infection system provides an opportunity to evaluate inhibitors of all stages of the HCV life cycle (9, 10, 11).

Currently, proof of concept has been obtained in clinical trials of three different HCV NS3 protease inhibitors, BILN 2061 (12, 13), Telaprevir (VX-950) (14), and SCH 503034 (15). However, because of many factors, including possible side effects and the emergence of drug-resistant mutants, there is still great need for improved therapies. We focused, therefore, on screening a set of licensed drugs which have not been recommended previously for antiviral use. Here, we found that the oral antifungal agent, griseofulvin, had a suppressive effect on HCV replication, assessed using the HCV-1b subgenomic replicon system and the particle-producing cell culture HCV-2a model of JFH-1. The mechanism of the anti-HCV activity of griseofulvin also was studied.

MATERIALS AND METHODS

Cell cultures and HCV replicon

The human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin For subgenomic replicon Huh7/Rep-Feo cells (7, 8), the culture medium was supplemented with 250 μg/ml G418. Huh 7.5.1 / JFH-1 cells (Huh 7.5.1 chronically infected HCV JFH-1) were maintained in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (16).

Reagents

Griseofulvin and Fluconazole were purchased from Wako Pure Chemical (Tokyo, Japan). Itraconazole was purchased from LKT Laboratories (St. Paul, MN). Recombinant human IFNα-2b was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell viability assays

For Griseofulvin and Fluconazole, viable cell growth was determined by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) reduction assay using the Cell Titer 96 Aqueous One solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer's protocol. For Itraconazole, viable cell growth was determined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega), according to the manufacturer's protocol.

Luciferase activity assays

Typically, Huh7/Rep-Feo cells were seeded in a 48-well plate at a density of 2×10^4 cells per well. Compounds were added to the culture medium at various concentrations. After 72 hours of culture, expression levels of the HCV replicon were measured by luciferase assay using the Luciferase Assay System (Promega) and the Luminescencer-JNR AB-2100 (Atto, Tokyo, Japan).

The Huh7 cells stably transfected with pEF Fluc IN vector were mock-treated (Control) or treated with 20 μM or 40 μM griseofulvin. After 72 hours of culture, luciferase assays were performed using the Luciferase Assay System (Promega) and the Luminescencer-JNR AB-2100 (Atto). Luciferase activity was normalized by the protein concentration, measured using a BCA Protein Assay Kit (Pierce, Rockford, IL).

The Huh7 cells stably transfected with pEF Rluc-HCV IRES Feo vector were mock-treated (Control) or treated with 20 μM griseofulvin. Dual luciferase activities were carried out at 8, 16, 24, and 32 hours after exposure to griseofulvin using the Dual-Luciferase Reporter Assay System (Promega) and the Luminescencer-JNR AB-2100 (Atto).

All assays were performed in triplicate, and the results were expressed as means ±SD relative light units.

RNA analysis

Total cellular RNA was extracted from Huh7/Rep-Feo cells using anRNAqueous-4PCR kit (Ambion, Austin, TX). RNA was reverse-transcribed with a Thermoscript^{TM} reverse transcriptase kit (Invitrogen, Carlsbad, CA).

Quantitative real-time Polymerase Chain Reaction (PCR) was carried out using an Applied Biosystems ABI Prism 7500 (Applied Biosystems, Foster City, CA). The forward and reverse primers for the 5'-untranslated region of HCV RNA were 5'-TGCGGAACCGGTGAGTACA-3' and 5'-CTTAAGGTTTAGGATTCGTGCTCAT-3', respectively. The fluorogenic probe used for quantification of HCV RNA was

5'-(FAM)-CACCCTATCAGGCAGTA-CCACAAGGCC-(TAMRA)-3'. Human 18S ribosomal RNA levels in the samples were analyzed by quantitative real-time PCR to normalize the RNA content. The forward and reverse primers for human 18S ribosomal RNA were 5'-ACTCTAGATAACCTCGGGCCGA-3' and 5'-GATGTGGTAGCCGTTTCTCAGG-3', respectively. The fluorogenic probe used for quantification of human 18S ribosomal RNA was 5'-(FAM)-CCATTCGAACGTCTGCCCTATCAACTTT - (TAMRA)-3'. The method has been described elsewhere (17).

