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Original article

Combination therapy for hepatitis C virus with heat-shock protein 90 inhibitor 17-AAG and proteasome inhibitor MG132

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Background: Hepatitis C virus (HCV) infection is a major cause of chronic liver disease. Here, we report a new and effective strategy for inhibiting HCV replication using an inhibitor of heat-shock protein 90, 17-AAG (17-allylamino-17demethoxygeldanamycin), and a proteasome inhibitor, MG132.

Methods: To explore the virological basis of combination therapy, we analysed the effects of 17-AAG and MG132, singly and in combination on HCV replication in an HCV replicon cell system.

Results: In HCV replicon cells, HCV RNA replication was suppressed by 17-AAG in a dose-dependent manner. As shown in the present study, the 50% inhibitory concentration values were 0.82 nM for 17-AAG and 0.21 nM for MG132. Low concentrations of MG132 had strong synergistic inhibitory effects with low toxicity on HCV replicon cells.

Conclusions: The results of this study suggest that the different effects and synergistic actions of 17-AAG and MG132 could provide a new therapeutic approach to HCV infection.

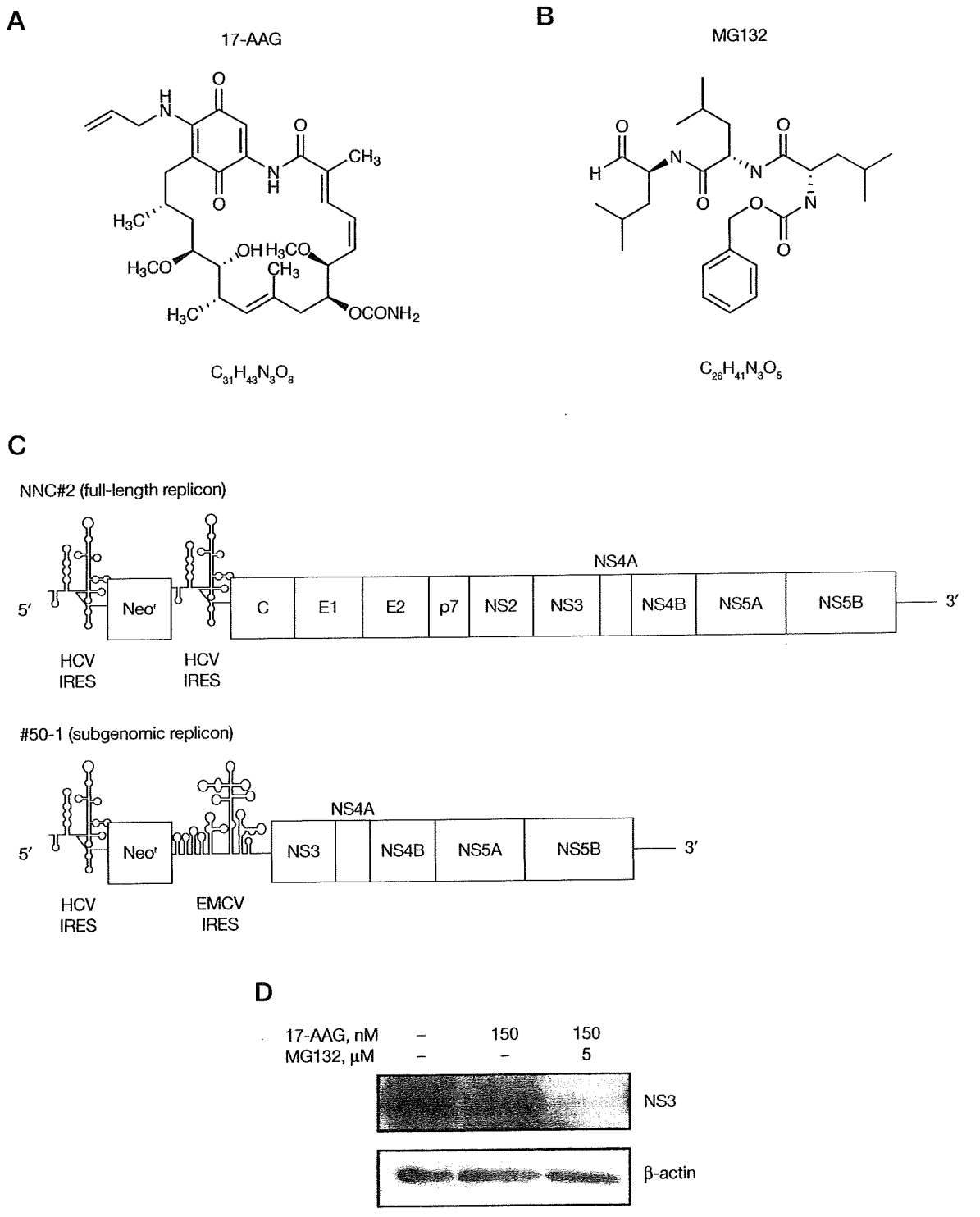
Introduction

Infection by the hepatitis C virus (HCV) is a major public health problem, with 170 million chronically infected people worldwide [1,2]. Combined treatment with interferon-α and ribavirin induces sustained antiviral response in 80% of patients with HCV genotype 2 or 3 [3,4]. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma [5,6]. Although interferon-α plus ribavirin therapy is effective for approximately 50–80% of patients with HCV, the development of improved therapies and preventative vaccines is urgently needed [7].

Heat-shock protein 90 (Hsp90) exerts chaperone activity together with a number of cochaperones, playing an important role in the folding of at least 200 specific proteins of various signalling pathways and in the refolding of proteins that have been denatured by stress [8–11]. Hsp90 is considered to be of prime importance to the survival of cancer cells. The constitutive expression of Hsp90 is 2- to 10-fold higher in tumour cells compared with their normal counterparts, suggesting that Hsp90 is critically important for tumour cell growth and/or survival. A small molecule inhibitor of Hsp90, the benzoquinone ansamycin 17-AAG (17-allylamino-17-desmethoxygeldanamycin; Figure 1A), exhibits antitumour activity in several human xenograft models, including colon, breast and prostate cancer [12–14]. The drug is currently completing multi-institution Phase I clinical trials, and Phase II trials are being planned.

The HCV non-structural protein 3 (NS3) forms a complex with Hsp90 [8,10,11] that is critical for HCV replication [15]. Treatment of the #50-1 HCV replicon cells with the Hsp90 inhibitor 17-AAG [8,16,17] suppresses HCV RNA replication and NS3 is the only HCV protein degraded in these cells [15]. This finding led us to suggest a crucial role for Hsp90–NS3 complexes in the HCV life cycle. By contrast, the proteasome is a large protein complex that also participates in protein degradation [18]. Among the 28 subunits of the 20S proteasome, the α-subunit 7 (PSMA7) is one of the α-ring subunits at the barrel centre of the proteasome complex. The 20S proteasome is activated

Figure 1. Schematic representation of the HCV replicon and the structures of 17-AAG and MG132



(A) Structure of the heat-shock protein 90 inhibitor 17-AAG. (B) Structure of the proteasome inhibitor MG132. (C) Structure of the hepatitis C virus (HCV) replicon RNAs comprising the HCV 5′-untranslated region, including the HCV internal ribosome entry site (IRES), the neomycin phosphotransferase gene (Neo^r), the encephalomyocarditis virus (EMCV) IRES or the HCV IRES, and the coding region for HCV proteins NS3 to NS5B (in the HCV subgenomic replicon) or core protein to NS5B (in the HCV full-length replicon). (D) Effect of 17-AAG and MG132 on expression of the NS3 protein. β-Actin was used as a lysate control. The HCV full-length replicon (NNC#2 cells) was analysed by western blotting after 17-AAG treatment with or without MG132.

upon association with its regulatory protein complex, classified as PA700 (19S regulator) and PA28 (11S regulator) [18]. Ribozymes and small interference RNAs that specifically target the putative HCV cofactor PSMA7 inhibit HCV expression [19,20].

In the present study, we describe our findings of HCV combination therapy with the Hsp90 inhibitor 17-AAG and the proteasome inhibitor MG132 (Figure 1B). Combining these different inhibitors produced significant synergistic inhibitory effects on HCV replicons, indicating that the inhibitors might be useful as an efficient dual strategy of molecular HCV therapeutics.

Methods

Cell culture and reagents

The HCV replicon cell line #50-1 (NN/1b/SG) [21], which carries a subgenomic replicon, and NNC#2 (NN/1b/FL) [22], which carries a full genome replicon, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, non-essential amino acids, L-glutamine, penicillin–streptomycin, and 300–1,000 μg/ml G418 (Invitrogen, Carlsbad, CA, USA) at 37°C in 5% CO_2. Huh-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. 17-AAG and MG132 were purchased from Sigma–Aldrich Chemical Co. (St Louis, MO, USA).

Real-time reverse transcriptase PCR analysis

HCV replicon cells were seeded at 1.5×10^5 cells in 24-well plates and cultured for 72 h. Total RNA was then isolated using Trizol (Invitrogen) according to the manufacturer's instructions. HCV RNA was quantified by real-time reverse transcriptase (RT)-PCR using an ABI 7700 sequence detector (Perkin-Elmer Applied Biosystems, Foster City, CA, USA), and the following primers and TaqMan probes located in the 5′-untranslated region: forward primer (nucleotides [nt] 130–146), 5′-CGGGAGAGCCATAGTGG-3′; reverse primer (nt 272–290), 5′-AGTACCACAAGGCCTTTCG-3′; and TaqMan probe (nt 148–168), 5′-CTGCGGAACCGGTGAGTACAC-3′ (all purchased from Applied Biosystems). The probe sequence was labelled with the reporter dye 6-carboxyfluorescein at the 5′-end and with the quencher dye TAMRA at the 3′-end [23].

Western blotting

NNC#2 cells were seeded at 1.5×10^5 cells in 24-well plates, treated with 17-AAG alone, MG132 alone, or combined 17-AAG and MG132, and cultured for 48 h. Western blotting analysis was performed using a previously described method [15]. The primary antibodies were monoclonal or polyclonal antibodies against PSMA7 (Abcam, Cambridge, MA, USA) and β-actine (SIGMA, Sigma–Aldrich). The anticore antibody was a kind gift from M Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Anti-NS3 antibody was a kind gift from Y Matsuura (Osaka University, Osaka, Japan).

Transfection and reporter assay

Huh-7 cells were seeded at 1.5×10^5 cells 24 h before transfection. Huh-7 cells were treated with MG132 (100 nM) following transfection with the plasmid DNA pHCV internal ribosome entry site (IRES) luciferase (luc) or pEMCV IRES luc using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luc activity was measured in the cell lysates using a luminometer (Berthold, Bad Wildbad, Germany).

