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Inhibitory Effect of a Triterpenoid Compound, with or without Alpha Interferon, on Hepatitis C Virus Infection▽†

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A lack of patient response to alpha interferon (-IFN) plus ribavirin (RBV) treatment is a major problem in eliminating hepatitis C virus (HCV). We screened chemical libraries for compounds that enhanced cellular responses to -IFN and identified a triterpenoid, toosendanin (TSN). Here, we studied the effects and mechanisms of action of TSN on HCV replication and its effect on -IFN signaling. We treated HCV genotype 1b replicon-expressing cells and HCV-J6/JFH-infected cells with TSN, with or without -IFN, and the level of HCV replication was quantified. To study the effects of TSN on -IFN signaling, we detected components of the interferon-stimulated gene factor 3 (ISGF3), phosphorylated signal transducer and activator of transcription 1 (STAT1), and STAT2 by Western blotting analysis; expression levels of mRNA of interferon regulatory factor 9 using real-time reverse transcription-PCR (RT-PCR); and interferon-stimulated response element reporter activity and measured the expression levels of interferon-inducible genes for 2′,5′-oligoadenylate synthetase, MxA, protein kinase R, and p56 using real-time RT-PCR. TSN alone specifically inhibited expression of the HCV replicon (50% effective concentration 20.6 nM, 50% cytotoxic concentration > 3 M, selectivity index > 146). Pretreatment with TSN prior to -IFN treatment was more effective in suppressing HCV replication than treatment with either drug alone. Although TSN alone did not activate the -IFN pathway, it significantly enhanced the -IFN-induced increase of phosphorylated STATs, interferon-stimulated response element activation, and interferon-stimulated gene expression. TSN significantly increased baseline expression of interferon regulatory factor 9, a component of interferon-stimulated gene factor 3. Antiviral effects of treatment with -IFN can be enhanced by pretreatment with TSN. Its mechanisms of action could potentially be important to identify novel molecular targets to treat HCV infection.

Hepatitis C virus (HCV) is one of the most important pathogens causing acute and chronic hepatitis, liver cirrhosis, and hepatocellular malignancies (29). Alpha interferon (α-IFN) combined with ribavirin (RBV) is the standard treatment for HCV infection (6, 10). However, virus elimination rates are about 50% among treated patients, and therapy is often accompanied by substantial side effects (6, 44). It was recently reported that genetic polymorphisms of the *IL28B* gene, which codes for lambda IFN, are critical for predicting responses to α-IFN plus RBV therapy (8, 35, 38). Patients with minor variants of *IL28B*, who comprise ~50% of Caucasian, 25% of Asian, and ~70% of African populations, showed poor responses to α-IFN treatment. Although new specific anti-HCV drugs are under development, many of them require combined use with α-IFN and RBV (26). Taken together, current difficulties in eliminating HCV are mostly attributable to the limited treatment options and to the limited activity of α-IFN against the virus. For this reason, the development of safe and effective agents that enhance antiviral actions against HCV has been a strong motivation in academia and industry.

To search for a new agent which enhances the effect of α-IFN, we used interferon-stimulated response element (ISRE) reporter screening. We screened a chemical library (60,500 compounds) for compounds that enhance ISRE activity when they are used in combination with α-IFN, using ISRE reporter screening, and identified several compounds that increased the ISRE reporter activities when they are used in combination with α-IFN and that did not show cytotoxicity. Among the hit compounds, toosendanin (TSN; $C_{30}H_{38}O_{11}$; molecular weight = 574) (Fig. 1), which is a triterpenoid derivative extracted from the bark of *Melia toosendan* Sieb et Zucc, was the strongest in enhancing α-IFN-induced ISRE reporter activation and the expression of interferon-stimulated genes (ISGs). TSN has been used as an anthelmintic vermifuge against ascaris (31). Although TSN has some other biological effects against toxin-producing anaerobic bacteria and against carcinoma cells (32, 45), antiviral activity has not been reported.

In this study, we showed, using an HCV replicon system, that TSN, with or without α-IFN, inhibits HCV replication in a cultured human hepatoma Huh7 cell line and that the combination of TSN and α-IFN shows synergistic effects on viral replication. We have investigated the mechanisms of action of

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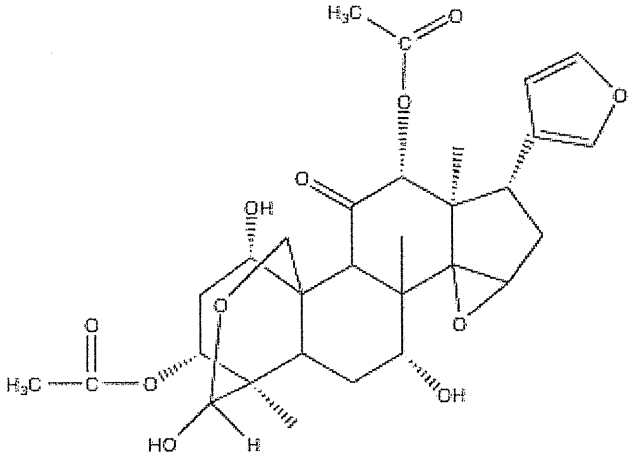

FIG. 1. Chemical structure of toosendanin.

TSN further and show that TSN induced activation of a component of interferon-stimulated gene factor 3 (ISGF3).

MATERIALS AND METHODS

Reagents. Alpha interferon was from Otsuka (Tokushima, Japan). TSN was from APIN Chemicals (Oxon, United Kingdom). Purity was over 77.32%. The designated concentration was achieved through dilution with cell culture medium (the final concentration of dimethyl sulfoxide [DMSO] in the medium was less than 0.3%). Beta-mercaptoethanol was from Wako (Osaka, Japan). The TSN used in this study was solubilized in DMSO.

Cells and cell culture. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. To maintain cell lines carrying an HCV subgenomic replicon (Huh7/Rep-Feo), G418 (Nakalai Tesque, Kyoto, Japan) was added to the culture medium at a final concentration of 500 μg/ml.

HCV subgenomic replicon construct. The HCV subgenomic replicon plasmid pRep-Feo expresses a fusion gene comprising the firefly luciferase and neomycin phosphotransferase (37, 43). RNA was synthesized *in vitro* from the plasmid and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.

Reporter constructs. We analyzed the effects of TSN, with or without α-IFN, on signal transduction of ISRE and nuclear factor-kappaB (NF-kappaB). A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (23). Plasmids pISRE-TA-Luc and pNF-kappaB-Luc (Clontech Laboratories, Franklin Lakes, NJ) contained consensus motifs upstream of the firefly luciferase gene. A plasmid, pTA-Luc (Clontech), which lacks the enhancer element, was used to determine the background. Plasmid pRL-CMV (Promega, Madison, WI), which expresses the Renilla luciferase protein, was used for normalization of transfection efficiency (17).

ISRE reporter screening. Huh7 cells were seeded in 384-well plates at a density of 3.0×10^3 cells/well. An ISRE-responsive firefly luciferase reporter was introduced using Lipofectamine 2000 (Invitrogen). Five hours after transfection, the cells were treated with 60,500 compounds from chemical libraries at a concentration of 3 μg/ml for 24 h and then treated with α-IFN at a concentration of 3 IU/ml. Six hours later, cells were lysed, and luciferase activities were quantified using a Steady Glo luciferase assay kit (Promega). The compounds were stored in 100% DMSO, and thus, the final concentration of DMSO was 0.3%. *Z'* factors were calculated as reported previously (46).

Luciferase assays and measurements of antiviral activity. Huh7/Rep-Feo cells were cultured with various concentrations of compound, such that the final DMSO concentration was 0.1%. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega) and the Bright-Glo luciferase assay system (Promega). Assays were performed in triplicate, and the results are expressed as mean percentage of the controls ± standard deviation (SD). The 50% effective concentration (EC_{50}) values were calculated using the probit method (2, 33). The determination of EC_{50}s was performed three times, and EC_{50}s are presented as means ± SDs for each compound.

MTS assays. To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays were performed using a Cell Titer 96 Aqueous One Solution cell proliferation assay (Promega) as previously reported (18, 22). Huh7/Rep-Feo cells and HCV-J6/JFH1-infected Huh7 cells were seeded in 96-well plates at a density of 8.0×10^3 cells/well. After treatment, to analyze the therapeutic index with the same concentration of the drug and administration time, 20 μl/well of Cell Titer 96 Aqueous One Solution reagent was added to the cells cultured in a 96-well plate, the plate was incubated at 37°C for 60 min, and then the absorbance at 490 nm was recorded with a 96-well plate reader. The cells were analyzed when the growth became confluent. Cell viability was expressed as the concentration required for 50% cytotoxicity (CC_{50}). The drug selectivity index was calculated as CC_{50}/EC_{50}. All experiments were performed in triplicate.

Analyses of drug synergism. The effects of treatment of Huh7/Rep-Feo cells with α-IFN, alone and in combination with TSN, were analyzed by using isobologram analysis as described previously (27, 37). Dose inhibition curves of α-IFN and TSN were drawn with the two drugs used alone or in combination. In each drug combination, EC_{50}s for α-IFN and TSN were plotted against the fractional concentration of α-IFN and TSN on the x and y axes, respectively. A theoretical line of additivity is drawn between plots of the EC_{50} for either drug that was used alone. The combined effects of the two drugs were considered additive, synergistic, or antagonistic if the plots of the drug combination were located on the line, below, or above the line of additivity, respectively.

HCV-J6/JFH1 cell culture. HCV-J6/JFH1 (21), which is a recombinant of HCV-JFH1 (42), was used. *In vitro*-synthesized HCV-J6/JFH1 RNA was transfected into naïve Huh7 cells (48), and the cells were cultured in the presence of drugs (34). Cellular viral RNA expression levels were measured using a real-time reverse transcription-PCR (RT-PCR) system.

Real-time RT-PCR analysis. Real-time RT-PCR was carried out as described previously (7). Total cellular RNA was extracted from cultured cells using Isogen (Nippon Gene, Tokyo, Japan), reverse transcribed, and subjected to real-time RT-PCR analyses. Expression of mRNA was quantified using TaqMan Universal PCR master mix, an ABI 7500 real-time PCR system (Applied Biosystems, CA), and a QuantiTect SYBR green PCR kit (Qiagen, CA). Some primers have been described elsewhere (30, 34). The primers used were -S (5'-TTT GAA ACA TCA AAG TTT TTC ACA GAC CTA-3'), -AS (5'-CAC AGT CAA GGT CCT TAG TAT TTC AGA TGT-3'), p56-S (5'-ACT TCG GAG AAA GGC ATT AGA TCT GGA AAG-3'), p56-AS (5'-TAA GGA CCT TGT CTC ACA GAG TTC TCA AAG-3'), Viperin-S (5'-GCT ACC AAG AGG AGA AAG CA-3'), Viperin-AS (5'-TTG ATC TTC TCC ATA CCA GC-3'), ISG20-S (5'-CTA CGA CAC GTC CAC TGA CAG G-3'), ISG20-AS (5'-CAT CGT TGC CCT CGC ATC TTC-3'), IRF9-S (5'-GCA GCA GCA GCC CTG AGC CAC AGG AAG TTA-3'), IRF9-AS (5'-TTA CCT GGA ACT TCG GTG GGG GGC CCA GGC-3'), IFNAR1-S (5'-CTT TCA AGT TCA GTG GCT CC-3'), IFNAR1-AS (5'-CAT CAG ATG CTT GTA CGC GGA G-3'), IFNAR2-S (5'-GCC AGA ATG CCT TCA TCG TCA G-3'), and IFNAR2-AS (5'-GTG AGT TGG TAC AAT GGA GTG G-3').