The primers used for reverse transcription-PCR (RT-PCR) were as follows: human 2',5'-oligoadenylate synthetase (2',5'-OAS): sense, 5'-CAATCAGCGAGGCCAGTAATC-3' antisense, 5'-TGGTGAGAAGTGCTGGGGTC-3'; MxA: sense, 5'-GTCAGGAGT-TGCCCTTCCCA-3', antisense, 5'-GGCCCCTTCCTTACCCTTA-3' and GAPDH: 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-CTTTAGGGTAGTGGTAGAAG-3', respectively. Each reaction mixture contained cDNA (3 μl), 1.5 mM $MgCl_2$, 200 μM dNTP, 1 μM each primer, 1.25 U AmpliTaq Gold (Applied Biosystems) with 1x supplied reaction buffer. After activation of AmpliTaq Gold activity at 95°C for 10 min, the temperature cycling conditions for MxA were 29

cycles consisting of denaturation at 95°C 30 sec, annealing at 56°C 1 min and extension at 72°C 1 min. For 2', 5'-OAS, the conditions were 32 cycles consisting of denaturation at 95°C 30 sec, annealing at 53°C 1 min and extension at 72°C 1 min. For GAPDH, the conditions were 30 cycles consisting of denaturation at 95°C 30 sec, annealing at 53°C 1 min and extension at 72°C 1 min. PCR products were subjected to electrophoresis in a 3% agarose gel.

Western blotting

Preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously (18). The antibodies used in this study were anti-NS3 antibody (Santa Cruz Biotechnology, Santa Cruz , CA) anti-NS5A antibody (Virogen, Watertown, MA) and anti-β-actin antibody (Cell signaling, Danvers, MA). Alkaline phosphatase-conjugated secondary antibodies and CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA) were used for detection.

Cell cycle analysis

Harvested cells were washed once with phosphate-buffered saline (PBS) and fixed

with 70% ethanol at 4°C for 1 hour. After an additional wash, the cells were treated with 250 μg/ml RNase A at 37°C for 1 hour and subsequently stained with 50 μg/ml propidium iodide at 4°C for 1 hour. The DNA content was then analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ) with ModFit LT software (Verity Software House, Topsham, ME).

Analyses of drug synergy.

The effects of treatment of Huh7/Rep-Feo cells with griseofulvin and IFN-α, alone and in combination, were analyzed with CalcuSyn, a computer program based on the method of Chou and Talalaly (19). After converting the dose-effect curves for each drug or drug combination to median-effect plots, the program calculated a combination index (CI). CIs of <1, 1, and >1 indicate synergy, an additive effect, and antagonism, respectively.

Plasmids and Stable transfection.

The plasmid pEF-Fluc-IN was constructed as follows: The fragment carrying the firefly luciferase was amplified from the pGL3 control vector (Promega) by PCR using a pair of primers (5'-GAATTCATGGAAGACGCCAAAAACATAAA-3' (Eco RI site)

and 5'-GCGGCCGCTTACACGGCGATCTTTCCGCCC-3' (Not I site)). The PCR product was cloned into the pGEM-T Easy vector (Promega). The EMCV IRES Neo fragment was excised from pMXs-IN vector by Not I and Sal I digestion (20). The Eco RI-Sal I fragment of the pCHO vector was excised from pGag-pol-IRES-bs^r vector by Eco RI and Sal I digestion (21). To construct pEF-Fluc-IB, the Eco RI-Not I fragment of firefly luciferase, and the Not I-Sal I fragment of the EMCV IRES Neo were inserted into the Eco RI and the Sal I site of pCHO by triple ligation.