MTS assay

HCV replicon cells were seeded in 96-well plates at 3×10^4 cells per well in a final culture volume of 100 μl for 72 h before adding increasing concentrations of 17-AAG and MG132. After incubation for 3 days, viable cell numbers were determined using the CellTitre 96® Aqueous non-radioactive cell proliferation assay (Promega Corp., Madison, WI, USA). The value of the background absorbance at 490 nm (A_{490}) of wells without cells was subtracted from the A_{490} value of wells with cells. The percentages of viable cells were then calculated using the following formula: (A_{490} 17-AAG-, MG132- or 17-AAG+MG132-treated cells/ A_{490} untreated cells)×100.

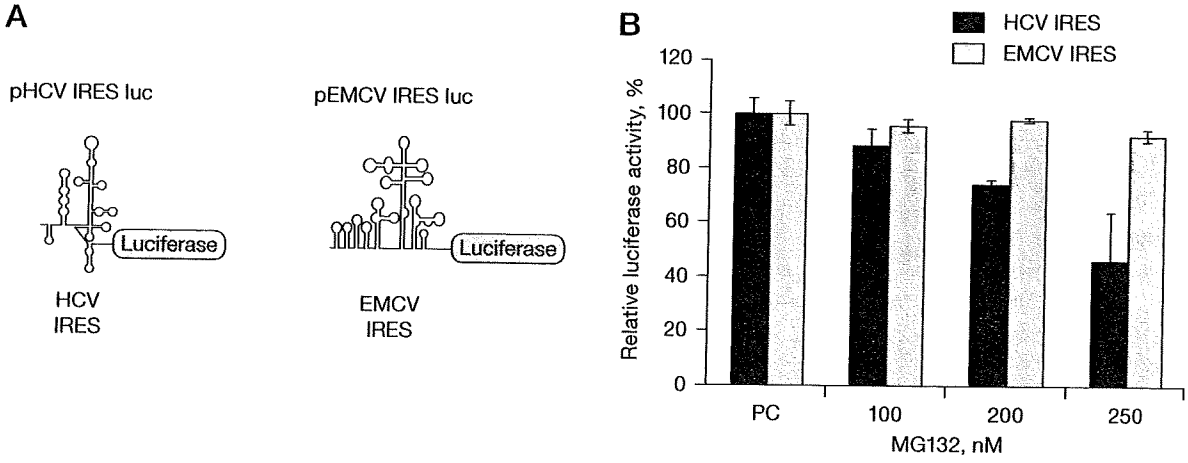
Drug synergism analysis

The effect of treatment of HCV replicon cells with 17-AAG and MG132, alone and in combination, was analysed by measuring HCV RNA with real-time PCR. The combination index (CI) for each combination of 17-AAG and MG132 treatment was calculated by the following formula using the 50% inhibitory concentration (IC_{50}): CI= IC_{50}(17-AAG combined)/IC_{50}(17-AAG alone) +IC_{50}(MG132 combined)/IC_{50}(MG132 alone). For such plots, the combined effects of the two drugs can be assessed as either additive (CI=1), synergistic (CI<1) or antagonistic (CI>1) [24].

Results

In a previous study, we examined the inhibitory effects of 17-AAG on HCV replication in an HCV replicon cell culture system. In HCV replicon cells treated with 17-AAG, HCV RNA replication was suppressed in a dose-dependent manner, and the only HCV protein degraded in these cells was NS3 [15]. To determine whether 17-AAG promoted the degradation of NS3,

Figure 2. Effect of proteasome inhibitor MG132 on HCV IRES activity



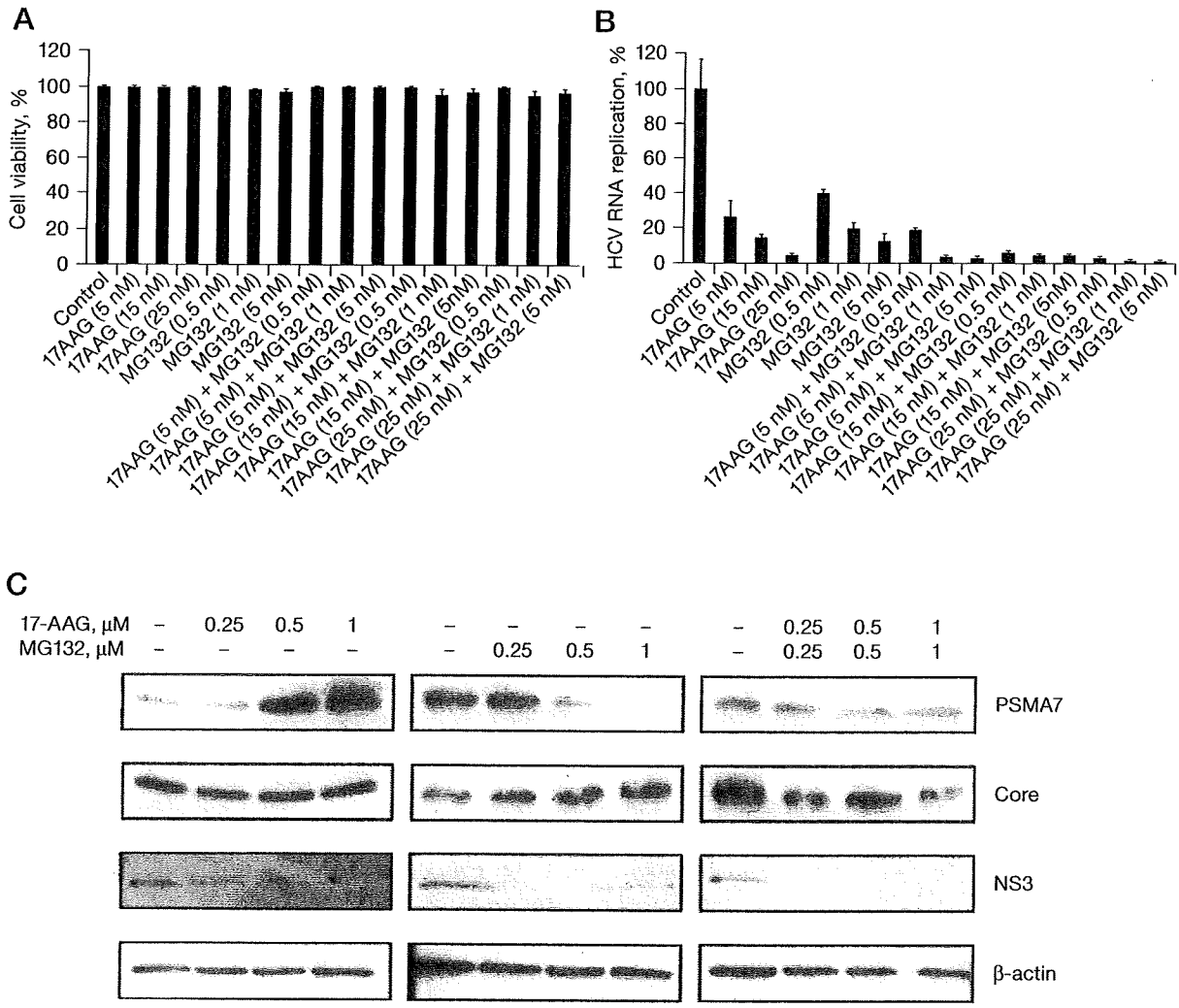
(A) Vector construct for hepatitis C virus (HCV) internal ribosome entry site (IRES)-mediated (pHCV IRES luc) or encephalomyocarditis virus (EMCV) IRES-mediated (pEMCV IRES luc) translation of firefly luciferase. (B) Huh-7 cells were transfected with pHCV IRES luc alone or pEMCV IRES luc alone (PC), or also treated with MG132 100, 200 and 250 nM. After 24 h, HCV IRES activity was determined by luciferase assay. Data are means ±SD of triplicate experiments.

we evaluated the effect of 17-AAG on #50-1 cells in which proteasomal degradation was also inhibited with the proteasome inhibitor MG132. Although 17-AAG treatment reduced NS3 levels in #50-1 cells, this NS3 degradation was completely blocked in the presence of MG132 [15]. In the present study, we used HCV full-length replicon NNC#2 cells (Figure 1C) [22] instead of the HCV subgenomic replicon #50-1 cells used in previous studies [15,21]. Treatment with 17-AAG decreased NS3 levels, but the presence of MG132 did not block the reduction in HCV NS3 (Figure 1D). Indeed, MG132 in combination with 17-AAG induced the complete disappearance of the NS3 (Figure 1D), suggesting that the reduction of HCV NS3 induced by treatment with the proteasome inhibitor MG132 was dependent on the HCV IRES. NNC#2 and #50-1 cells have different virus IRESs, known as the HCV IRES [25–27] and the encephalomyocarditis virus (EMCV) IRES [28], and HCV IRES-mediated translation is induced by PSMA7 [29]. The PSMA7 activity is blocked by MG132 [30]. Similar effects were observed when PSMA7-directed ribozyme or small interfering RNAs inhibited HCV replication [19,20].

We examined the effects of combination therapy with 17-AAG and MG132 on HCV. To evaluate the MG132-mediated effect on HCV IRES activity, Huh-7 cells were treated with MG132 (100, 200 and 250 nM) and then transfected with pHCV IRES luc or pEMCV IRES luc (Figure 2A). MG132 inhibited luc activity of HCV IRES in dose-dependent manner, but did not inhibit translation derived from EMCV IRES (Figure 2B), indicating a preferential effect of MG132 on HCV IRES activity [19]. NNC#2 cells were then treated with 17-AAG or MG132 for 3 days. HCV RNA was quantified by real-time RT-PCR using an ABI 7500 Fast (Perkin-Elmer, Applied Biosystems). Dose-dependent cytotoxicity was not observed upon application of MG132 and/or 17-AAG (99% at concentrations ranging between 5 and 25 nM; Figure 3A), but was observed at MG132 concentrations of 10 μM (data not shown). Cells treated with increasing doses of 17-AAG or MG132 showed reduced levels of HCV RNA (Figure 3B). Low concentrations (1 nM) of MG132 had inhibitory effects similar to those of 15 nM 17-AAG (Figure 3B). In NNC#2 cells treated with 5 nM MG132, HCV RNA replication was suppressed by 85%, and this effect was dose-dependent (Figure 3B). The IC_{50} values were 0.82 nM for 17-AAG and 0.21 nM for MG132 (Table 1 and Figure 3B).