Western blotting. Twenty micrograms of total cell lysate was separated by SDS-PAGE and blotted onto a polyvinylidene fluoride Western blotting membrane. The membrane was incubated with the primary antibodies, followed by incubation with a peroxidase-labeled anti-IgG antibody, and were visualized by chemiluminescence using an enhance chemiluminescence Western blotting analysis system (Amersham Biosciences, Buckinghamshire, United Kingdom). The antibodies used were mouse anti-NS5A (BioDesign, ME), rabbit anti-signal transducer and activator of transcription 1 (anti-STAT1) p84/p91, rabbit anti-phospho-STAT1 (Tyr 701), rabbit anti-STAT2, rabbit anti-phospho-STAT2 (Tyr 690) (Santa Cruz, CA), and anti-beta-actin antibody (Sigma). NIH image software was used to analyze the densitometry of the Western blot analysis. Quantification of STAT phosphorylation was done using NIH image software, and the results correspond to the ratio between the phosphorylated STAT1 (p-STAT1) or p-STAT2 amount and the STAT1 or STAT2 amount normalized to the amount for the control without α-IFN and TSN. The results correspond to the ratio between the NS5A amount and the beta-actin amount normalized to the amount for the control without α-IFN and TSN.

Statistical analyses. Statistical analyses were performed using Student's t test. P values of less than 0.05 were considered statistically significant.

RESULTS

ISRE reporter screening. At the primary screening (n = 1), we defined a 1.5-fold induction in response to α-IFN to be a hit compound, and the hit rate was about 1%. At the secondary screening (n = 4), we selected the compound whose cps were 2 SDs larger than that for the drug used as a negative control, and the hit compound rate was 0.2% of the original library. Both assays were highly reproducible, and reflecting this, the Z' factor (46) for the ISRE reporter screen was 0.97.

TSN has activity against HCV RNA replication. Huh7/Rep-Feo cells were cultured with various concentrations of TSN, and the effect was measured using a luciferase assay. TSN caused a marked suppression of HCV RNA replication in a dose-dependent manner (Fig. 2A). The EC_{50} of TSN was 20.6 nM. In contrast, MTS assays showed that treatment with TSN had little effect on cellular viability and replication, with a CC_{50} of over 3 μM and a selectivity index of more than 146. These results indicated that TSN had an effect against HCV RNA replication when it was used alone and that the effect was specific for HCV replication and not attributable to nonspecific cytotoxicity (Fig. 2B). Similarly, by Western blotting (Fig. 2C), the expression of HCV NS5A protein was shown to be reduced by corresponding amounts following treatment with TSN. To determine whether TSN suppresses HCV IRES-dependent translation, we used a Huh7 cell line that had been stably transfected with pCIneo-Rluc-IRES-Fluc (Fig. 2D). Treatment of these cells with TSN resulted in no significant change of the internal luciferase activities at concentrations of TSN that suppressed expression of the HCV replicon.

TSN increased ISRE reporter activity with -IFN. Because we identified TSN originally through ISRE reporter-based drug screening, we analyzed the effects of TSN on the cellular responses to α-IFN following pretreatment with TSN. First, we treated ISRE-TA-Luc-transfected Huh7 cells with TSN and α-IFN simultaneously or pretreated the cells with 10 to 100 nM TSN at 24 or 48 h prior to α-IFN treatment. Luciferase assays were performed 6 h after addition of α-IFN at concentrations of 0.1 to 100 IU/ml (Fig. 3A and B). Treatment with TSN alone did not increase ISRE reporter activity. Similarly, simultaneous treatment with TSN and α-IFN did not enhance α-IFN-induced ISRE reporter activation more than treatment with α-IFN alone. In contrast, pretreatment with TSN 24 or 48 h before addition of α-IFN significantly increased ISRE activation compared to that achieved by treatment with α-IFN alone (Fig. 3A). On the basis of these results, we performed the subsequent experiments with addition of TSN 24 h before α-IFN treatment.

We next quantified the expression levels of ISGs, including those for 2′,5′-oligoadenylate synthetase (25AS), MxA, protein kinase R, p56, viperin, and ISG20, which encode proteins with direct antiviral activity (14, 15, 25). Naïve Huh7 cells were treated with TSN for 24 h, followed by treatment with 100 IU/ml α-IFN for 24 h. The expression of each ISG was significantly elevated in a dose-dependent manner following pretreatment with TSN and α-IFN stimulation (Fig. 3C). These results indicated that TSN pretreatment significantly enhanced the cellular response to α-IFN-induced, ISRE-regulated expression of ISGs.

It has been reported that α-IFN receptor-mediated signaling

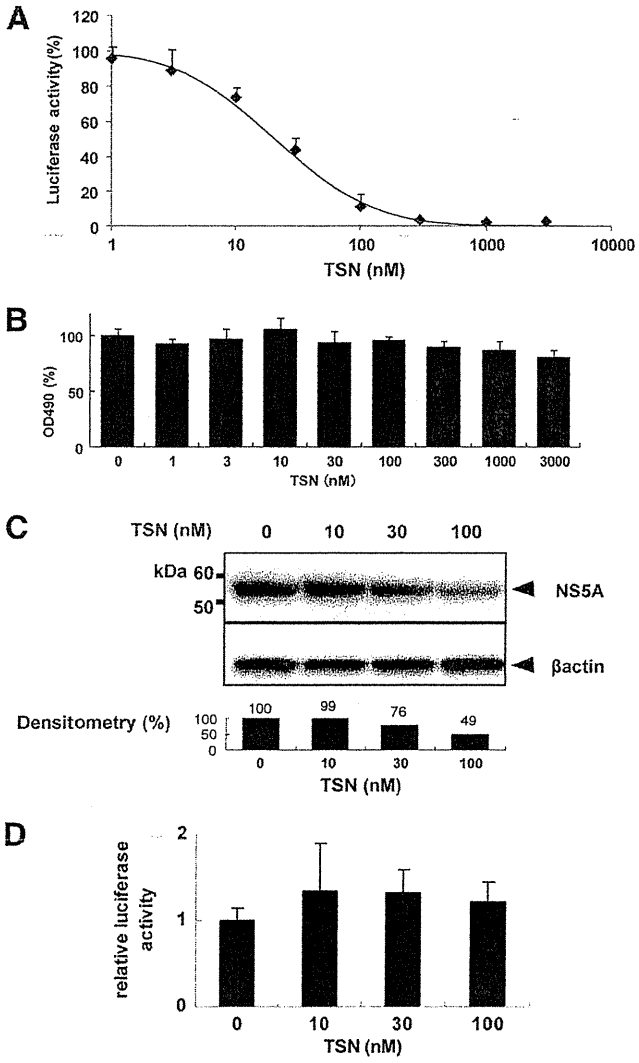

FIG. 2. Effect of TSN on expression of HCV replicon. (A) HCV replicon cells were treated with various concentrations of TSN for 48 h. Replication levels of HCV RNA were analyzed by luciferase assay. Bars indicate luciferase activities relative to that of the drug-negative control. (B) Cell viability was determined by MTS assay. Bars indicate the value relative to that of the drug-negative control. (C) Western blotting analyses. The expression of NS5A and beta-actin was detected using anti-NS5A and anti-beta-actin antibodies. Densitometry of NS5A protein was performed, and the result is indicated as a percentage of the result for the drug-negative control. The assay was repeated three times, and a representative result is shown. (D) A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was transfected into Huh7 cells. The cells were cultured with TSN at the concentrations indicated, and dual luciferase activities were measured after 24 h of treatment. Values are displayed as ratios of Fluc to Rluc. In panels A, B, and D, the assays were done in triplicate and repeated three times. Error bars indicate means ± SDs.

cross talks with several alternative pathways, including the NF-kappaB, gamma IFN, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (9, 16, 24, 28). Therefore, we analyzed the effect of TSN on the signaling pathways indicated above. Cells were transfected with various reporter plasmids, including NF-kappaB, gamma

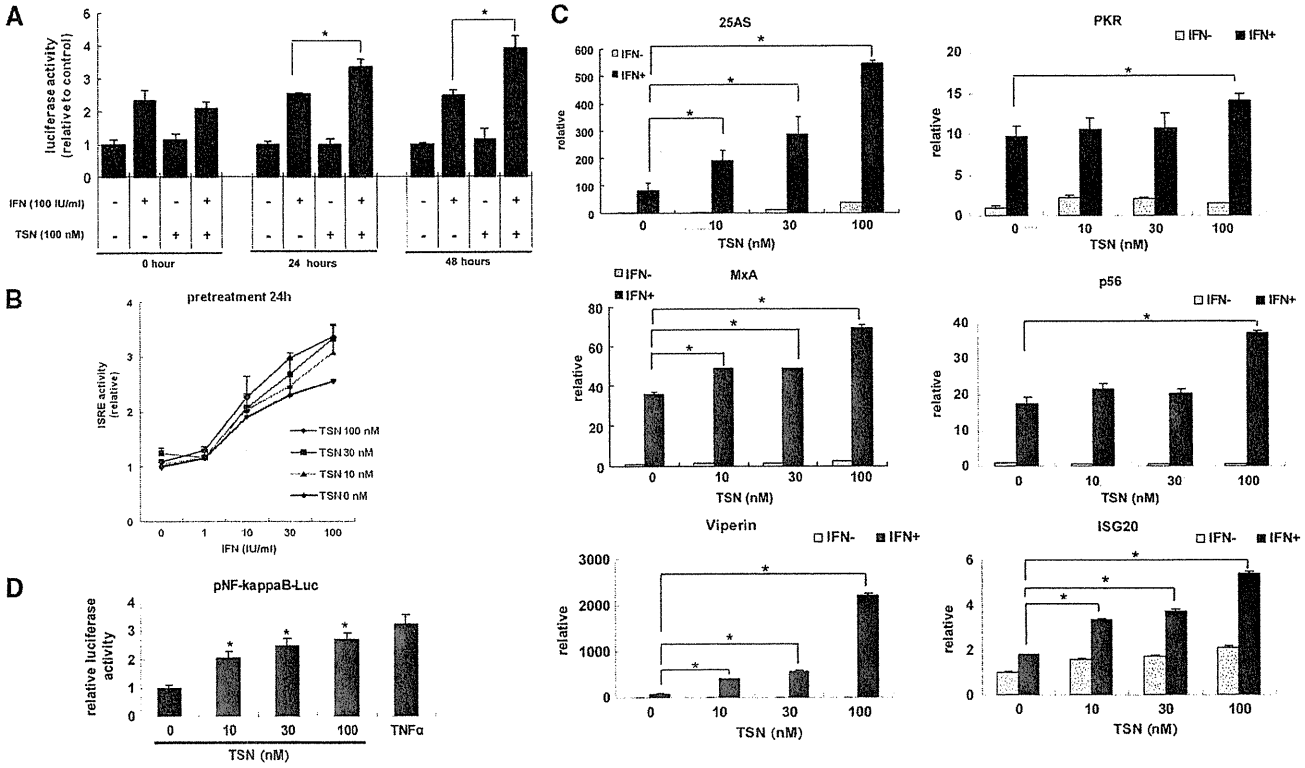

FIG. 3. ISRE reporter screening and aberrant pathway of α-IFN. (A) Pretreatment with TSN. Huh7 cells transfected with a reporter gene (pISRE-Luc and pRL-CMV) were pretreated with TSN (0 or 100 nM) for 0, 24, or 48 h, followed by treatment with α-IFN (0 or 100 IU/ml). Six hours later, the relative ISRE-luciferase activity ($n = 4$) was determined as described in Materials and Methods. The data are expressed as means ± SDs and are a representative example of the data from three similar experiments. (B) Pretreatment with TSN at the concentrations indicated for 24 h, followed by treatment with α-IFN (0 to 100 IU/ml). The ISRE reporter assay was performed as described for panel A. (C) Type I IFN-induced antiviral ISG expression in Huh7 cells. Huh7 cells were treated with TSN for 24 h, followed by treatment with α-IFN at 100 IU/ml for 24 h. The total cellular RNA was then isolated for real-time RT-PCR analysis of the mRNAs of 25AS, MxA, p56, viperin, and ISG20. Beta-actin was used as a control. The data are expressed as means ± SDs and are a representative example of data from three similar experiments. *, $P < 0.05$. (D) Analysis of aberrant pathways of α-IFN signaling under the influence of TSN. Promoter activities of NF-kappaB were analyzed by luciferase reporter assays. These cells were transfected with pNF-kappaB-TA-Luc, pTA-Luc which lacks the enhancer element and which was used as a negative control, and pRL-CMV to normalize transfection efficiency. At 24 h after transfection with these reporters, treatment with TSN (0, 10, 30, 100 nM) was carried out. After 24 h, the relative levels of induction of NF-kappaB activity for each treatment were calculated. TNF-α (50 ng/ml), which was used as a positive control for NF-kappaB, was added 6 h before analysis. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. *, $P < 0.05$.

interferon activation site (GAS), or activator protein 1 (AP1) Fluc plasmids. The reporter activities were measured after culture with or without TSN. As shown in Fig. 3D, there was no significant effect of TSN on GAS or AP1 reporter activities (data not shown). In contrast, NF-kappaB reporter activity was significantly elevated by TSN in a dose-dependent fashion.