The plasmid pEF Rluc-HCV IRES Feo was constructed as follows: The fragment carrying the Renilla luciferase was amplified from the phRL-TK vector (Promega) by PCR using a pair of primers (5'-GAATTCATGGCTTCCAAGGTGTACGACCC-3' (Eco RI site) and 5'-GGATCCTTACTGCTCGTTCTTCAGCACGC-3' (Bam HI site)). The fragment carrying the HCV IRES Feo was amplified from the pRep-Feo vector (7) by PCR using a pair of primers (5'-GGATCCGCCAGCCCCCGATTGGGGGCGAC-3' (Bam HI site) and 5'-GTCGACTCAGAAGAACTCGTCAAGAAGGC-3' (Sal I site)). Each PCR product was cloned into the pGEM-T Easy vector (Promega). To construct pEF Rluc-HCV IRES Feo, the Eco RI-Bam HI fragment of Renilla luciferase, and the Bam HI-Sal I fragment of HCV IRES Feo were inserted into the Eco RI and Sal I site of pCHO by triple ligation.

The pEF-Fluc-IB and pEF Rluc-HCV IRES Feo was transfected into Huh7 cells using the Effectene Transfection Reagent (QIAGEN, Hilden, Germany) according to the manufacturer's recommendation. Two days after transfection, Huh7 cells were selected in medium containing 250 μg/ml G418.

Immunofluorescent staining.

After treatment with griseofulvin for 72 hours, HCV-JFH-1 infected cells were fixed with cold methanol and blocked using Blocking One (Nacalai Tesque, Kyoto, Japan). For detection of NS3 protein, the cells were incubated with anti-NS3 antibody (Virogen) for 1 hour at room temperature. After washing with PBS, the cells were incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. After washing with PBS, the cells were stained with 7-Aminoactinomycin D (7-AAD) for nuclear counterstaining and analyzed using fluorescence microscopy.

RESULTS

Replication of a subgenomic HCV-1b replicon is suppressed by griseofulvin.

We investigated the anti-HCV effect and cell toxicity of griseofulvin in the HCV subgenomic replicon cells, Huh7/Rep-Feo. The luciferase activities of the Huh7/Rep-Feo cells showed that replication of the HCV replicon was suppressed by griseofulvin in a dose-dependent manner (Fig. 1A). Next, we performed a time-course experiment in which the luciferase activities of Huh7/Rep-Feo cells were measured at various time-points after treatment with griseofulvin. As shown in Figure 1B, griseofulvin induced a decrease in the luciferase activities of Huh7/Rep-Feo cells over time. On the other hand, treatment with griseofulvin had little effect on cellular viability at this range of concentrations, as revealed by MTS assay (Fig. 1C). The 50% effective concentration (EC_{50}) of griseofulvin was 6.13 ± 0.17 μM. The 50% cytotoxic concentration of this compound (CC_{50}) was 217.93 ± 3.49 μM. Thus, the selectivity index (ratio of CC_{50} to EC_{50}) was 35.5 (Table 1). Furthermore, we examined the effect of other antifungal agents, fluconazole and itraconazole, on HCV RNA replication. In contrast, fluconazole and itraconazole had little effect on HCV RNA replication (Table 1).

We analyzed HCV RNA levels in Huh7/Rep-Feo cells treated with griseofulvin, or not treated, using real time RT-PCR. As shown in Figure 2A, treatment with griseofulvin decreased the replicon RNA titer in a dose dependent manner. Similar results were seen at the protein level by monitoring the HCV nonstructural proteins NS3 and NS5A. Western blot analysis demonstrated that griseofulvin treatment results in reduced levels of these viral proteins (Fig. 2B).