We also investigated combination therapy with 17-AAG and MG132 on HCV in NNC#2 cells. In combination with 5 nM, 15 nM or 25 nM of 17-AAG, 0.5 nM, 1 nM or 5 nM of MG132 was applied to NNC#2 cells, respectively. The combination of 5 nM 17-AAG and 1 nM MG132 suppressed HCV RNA replication by 90% in the NNC#2 cells (Figure 3B). The CI was 0.42, demonstrating that 17-AAG and MG132 had synergistic inhibitory effects on HCV replicon cells (Table 1). The combination of 17-AAG with MG132 inhibited HCV RNA replication with an approximately 2.5-fold reduction in the dose (Table 1). To determine the effects of 17-AAG and MG132 on the expression of core and PSMA7 proteins, NNC#2 cells were treated

Figure 3. MG132 and 17-AAG inhibition of HCV RNA replication in HCV replicon cells



(A) Cytotoxic effects of 17-AAG and MG132, singly or in combination, on NNC#2 cells, shown as the percentage reduction of the number of viable cells assessed by MTS assay. Data are means ±SD of triplicate experiments. (B) Inhibition of hepatitis C virus (HCV) replication by 17-AAG and MG132, singly or in combination, in NNC#2 cells. Measurement of HCV replication by real-time reverse transcriptase PCR. Data are means ±SD of triplicate experiments. (C) Effect of 17-AAG and MG132 on the expression of core, non-structural protein 3 (NS3) and the proteasome α-subunit 7 (PSMA7) protein. The core, NS3 and PSMA7 protein were analysed by western blotting after treatment of NNC#2 cells with various concentrations of 17-AAG or MG132, alone or in combination. β-Actin was used as a lysate control.

with various concentrations of 17-AAG or MG132 alone, and 17-AAG plus MG132 at day 2. Treatment with 17-AAG alone increased the expression of PSMA7, but did not reduce core protein expression. By contrast, treatment with MG132 alone reduced the expression of PSMA7, but not the core protein (Figure 3C), whereas expression of NS3 was reduced by treatment with 17-AAG or MG132 alone, and 17-AAG plus MG132. This result was consistent with our previous findings that NS3 levels were reduced in NNC#2 cells treated with 17-AAG for 3 days, but that core protein

Table 1. Combination index and dose reduction in inhibition of hepatitis C virus RNA replication by combining 17-AAG with MG132

Drug	IC_{50}, nM		Fold dose reduction[a]
	Alone	In combination	
17-AAG	0.82	0.33	2.52
MG132	0.21	0.01	21.00

The combination index for 17-AAG and MG132 was 0.42. [a]Dose reduction is the 50% inhibitory concentration (IC_{50}) of the drug in combination. Each sample was tested in triplicate and the mean values are presented

was detected for up to 6 days [15]. Other researchers, however, have reported that MG132 blocks the degradation of HCV core protein [31]. Interestingly, combined treatment with MG132 and 17-AAG reduced the expression of both the core and PSMA7 proteins in NNC#2 cells (Figure 3C). These results suggest that MG132 and 17-AAG are potent anti-HCV agents, and are more effective in combination therapy than as single monotherapeutic agents.

Discussion

HCV is a major cause of chronic liver disease. The results of the present study indicate that the individual effects of the Hsp90 inhibitor 17-AAG on HCV replicon cells and the synergistic action of the proteasome inhibitor MG132 might account for the improved clinical response to combination therapy. We previously reported a new and effective strategy for inhibiting HCV replication using 17-AAG to inhibit Hsp90 [15]. The mechanism by which 17-AAG so effectively suppresses HCV replication is the destabilization of NS3, which disrupts the Hsp90 chaperone complex. A previous study demonstrated that HCV NS3 degradation is greatly increased by treatment of HCV subgenomic replicon #50-1 cells with 17-AAG, but this degradation is completely blocked in the presence of the proteasome inhibitor MG132 [15]. By contrast, in the present study we used HCV full-length replicon NNC#2 cells in place of HCV subgenomic replicon #50-1 cells used in previous studies [15]. The resulting 17-AAG treatment reduced NS3 levels, but MG132 treatment of NNC#2 cells did not block the degradation of HCV NS3 (Figure 1D). This blocking efficiency significantly influenced the different activities of the virus IRES (that is, HCV IRES and EMCV IRES) [25–28]. In our assays, when Huh-7 cells exposed to MG132 were transfected with pHCV IRES luc or pEMCV IRES luc, HCV IRES activity was inhibited, but EMCV IRES was not (Figure 2B). Apcher *et al.* [29] reported that HCV IRES-mediated translation can be induced by PSMA7. The proteasome inhibitor MG132 inhibits PSMA7 activity. These findings demonstrate that HCV IRES activity is also reduced by MG132. By contrast, degradation of NS3 by 17-AAG is dependent on the proteasome system [15].

The present study demonstrated that NNC#2 cells containing a full HCV genome replicon treated with 17-AAG or MG132 for 3 days did not show dose-dependent cytotoxicity (Figure 3A), but 10 μM MG132 was cytotoxic (data not shown). As shown in the present study, the IC_{50} values were 0.82 nM for 17-AAG and 0.21 nM for MG132 (Table 1 and Figure 3B). These data provide evidence that a dual treatment strategy with 17-AAG and MG132 inhibits HCV replication. The combination of 5 nM 17-AAG and 1 nM MG132 suppressed HCV RNA replication by 90% in NNC#2 HCV replicon cells (Figure 3B). The two drugs, 17-AAG and MG132, had synergistic inhibitory effects on HCV replicon (Table 1). Importantly, the combined use of these different groups of inhibitors showed strong synergistic inhibitory effects on HCV replication, indicating that combining these inhibitors might be a useful and efficient strategy for anti-HCV chemotherapy.

Given the absence of a single effective and proven antiviral agent against HCV, the combination of 17-AAG with agents that possess potential antiviral effects will continue to dominate novel therapeutic approaches. The present study demonstrated a strong synergistic effect of 17-AAG and MG132 on intracellular HCV replication, and these effects are attributable to the direct and specific inhibition of viral replication. Our results indicate that antiviral treatment with 17-AAG might be improved by combining 17-AAG with MG132, and that this combination therapy might be a feasible strategy for the treatment of HCV infection. Modifications of these inhibitors might also result in the development of more effective antiviral compounds.

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Disclosure statement

The authors declare no competing interests.

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Heat-shock Protein 90 Is Essential for Stabilization of the Hepatitis C Virus Nonstructural Protein NS3*

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The hepatitis C virus (HCV) is a major cause of chronic liver disease. Here, we report a new and effective strategy for inhibiting HCV replication using 17-allylaminogeldanamycin (17-AAG), an inhibitor of heat-shock protein 90 (Hsp90). Hsp90 is a molecular chaperone with a key role in stabilizing the conformation of many oncogenic signaling proteins. We examined the inhibitory effects of 17-AAG on HCV replication in an HCV replicon cell culture system. In HCV replicon cells treated with 17-AAG, we found that HCV RNA replication was suppressed in a dose-dependent manner, and interestingly, the only HCV protein degraded in these cells was NS3 (nonstructural protein 3). Immunoprecipitation experiments showed that NS3 directly interacted with Hsp90, as did proteins expressed from ΔNS3 protease expression vectors. These results suggest that the suppression of HCV RNA replication is due to the destabilization of NS3 in disruption of the Hsp90 chaperone complex by 17-AAG.

Infection by the hepatitis C virus (HCV)[2] is a major public health problem, with 170 million chronically infected people worldwide (1, 2). The current treatment by combined interferon-ribavirin therapy fails to cure the infection in 30–50% of cases (3, 4), particularly those with HCV genotypes 1 and 2. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma (5, 6). Although an effective combined interferon-α-ribavirin therapy is available for about 50% of the patients with HCV, better therapies are needed, and preventative vaccines have not yet been developed.

HCV is a member of the *Flaviviridae* family and has a positive strand RNA genome (7, 8) that encodes a large precursor polyprotein, which is cleaved by host and viral proteases to generate at least 10 functional viral proteins: core, E1 (envelope 1), E2, p7, NS2 (nonstructural protein 2), NS3, NS4A, NS4B, NS5A, and NS5B (9, 10). NS2 and the amino terminus of NS3 comprise the NS2-3 protease responsible for cleavage between NS2 and NS3 (9, 11), whereas NS3 is a multifunctional protein consisting of an amino-terminal protease domain required for processing NS3 to NS5B (12, 13). NS4A is a cofactor that activates the NS3 protease function by forming a heterodimer (14–17), and the hydrophobic protein NS4B induces the formation of a cytoplasmic vesicular structure, designated the membranous web, which is likely to contain the replication complex of HCV (18, 19). NS5A is a phosphoprotein that appears to play an important role in viral replication (20–23), and NS5B is the RNA-dependent RNA polymerase of HCV (24, 25). The 3′-untranslated region consists of a short variable sequence, a poly(U)-poly(UC) tract, and a highly conserved X region and is critical for HCV RNA replication and HCV infection (26–29).

Hsp90 (heat-shock protein 90) is a molecular chaperone that plays a key role in the conformational maturation of many cellular proteins. Hsp90 normally functions in association with other co-chaperone proteins, which together play an important role in folding newly synthesized proteins and stabilizing and refolding denatured proteins in cells subjected to stress (30–34). Its expression is induced by cellular stress and is also associated with many types of tumor. Hsp90 inhibitors are currently showing great promise as novel pharmacological agents for anticancer therapy.

Hsp90 inhibitors have two major modes of action as preferential clients for protein degradation or as Hsp70 inducers. The benzoquinone ansamycin antibiotic geldanamycin and its less toxic analogue 17-allylamino-17-demethoxygeldanamycin (17-AAG) directly bind to the ATP/ADP binding pocket of Hsp90 (34–36) and thus prevent ATP binding and the completion of client protein refolding. Recently, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protease, using NS2/3 translated by rabbit reticulocyte lysate. Nakagawa *et al.* (38) also reported that inhibition of Hsp90 is highly effective in suppressing HCV genome replication. Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. More recently, Okamoto *et al.* (39) reported that Hsp90 could bind to FKBP8 (FK506-binding protein 8) and form a complex with NS5A. The interaction with FKBP8 has also been shown to be the mechanism by which Hsp90 regulates HCV RNA replication, a process in which Hsp90 clearly plays an important role.

In this study, we have demonstrated that NS3 also forms a complex with Hsp90, which is critical for HCV replication. On the basis of the findings that treating HCV replicon cells with

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[2] The abbreviations used are: HCV, hepatitis C virus; 17-AAG, 17-allylamino-17-demethoxygeldanamycin.