Synergistic inhibitory effects of TSN and -IFN on the replicon. We next assessed the effects of TSN combination with α-IFN on the intracellular replication of the HCV genome. Huh7/Rep-Feo cells were treated with various concentrations of TSN (0, 0.01, and 0.03 μg/ml) and α-IFN (0 to 100 IU/ml). Replication of the HCV replicon was suppressed by pretreatment with TSN, followed by treatment with α-IFN, in a dose-dependent manner (Fig. 4A; see Fig. S1 in the supplemental material). The EC_{50} of α-IFN in the absence of TSN was 7.61 IU/ml, while that after pretreatment with 0.03 μg/ml (41 nM) TSN was 3.16 IU/ml. These results indicated that pretreatment with TSN before α-IFN treatment is more effective in inhibiting HCV replication than treatment with α-IFN alone.

Subsequently, we conducted the following assay to determine whether TSN and α-IFN have a synergistic inhibitory effect on the replicon. The relative dose-inhibition curves of α-IFN were plotted for several concentrations of TSN and α-IFN. The curves shifted to the left with increasing concentrations of TSN (Fig. 4B), demonstrating that HCV replication was considerably reduced by the combination compared with that by either TSN or α-IFN alone. An MTS-based cell viability assay did not show significant cytotoxicity from TSN (Fig. 4C). Western blot analysis and densitometry of each blot showed results essentially identical to those from the luciferase assay (Fig. 4D).

We used isobologram analysis to determine whether the anti-HCV effect of TSN is synergistic with that of α-IFN (27, 37). Huh7/Rep-Feo cells were treated with a combination of α-IFN and TSN at an EC_{50} ratio of 1:0, 2:3, 1:4, or 0:1, and the dose-effect plots were drawn (Fig. 4E). The fractional EC_{50}s for α-IFN and TSN were plotted on the x and y axes, respectively, to generate an isobologram (Fig. 4F). Each plot showing

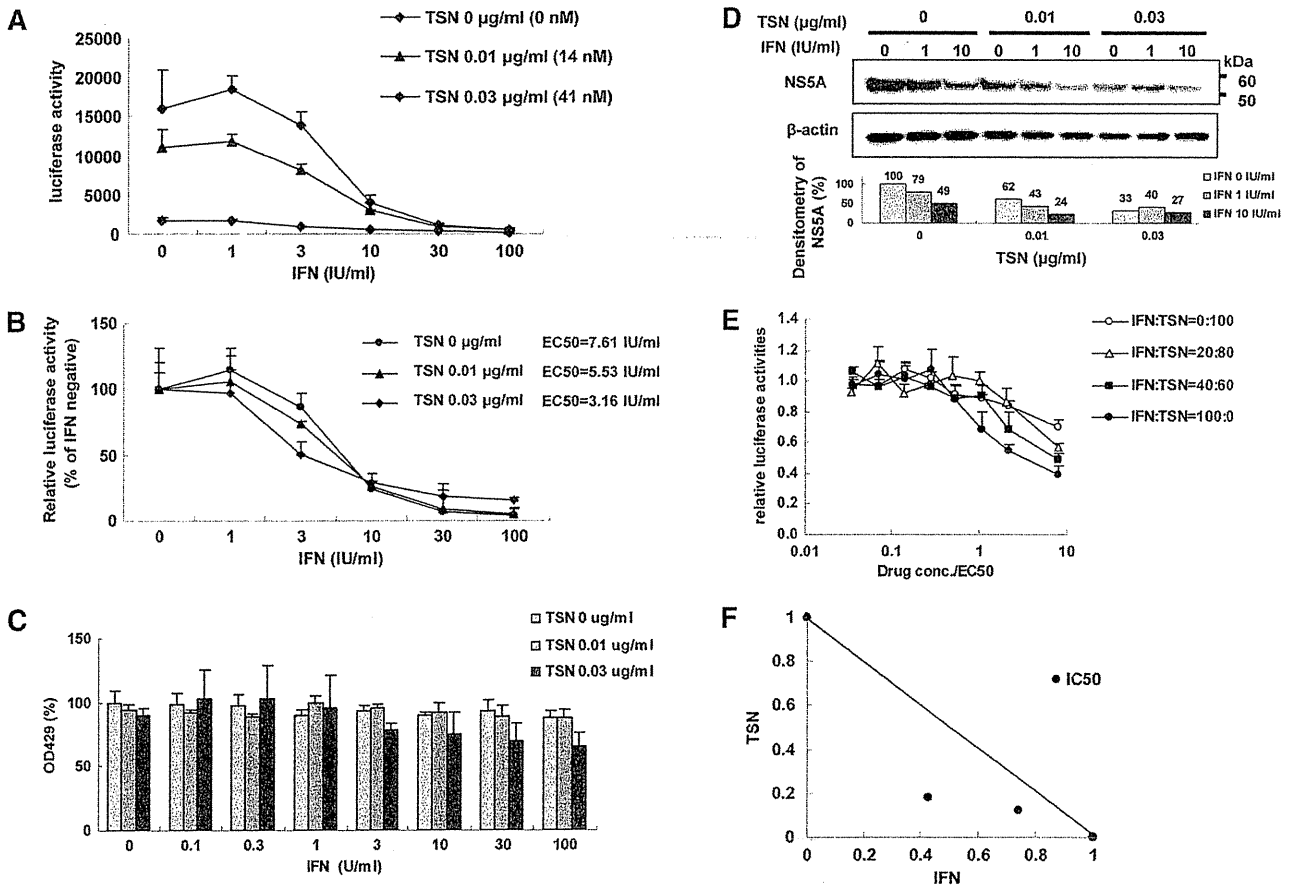

FIG. 4. Suppression of HCV RNA replication by TSN combined with α-IFN. (A and B) Luciferase activity (A, absolute value; B, relative value). Huh7/Rep-Feo cells, which constitutively express an HCV replicon, enable the quantification of replication levels through the measurement of luciferase activity. Absolute and relative dose-response curves in the presence of 24 h of pretreatment of various concentrations of TSN (0, 0.01, 0.03 μg/ml) and α-IFN (0, 100 IU/ml). (A) Bars indicate luciferase activities. (B) Bars indicate luciferase activities relative to the activity of each α-IFN-negative control. Luciferase assays were performed in triplicate. Error bars indicate means ± SDs. (C) MTS assay of Huh7/Rep-Feo cells cultured with the indicated concentrations of TSN and α-IFN. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. (D) Western blotting. Ten micrograms of total cellular protein was separated by polyacrylamide gel electrophoresis and transferred onto the membrane. Monoclonal anti-NS5A antibody or an anti-beta-actin antibody was used as the primary antibody. Densitometry of NS5A or beta-actin protein was performed and the result is indicated as a percentage of that for the drug-negative control. The assay was repeated three times, and representative results are shown. (E) Dose-inhibition curves of α-IFN and TSN when they were combined at the indicated ratios, adjusted by the EC_{50} of the individual drug. Assays were done in triplicate, and mean values were plotted and indicated as means ± SDs. (F) Graphical representation of the isobologram analysis. For each drug combination in panel E, the EC_{50}s of α-IFN and TSN for inhibition of HCV replication were plotted against the fractional concentrations of α-IFN and TSN, which are indicated on the *x* and *y* axes, respectively. A theoretical line of additivity is drawn between the EC_{50} for each drug alone. All of the fractional EC_{50} plots for the TSN and α-IFN combinations fell below the line of additivity, indicating synergy.

the fractional EC_{50} of each drug ratio fell below the line showing additivity, indicating that the effect of the drug combination on intracellular HCV RNA replication was synergistic. The MTS values at the drug concentrations used in this isobologram analysis did not show any significant decrease, suggesting that the synergistic action of α-IFN and TSN on HCV replication is through their pharmacological effects and is not due to augmentation of cytotoxicity.

Suppression of HCV-J6/JFH1 infection by pretreatment of TSN with -IFN. The inhibitory effects of pretreatment with TSN prior to α-IFN treatment demonstrated on HCV subgenomic replication were validated further using HCV-J6/JFH1 cell culture (21, 42). Various concentrations of TSN and α-IFN were added to HCV-J6/JFH1-infected Huh7 cells, and intracellular HCV RNA was quantified after 48 h of incubation. As shown in Fig. 5A, TSN with or without α-IFN suppressed expression of intracellular HCV RNA in a dose-dependent manner. The EC_{50}s of α-IFN with TSN at 0, 10, and 30 nM were 4.71 IU/ml, 3.83 IU/ml, and 3.52 IU/ml, respectively. An MTS-based cell viability assay did not show significant cytotoxicity from TSN (Fig. 5B). These data indicate that pretreatment with TSN also augmented the α-IFN effect on the JFH1 system.

TSN upregulates ISGF3 in combination with -IFN. Subsequently, we performed experiments to investigate the mechanisms of action of TSN. First, we quantified expression of alpha/beta IFN receptor subunit (IFNAR) 1 and IFNAR2 and the effect of TSN. Real-time RT-PCR analysis showed no

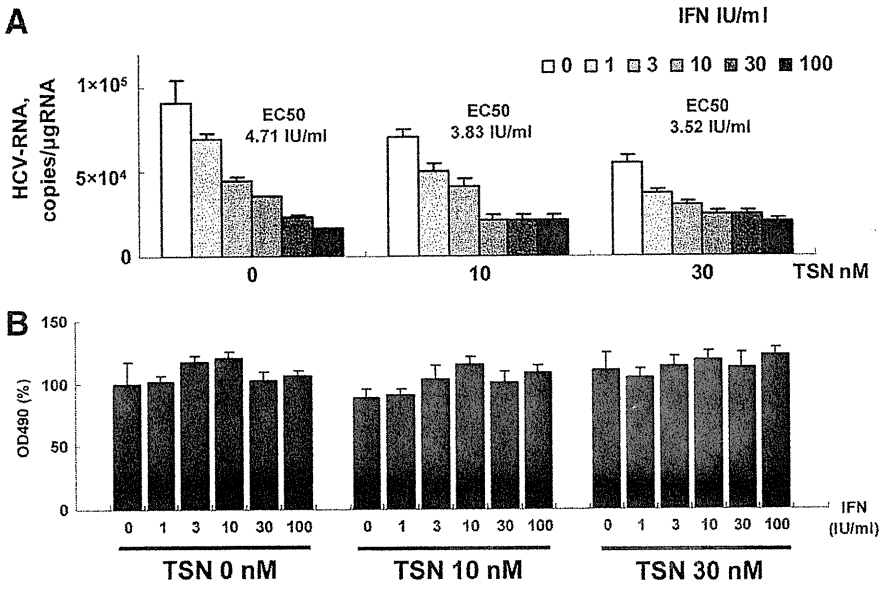

FIG. 5. Suppression of full HCV-J6/JFH1 replication by pretreatment of TSN with α-IFN. Ten micrograms of HCV-J6/JFH1 RNA was transfected into Huh7 cells. At 48 h after transfection, cells were pretreated with TSN for 24 h, followed by treatment with α-IFN (0, 1, 3, 10, 30, 100 IU/ml). At 48 h after α-IFN addition, cells were harvested. (A) Real-time RT-PCR analysis; (B) effect of pretreatment TSN with α-IFN on cell viability. MTS assays were performed 48 h after culture in the presence of pretreatment TSN with α-IFN. Bars indicate values relative to that of the drug-negative control. In panels A and B, the assays were done in triplicate and repeated three times. Error bars indicate means ± SDs.

change in levels of IFNAR1 and IFNAR2 mRNA expression with or without TSN (Fig. 6).