However, it remained to be clarified whether the griseofulvin inhibits firefly luciferase directly. To investigate this possibility, we examined the effect of griseofulvin on firefly luciferase activity using Huh7 cells expressing firefly luciferase constitutively. Treatment of these cells with griseofulvin resulted in no significant change in the firefly luciferase activity (Fig.3). This result excludes the possibility that griseofulvin inhibits firefly luciferase activity directly.

The anti-HCV activity of griseofulvin is not mediated by the IFN signaling pathway.

It has been reported that the HCV replicon is highly sensitive to IFN (22, 23). To determine whether the action of griseofulvin on the HCV subgenomic replicon involves activation of IFN stimulated gene responses, we analyzed the expression of IFN inducible genes in HCV replicon cells. RT-PCR analysis showed that the messenger

RNAs for MxA and 2', 5'-oligoadenylate synthetase (2', 5'-OAS), which are both IFN inducible genes, were induced by IFN α-2b but not by griseofulvin (Fig. 4). These results suggest that the action of griseofulvin on the intracellular replication of HCV replicon is independent of the IFN signaling pathway.

Synergistic inhibitory effect of griseofulvin and IFN-α on HCV replicon

Whether a combination of griseofulvin and IFN-α exhibits a synergistic, additive, or antagonistic effect was assessed using an isobologram method (19). Isobologram analysis is an approach used in pre-clinical studies to quantify the extent of synergistic, additive, or antagonistic effects between drugs used in combination. For instance, a representation of an isobologram to evaluate a drug-drug interaction is shown in Figure 5A. It is understood that synergy, additivity, and antagonism are represented by concave, liner, and convex isoeffective curves (isoboles), respectively. The combined anti-HCV effects of griseofulvin and IFN-α were evaluated. Prior to the combination experiments, the optimal concentration ratio of two compounds (combination ratio) had to be determined. After preliminary experiments, three different rations were chosen for each combination (data not shown). The ratios of griseofulvin and IFN-α were 9:1, 1:1, and 1:9. Each concentration of griseofulvin and IFN-α at 50% inhibition were plotted on the

X- and Y-axes, respectively, to generate an isobologram (Fig. 5B). As shown in Figure 5B, each plot fell far below the line showing additivity, indicating that the effect of the griseofulvin and IFN-α combination on HCV RNA replication is strongly synergistic.

Griseofulvin induces G2/M cell cycle arrest in HCV replicon cells.

As described previously, griseofulvin blocks cell cycle progression at G2/M in several human cell lines (24). Here, we examined the effect of griseofulvin on cell cycle progression in Huh7/Rep-Feo cells. As shown in Figure 6, the population of griseofulvin treated Huh7/Rep-Feo cells in G2/M phase at 12 hours was 40.0%, compared to 15.3% for the control cell populations. These data imply that griseofulvin might have the potential to arrest Huh7/Rep-Feo cells in G2/M phase.

As described above, treatment of Huh7/Rep-Feo cells with 20 μM griseofulvin for 12 hours results in G2/M arrest (Fig. 6), while treatment for 72 hours had no effect on cell growth (Fig. 1C). To explain this discrepancy, we examined the growth kinetics of griseofulvin treated Huh7/Rep-Feo cells. The cells were cultured with 20 μM griseofulvin and cell growth was monitored by MTS assay. The cell viability declined gradually until 48 hours after treatment with 20 μM griseofulvin, but increased from 48 hours to 72 hours (Fig. 7). These data indicated that treatment with 20 μM griseofulvin

arrests Huh7/Rep-Feo cells in G2/M phase, but dose not inhibit cell growth completely.

Griseofulvin does not inhibit HCV IRES-dependent translation.