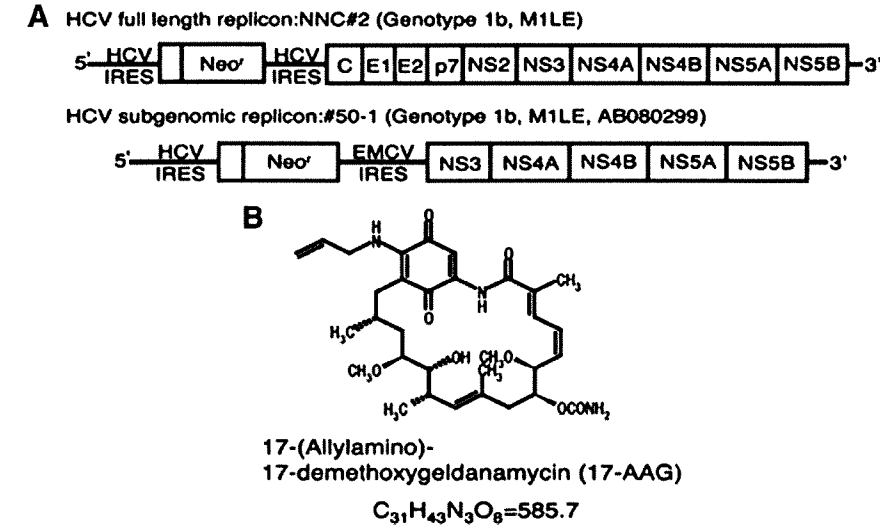

FIGURE 1. **Schematic representation of HCV replicon and structure of 17-AAG.** *A*, structure of the HCV replicon RNAs, comprising the HCV 5′-untranslated region, including the HCV internal ribosome entry site (*IRES*), the neomycin phosphotransferase gene (*Neo'*), the encephalomyocarditis virus (*EMCV*) IRES or HCV IRES, and the coding region for HCV proteins NS3 to NS5B (in the HCV subgenomic replicon) or core to NS5B (in the HCV full-length replicon). *B*, structures of the Hsp90 inhibitor, 17-AAG.

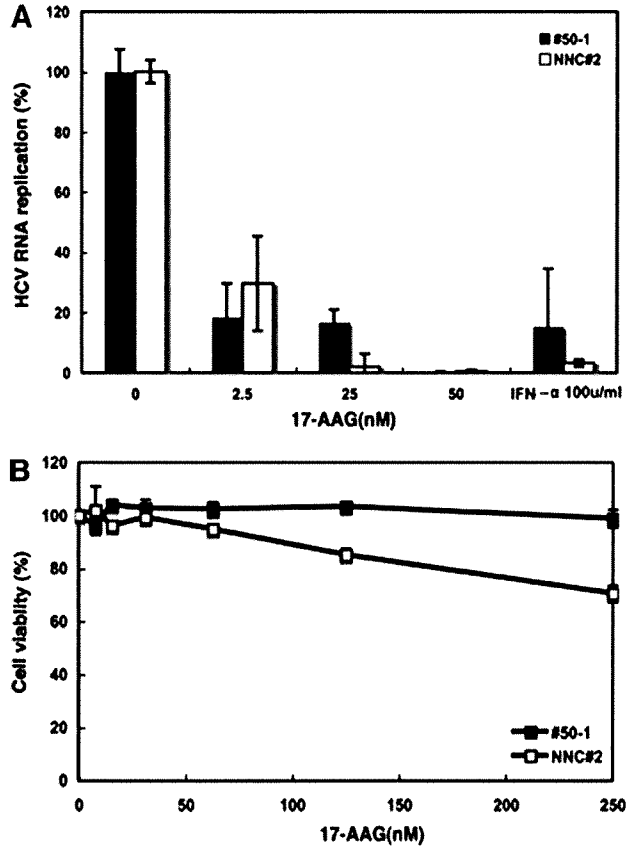

FIGURE 2. **Hsp90 inhibits HCV RNA replication in HCV replicon cells.** *A*, inhibition of HCV replication by 17-AAG in NNC#2 (*white squares*) and #50-1 cells (*black squares*) measured by real time reverse transcription-PCR after 72 h. Interferon-α was used as a positive control. The data are means ± S.D. from triplicate experiments. *B*, cytotoxic effects of 17-AAG in NNC#2 (*white squares*) and #50-1 (*black squares*), shown as the percentage reduction in viable cell numbers in an [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (inner salt)] assay. The data are means ± S.D. from triplicate experiments.

the Hsp90 inhibitor, 17-AAG, suppressed HCV RNA replication, and that the only HCV protein degraded in these cells was NS3, we suggest a crucial role for Hsp90-NS3 protein complexes in the HCV life cycle.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—The HCV replicon cell lines #50-1 (NN/1b/SG) (40), which carries a subgenomic replicon, and NNC#2 (NN/1b/FL) (41), which carries a full genome replicon, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, penicillin/streptomycin, and 300–1,000 μg/ml G418 (Invitrogen) at 37 °C in 5% CO_2. The human embryonic kidney-derived cell line 293T was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. 17-AAG was purchased from Sigma.

Measuring HCV RNA by Real Time PCR—HCV replicon cells were seeded at 1.5×10^5 cells in 24-well plates and cultured for 72 h. Total RNA was then isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. HCV RNA was quantified by real time reverse transcription-PCR using an ABI 7700 sequence detector (PerkinElmer Life Sciences) and the following primers and TaqMan probes located in the 5′-untranslated region: forward primer (nucleotides 130–146), 5′-CGGGAGAGCCATAGTGG-3′; reverse primer (nucleotides 272–290), 5′-AGTACCACAAGGCCTTTCG-3′; and TaqMan probe (nucleotides 148–168), 5′-CTGCGGAACCG-GTGAGTACAC-3′ (all purchased from Applied Biosystems). The probe sequence was labeled with the reporter dye, 6-carboxyflurorescein, at the 5′-end and with the quencher dye TAMRA at the 3′-end (42).

Western Blotting and Immunoprecipitation Analyses—Cells were lysed in 1× CAT enzyme-linked immunosorbent assay buffer (Roche Applied Sciences). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, and these were blocked with 5% skimmed milk. The primary antibodies used were monoclonal or polyclonal antibody against FLAG-M5 (Sigma), Hsp70 (Sigma), Hsp90 (Cell Signaling Technologies, Danvers, MA), Hsp90α (Calbiochem), Hsp90β (Calbiochem), and Hsf-1 (Calbiochem). Core, NS4A, and NS4B were a gift from Dr. M. Kohara (Tokyo Metropolitan Institute of Medical Science). E1, E2, NS3, NS5A, and NS5B were a gift from Prof. Y. Matsuura (Osaka University, Japan). Immunoprecipitation from cell lysates was carried out using anti-FLAG M5 antibody (Sigma) and the Protein G immunoprecipitation kit (Sigma), according to the manufacturer's instructions, and the immunoprecipitates were analyzed by Western blotting.

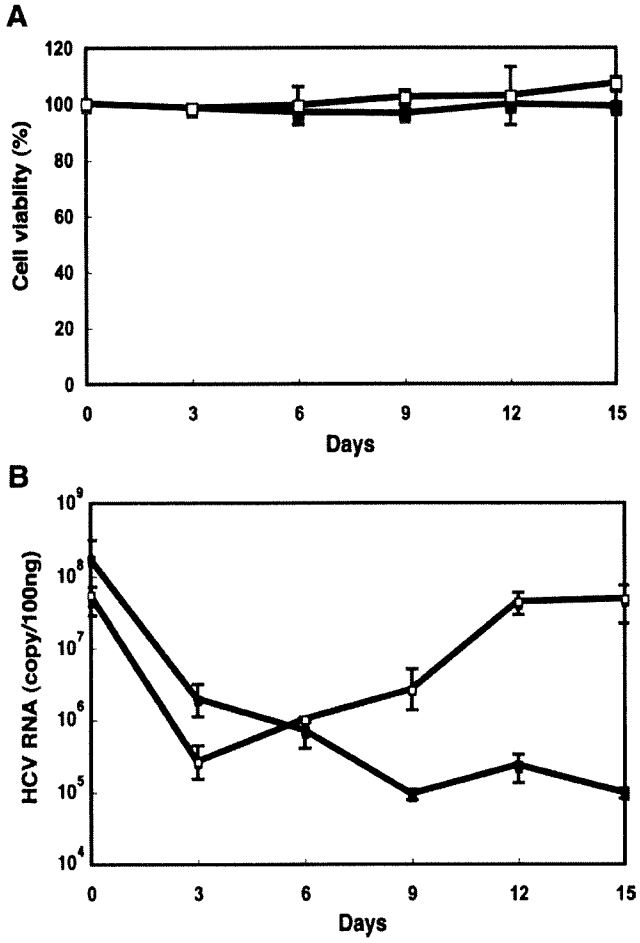

FIGURE 3. **Long term inhibition of HCV replication in NNC#2 cells.** *A*, cytotoxic effect of 17-AAG in NNC#2 cells, shown as the percentage reduction of viable cell numbers assessed by trypan blue staining. NNC#2 cells were treated with 50 nM 17-AAG on day 0 only (*white squares*) or at 3-day intervals for 15 days (*black squares*). The data are means ± S.D. from triplicate experiments. *B*, measurement of HCV replication by real time reverse transcription-PCR. Inhibition of HCV RNA replication in NNC#2 cells treated with 50 nM 17-AAG on day 0 only (*white squares*) or at 3-day intervals for 15 days (*black squares*). *Day 0*, mock. The data are means ± S.D. from triplicate experiments.

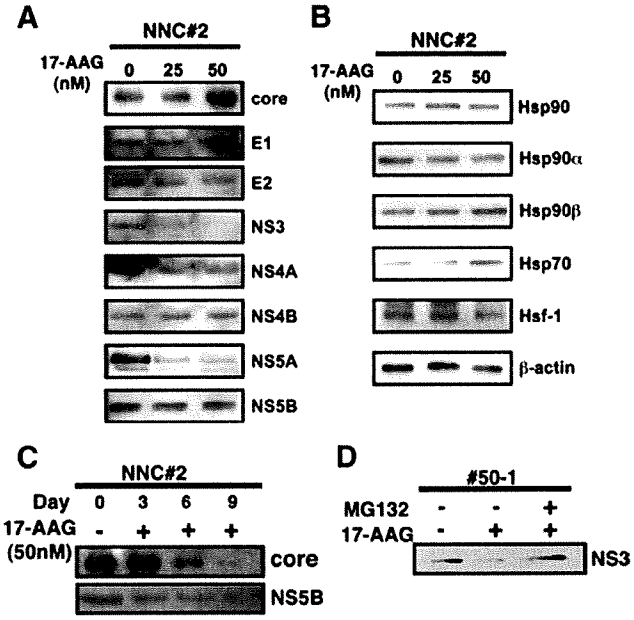

FIGURE 4. **Effect of 17-AAG on HCV NS3 protein levels.** *A*, Western blot analysis of HCV protein expression in NNC#2 or #50-1 cells treated with 17-AAG. NNC#2 or #50-1 cells were treated with 25 and 50 nM 17-AAG for 3 days. Cell lysates were separated by SDS-PAGE, immunoblotted, and probed with antibodies specific for HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and NS5B. *B*, Western blot analysis of Hsp90, Hsp70, and other chaperone expression in NNC#2 cells treated with 17-AAG (25 and 50 nM, as indicated) for 3 days. *C*, expression of HCV core and NS5B protein in cells treated with 50 nM 17-AAG for 9 days. *D*, effect of 50 nM 17-AAG on NS3 expression in #50-1 cells simultaneously treated with 100 nM MG132.

