inhibition	Cyp binding	Immuno-suppression
[MeIle⁴]Cs (NIM811)	0.10	0.22	0.59	>1,700
[O-acetyl-D-MeSer³]Cs	0.14	0.31	0.75	2.4
[MeVal⁴]Cs	0.15	0.33	0.54	>2,500
Cyclosporine	0.45	1	1	1
[D-Lys⁸]Cs	2.07	4.60	2.6	150
[MeAla¹⁰]Cs	8.48	18.84	26	15
[O-Acetyl-MeBmt¹]Cs	>10	>22	>97	270

[a] Anti-HCV activity was determined in the 48-h luciferase-based HCV replicon assay. Cyclophilin-binding activity was determined in a competition assay with cyclosporine (1). The binding affinities to cyclophilin A are shown; however, these compounds also bind to cyclophilin B with similar affinities (unpublished results). Immunosuppressive activity was measured in an interleukin-2 reporter gene assay in Jurkat cells (1). IC_{50}s of various cyclosporine derivatives (Cs) in three assays were compared to those of cyclosporine, and results are shown as *n*-fold increase over those for cyclosporine. Me, methyl; Bmt, 4R-4-[(E)-2-butenyl]-4-methyl-L-threonine.

TABLE 3. Serum protein-binding effect on anti-HCV
activity of NIM811[a]

Compound	IC$_{50}$ of compound at human serum level of:			
	0%	10%	20%	40%
NIM811	0.12 μM	0.23 μM	0.30 μM	0.63 μM
BILN2061	0.53 nM	3.52 nM	7.13 nM	28.88 nM

[a] HCV replicon cells (Huh-Luc/neo-ET) were treated with various concentrations of NIM811 or BILN2061, a specific inhibitor of HCV NS3-4A protease, for 48 h in the absence or presence of 10%, 20%, or 40% human serum. IC$_{50}$ is the concentration of a compound at which the luciferase activity in the replicon cells is reduced by 50%.

NIM811 alone induced multilog HCV RNA reduction in the replicon cells after prolonged treatment. Current standard HCV therapy requires treating patients for 6 to 12 months, during which the viral load is progressively reduced by multiple logs and eventually becomes undetectable if the therapy is successful. Therefore, in addition to the standard IC$_{50}$ determination, it is highly relevant to evaluate potential novel antiviral agents in vitro for their abilities to induce multilog viral RNA reduction after prolonged treatment. Here, HCV replicon cells were treated with various concentrations of NIM811 for three, six, or nine consecutive days. At the end of each treatment, total RNA was extracted from the cells, and the quantity of HCV RNA was determined by QRT-PCR and normalized against the amount of total RNA extracted. The level of remaining HCV RNA with each compound treatment was compared to that for untreated control cells at the same time point to determine the log reduction of HCV RNA over time. As shown in Fig. 3, there was a concentration- and time-dependent reduction of HCV RNA with NIM811 treatment. Importantly, 1 μM NIM811 was able to reduce HCV RNA in the replicon cells by more than 3-log after 9 days of treatment, which was comparable to what has been reported with HCV protease inhibitors under similar conditions (13, 18). No significant cytotoxicity was observed in these experiments.

Combination of NIM811 with IFN-α significantly enhanced anti-HCV activity without increasing cytotoxicity. Current standard therapy for chronic hepatitis C is the combination of pegylated IFN-α and ribavirin. Although ribavirin does not exhibit a strong antiviral effect itself, it improves the sustained virological response when used in combination with IFN-α in hepatitis C patients. It is most likely that the future HCV therapy will continue be a combination of multiple drugs with different mechanisms of action. Therefore, it is important to investigate the effect of potential novel therapeutic agents when used together with IFN-α, the basis of current treatment.