Next, we investigated the ISGF3 components, STAT1, and STAT2, using Western blotting, and interferon regulatory factor 9 (IRF9), using real-time RT-PCR. Huh7 cells were treated with various concentrations of TSN or 0.01% DMSO. Twenty-four hours after TSN treatment, 100 IU/ml of α-IFN was added, and STATs and IRF9 were detected. Western blot analysis demonstrated that phosphorylated STAT1 and STAT2 levels were increased more by treatment with α-IFN and TSN than by α-IFN treatment alone (Fig. 7A and B). In addition, IRF9 mRNA expression was significantly higher following pretreatment with TSN prior to α-IFN therapy than by α-IFN monotherapy (Fig. 8). These findings are consistent with the hypothesis that TSN activates ISGF3 components in combination with α-IFN.

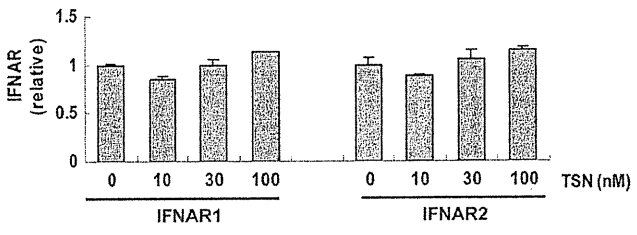

FIG. 6. IFNAR expression. Huh7 cells were pretreated with TSN for 24 h, followed by treatment with 100 IU/ml α-IFN for 6 h. The total cellular RNA was then isolated for real-time RT-PCR analysis of the mRNAs of IFNAR1 and IFNAR2. The assays were done in triplicate and repeated three times. The data are shown as means ± SDs.

DISCUSSION

In this study, we investigated the molecular actions of TSN on HCV replication and on α-IFN-mediated cellular antiviral responses. Treatment of cells expressing an HCV subgenomic replicon with TSN alone specifically inhibited HCV replication with a selectivity index of more than 146 (Fig. 2). In addition, pretreatment of cells with TSN prior to addition of α-IFN augmented α-IFN receptor-mediated, ISRE-regulated gene expression (Fig. 3). Consistent with these findings, TSN pretreatment significantly enhanced the suppressive effects of α-IFN on the HCV replicon and HCV cell culture (Fig. 4 and 5). Finally, we demonstrated that the α-IFN-enhancing effects of TSN are through increased transcriptional activation of a component of ISGF3 (Fig. 7 and 8). Taken together, out results demonstrate that TSN is potentially an effective antiviral agent when it is used alone and especially when it is used in combination with α-IFN and that screening for such α-IFN-enhancing agents may identify promising antiviral therapeutics. Because TSN treatment alone or simultaneous treatment with TSN and α-IFN did not increase ISRE activity or augment α-IFN-mediated ISRE activation, TSN may affect α-IFN sensitivity by upregulating molecules that affect α-IFN receptor-mediated signaling without activating ISRE signaling directly.

Type I interferon plays a central role in eliminating viruses through its innate antiviral activity or following therapeutic application. Binding of α-IFNs to their receptors activates the Jak-STAT pathway to form a complex with ISGF3, which translocates to the nucleus, binds the ISRE located in the promoter/enhancer region of the ISGs, and activates expression of ISGs (28, 39, 40). In this study, we demonstrated that TSN enhanced α-IFN effects by upregulating ISGF3, which

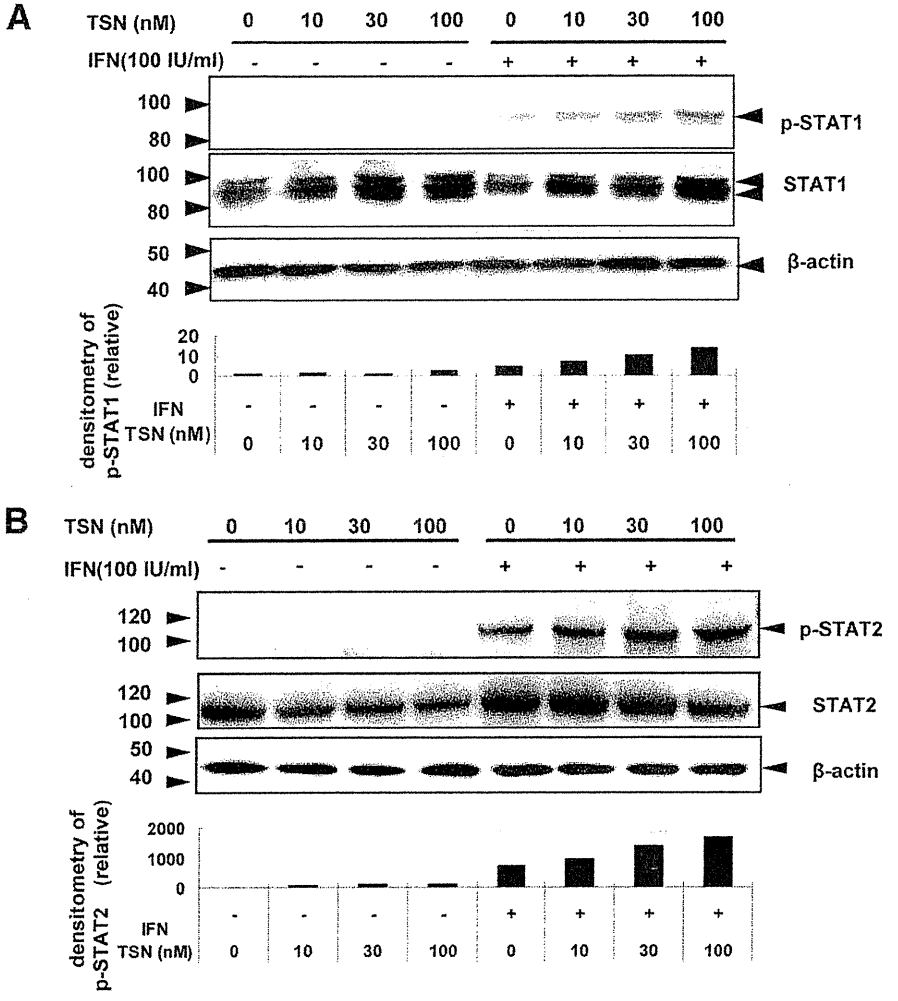

FIG. 7. TSN with α-IFN treatment of Huh7 cells increases phosphorylation of STAT1 and STAT2. (A) Western blotting. Alteration in the distribution of α-IFN-induced phosphorylation of STAT1 and STAT2 by TSN. Huh7 cells were treated with TSN or 0.01% DMSO for 24 h. After that, the cells were stimulated by 100 IU/ml α-IFN for 30 min. Cells were harvested, and the resulting lysates were analyzed for phosphorylated and total STAT1 or STAT2. The relative amounts of phosphorylated STAT1 or STAT2 were normalized to the amount of total STAT1 or STAT2 and expressed relative to the amount for the drug-negative control. The assay was repeated three times, and a representative result is shown.

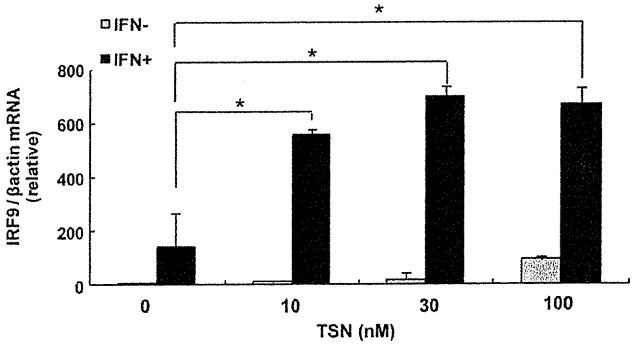

FIG. 8. IRF9 mRNA expression after combination treatment with TSN and α-IFN. Real-time RT-PCR analysis. Huh7 cells were treated with TSN for 24 h. After 6 h, the cells were stimulated by α-IFN (100 IU/ml). We used the method described in the legend to Fig. 6 to analyze the mRNA of IRF9. The assays were done in triplicate and repeated three times. Error bars indicate means ± SDs. *, $P < 0.05$.

may cancel the suppressive effect of HCV gene products on the α-IFN signaling pathway.

In our study, it was not proved that increasing ISRE activities had direct relevance to inhibition of HCV replication. In Fig. 3C, we showed that TSN with α-IFN treatment had elevated the level of expression of mRNA of ISGs. Previous studies suggested that overexpression of known ISGs inhibited HCV replication in HCV replicon-containing Huh7 cells (13, 14). These findings may support the possibility that TSN had the potential to augment the α-IFN effect.

Other than the canonical Jak/STAT-mediated α-IFN signaling pathway, several alternative α-IFN pathways have been reported, including the NF-kappaB, gamma IFN, PI3K, and MAPK pathways (9, 16, 24, 28). We carried out reporter assays using NF-kappaB, AP1, and GAS reporter plasmid constructs and treated the cells with TSN. As shown in Fig. 3D, TSN activated NF-kappaB-regulated gene expression significantly. NF-kappaB is a sequence-specific transcription factor which

regulates the expression of numerous cellular and viral genes and plays important roles in inflammation, innate immune responses, tumorigenesis, and cell survival (3, 19). Activation of NF-kappaB is principally regulated by tumor necrosis factor alpha (TNF-α), Toll-like receptors (TLRs), and RIG-I, which may possibly be associated with the molecular mechanisms of TSN monotherapy. Horsmans et al. (12) and Agrawal and Kandimalla (1) reported that TLR7, -8, and -9 agonists have the ability to modulate TLR-mediated immune responses in targeting a broad range of disease vectors, including HCV, alone or in combination with other therapeutic agents. These reports support the hypothesis that activation of NF-kappaB may be one of the mechanisms of action of TSN.

It has been reported that TSN exhibits cytotoxic/antiproliferative potential at high concentrations (36, 47). In our study, the selectivity index of TSN against HCV was sufficient to ascertain that the antiviral effects are not simply due to the cytotoxicity of TSN. A recent study showed that a triterpenoid compound, dammarenolic acid, inhibits retrovirus, human immunodeficiency virus, simian immunodeficiency virus, murine leukemia virus, and respiratory syncytial virus infections *in vitro* (4) (5). We have analyzed the effects of dammarenolic acid on antiviral actions on Huh7/Rep-Feo cells, cytotoxicity, and ISRE reporter activation. However, dammarenolic acid did not inhibit HCV replication or enhanced α-IFN-induced ISRE activity (data not shown). These findings suggest that the anti-HCV and α-IFN enhancer effects are distinctive features of TSN among triterpenoid compounds. Hiasa et al. have reported that ME3738, a triterpenoid saponin, suppressed HCV replication through production of endogenous beta interferon (11). ME3738 is now in clinical trials for treatment of HCV-infected patients. Taking these findings together, despite reports on the cell-suppressive effect of triterpenoids, properly selected or designed compounds might be used as drugs against HCV infection.

Because the mechanisms of action of these triterpenoid compounds against these viruses are poorly understood, further investigation of the mechanism of action of TSN on HCV may be valuable to implement antiviral strategies against other viruses. It would be important to assess drug resistance after continuous treatment with TSN. There is no *in vitro* or *in vivo* report on resistance of TSN or cellular attenuation of responses to TSN. Such information, if any is found, would help elucidate the mechanism of action of TSN.