[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt Assay—HCV replicon cells were seeded in 96-well plates at 3×10^4 cells/well in a final culture volume of 100 μl for 72 h before the addition of increasing concentrations of 17-AAG. After incubation for 3 days, viable cell numbers were determined using the Celltiter 96 Aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The value of the background absorbance at 490 nm (A_{490}) of wells without cells was subtracted. The percentages of viable cells were then calculated using the formula, (A_{490} of 17-AAG-treated sample/A_{490} of untreated cells) × 100.

Plasmids and Transfection—The pFLAG-CMV-NS3 vector was constructed by subcloning a DNA fragment encoding full-length NS3, Δhelicase, Δprotease, ΔPH 1, ΔPH 2, and ΔH 1 into the EcoRI and XbaI sites of the pFLAG-CMV™-2 expression vector (Sigma), so that the amino-terminal FLAG epitope was fused in frame with NS3. The core expression vector was a gift from Dr. M. Kohara. The vector was transfected into 293T cells using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions.

RESULTS

Hsp90 Inhibitor 17-AAG Suppresses HCV RNA Replication—To investigate the effect of 17-AAG on HCV replication, cells containing a full HCV genome replicon (NNC#2) or a subgenomic replicon (#50-1) were treated with 17-AAG (Fig. 1, *A* and *B*). Both of the HCV replicon cell lines were treated for 72 h with different concentrations of 17-AAG or with DMSO as a control. In cells treated with 50 nM 17-AAG, HCV RNA replication was suppressed by 99% in both of the HCV replicon cell lines, and the inhibition of RNA replication occurred in a dose-dependent manner (Fig. 2*A*). The half-maximal inhibitory concentration (IC_{50}) values of 17-AAG for HCV replication were 0.3 nM in NNC#2 cells and 0.1 nM in #50-1 cells. Furthermore, we used a tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay to determine the viability of NNC#2 and #50-1 cells in the presence of 17-AAG. 17-AAG showed no toxicity to NNC#2 and #50-1 cells at 50 nM, (Fig. 2*B*). These results suggested that 17-AAG had a greater inhibitory effect on HCV RNA replication than 100 units/ml interferon-α.

Long Term Suppression of HCV RNA Replication—We next examined the effect of 17-AAG on HCV replication over time. When NNC#2 cells were cultured with 50 nM 17-AAG only on day 0 (*white squares*), the level of HCV RNA was reduced by 2 log on day 3 but had increased to control levels by day 12 (Fig.

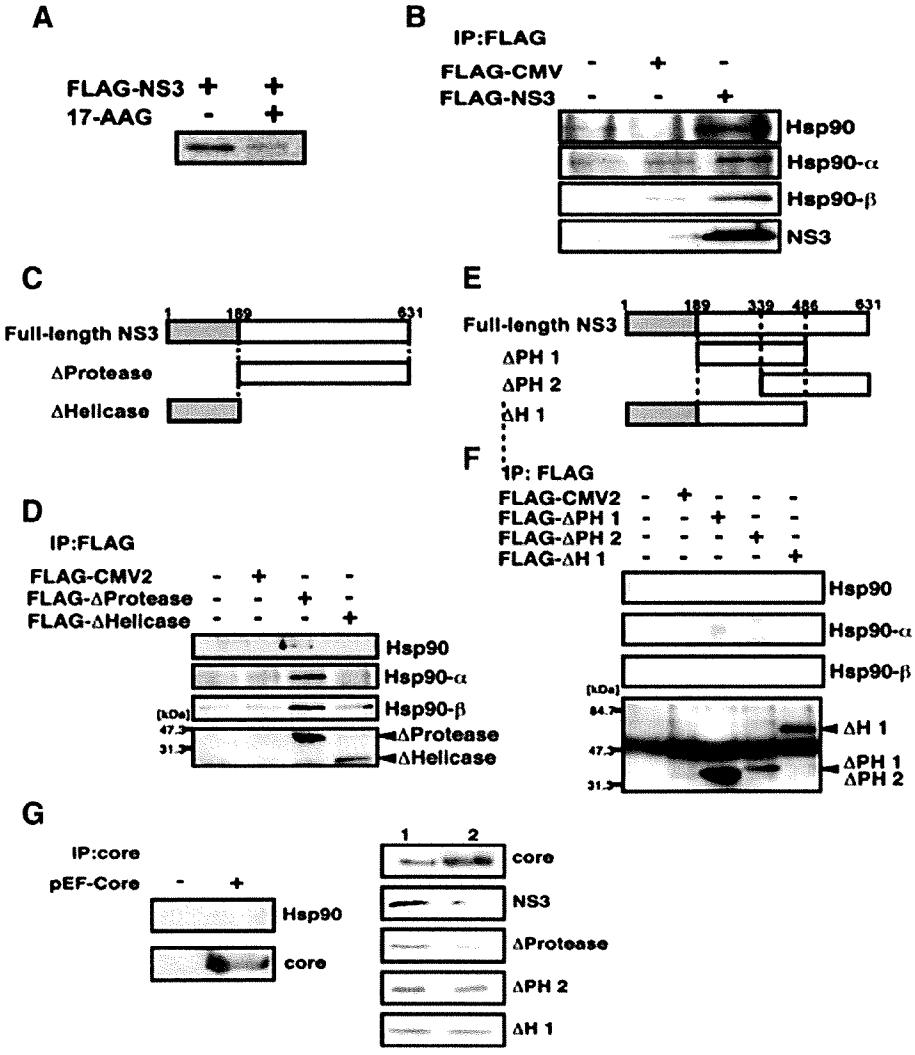

FIGURE 5. **Hsp90 regulates HCV NS3 protein stability.** *A*, Western blot showing the inhibition of NS3 protein expression in 293T cells caused by 17-AAG. Cells were transfected with pFLAG-NS3 in the presence of 250 nM 17-AAG for 48 h. *B*, FLAG-NS3 was expressed in 293T cells and immunoprecipitated (*IP*) from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated were analyzed by Western blotting using anti-Hsp90, anti-Hsp90α, anti-Hsp90β, and anti-FLAG antibodies. The data shown in each *panel* are representative of three independent experiments. *FLAG-CMV*, empty plasmid vector. *C*, schematic representations of HCV NS3 protein and its deletion mutants. *D*, FLAG-NS3, FLAG-Δprotease, and FLAG-Δhelicase were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90α, Hsp90β, and FLAG antibodies were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments. *E*, schematic representations of HCV NS3 protein and further deletion mutants. *F*, FLAG-ΔPH 1, FLAG-ΔPH 2, and FLAG-ΔH 1 were expressed in 293T cells and immunoprecipitated from cell lysates with anti-FLAG antibody. Proteins immunoprecipitated with anti-Hsp90, Hsp90α, Hsp90β, and FLAG antibodies were analyzed by Western blotting. The data shown in each panel are representative of three independent experiments. *G*, FLAG-NS3, pEF-Core, FLAG-Δprotease, FLAG-ΔPH 2, and FLAG-ΔH 1 were expressed in 293T cells treated with 17-AAG. Proteins immunoprecipitated with anti-core, Hsp90 antibody were analyzed by Western blotting. 17-AAG-treated cell lysates were analyzed on Western blots, using the specific antibodies shown to the *right* of the panels. *Lane 1*, control; *lane 2*, 17-AAG (1 μM).

3*B*). However, when 50 nM 17-AAG was added to the cells at 3-day intervals for 15 days (*black squares*), the observed significant reduction in HCV RNA (by 3 log) was sustained from day 3 to day 15. We used trypan blue staining to check that long term treatment with 17-AAG did not induce cellular toxicity (Fig. 3*A*). Our results suggested that 17-AAG has the potential to safely induce long term suppression in HCV replication.

Reduced Expression of NS3 Protein in 17-AAG-treated HCV Replicon Cells—To investigate the mechanism by which 17-AAG inhibited HCV replication, we analyzed the expression of HCV core, E1, E2, NS3, NS4A, NS4B, NS5A, and NS5B proteins by Western blotting. NNC#2 cells treated with increasing doses of 17-AAG showed a marked reduction in the expression of NS3 (Fig. 4*A*) after 3 days, in common with the level of HCV RNA (Fig. 2*A*). However, levels of the other proteins were unchanged. This dose-dependent inhibition suggested that NS3 was more sensitive to 17-AAG than the other proteins. Similar effects on NS3 expression and RNA replication were seen in #50-1 cells treated with 17-AAG (Fig. 4*A*).

Another effect of 17-AAG treatment seen in these cells was an increase in Hsp70 expression and a slight increase in Hsp90 expression (Fig. 4*B*). The induction of Hsp70 expression suggested that Hsp90 inhibition by 17-AAG strongly activated HSF-1 (heat-shock transcription factor 1) (43). We also examined the levels of HCV core and NS5B protein expression in NNC#2 cells treated with 50 nM 17-AAG. Reduced levels of these proteins were seen in NNC#2 cells on day 6, and both HCV core and NS5B protein were undetectable on day 9 (Fig. 4*C*). To determine whether 17-AAG promoted the degradation of NS3, we next looked at the effect of 17-AAG on #50-1 cells in which proteasomal degradation was also inhibited. Although 17-AAG treatment still induced a reduction in the NS3 protein level in #50-1 cells (Fig. 4*D*), the degradation of NS3 was completely blocked in the presence of the proteasome inhibitor, MG132. This suggested that the pharmacological effect of 17-AAG was dependent on the proteasome system (44, 45).

Protein Folding in Hsp90-NS3 Interaction—To investigate the role of Hsp90 in HCV NS3 activation, the FLAG-NS3 protein was transfected into 293T cells, with or without 17-AAG, and the cell lysates were analyzed by Western blotting. The expression of NS3 from FLAG-NS3 was reduced in the presence of 17-AAG (Fig. 5*A*), suggesting that Hsp90 is involved in HCV NS3 degradation, possibly through a physical interaction.

