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Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae* radix. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneo-delS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

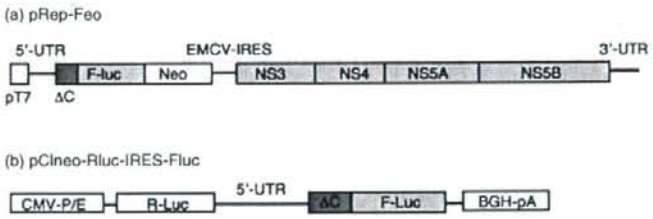

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3′ UTR, 3′ untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency. The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[3] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

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

RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

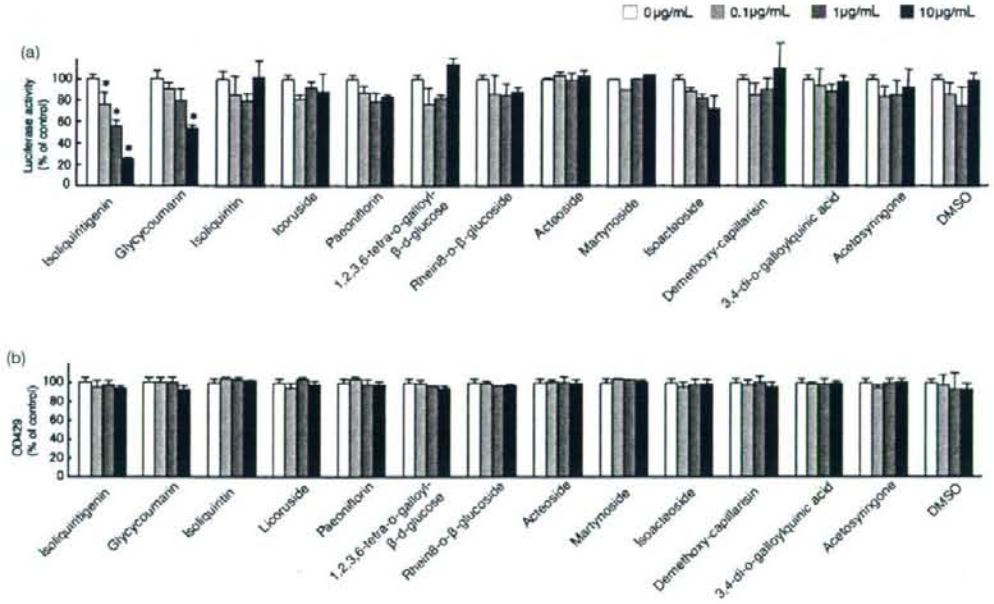

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

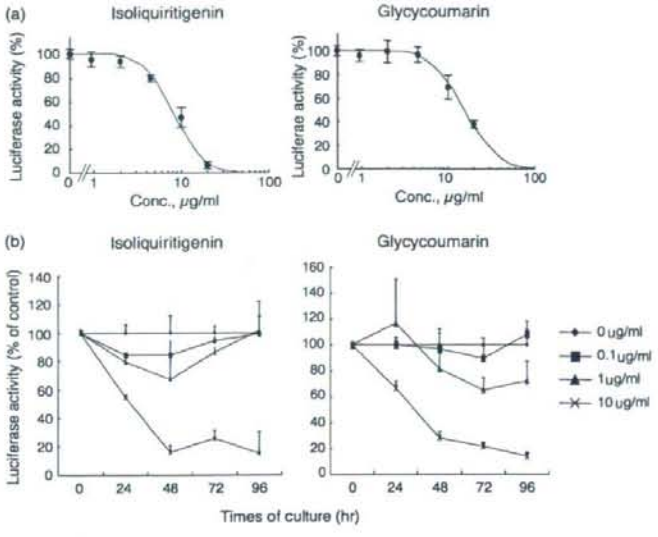

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