Given the current situation of limited therapeutic options against HCV, the search for more potent and less toxic antiviral drugs is needed to improve clinical anti-HCV chemotherapeutics. Several direct antiviral agents with activity against HCV are currently undergoing clinical trials. These include NS3 protease inhibitors and NS5B polymerase inhibitors (41). However, the frequent emergence of drug-resistant viruses is a major weakness of such agents (20). Our results indicate that TSN is also effective at suppressing HCV infection and replication. Future studies with TSN, its derivatives, and other chemicals that target the α-IFN pathway could be directed toward developing a new class of antiviral treatment regimens and drugs.

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Original Article

Studies on virus kinetics using infectious fluorescence-tagged hepatitis C virus cell culture

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Aim: Studies of the complete hepatitis C virus (HCV) life cycle have become possible with the development of a HCV-JFH1 cell culture system.

Methods: In this study, we constructed two fluorescence protein-tagged recombinant JFH1 virus clones, JFH1-EYFP and JFH1-AsRed, as well as two corresponding clones with adaptive mutations, JFH1-EYFP mutant and JFH1-AsRed mutant, that and were as effective as JFH1 in producing infectious virus particles, and investigated their viral infection life cycles.

Results: After infection of the fluorescence-tagged mutant viruses, infected cells increased exponentially. In cells, EYFP or AsRed and NS5A were expressed as a fusion protein and co-localized in core proteins. The rate of the cell–cell spread was dependent on the cell densities with a maximum of $10^{2.5}$/day. Treatment of cells with interferon or a protease inhibitor suppressed expansion of virus-positive cells.

Conclusion: Taken together, these results indicate that fluorescence-tagged HCV is a useful tool to study virus infection life cycles and to assist in the search for novel antiviral compounds.

Key words: AsRed, confocal laser microscopy, HCV-JFH1 cell culture, protease inhibitor, yellow fluorescence protein

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is characterized frequently by chronic inflammation of the liver, leading to decompensated liver cirrhosis and hepatocellular cancers.[1] Interferon (IFN)-α has been the mainstay of HCV therapy.[2] However, the most effective therapy, pegylated IFN plus ribavirin in combination, can eliminate HCV from only half of the patients treated[3,4] and often is accompanied by substantial side-effects.[5,6] These difficulties in eliminating the virus are attributable mostly to the limited treatment options.[7]

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Hepatitis C virus belongs to the family Flaviviridae. The viruses have positive-strand RNA genomes of approximately 10 kb that encode polyproteins of approximately 3000 amino acids. The protein is post-translationally processed by cellular and viral proteases into at least 10 mature proteins. The viral non-structural (NS) proteins accumulate in the endoplasmic reticulum (ER) and they direct genomic replication and viral protein synthesis.[8,9] Studies of the HCV life cycle and the development of new drugs have long been hampered by the lack of cell culture systems. These problems have been greatly overcome by the development of the HCV subgenomic replicon[10] and HCV-JFH1 cell culture[11] systems.

After the development of HCV-JFH1 cell culture, many variations of reporter protein-tagged HCV systems have been described.[12–15] These reporter systems, however, feature poor or absent virus propagation, secretion and re-infection. The C-terminal end of the NS5A region, which has been used for insertion of

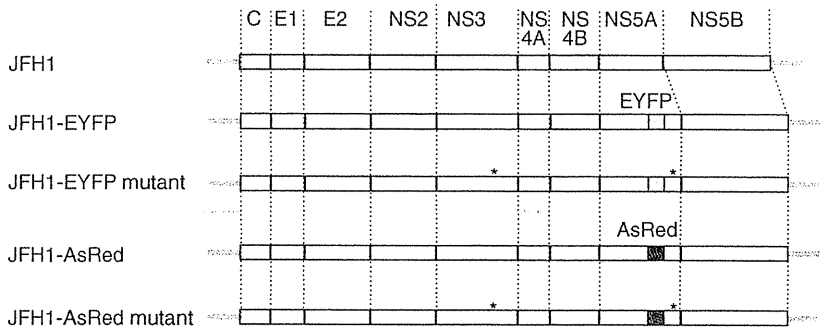


Figure 1 Schematic of the JFH1-based hepatitis C virus constructs. EYFP or AsRed was inserted in-frame into the *Cpo*I site of NS5A (JFH1-EYFP or JFH1-AsRed). The adaptive mutations in NS3 and NS5A (T4209A and C7653T) are indicated by asterisks (JFH1-EYFP mutant or JFH1-AsRed mutant).

reporter sequences, does not affect viral genomic replication,[12] while manipulation of the domain resulted in the loss of viral particle secretion possibly through abrogation of NS5A-core protein–protein association that is crucial for the viral particle assembly.[16] These problems have made it to difficult to analyze the entire viral life cycle in living cells. Recently, Han *et al.* reported a mutant HCV-JFH1 clone that expressed green fluorescent protein (GFP)-tagged NS5A and which could propagate and secrete infectious virus particles.[17]

In this study, we took advantage of the mutant fluorescence protein-tagged HCV and investigated the life cycles of HCV infection. We have demonstrated the rate of expansion of HCV infection using flow cytometry. Moreover, we have shown the kinetics of co-infection with two virus strains, which differed in their ability to secrete infectious virus particles.

METHODS

Reagents

RECOMBINANT HUMAN IFN-α-2b was from Schering-Plough (Kenilworth, NJ). Protease inhibitor (BILN2061) was from Boehringer Ingelheim (Ingelheim, Germany). BILN2061 is a macrocyclic NS3 protease inhibitor, the antiviral effects of which have been reported in a phase I clinical study.[18] Although further development of BILN2061 has been abandoned, this compound has structural homology with other protease inhibitors that are currently being evaluated in clinical trials, such as TMC435 and MK7009.[7,19]

Cell culture

Huh7.5.1 cells[20] and their derivatives were maintained in Dulbecco's modified minimal essential medium (DMEM; Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA, USA) at 37°C under 5% CO_2.

Plasmid constructs (Fig. 1)

In order to produce pJFH1-EYFP, the EYFP gene was amplified from pEYFP-C1 (Clontech, Mountain View, CA, USA) by polymerase chain reaction (PCR). The PCR products were then inserted into the *Cpo*I site (7484) of pJFH1. Similarly, pJFH1-AsRed was produced using pAsRed2 (Clontech). The JFH1-EYFP and JFH1-AsRed mutants, which are JFH1-based mutants with robust virus production capability, have two point mutations (T4209A, C7653T).[17] In order to introduce these mutations into pJFH1-AsRed, the *Avr*II/*Nsi*I and *Bsr*GI fragments of pJFH1-AsRed were amplified by PCR. The PCR products were subcloned into the T-Vector (pGEM-T Easy Vector Systems; Promega, Madison, WI, USA) and the mutations were introduced by site-directed mutagenesis (Quick-Change II Site-Directed Mutagenesis Kit; Stratagene, La Jolla, CA, USA), as reported previously.[21] Finally, these *Avr*II/*Nsi*I and *Bsr*GI fragments were reinserted into the parental plasmid, pJFH1-AsRed. The pJFH1-AsRed mutant was digested with *Cpo*I and the DNA fragment was subcloned into pJFH1-EYFP, producing the mutant pJFH1-EYFP. All nucleotide numbers refer to pJFH1.[11]

Constitutive expression of ER-fluorescence protein in Huh7.5.1 cells

Huh7.5.1 cells were seeded into 6-cm diameter dishes and transfected with pDsRed2-ER (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Isolated clones (ER-Huh7.5.1 cells) were maintained under selection with 0.75 mg/mL G418 (Nacalai Tesque, Kyoto, Japan) and

screened for DsRed protein expression using fluorescence microscopy (BZ-8000; Keyence, Tokyo, Japan).

RNA transcription and transfection

Recombinant HCV RNA was synthesized and transfected as previously described.[22,23] Briefly, the plasmids were linearized by digestion with *XbaI* and subjected to *in vitro* transcription using RiboMax Large Scale RNA Production System (Promega). For the RNA transfection, Huh7.5.1 cells were suspended in Opti-MEM (Invitrogen) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1050 μF and 270 V) using the Gene Pulser II apparatus (Bio-Rad, Richmond, CA, USA). After electroporation, the cell suspension was left for 5 min at room temperature and then incubated under normal culture conditions in a 10-cm diameter dish. The transfected cells were split every 3–5 days. The culture media were transferred subsequently onto uninfected Huh7.5.1 cells.

Immunofluorescence microscopy

Immunofluorescence microscopy was performed as described previously.[24] Cells were cultured on 18-mm round cover slips (Matsunami, Osaka, Japan) and fixed using 4% paraformaldehyde for 10 min at room temperature. Cells were incubated with the primary antibodies for 1 h at 37°C and with Alexa Fluor 488 goat antimouse immunoglobulin (Ig)G antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature in the dark. Mouse anti-NS5A antibody (Biodesign, Saco, ME, USA) and mouse anti-core antibody (Abcam, Cambridge, MA, USA) were used as primary antibodies. Cells were mounted with VECTA SHIELD Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and visualized by confocal laser fluorescent microscopy (BZ-8000 [Keyence] and FLUOVIEW FV10i [Olympus, Tokyo, USA]).[25]

Flow cytometry

JFH1-EYFP and JFH1-EYFP mutant-transfected Huh7.5.1 cells and uninfected Huh7.5.1 cells were cultured in 12-well plates (Becton Dickinson, Franklin Lakes, NJ, USA). The cells were collected on the days, post-transfection or post-infection, indicated. After washing with phosphate buffered saline (PBS; Nacalai Tesque) supplemented with 3% FBS and staining of dead cells with propidium iodide, the cells were analyzed using a FACS Calibur with CellQuest software (Becton Dickinson).

Quantification of HCV core antigen in the culture medium

The culture media from Huh7.5.1 cells transfected with JFH1 and its derivatives were collected on the days indicated and stored at 80°C. The levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics).