We confirmed this specific interaction by immunoprecipitating 293T cell lysates with anti-FLAG antibody. This clearly showed that FLAG and Hsp90 co-precipitated, suggesting that NS3 was bound to the chaperone complex formed with Hsp90 (Fig. 5*B*). NS3 mutants lacking the protease and helicase regions were generated in order to identify the region responsible for the interaction with Hsp90 (Fig. 5*C*). FLAG-NS3, FLAG-NS3-Δhelicase, or FLAG-NS3-Δprotease were transfected into 293T cells, and anti-FLAG antibody immunoprecipitates were analyzed by Western blotting (Fig. 5*D*). Although FLAG-NS3-Δprotease was clearly co-immunoprecipitated with Hsp90, no protein band corresponding to FLAG-NS3-Δhelicase was detected (Fig. 5*D*), suggesting that the NS3 helicase region mediates binding to Hsp90. To confirm this finding, plasmids expressing different NS3 helicase mutants fused with FLAG (ΔPH 1, ΔPH 2, and ΔH 1) were constructed (Fig. 5*E*). Expressing these NS3 helicase mutants in 293T cells and analyzing their immunoprecipitates with anti-FLAG antibody by Western blotting showed that, although all of the NS3 helicase mutant proteins were immunoprecipitated by anti-FLAG-antibody, no Hsp90 was co-precipitated (Fig. 5*F*).

We also confirmed that the NS3 helicase region mediated the specific interaction with Hsp90 by transfecting FLAG-NS3 and FLAG-NS3 deletion mutants into 293T cells pretreated with 17-AAG (Fig. 5*G*). The proteins expressed by FLAG-NS3 and FLAG-NS3-Δprotease were degraded in cells pretreated with 17-AAG, whereas no degradation of the ΔPH 2 and ΔH 1 NS3 mutants lacking helicase regions was seen (Fig. 5*G*). Further, when pEF-core was expressed in 293T cells, core was unable to co-immunoprecipitate Hsp90, and no degradation of core protein was observed (Fig. 5*G*). Our data demonstrate that 17-AGG destabilizes several binding proteins (NS3 and NS3-Δprotease) to Hsp90 but stablilizes some nonbinding proteins (the ΔPH 2 and ΔH 1 NS3 mutants lacking helicase regions and core) to Hsp90. In previous reports (46), similar effects were observed when wild-type and mutated p53 were translated in the presence of geldanamycin. These results further supported the hypothesis that Hsp90 has a role in folding the NS3 helicase domain and that this has an important role in stabilizing the full-length NS3 protein. A protein complex that includes NS3 and Hsp90 is therefore implicated in the control of HCV replication.

DISCUSSION

The Hsp90 inhibitor, 17-AAG, is known to have highly selective effects on tumor cells that are a result of its high affinity for Hsp90 client oncoproteins, which are incorporated into the Hsp90-dependent multichaperone complex, thereby increasing their binding affinity for 17-AAG more than 100-fold (47). This high selectivity effectively minimizes the toxic side effects of 17-AAG so that it is a good candidate for clinical application, especially in treating neurodegenerative diseases. In this study, we observed the inhibitory effects of 17-AAG on the replication of an HCV subgenomic replicon that lacked NS2. On the other hand, Waxman *et al.* (37) demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protein using NS2/3 translated in rabbit reticulocyte lysate and expressed in Jurkat cells. Because the replicon cells used in our study genetically lacked NS2, our results suggest that Hsp90 may directly interact with the NS3 protein in the HCV replicon.

In cell lines in which 17-AAG was a potent inhibitor of HCV replication, with IC_{50} values of 3–10 nM, we also found strong evidence that the association between HCV Hsp90 and NS3, but not other NS proteins, was the essential mechanism controlling the preferential degradation of NS3 after 17-AAG treatments. Furthermore, we showed that NS3 interacted with Hsp90 through the NS3 helicase domain. It was also clear that the expression of NS3 protein with helicase activity in 293T cells pretreated with 17-AAG was reduced, but the expression of NS3 mutants lacking the helicase regions (ΔPH 2 and ΔH 1) was not. The role of Hsp90 in achieving and/or stabilizing the NS3 protein was suggested by the fact that only 17-AGG bound to Hsp90 was capable of affecting NS3. The use of Hsp90 inhibitors represents a novel strategy for the development of anti-HCV therapies.

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Stable replication of the EBNA1/OriP-mediated baculovirus vector and its application to anti-HCV gene therapy

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Abstract

Background: Hepatitis C virus (HCV) is one of the main causes of liver-related morbidity and mortality. Although combined interferon-α-ribavirin therapy is effective for about 50% of the patients with HCV, better therapies are needed and preventative vaccines have yet to be developed. Short-hairpin RNAs (shRNAs) inhibit gene expression by RNA interference. The application of transient shRNA expression is limited, however, due to the inability of the shRNA to replicate in mammalian cells and its inefficient transduction. The duration of transgene (shRNA) expression in mammalian cells can be significantly extended using baculovirus-based shRNA-expressing vectors that contain the latent viral protein Epstein-Barr nuclear antigen I (EBNA1) and the origin of latent viral DNA replication (OriP) sequences. These recombinant vectors contain compatible promoters and are highly effective for infecting primary hepatocyte and hepatoma cell lines, making them very useful tools for studies of hepatitis B and hepatitis C viruses. Here, we report the use of these baculovirus-based vector-derived shRNAs to inhibit core-protein expression in full-length hepatitis C virus (HCV) replicon cells.

Results: We constructed a long-term transgene shRNA expression vector that contains the EBV *EBNA1* and *OriP* sequences. We also designed baculovirus vector-mediated shRNAs against the highly conserved core-protein region of HCV. HCV core protein expression was inhibited by the EBNA1/OriP baculovirus vector for at least 14 days, which was considerably longer than the 3 days of inhibition produced by the wild-type baculovirus vector.

Conclusion: These findings indicate that we successfully constructed a long-term transgene (shRNA) expression vector (Ac-EP-shRNA452) using the EBNA1/OriP system, which was propagated in *Escherichia coli* and converted into mammalian cells. The potential anti-HCV activity of the long-term transgene (shRNA) expression vector was evaluated with the view of establishing highly effective therapeutic agents that can be further developed for HCV gene therapy applications.

Background

Infection by the hepatitis C virus (HCV) is a major public-health problem, with 170 million people chronically infected worldwide [1,2]. The current treatment with combined interferon-ribavirin therapy fails to cure the infection in 30% to 50% of cases [3,4], particularly those with HCV genotypes 1 and 2. Chronic infection with HCV results in liver cirrhosis and can lead to hepatocellular carcinoma [5,6]. Although combined interferon-α-ribavirin therapy is effective for about 50% of the patients infected with HCV, better therapies are needed and preventative vaccines have yet to be developed. In an effort to develop an alternative to combined interferon-ribavirin treatment, we used RNA interference based on short-hairpin RNA (shRNA), which is a powerful tool for suppressing gene function [7]. Small interference RNAs (siRNAs) directed against HCV are likely to successfully block the replication cycle because HCV is an RNA virus and replicates in the cytoplasm of liver cells without integration into the host genome.

The ability of baculoviruses, including *Autographa californica multiple nuclear polyhedrosis virus* (AcMNPV), to infect insect cells has led to their use in multiple protein expression systems [8,9] and as plant insecticides [10]. AcMNPV, the genome of which comprises a circular, double-stranded DNA that contains ~130 Kbp [11] surrounded by a large envelope, infects a variety of mammalian cell types, with the exception of certain hematopoietic cell lines, although its genome does not replicate or integrate into mammalian chromosomes [12,13]. In particular, the inability of baculoviruses to replicate in mammalian cells makes them attractive candidate vectors for *in vitro* gene therapy studies [14,15]. These recombinant vectors contain compatible promoters and are highly effective in infecting primary hepatocyte and hepatoma cell lines, making them very useful tools for studies of hepatitis B and hepatitis C viruses [16-18].

A major limitation of the baculoviral transduction vector, however, is the short duration of transgene expression. Because the baculovirus genome cannot replicate in mammalian cells, it is usually lost or diluted soon after infection. The efficiency of transgene expression must be substantially increased to be applicable for human gene therapy [19]. The Epstein Barr virus (EBV) plasmid is a replicating episomal vector that has been developed to overcome the problem of rapid elimination of intracellularly-delivered plasmid DNA in nonviral vector-mediated gene transfer. EBV is a gamma herpes virus that is maintained as a ~172-kb episome in a small ratio of resting B cells and epithelial cells in most of the human population. EBV induces latent infection in human B cells [20]. When EBV infects cells, the linear and double-stranded genomes are circularized and sustained as a stable episome. The EBV replication system is present at about 1~100 copies per cell [21], and separates by non-covalent attachment to the host chromosome. The EBV replicon vector system has been used to study long-term transgene expression [22,23]. The origin for latent viral DNA replication (OriP) [24] and the latent viral protein Epstein-Barr nuclear antigen 1 (EBNA1) [21] are essential for the replication of EBV [25]. The *EBNA1/OriP* elements have been successfully exploited to achieve durable expression of foreign genes with plasmid- or virus-based expression systems [26-30].

Previously, we demonstrated efficient inhibition of intracellular HCV replication by baculovirus-based shRNA-expressing vectors [31]. This expression system is transient, however, and therefore unable to provide long-term expression of the shRNA. We hypothesized that long-term transgene (shRNA) expression can be significantly improved in mammalian cells using baculovirus-based shRNA-expressing vectors containing *EBNA1/OriP* sequences.

In the present study, we constructed a long-term transgene (shRNA) expression vector (Ac-EP-shRNA452) using the EBNA1/OriP system, which was propagated in *Escherichia coli* and converted into mammalian cells. The potential anti-HCV activity of the long-term transgene (shRNA) expression vector was evaluated with the view of establishing highly effective therapeutic agents that can be further developed for HCV gene therapy applications.

Results

Construction of baculovirus transfer vectors carrying shRNA-synthesizing cassettes

The core-protein forms the nucleocapsid and modulates gene transcription, cell proliferation, and apoptosis. HCV functions as an mRNA with a single-stranded RNA genome; thus, we hypothesized that cleavage of the core-protein mRNA would inhibit nuclear transport and virus duplication. We previously reported the design of baculovirus vectors expressing shRNA against the following region of the HCV: 452-472, which contains the nuclear localization signal site of the HCV core region (Figure 1A, B) [31]. This vector cannot, however, induce long-term shRNA expression. Therefore, we constructed a long-term transgene shRNA expression vector that contains the EBV *EBNA1* and *OriP* sequences (Figure 1C). Recombinant baculovirus containing the shRNA genome (Ac-shRNA and Ac-EP-shRNA) was generated by homologous recombination of the transfer vector and linearized baculovirus DNAs (BD Biosciences, San Jose, CA) in Sf9 cells. Viruses were produced at high titers, ranging from 2.0×10^8 to 4.5×10^8 pfu/ml.