The combination of NIM811 and IFN-α was first evaluated with the standard 48-h luciferase-based HCV replicon assay. HCV replicon cells, Huh-luc/neo-ET, were treated with vari-
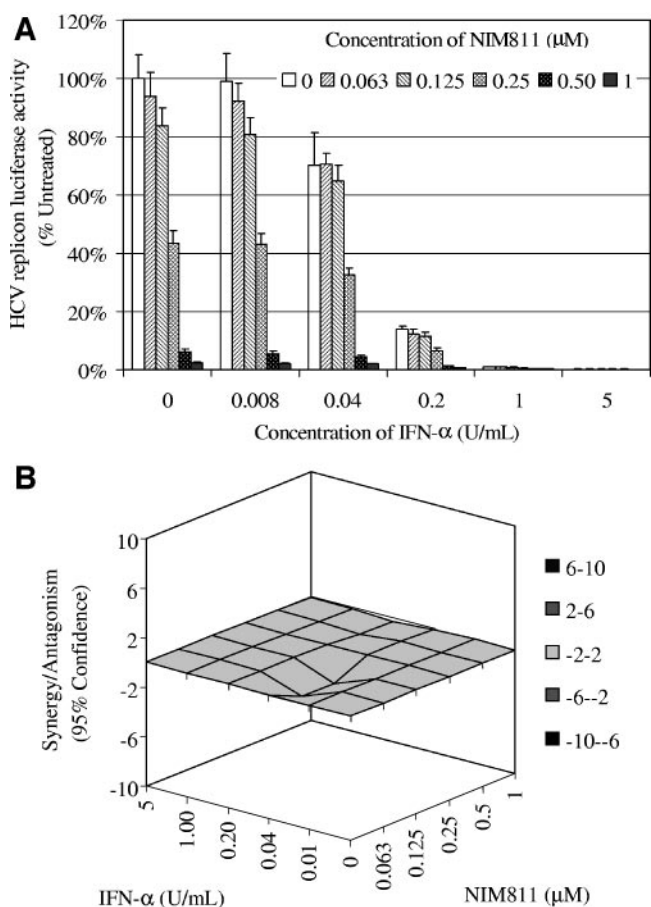
FIG. 4. Combination of NIM811 and IFN-α in the 48-h HCV replicon assay. (A) Inhibition of HCV replicon. HCV replicon cells (Huh-Luc/neo-ET) were treated with various concentrations of NIM811 alone, IFN-α alone, or the two in combination for 48 h. Antiviral activities were determined by measuring the reduction of luciferase activities in the cells. The average percentages of inhibition with all compound treatments compared to those for untreated control are shown as a three-dimensional response surface plot. Each data point represents the average for eight replicates in cell culture. (B) Synergy analysis. The results of combination of NIM811 and IFN-α in the 48-h HCV replicon assay were analyzed using a mathematical model, MacSynergy, as described Materials and Methods. The three-dimensional surface plot shown represents the differences (within 95% confidence interval) between actual experimental effects and theoretical additive effects of the combination at various concentrations of the two compounds.
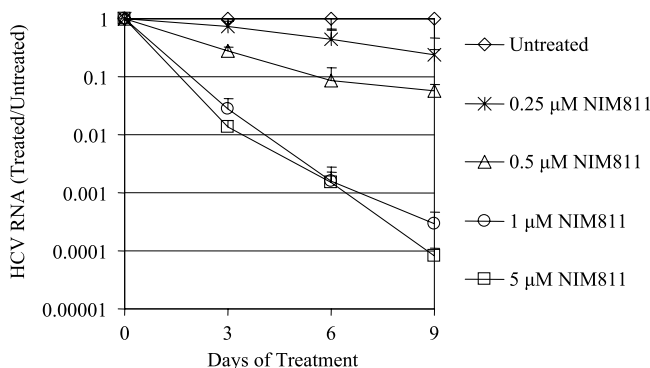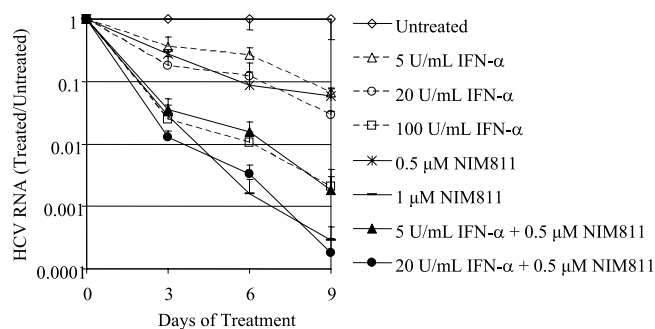

FIG. 3. Multilog reduction of HCV RNA in the replicon cells over 9 days of treatment with NIM811. HCV replicon cells (clone A) were treated with four different concentrations of NIM811 for 3, 6, or 9 days. Medium and compounds were refreshed every 3 days. At the end of each treatment, the quantity of HCV RNA was determined by QRT-PCR and was normalized against the amount of total RNA extracted for each sample. Each data point represents the average for five replicates in cell culture. The level of remaining HCV RNA in compound-treated cells was compared to that in untreated cells at the same time point to calculate the log reduction of HCV RNA.