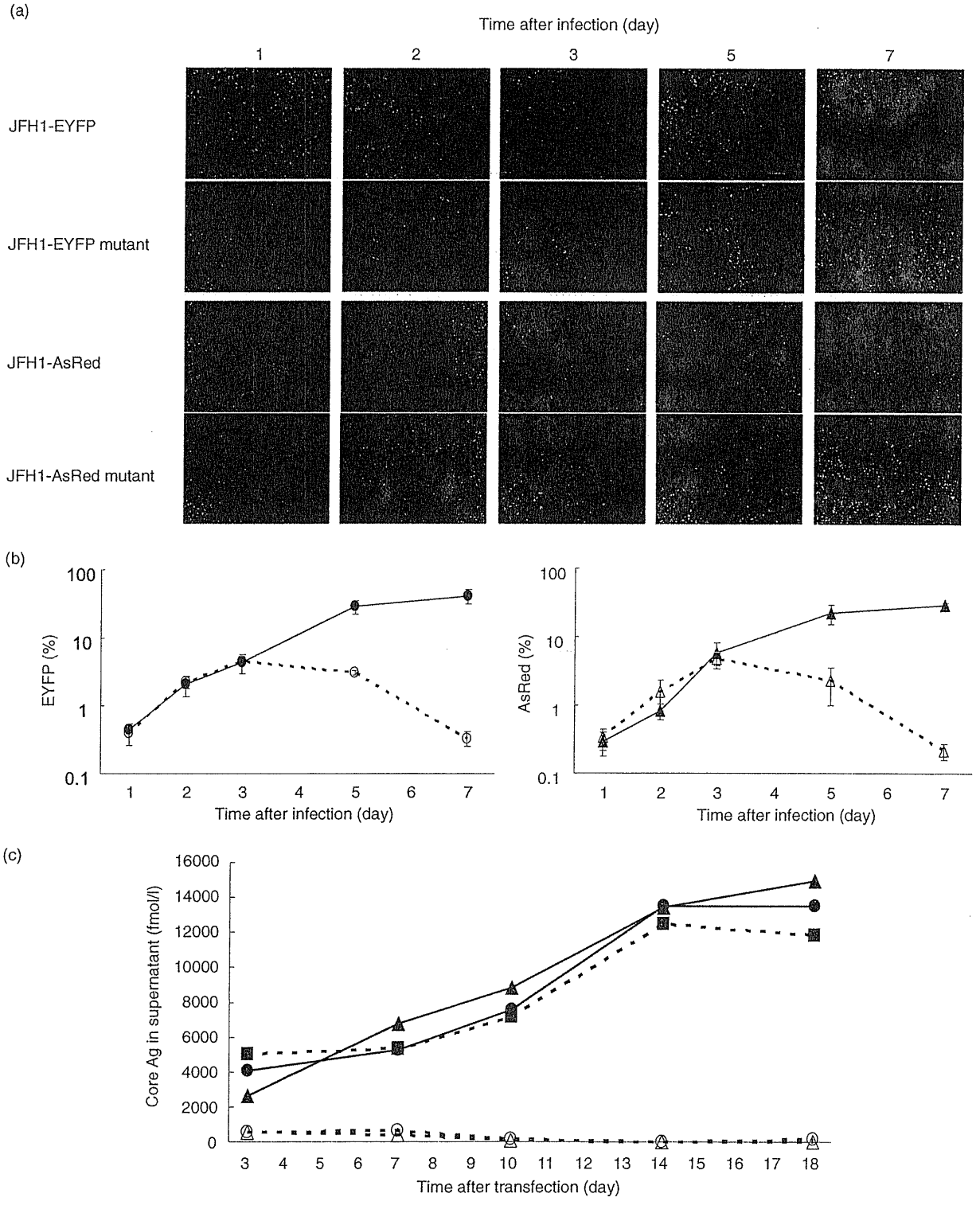
Western blotting

Western blotting was carried out as described previously.[21] Briefly, 10 μg of total cell lysate was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and blotted onto a polyvinylidene fluoride membrane. The membrane was incubated with primary antibodies followed by peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Biosciences). The antibodies used were anti-core mouse monoclonal antibody 2H9, anti-NS5A mouse monoclonal antibody 9E10 (provided by Dr Rice), and anti-GFP rabbit polyclonal antibody (Invitrogen). The anti-GFP is able to detect EYFP. To confirm expression of EGFP in Huh 7.5.1 cells, Huh7.5.1 cells were seeded into a 6-cm diameter dish and transfected with pEGFP (Invitrogen) using Lipofectamine 2000 (Invitrogen).

Statistical analyses

Statistical analyses were performed using Welch's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Figure 2 Infectious hepatitis C virus (HCV) reporter virus with robust virus production capability. (a) Huh7.5.1 cells were transfected with JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed or JFH1-AsRed mutant HCV RNA. At 3 days post-transfection, culture media were collected and added onto uninfected cells. At the days indicated, EYFP or AsRed-directed-fluorescence was visualized directly. (b) The ratio of EYFP or AsRed-positive cells in (a) is counted in each image and plotted vs time. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. —●—, JFH1-EYFP mutant; ·✧·, JFH1-EYFP; —▲—, JFH1-AsRed mutant; ·✧·, JFH1-AsRed. (c) The levels of core antigen in the culture medium of JFH1, JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, and JFH1-AsRed mutant-transfected Huh7.5.1 cells collected on the days indicated. Ag, antigen. ·■·, JFH1; ·✧·, JFH1-EYFP; —●—, JFH1-EYFP mutant; ·✧·, JFH1-AsRed; —▲—, JFH1-AsRed mutant.



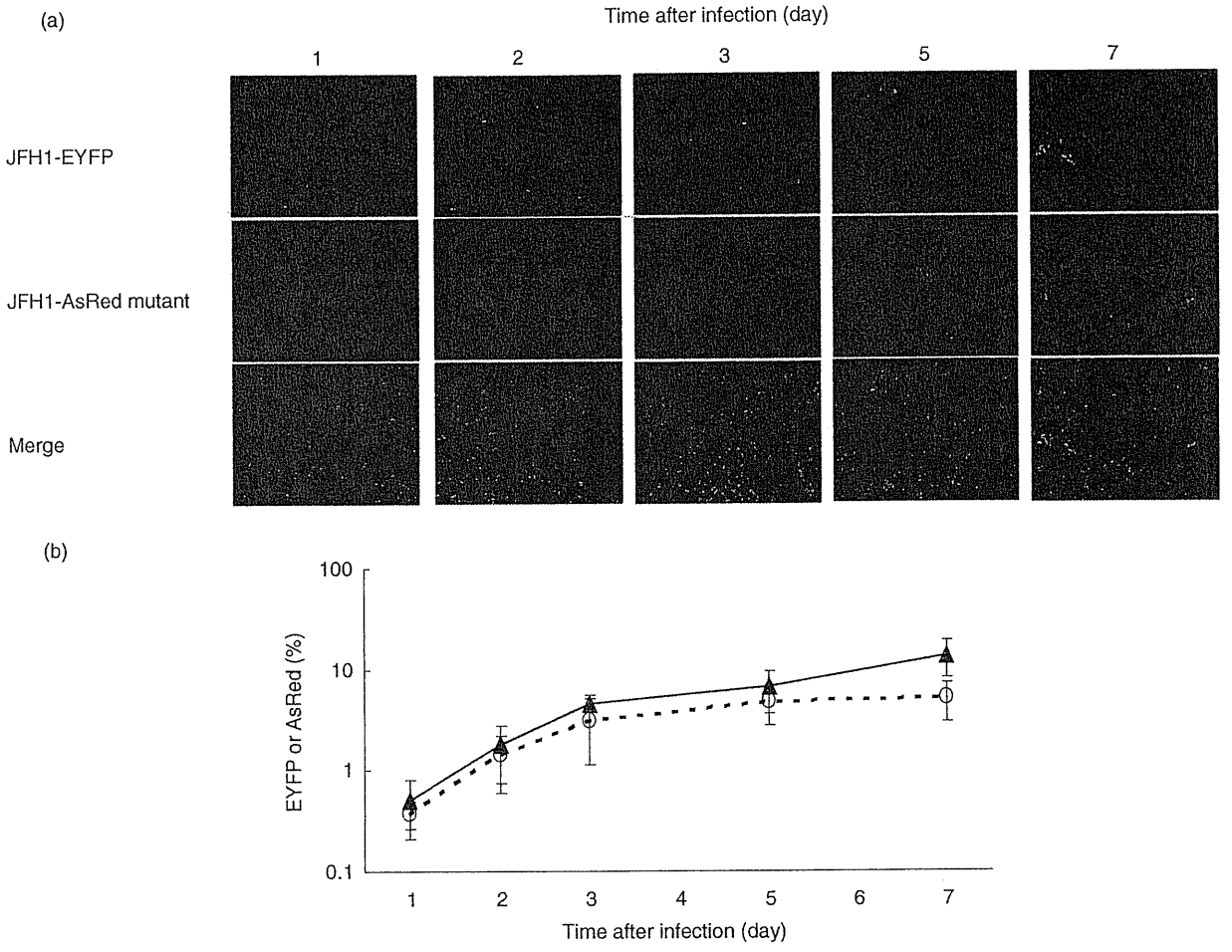


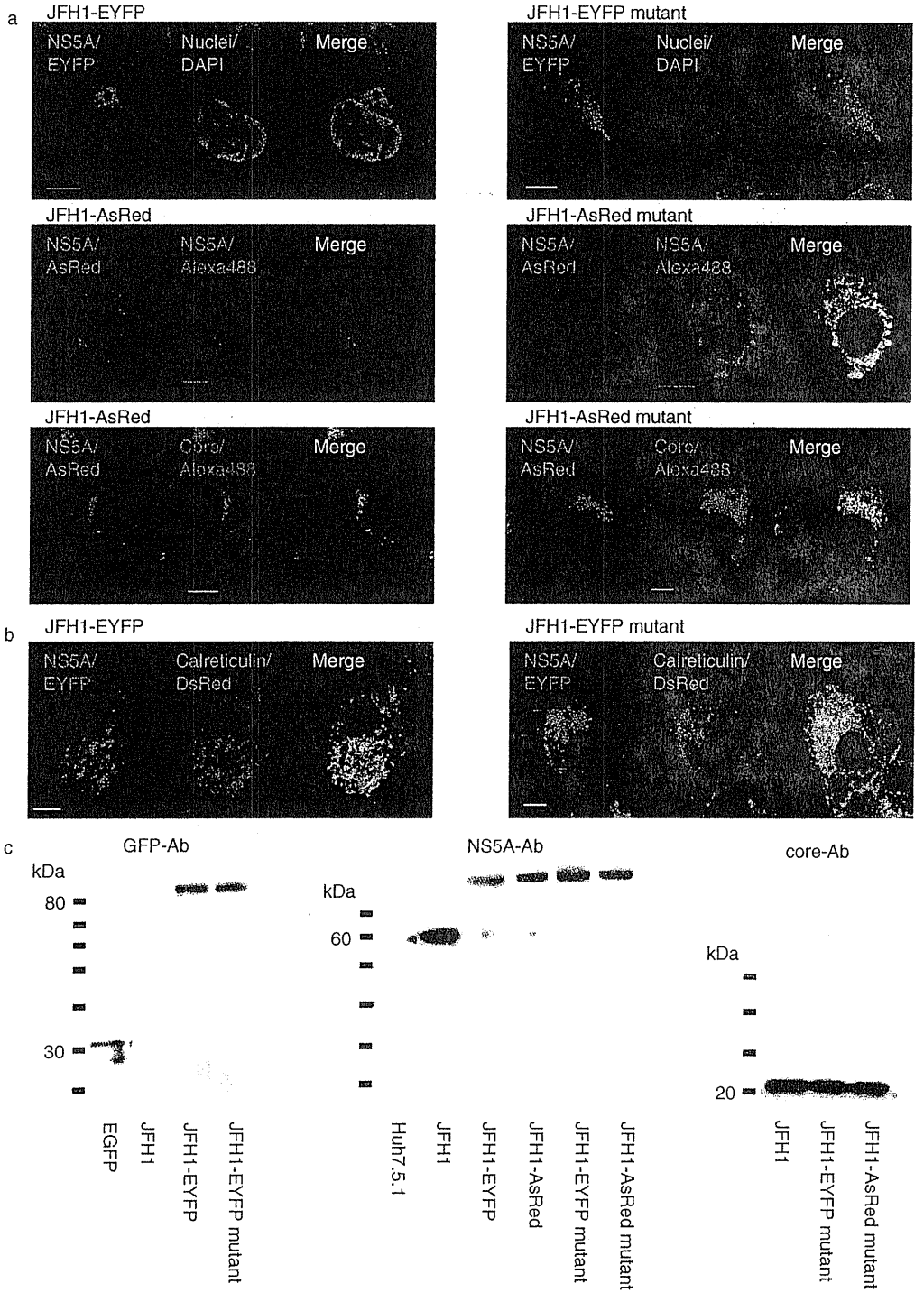
Figure 3 Intracellular *trans*-complementation of virus proteins. (a) Culture media from JFH1-EYFP and JFH1-AsRed mutant-transfected cells at 3 days post-transfection were added onto uninfected Huh7.5.1 cells. At the days indicated, EYFP or AsRed-directed-fluorescence was visualized directly. (b) The ratio of EYFP or AsRed-positive cells in Fig. 2a is calculated and plotted vs time. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —▲— JFH1-AsRed mutant; ···○··· JFH1-EYFP.