Inhibition of HCV RNA replication of EBNA1/OriP baculovirus-mediated shRNA-expression vectors in the HCV replicon

We investigated whether the intracellular expression of shRNA inhibited viral replication and affected HCV RNA

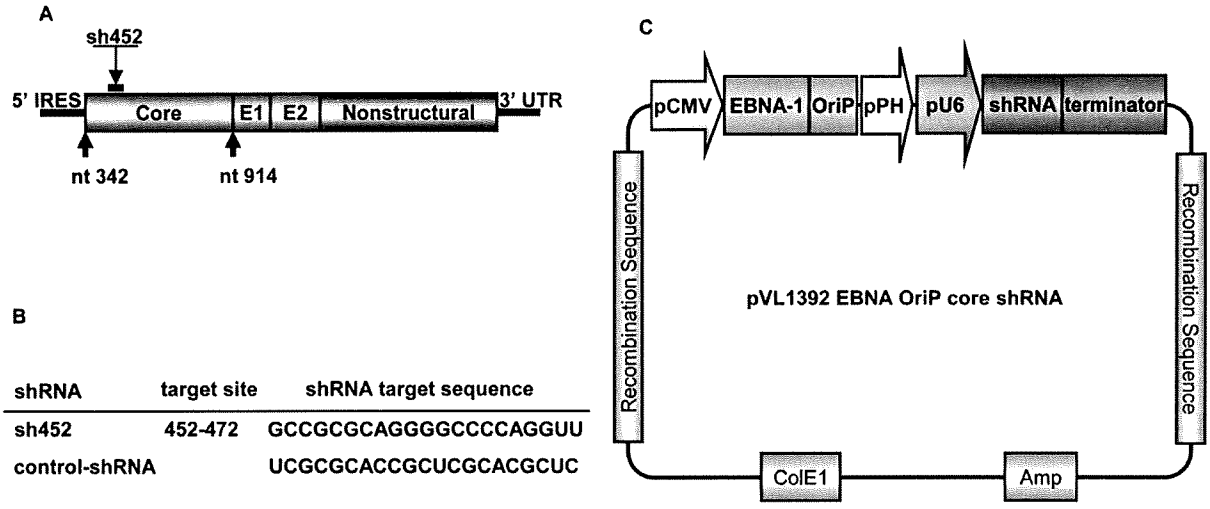

Figure 1
A Genomic profile of HCV showing both coding and non-coding genes. B HCV core region target sites and sequences used for the design of shRNAs. **C** Construction and schematic representation of EBNA1/OriP baculovirus transfer vector expressing HCV core shRNA.

levels in NNC#2 cells. The baculovirus-infection efficiency of NNC#2 cells ranged from 80% to 90% [31]. Real-time reverse transcription polymerase chain reaction (RT-PCR) was used to examine the ability to silence RNA in NNC#2 cells 3 days post-infection. When NNC#2 cells were infected with Ac-shRNAs at a multiplicity of infection (MOI) of 50 and 100, HCV RNA levels were significantly reduced compared with a scrambled shRNA control. Two of the constructs, Ac-shRNA452 (55%, MOI 50; 71%, MOI 100) and Ac-EP-shRNA452 (55%, MOI 50; 67%, MOI 100), inhibited the HCV RNA levels (Figure 2A). In contrast, the control baculovirus vector (Ac-EP-control-shRNA) did not inhibit HCV replication (Figure 2A). These findings indicated that the shRNA had a sequence-specific inhibitory effect on HCV replication. We next used the CLEIA assay to examine whether shRNA against the HCV core protein inhibited viral replication. When NNC#2 cells were infected with Ac-shRNAs at MOIs of 50 and 100, core-protein expression was significantly reduced compared with a non-related shRNA control (Figure 2B). The Ac-EP-control-shRNA baculovirus vectors had no inhibitory effect on HCV replication.

Enhanced baculovirus-mediated shRNA effects were observed in the presence of EBNA1/OriP

To investigate the effect of EBNA/OriP on shRNA expression, we examined the inhibition of HCV replication by Ac-shRNA452 and Ac-EP-shRNA452 in NNC#2 cells for 14 days. When NNC#2 cells were infected with either Ac-shRNA452 or Ac-EP-shRNA452 at an MOI of 100, core-protein expression was significantly reduced compared with a scrambled shRNA control (Ac-EP-control-shRNA) for 3 days (data not shown). Both Ac-shRNA452 and Ac-EP-shRNA inhibited HCV replication for 3 days (Figure 3A). After 3 days, however, cells infected with Ac-shRNA452 exhibited a steady increase in HCV RNA expression while those infected with Ac-EP-shRNA452 continued to have low HCV RNA expression for at least 14 days (Figure 3A). Infection of the NNC#2 cells with recombinant baculovirus vectors containing genetic elements from EBV, EBNA1, and OriP did not induce cellular toxicity, as determined with a bromodeoxyuridine (BrdU)-based colorimetric assay (Figure 3B). These results suggest that HCV RNA expression was more effectively inhibited by the EBNA/OriP baculovirus vector than by the wild-type baculovirus vector.

Production of EBNA1 protein and siRNA by baculovirus-based shRNA-expressing vectors containing EBNA1/OriP sequences

We first used Western blot analysis to detect EBNA1 protein in Ac-EP-shRNA-infected cells (Figure 4A). EBNA1 protein was detected in the Ac-EP-shRNA-infected cells. Then, to investigate whether HCV core gene-targeting shRNAs can be digested to mono-specific products of the expected size, siRNAs were analyzed by Northern blot analysis of shRNA-expressing NNC#2 cells. The siRNAs from both Ac-shRNA452 and Ac-EP-shRNA452 yielded products of ~20 nt, which is the expected size of monomeric siRNAs, for 3 days (Figure 4B). The siRNA band in

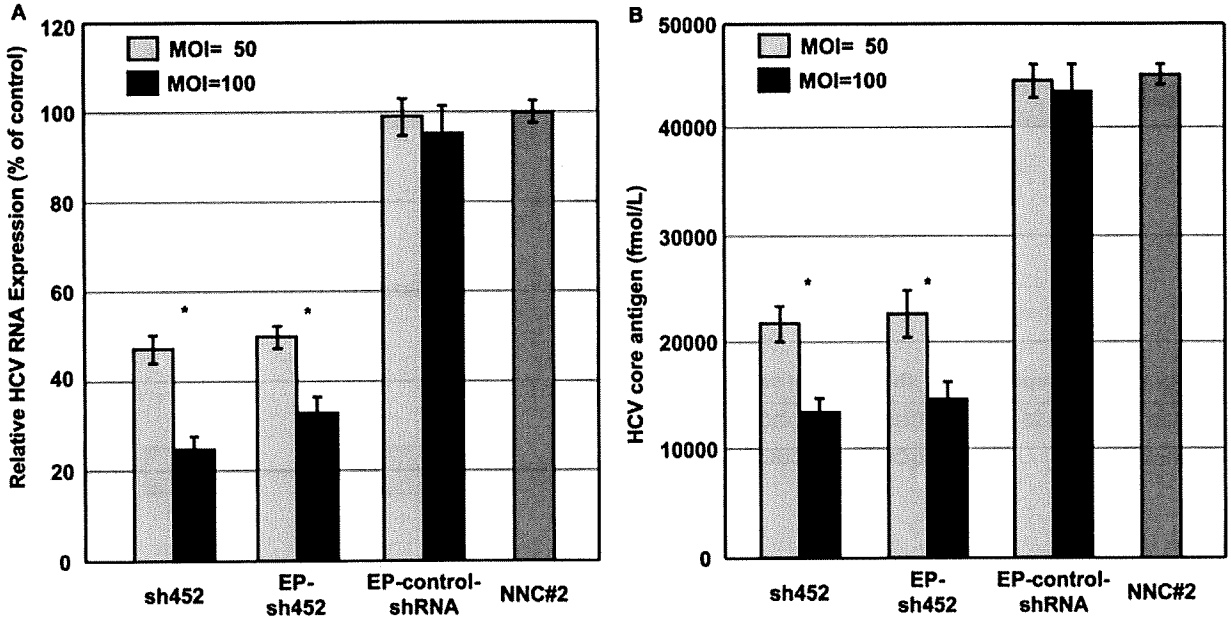

Figure 2
Inhibition of HCV RNA by EBNA1/OriP and wild-type baculovirus-mediated sh452. A Real time PCR analysis of HCV RNA expression after transduction of HCV full replicon cells (NNC#2, 4 × 10^4 cells/well) with an MOI 50 and 100 baculovirus-mediated shRNA. HCV RNA values relative to the scrambled shRNA control are shown. **B** Inhibition of HCV replication by baculovirus-mediated core shRNAs. Ac-shRNAs were used to infect HCV replicons and intracellular HCV core protein levels measured after 3 days by an HCV protein antigen CLEIA assay. Error bars represent standard errors of the mean from three experiments. *$p < 0.01$.

Ac-shRNA452-infected cells, however, became undetectable after 5 days. In contrast, siRNA in Ac-EP-shRNA452-infected cells could be detected for at least 14 days.

Discussion

There is high demand for the development of effective anti-HCV drugs. Gene silencing by RNA interference is a promising approach to elucidate gene function and to inhibit certain RNA viruses such as HCV [32-34]. Delivery of siRNA to the appropriate cells or tissues, however, is a major challenge. Several approaches have been described for generating loss-of function phenotypes in mammalian systems using siRNA, but these techniques are limited and are not suitable for generating a long-term silencing effect *in vivo* [35,36]. Efficient and safe delivery systems have not yet been established for the suppression of HCV replication. Baculoviruses appear to be useful viral vectors, not only for the abundant expression of foreign genes in insect cells, but also for efficient gene delivery to the hepatoma lines HepG2 and Huh7 [37]. One of the major limitations of the baculoviral transduction vector is the short duration of transgene expression. The EBNA1/OriP system has been widely exploited in many different vectors and cell lines. The findings suggest that the EBNA1/OriP system is effective and useful for long-term and high-level transgene expression.

In this study, recombinant baculovirus vectors containing genetic elements from EBV, EBNA1/OriP, which are essential for the episomal maintenance of the EBV genome in latently infected cells, were constructed and tested for their ability to sustain and express the transgene (enhanced HCV core gene-targeting shRNAs) in HCV replicon cells. The introduction of wild-type or EBNA1/OriP-baculovirus-mediated sh452 into target cells containing HCV replicon RNA induced a dose-related reduction in the level of HCV RNA at 3 days. The effectiveness of the inhibition of HCV replication, however, did not differ under the control of the two different vectors (Ac-shRNA452 or Ac-EP-shRNA452).