FIG. 5. Combination of NIM811 with IFN-α facilitated multilog HCV RNA reduction. HCV replicon cells were treated with various concentrations of NIM811 alone, IFN-α alone, or the two in combination for 3, 6, or 9 days. Medium and compounds were refreshed every 3 days. At the end of each treatment, the quantity of HCV RNA was determined by QRT-PCR and was normalized against the amount of total RNA extracted for each sample. Each data point represents the average for five replicates in cell culture. The level of remaining HCV RNA in compound-treated cells was compared to that in untreated cells at the same time point to calculate the log reduction of HCV RNA.

ous concentrations of NIM811 and IFN-α either alone or in combination for 48 h. As shown in Fig. 4A, there was a concentration-dependent inhibition of HCV replicon with NIM811 alone, IFN-α alone, or the two in combination. Importantly, the combinations of the two compounds at various concentrations always resulted in a greater inhibition than either compound alone at the same concentration. To determine whether the effect of the combination was synergistic, additive, or antagonistic, the data were analyzed using a mathematic model, MacSynergy, as described in Materials and Methods (20). The result of the analysis is shown as a three-dimensional synergy plot in Fig. 4B. There was no significant difference between experimental effects and theoretical additive effects at various concentrations of the two compounds tested, indicating that the effect of the combination was largely additive based on this mathematical model. Moreover, the effect on cell viability was also examined to make sure that the inhibition of HCV replicon was not due to the cytotoxicity of the compounds. Importantly, no significant cytotoxicity was observed with NIM811 and IFN-α either alone or in combination in these experiments (data not shown).

The combination of NIM811 and IFN-α was further evaluated in the 9-day HCV RNA reduction assay. HCV replicon cells were treated with various concentrations of NIM811 alone, IFN-α alone, or the two in combination for 3, 6, or 9 days. As shown in Fig. 5, there was a time-dependent reduction of HCV RNA with all different compound treatments. After 9 days, 5 U/ml IFN-α resulted in a 1.2-log reduction of HCV RNA in the replicon cells, and 0.5 μM NIM811 also resulted in a 1.2-log HCV RNA reduction. In contrast, the combination of the two led to a 2.7-log viral RNA reduction, which was significantly greater than the effect of either agent alone. Moreover, 20 U/ml IFN-α alone resulted in a 1.5-log reduction of HCV RNA after 9 days, whereas the combination of 20 U/ml IFN-α and 0.5 μM NIM811 led to a 3.7-log viral RNA reduction, which was greater than that with a much higher concen-

tration (100 U/ml) of IFN-α alone (2.7-log) or a higher concentration (1 μM) of NIM811 alone (3.5-log).

DISCUSSION

Current drug discovery efforts for HCV focus primarily on two viral targets, NS3-4A protease and NS5B polymerase. However, drug-resistant mutations in the viral genome are likely to arise during therapy and reduce the effectiveness of specific inhibitors of viral enzymes. An alternative and complementary strategy is to target the cellular machinery that is also required for viral replication. It was reported recently that cyclosporine has inhibitory activities against HCV (16, 23). In the studies presented here, the anti-HCV activity of NIM811, a nonimmunosuppressive cyclosporine derivative, was demonstrated using both subgenomic and genomic replicon systems. NIM811 and other cyclosporine derivatives bind to a family of host proteins, cyclophilins, which have peptidyl-prolyl isomerase activity and are involved in various cellular processes. It has also been shown that cyclosporins are substrates and inhibitors of p-glycoprotein. One concern of targeting host factors directly is the potential side effects it may cause if the normal biological functions of these host factors are also blocked. Importantly, it was shown here that NIM811 did not affect cell viability or cell growth at the concentrations where it exhibited potent anti-HCV activity, suggesting a good therapeutic window. Nevertheless, the potential side effects associated with targeting host factors should be carefully monitored in vivo.