RESULTS

Infectious HCV reporter virus with robust virus production capability

FIRST, WE STUDIED whether the JFH1-EYFP mutant viruses are able to secrete sufficient amounts of infectious virus particles. Full-length HCV RNA was transcribed *in vitro* and transfected into Huh7.5.1 cells. Culture media were collected from cells transfected with JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, or JFH1-AsRed mutant, respectively, and inoculated into uninfected Huh7.5.1 cells. EYFP- or AsRed-positive cells were

Figure 4 Localization and expression of NS5A-EYFP and NS5A-AsRed fusion proteins. (a,b) Huh7.5.1 cells transfected with JFH1-EYFP, AsRed and their mutant RNA genomes were fixed at 3 days post-transfection. NS5A-EYFP and NS5A-AsRed fusion proteins were visualized using EYFP and AsRed, respectively. DsRed auto-fluorescence was observed directly. NS5A and core proteins were immunostained with Alexa Fluor 488-labeled goat antimouse immunoglobulin G (green). 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) staining revealed the nuclear chromatin. Bars represent 10 μm. (c) Cells were transfected with EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant or JFH1-AsRed mutant. Cells were harvested at 3 days post-transfection, and western blotting was performed by using anti-GFP, NS5A or core antibodies.



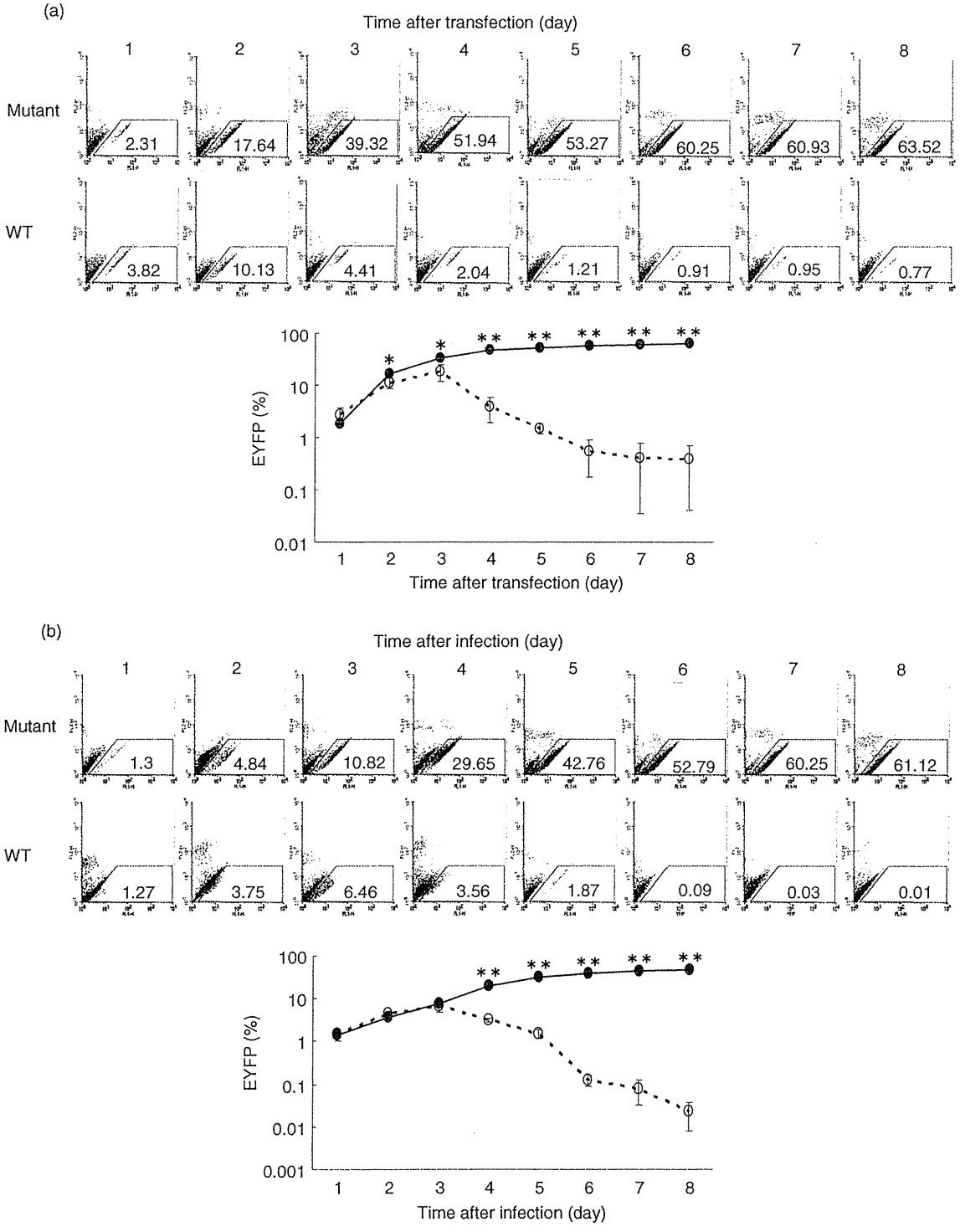
directly visualized by fluorescence microscopy on days 1–7. As shown in Figure 2(a), the number of cells positive for both JFH1-EYFP and JFH1-AsRed mutants, but not for JFH1-EYFP and JFH1-AsRed-infected cells, increased in a time-dependent manner. In JFH1-EFYP mutant-transfected cells, the proportion of EYFP-positive cells on days 3, 5 and 7 post-infection was 4.4%, 29% and 41%, respectively. In contrast, only 4.9% of JFH1-EYFP-transfected cells became EYFP-positive at 3 days post-infection, and the percentage of these fluorescence-positive cells decreased rapidly thereafter (Fig. 2b). Similarly, the percentage of cells infected with JFH1-AsRed mutant but not JFH1-AsRed increased exponentially. These results indicated that the two fluorescence virus clones with mutations are able to secrete infectious virus particles. We next compared levels of HCV core antigen in culture medium of cells infected with JFH1, JFH1-EYFP, JFH1-EYFP mutant, JFH1-AsRed, and JFH1-AsRed mutant viruses. The mutant viruses, but not the wild-type, produced amounts of core protein comparable to that of the parental JFH1 (Fig. 2C). In HCV-JFH1, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells, the core protein reached a peak of 1.25, 1.35 and 1.34 fmol/L, respectively, at 14 days post-transfection, while that of JFH1-EYFP JFH1-AsRed-transfected cells became undetectable at 10 days post-transfection. These results indicated that the mutant type is capable of producing an amount of viral particles comparable to that of the parental JFH1.

Using the two fluorescence-tagged viruses, we conducted co-infection of two virus strains, JFH1-AsRed mutant, which secreted infectious virus particles, and JFH1-EYFP, in which virus particle secretion was impaired. We collected culture media from cells transfected with JFH1-AsRed-mutant or JFH1-EYFP on day 2 post-transfection and infected both media onto uninfected Huh7.5.1 cells at a multiplicity of infection (moi, focus forming unit per cell) of 0.01. The number of JFH1-AsRed mutant-infected cells increased exponentially until day 3 but reached a plateau on days 5 and 7 post-infection. Interestingly, the number of cells positive for viral secretion-impaired JFH1-EYFP also increased in a manner similar to that of the JFH1-AsRed mutant (Fig. 3a). The percentage of AsRed mutant-positive cells was 4.6%, 6.7% and 14.8% at days 3, 5 and 7, respectively, while the percentage of EYFP-positive cells at the corresponding days was 3.1%, 4.8% and 5.1%, respectively (Fig. 3b). These results suggest that, in the co-culture of two HCV clones with and without virus particle secretion, a secretion-impaired virus clone is able to replicate and produce infectious particles possibly through the complementation of the intact virus.

Expression and subcellular localization of NS5A-fluorescence proteins

We next used fluorescence microscopy to study the subcellular localization of fluorescence and viral proteins. In cells transfected with JFH1-EYFP, JFH1-AsRed, and the respective mutants, EYFP and AsRed, were clearly visualized as dot-like structures in the perinuclear area (Fig. 4a). To determine if the NS5A-AsRed fusion protein indicates the subcellular localization of NS5A, we performed immunofluorescence staining of JFH-AsRed- and JFH1-AsRed mutant-infected cells using NS5A and HCV-core antibodies. Fluorescence of AsRed was co-localized precisely with NS5A and partially with core proteins. The fluorescence intensities of the JFH1-EYFP and -AsRed mutants within the cells were equal to that of the wild-type constructs. EYFP-NS5A of wild type and mutant JFH1 were localized in the ER (Fig. 4b). Western blotting was performed by using anti-GFP and anti-HCV-NS5A antibodies. As shown in Figure 4(c), three bands of the expected molecular weights of 27, 58 and 85 kDa, which corresponded to EGFP, NS5A and NS5A-EYFP fusion protein, were detected in EGFP, JFH1, JFH1-EYFP, JFH1-AsRed, JFH1-EYFP mutant, and JFH1-AsRed mutant-transfected cells. The expression levels of core protein in JFH1-EYFP mutant- and JFH1-AsRed mutant-transfected cells were almost the same as those transfected with parental JFH1. These results indicate

Figure 5 Kinetics of hepatitis C virus (HCV)-infected cells. (a) Huh7.5.1 cells were infected with JFH1-EYFP or JFH1-EYFP mutant HCV RNA. At the days indicated, cells were harvested and subjected to flow cytometry. EYFP-positive cells were sorted based on EYFP activating (x-axis) and staining with a marker of dead cells (y-axis). The results are depicted as density plots. The ratios of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. $^{**}P < 0.01$. —•—, JFH1-EYFP mutant; ··◇··, JFH1-EYFP. (b) Culture media from JFH1-EYFP or mutant-transfected cells were added onto uninfected Huh7.5.1 cells at a moi of 0.01. At the days indicated, infected cells were analyzed using flow cytometry. The results are depicted as density plots. The ratio of EYFP-positive cells vs time are shown below. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. $^{**}P < 0.01$. WT, wild type. —•—, JFH1-EYFP mutant; ··◇··, JFH1-EYFP.



that the fusion proteins of NS5A and the fluorescent proteins remain intact within cells and serve as accurate markers of infection and as indicators of the subcellular localization of HCV-NS5A proteins.

Kinetics of HCV infection

Using those fluorescence-tagged HCV constructs, we analyzed more precisely the ratio and kinesics of HCV RNA-transfected cells and virus-infected cells by flow cytometry. After HCV RNA transfection, the percentages of JFH1-EYFP and JFH1-EYFP mutant-transfected cells were almost equivalent up to 2 days. Thereafter, JFH1-EYFP-positive cells began to decrease in number, while the mutant-transfected cells increased exponentially until 5 days post-transfection and then reached a plateau, when 52.2% of the cells were EYFP-positive (Fig. 5a). We collected the media from JFH1-EYFP and mutant-transfected cells at 2 days post-transfection, and added it onto uninfected Huh7.5.1 cells at a moi of 0.01. Similar to the results of the transfection assay, the population of JFH1-EYFP mutant-infected cells increased exponentially and reached a stable state at 6 days post-infection, when 39.2% of the cells were EYFP-positive (Fig. 5b). Calculating from the above data, the rate of expansion of HCV-infected cells was $2^{1.5}$/day. The cell-to-cell expansion of the JFH1-EYFP mutant infection was blocked by prior treatment of cells with anti-CD81 antibody (data not shown). This finding indicated that the expansion of the EGFP-positive cells was due to cell–cell spread of EYFP-tagged HCV and not the division of the virus-positive cells.

To further refine the calculation of the rate of cell-to-cell spread of infection, we carried out JFH1-EYFP mutant infection of uninfected Huh7.5.1 cells seeded at various densities from 2×10^3 to 2×10^5 cells/mL (Fig. 6). Flow cytometry showed that the rates of expansion of HCV-infected cells were $2^{1.5}$, $2^{2.3}$ and $2^{2.5}$/day at 2×10^3, 2×10^4 and 2×10^5 cells/cm^2, respectively. The ability of JFH1-EYFP to spread is greater in cells seeded at higher density. The maximum rate of expansion of HCV-infected cells was calculated as $2^{2.5}$/day.