To investigate the long-term effect of EBNA/OriP on shRNA expression, we examined the inhibition of HCV replication by Ac-shRNA452 and Ac-EP-shRNA452 in NNC#2 cells for 14 days. Both Ac-shRNA452 and Ac-EP-shRNA inhibited HCV replication for 3 days. After 3 days, however, cells infected with Ac-shRNA452 exhibited a steady increase in HCV RNA expression while those

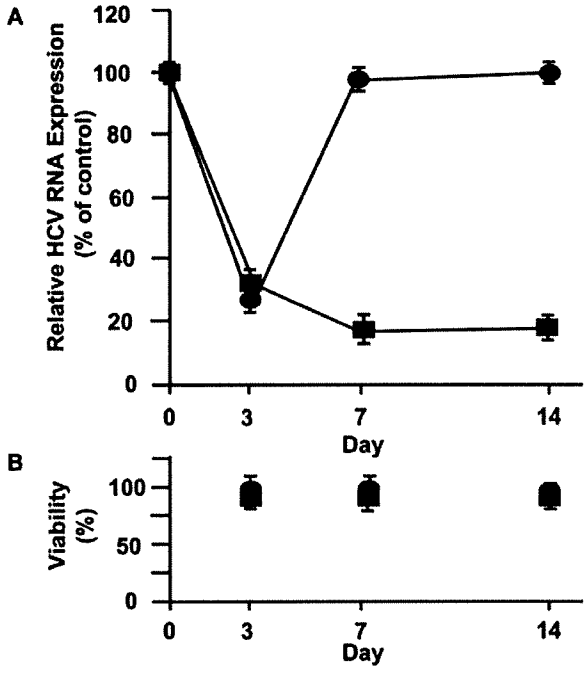

Figure 3
Long-term inhibition of HCV RNA by EBNA1/OriP and wild-type baculovirus-mediated sh452. A Real-time RT-PCR analysis of HCV RNA expression after transduction of HCV full replicon cells (NNC#2, 4 × 10^4 cells/well) with Ac-shRNA452 (MOI = 100 [circle]), Ac-EP-shRNA452 (MOI = 100 [square]). **B** The cytotoxicity of Ac-EP-shRNA452 (square) and Ac-shRNA452 (circle) represented as the percentage reduction of viable Huh-7 cells. A cytotoxicity assay was performed using a BrdU Cell Proliferation ELISA kit according to the manufacturer's instructions (Roche Diagnostics GmbH). The toxicity results are representative of three independent experiments.

infected with Ac-EP-shRNA452 continued to have low HCV RNA expression for at least 14 days. These recombinant baculovirus vectors containing genetic elements from EBV, EBNA1, and OriP did not induce cellular toxicity in the NNC#2 cells, as determined with a BrdU-based colorimetric assay. The HCV RNA was inhibited by EBNA1/OriP baculovirus-mediated shRNA452 for a longer time by the EBNA1/OriP baculovirus vector than by the wild-type baculovirus vector.

To investigate whether EBNA1/OriP baculovirus-mediated shRNA452 can be digested to mono-specific products of expected size, monomeric siRNAs were performed by Northern blot analysis in AB1-shRNA expressing NNC#2 cells. The shRNAs yielded products ~20 nt, the expected size of monomeric siRNAs, over the long term. Furthermore, EBNA1 protein was also detected in the Ac-EP-shRNA-infected cells. These findings indicated a direct correlation between the level of the virus and siRNA or EBNA1 production.

Conclusion

The results of the present study indicate that we have successfully constructed a long-term transgene (shRNA) expression vector (Ac-EP-shRNA452) using the EBNA1/OriP system, which was propagated in *Escherichia coli* and converted into mammalian cells. The potential anti-HCV activity of the long-term transgene (shRNA) expression vector was evaluated with the view of establishing highly effective therapeutic agents that can be further developed for HCV gene therapy applications.

Methods

Cell culture

NNC#2 (NN/1b/FL) cells [38] carrying a full genome replicon were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, non-essential amino-acids, L-glutamine, and 1 mg/ml G418 (Invitrogen, Carlsbad, CA).

Northern blot analysis

Total RNA was extracted from Ac-shRNA452 infected Huh7 cells using a mirVana™ miRNA Isolation Kit, according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). Small RNAs (5 μg) were loaded onto a 15% (w/v) polyacrylamide/7 M urea gel. After transfer to a Hybond-N™ nylon membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), synthetic locked nucleic acid (LNA)/DNA oligonucleotides (sh452: 5'-DIG-CCGCGCAGGGGCCCCAGG-3') complementary to the antisense strand of the shRNA452 were used as probes. The membranes were prehybridized for 1 h in DIG EASY hybridization buffer (Roche Diagnostics GmbH) at 60°C and hybridized overnight to the 5'-DIG labeled LNA/DNA probe (10 ng/ml of hybridization buffer). Four post-hybridization washes were performed for 20 min each at 60°C with 2× SSC (1× SSC = 0.15 M NaCl plus 0.015 M sodium citrate-0.1% sodium dodecyl sulfate). LNA/DNA/RNA hybrids were detected using the CSPD chemiluminescent detection system (Roche Diagnostics GmbH).

Western blot analysis

Cells were lysed in 1× CAT enzyme-linked immunosorbent assay buffer (Roche Diagnostics GmbH). Cell lysates were separated by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, and these were blocked with PVDF Blocking Reagent (TOYOBO, Ohsaka, Japan). The primary antibodies used were monoclonal antibodies against EBNA1 (Acris Antibodies GmbH) and G3PDH (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Horseradish peroxidase-conju-

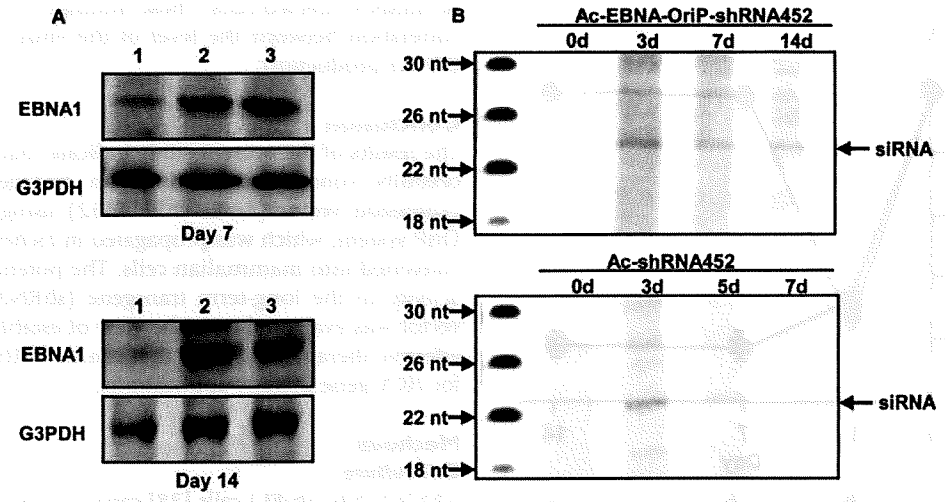

Figure 4
Detection of EBNA1 protein and siRNA in Ac-EP-shRNA452 infected cells. A Western blot analysis of EBNA1 expression in baculovirus-infected Huh7 cells. Cell lysates were prepared 7 days and 14 days post-infection from cells infected with different viruses. Lane 1: Ac-sh452; lane 2: Ac-EP-sh452; lane 3: Ac-EP-control-shRNA. **B** Expression of siRNA by a baculovirus vector. To demonstrate the intracellular expression of the shRNA construct in the respective siRNA, Huh-7 cells were infected with Ac-EP-shRNA452. The mixture was run on a 15% polyacrylamide TBE urea gel after 3, 7, and 14 days.

gated anti-goat antibody (Sigma Chemical Co., St. Louis, MO) was used as the secondary antibody.

RNA purification and real-time RT-PCR

Total RNA was isolated from the cells using a mirVana miRNA Isolation Kit (Ambion, Austin, TX). Real-time RT-PCR was performed using the following primers located in the HCV core region: forward primer (813-833 nt), 5'-CTGGAGGACGGCGTGAATTAT-3'; reverse primer (938-957 nt), 5'-CGTTCGTGACATGGTATATC-3'. HCV-specific RNA was detected by real-time PCR as an increase in SYBR Green I fluorescence on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA). The 18S rRNA housekeeping gene was used as a control for normalization. Each real-time PCR assay was performed in triplicate.

Cytotoxicity assay

NNC#2 cells (2×10^4 cells/mL) were seeded into 96-well microtiter plates and incubated in the presence of various concentrations of the test compounds. The dilutions ranged from 1 to 5-fold, and 9 concentrations were examined. All of the experiments were performed in triplicate. After 3 days culture at 37°C in a CO_2 incubator, cell viability was quantified using a colorimetric BrdU Cell Proliferation enzyme-linked immunosorbent assay according to the manufacturer's instructions (Roche Diagnostics GmbH). The absorbances were read by a microcomputer-controlled photometer (Titertec MultiscanR; Labsystem Oy, Helsinki, Finland) at 405 nm. These values were then translated into percentages per well.

Baculovirus transfer vector constructs

We designed baculovirus transfer vectors expressing shRNAs against the following region of the HCV core-protein sequence: nucleotides 452-472, which contains the nuclear localization signal site (pU6-core-shRNA452) [31]. The following site in the core region of the common sequence of the HCV strain M1LE (GenBank accession number AB080299) was chosen as the target for the shRNA: 5'-GCCGCGCAGGGGCCCCAGGUU-3' (shRNA452). Sense and antisense strands of shRNA oligonucleotides were synthesized, annealed at 95°C for 3 min, and then slowly cooled in phosphate-buffered saline (pH 7.4, containing 50 mM NaCl). The oligonucleotides contained the loop CCACACC sequence, and KpnI and BamHI ends, which were inserted into a pU6 vector, based on pSV2-neo. A Pol III-type U6 promoter allowed for constant expression of the shRNAs. Fragments of U6-core-sh452, ranging from the *Eco*RI site upstream of the U6 promoter to the BamHI site downstream of the terminating sequence, were sequenced and then inserted into the cloning site of the baculovirus transfer vectors pVL1392 and pVL1393 (BD Biosciences, San Jose, CA) in an opposite orientation to the polyhedrin promoter to create pVL1392-core-shRNA452 and pVL1393-core-shRNA452. A spacer was inserted between the inverted sequences to form a hairpin structure, and to enhance its stability.

The EBV *EBNA1* and *OriP* gene sequences were obtained from the pCEP4 plasmid (Invitrogen). The *EBNA1/OriP* sequence was digested with restriction enzymes *Eco*RI and