NIM811 has been shown previously to be a potent inhibitor of HIV (1, 21). HIV specifically incorporates cyclophilin A into virions via contacts with the capsid domain of the Gag polyprotein Pr55gag. NIM811 binds to cyclophilin A and inhibits its incorporation into HIV-1 virions (8). In contrast, NIM811 exhibited similar anti-HCV activities in genomic replicons versus subgenomic HCV replicons that lack viral structural proteins, suggesting that the anti-HCV effect of NIM811 is mediated by a mechanism different from that used in HIV. Interestingly, K. Watashi et al. recently reported that cyclophilin B bound to HCV NS5B polymerase directly and increased its RNA-binding activity, the functions of which were blocked in the presence of cyclosporine (24). On the other hand, since there has been no evidence that virions or virus-like particles are produced in the genomic genotype 1 HCV replicon systems used in this study and the functions of viral structural proteins are not clear (19), it cannot be ruled out that the HCV structural proteins may play additional roles in mediating the anti-HCV effect of NIM811, which remains to be investigated in a true viral infection model.

There are six different genotypes of HCV, of which genotype 1 is the predominant genotype in North America, Europe, and Japan. Patients with genotype 1 virus also have the lowest response rate to the current alpha interferon-based therapy and therefore represent a major unmet medical need. There are two main subtypes of genotype 1 virus, 1a and 1b. The antiviral activities of NIM811 were demonstrated with both 1a and 1b replicons in this study, with slightly better activities against genotype 1a. Interestingly, while the manuscript was being reviewed, Ishii et al. reported that the replication of a genotype 2a HCV (JFH1 strain) was not mediated by cyclophilin B and was less sensitive to cyclosporine or NIM811 treatment than

genotype 1b HCV (10). The clinical significance of these findings remain to be investigated.

Most if not all drugs bind to human serum proteins to a certain extent. It has been suggested that high protein-binding activity may significantly limit the availability of free drug in circulation where high concentration of serum proteins are present and thus decrease efficacy in patients. Here, we showed that there was only a moderate (fivefold) increase of IC_{50} with NIM811 in the presence of 40% human serum compared to >50-fold with the HCV NS3-4A protease inhibitor, BILN2061 (12). This observation is consistent with the moderate protein-binding activity (87.8 to 89.3%) of NIM811 compared to >99% protein-binding activity reported for BILN2061. However, the significance of serum protein binding for the clinical efficacy of HCV drugs remains unclear, since their main site of action in patients is the liver, where the virus predominantly replicates.

Current standard therapy for chronic hepatitis C is the combination of pegylated IFN-α and ribavirin. Given the high replication efficiency of the virus in patients and likely emergence of resistant mutations during long-term treatment, it is most likely that the future therapy for HCV, like that for HIV, will be combinations of multiple drugs with different mechanisms. NIM811, an HCV inhibitor with a novel mechanism of action, presents an opportunity for new combinations in future HCV therapy. Although the ultimate test will be in the clinic, it is beneficial to investigate the combinations in vitro beforehand to determine their potential effects on antiviral activity as well as toxicity. Data presented here first demonstrated that the combination of NIM811 and IFN-α led to a significantly greater inhibition of HCV replication than either agent alone in the standard 48-h HCV replicon assay. Moreover, the effect of the combination was determined to be additive when analyzed in a mathematical model based on the Bliss independence theory. Similar results were also obtained with the combination of cyclosporine and IFN-α, as well as in the QRT-PCR based HCV replicon assay measuring viral RNA reduction after 48 h (not shown). It is important to note that none of the combinations tested resulted in any significant increase of cytotoxicity. The combination of NIM811 and IFN-α was further evaluated in the 9-day HCV RNA reduction assay, in which the combination of the two agents led to a much higher degree of viral RNA reduction than either agent alone. In fact, the effect of the combination was even better than those of the two single treatments added together (2.7 log versus 1.2 log plus 1.2 log or 3.7 log versus 1.2 log plus 1.5 log), suggesting a potentially synergistic effect after 9 days of treatment. Moreover, the combination of NIM811 and IFN-α resulted in stronger antiviral effects than those of single treatments at much higher concentrations, which supports the rationale of combination therapy aimed to reduce the drug dosages and thus potential side effects associated with high doses of either drug.

Taken together, we have demonstrated that NIM811 is a potent inhibitor of HCV RNA replication in vitro. A greater than 3-log reduction of HCV RNA can be achieved after treating the replicon cells for 9 days with as low as 1 μM NIM811, which is likely attainable based on its favorable pharmacokinetics and safety profile. NIM811 represents an inhibitor of a novel mechanism targeting host factors and can be used in combination with other anti-HCV agents as illustrated

in this study. These data warrant further clinical investigation of NIM811 in patients with chronic hepatitis C.

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