Effects of antiviral drugs on HCV-infected cells

We next investigated the effects of antiviral agents on the infection kinetics of tagged-HCV. Eighteen hours after transfection of EYFP-tagged HCV RNA, the cells were treated with 10, 30 or 50 U/mL of IFN-α-2b or with 10 μM of protease inhibitor, BILN2061. JFH1-EYFP mutant-transfected cells were analyzed using flow

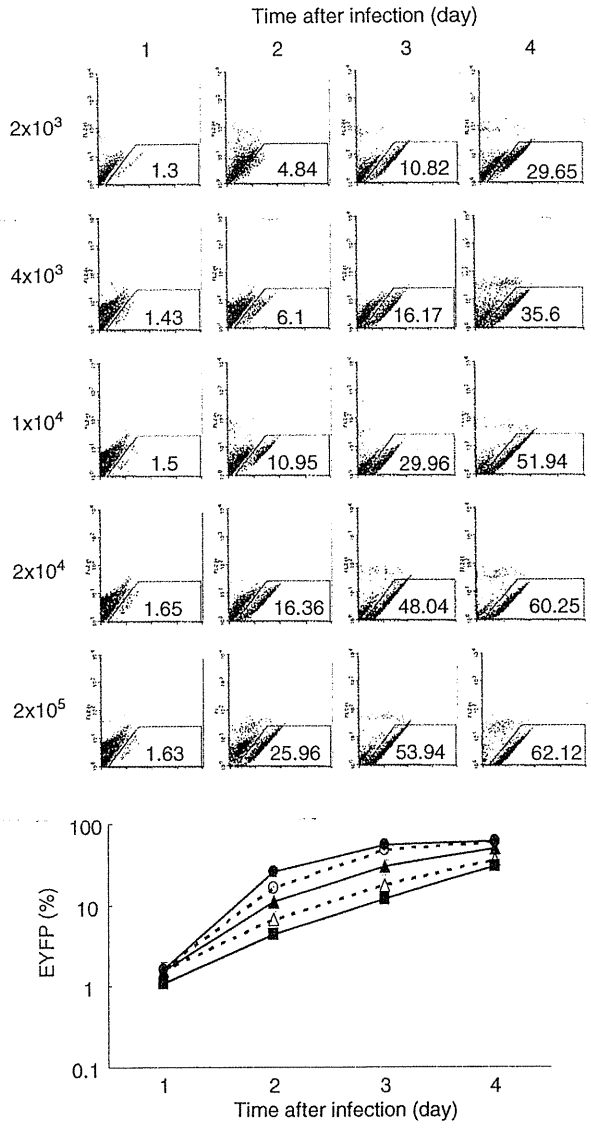


Figure 6 Rate of expansion of hepatitis C virus (HCV)-infected cells. The medium from JFH1-EYFP mutant was inoculated onto Huh7.5.1 cells seeded at different densities (2×10^3, 4×10^3, 1×10^4, 2×10^4 and 2×10^5 cells/cm^2) with core antigen adjusted doses. The results of flow cytometric analysis are depicted as density plots. The ratios of EYFP-positive cells vs time are shown beneath. Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. —■—, 2×10^3; ···☆···, 4×10^3; —▲—, 1×10^4; ···◇···, 2×10^4; —●—, 2×10^5.

cytometry. As shown in Figure 7, treatment of cells with the two compounds suppressed the time-dependent increase of HCV propagation. In addition, IFN-α-2b suppressed the dose-dependent increase of HCV

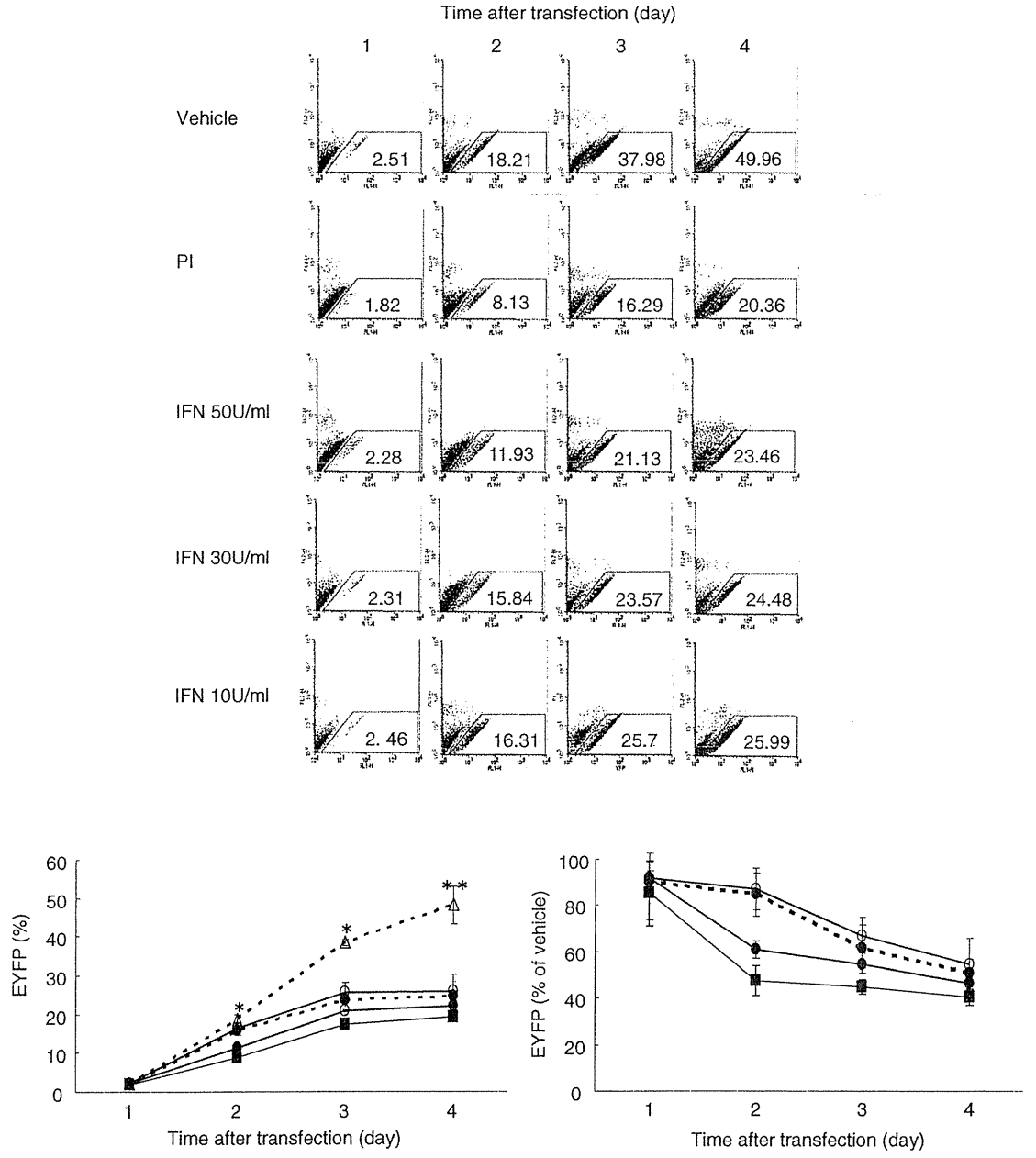


Figure 7 Effect of antiviral drugs on hepatitis C virus (HCV)-infected cells. Huh7.5.1 cells were transfected with JFH1-EYFP mutant RNA. Eighteen hours after transfection, cells were cultured with 10, 30 or 50 U/mL of interferon (IFN)-α-2b or 10 μM of the protease inhibitor BILN-2061. The cells were harvested at the days indicated and flow cytometry was performed. Ratios of EYFP-positive cells over time are shown at lower left. Plot values of 100% in each curve represent the EYFP expression levels in untreated cells (lower right). Assays were carried out in triplicate and the results are expressed as mean ± standard deviation. $^*P < 0.05$. $^{**}P < 0.01$. PI, protease inhibitor. ·-·-·, vehicle; —●—, 50 U/mL; ·-●-·, 30 U/mL; —○—, 10 U/mL; —■—, PI.

propagation. At all time points, the number of infected cells was significantly lower in the culture treated with the two compounds than in the untreated culture. The protease inhibitor suppressed infection faster than did IFN-α-2b. These data indicate that IFN and the protease inhibitor were not only able to suppress intracellular HCV replication levels but also to inhibit virus particle secretion and expansion of HCV-infected cell populations.

DISCUSSION

IN THIS STUDY, we used fluorescence-tagged HCV, in which virus assembly, particle secretion and re-infection functions are fully preserved (Fig. 1).[17] Utilizing the fluorescence-tagged HCV, we analyzed the rate of expansion of HCV infection using live-cell flow cytometric analyses (Figs 5–7). In the early periods of infection, the expansion of the virus-positive cell population increased exponentially and the maximum rate of expansion was calculated as $2^{2.5}$/day. It is not clear why HCV propagation reaches a plateau, but this observation, where HCV replication is limited in confluent cells, has been made previously.[26] Possible explanations include cell death due to over-confluence, depletion of the nucleoside triphosphate pools in resting cells, and/or cell cycle-dependent effects on virus RNA replication and translation.

Co-infection of the two virus clones, EYFP-JFH1 and AsRed mutant JFH1, showed that viruses with impaired particle secretion were able to replicate and expand virus-infected cells (Fig. 3). Although we have found no clear mechanism to explain those effects, we speculate that the secretion-defective virus (JFH1-EYFP) may assemble into infectious virus through *trans*-complementation of virus proteins via co-infection in a single cell or recombination of mutant and wild-type virus genomic RNA. The co-infection experiment showed that the increase of JFH1-AsRed mutant-positive cells was slower than in the single clone infection experiment (Fig. 2a). These findings suggest that viruses with impaired particle secretion (JFH1-EYFP) partially suppressed expansion of viruses with intact particle secretion (JFH1-AsRed mutant) through *trans*-suppression of cellular virus replication or competitive binding to cellular virus entry receptors.

After the development of HCV-JFH1 cell culture,[11] many variations of HCV cell culture systems have been developed. Lindenbach *et al.* developed a genotype 2a intragenotypic chimera, J6/JFH, in which the JFH1 structural region was replaced with that of J6, isolated from a patient with chronic hepatitis.[27] The J6/JFH chimera is able to produce virus particles more efficiently than JFH1 but does not produce virus-induced cytopathic effects (CPE). Several marker protein-tagged viruses have been reported, in which viral infection could be visualized readily in living cells. A subgenomic replicon that expressed an NS5A-GFP fusion protein was reported first.[12] However, the clone lacked the structural regions that are required for virus propagation. Subsequently, full-length HCV reporter viruses were developed in which the EGFP gene was inserted into the NS5A-C-terminus of JFH1[13] or JC-1.[14] Jones *et al.* inserted the *renilla* luciferase gene into P7 of J6/JFH.[15] Unfortunately, the efficiency of virus production by the recombinant reporter viruses was greatly reduced compared to wild-type viruses. Very recently, it has been reported that a JFH1-based adaptive strain of a HCV reporter virus can produce infectious HCV particles as robustly as the JFH1 wild-type strain.[17] This virus system has overcome the serious limitations associated with the use and application of other reporter viruses.

Compared with the other HCV reporter viruses, the JFH1-EYFP/AsRed mutant is capable of producing amounts of HCV virus equivalent to that of the parental JFH1, which enables continuous passage of infection in cell culture and analyses using various research modalities, including flow cytometry and live-cell microcopy. Considering the current situation regarding the lack of singly effective, proven antiviral agents against HCV, other than IFN formulations, the search for potential antiviral agents will continue to be a dominant goal of research to improve clinical anti-HCV chemotherapeutics. This tagged HCV culture system may provide a very convenient tool for studies of the complete virus life cycle in live cells and of virus–host interactions, and it may be useful for high-throughput screening of drugs.

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