Previous studies have shown that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization and the cell cycle in G2/M, inhibit HCV replication but not HCV IRES-dependent translation (25). Therefore, we determined whether G2/M cell cycle arrest by griseofulvin affects HCV IRES-dependent translation using Huh7 cells transfected with pEF Rluc-HCV IRES Feo (Fig. 8A). Treatment of these cells with 20 μM griseofulvin resulted in no significant change of the internal luciferase activities, a concentration that suppressed expression of the HCV replicon and arrested the HCV replicon cells in G2/M phase (Fig. 8B). These results suggested that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation, as shown previously for vinblastine sulfate and nocodazole.

Griseofulvin suppressed JFH-1 HCV replication.

The studies described thus far were carried out using the subgenomic HCV-1b replicon system. Recently, Wakita et al. established a cell culture model for HCV. This

system, known as the JFH-1 system and based on genotype 2a HCV, allows the production of virus that can be efficiently propagated in cell culture (HCVcc) (10). Therefore, we examined the effect of griseofulvin using the JFH-1 system. The Huh 7.5.1/JFH-1 cells (cells persistently infected with HCV JFH-1) were cultured with 10 μM or 20 μM griseofulvin for 72 hours. We detected the HCV NS3 protein in Huh-7.5.1 / JFH-1 HCV cells by immunostaining. As shown in Figure 9, in the absence of griseofulvin treatment, the NS3 protein was localized predominantly in the perinuclear region. After treatment of griseofulvin, the NS3 protein expression level was reduced substantially (Fig 9). This result indicates that griseofulvin also suppressed HCV replication in the JFH-1 HCVcc system.

DISCUSSION

We have shown here that griseofulvin inhibits the replication of HCV in the HCV subgenomic replicon cells, Huh7/Rep-Feo. In this reporter-based subgenomic replicon system, the EC_{50} of griseofulvin for inhibition of HCV replication, determined by measurement of the luciferase activity, was approximately 6.13 μM. The real-time RT-PCR and western blot analyses revealed that both RNA synthesis and its translation were inhibited by griseofulvin in a dose-dependent manner. Treatment of Huh7/Rep-Feo cells with griseofulvin did not activate the IFN inducible gene responses, suggesting that the inhibitory mechanism of griseofulvin in HCV replication is independent of the IFN signaling pathway. Moreover, we demonstrated that combination treatment of griseofulvin and IFN-α had a synergistic inhibitory effect in Huh7/Rep-Feo cells. We also demonstrated that griseofulvin suppressed replication of JFH-1 HCV.

A previous study demonstrated that griseofulvin induces G2/M arrest in several human cell lines (24). Here, we show that griseofulvin arrested the Huh7/Rep-Feo cells in G2/M. Recently, several studies have shown a correlation between HCV IRES-mediated translation and the cell cycle. Honda et al. reported that the HCV IRES activity was highest in G2/M (26). In contrast, Venkatesan et al. reported that the HCV

IRES activity was lowest in G2/M (27), whilst other studies reported that the HCV IRES activity was independent of the stage of the cell cycle (28, 29).In addition, Bost et al. reported that several cell cycle inhibitors (vinblastine sulfate, colchicine, nocodazole, and cytochalasin D) did not affect HCV IRES-dependent translation (25). We also have shown that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation. Accordingly, our results support the hypothesis that the HCV IRES activity is independent of the cell cycle.

Previous studies have demonstrated that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization, are able to inhibit HCV RNA replication in HCV subgenomic replicon cells (25). These findings indicated that microtubule polymerization is required for formation of the HCV replication complex. Griseofulvin has been shown to arrest human cells in G2/M phase by acting on microtubule polymerization (30). Thus, it is speculated that the inhibition of microtubule polymerization by griseofulvin may influence on the formation of the HCV RNA replication complex. Further, defining the mechanism of action of griseofulvin against HCV replication may be important for defining a novel target for anti-HCV therapy.

Griseofulvin has been used for many years for the treatment of ringworm and other

dermatophyte infections. Moreover, griseofulvin does not have significant toxicity for humans. Consequently, the development of derivatives of this compound may be a useful strategy for future therapeutic intervention in chronic hepatitis C.

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