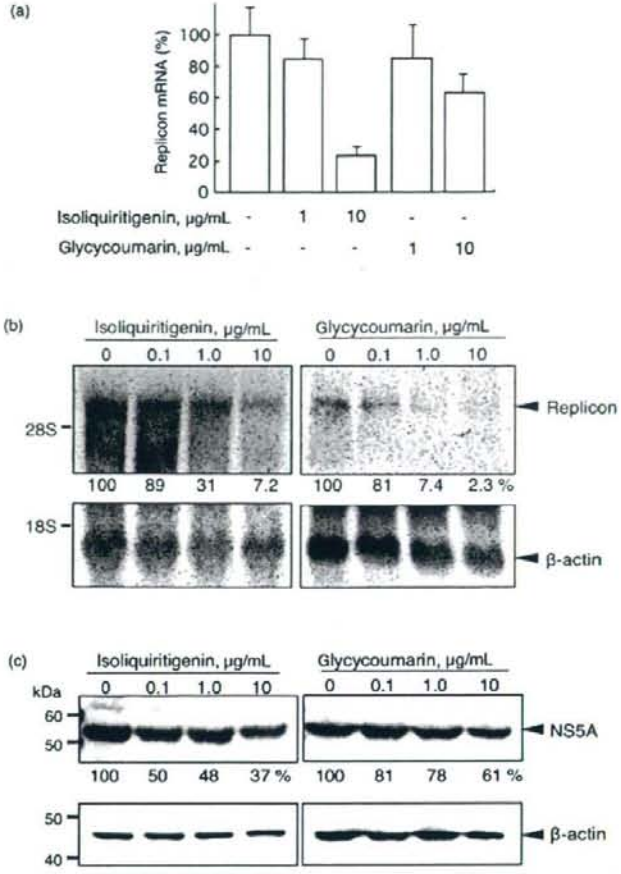

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycycoumarin than the untreated culture. The effect of glycycoumarin was partly reversed on day six probably due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycycoumarin (Fig. 7b).

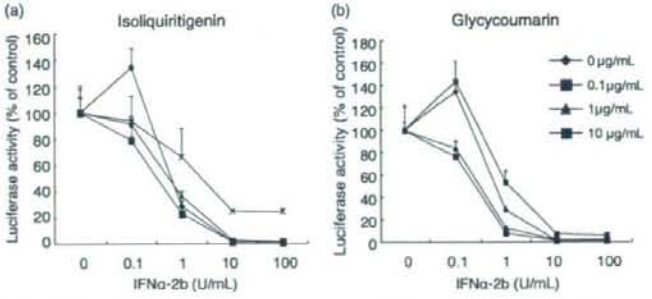

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

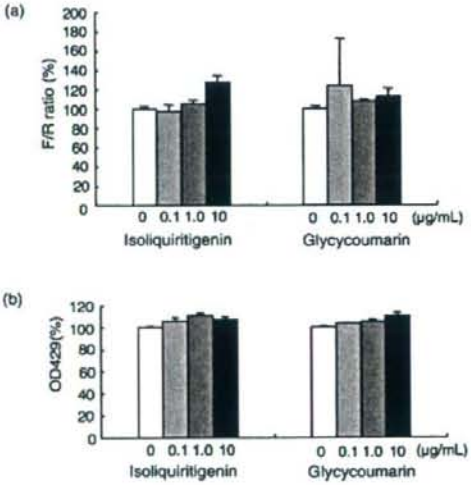

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.

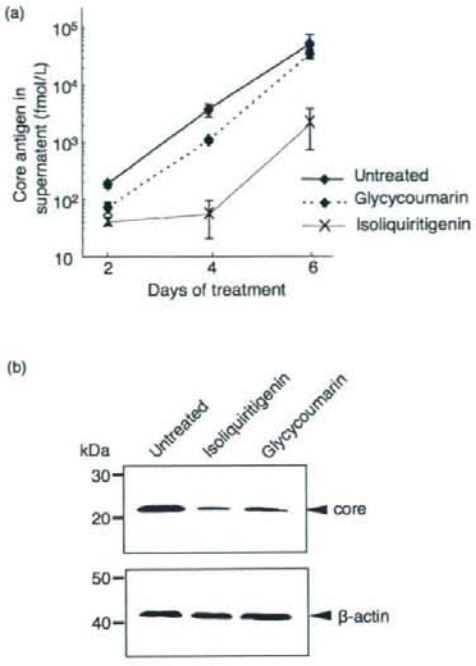

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes, Haemophilus infuenzae* and *Moraxella catarrhalis*,[36] and methicillin-resistant *Staphylococcus aureus*,[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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Original Article

Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication *in vitro*

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Aim: Hepatitis C virus (HCV), which infects an estimated 170 million people worldwide, is a major cause of chronic liver disease. The current standard therapy for chronic hepatitis C is based on pegylated interferon (IFN)α in combination with ribavirin. However, the success rate remains at approximately 50%. Therefore, alternative agents are needed for the treatment of HCV infection.

Methods: Using an HCV-1b subgenomic replicon cell culture system (Huh7/Rep-Feo), we found that griseofulvin, an oral antifungal agent, suppressed HCV-RNA replication and protein expression in a dose-dependent manner. We also found that griseofulvin suppressed the replication of infectious HCV JFH-1. A combination of IFNα and griseofulvin exhibited a synergistic inhibitory effect in Huh7/Rep-Feo cells.

Results: We found that griseofulvin blocked the cell cycle at the G_2/M phase in the HCV subgenomic replicon cells, but did not inhibit HCV internal ribosome entry site-dependent translation.

Conclusion: Our results suggest that griseofulvin may represent a new approach to the development of a novel therapy for HCV infection.

Key words: cell cycle, griseofulvin, hepatitis C virus internal ribosome entry site, hepatitis C virus replicon, JFH-1

INTRODUCTION

HEPATITIS C VIRUS (HCV) is an etiologic agent of chronic liver disease,[1,2] and it is estimated that approximately 170 million people worldwide are infected with the virus. Chronic hepatitis C can lead to severe liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma.[3] With advancements in HCV therapy, including the most recent combination of pegylated interferon (IFN)α and ribavirin, up to one-half of patients achieve a sustained virological response. However, the remainder cannot clear the virus, demonstrating a great need for more powerful therapeutic modalities.[4]

Investigations have been hampered by the lack of an efficient HCV cell culture system. In 1999, the establishment of an HCV subgenomic replicon cell culture system improved the situation. The subgenomic replicon RNA is composed of the HCV 5′ untranslated region (UTR) containing the internal ribosomal entry site (IRES), a neomycin phosphotransferase (neo) gene and the HCV non-structural (NS) proteins through 3–5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′ UTR.[5] A HCV replicon carrying, in addition to the selectable marker, a gene encoding luciferase, can be used to screen a large number of compounds for antiviral activity.[6–8] The recent development of an *in vitro* HCV infection system provides an opportunity to evaluate inhibitors of all stages of the HCV life cycle.[9–11]

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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.

METHODS

Cell cultures and HCV replicon

THE HUMAN HEPATOMA cell line, Huh7, was maintained in Dulbecco's modified Eagle's 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 (HCV 1b replicon that expresses a chimeric protein consisting of neomycin phosphotransferase and firefly luciferase) cells,[7,8] the culture medium was supplemented with 250 g/mL G418. Huh 7.5.1/JFH-1 cells (Huh 7.5.1 chronically infected HCV JFH-1) were maintained in 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, USA). Recombinant human IFNα-2b was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

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, USA), according to the manufacturer's protocol.

For itraconazole, viable cell growth was determined using the CellTiter-Glo luminescent cell viability assay (Promega, USA), 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 h of culture, the expression levels of the HCV replicon were measured by luciferase assay using the luciferase assay system (Promega, USA) and the Luminescencer-JNR AB-2100 (Atto, Tokyo, Japan).

The Huh7 cells stably transfected with the pEF Fluc IN vector were mock treated (control) or treated with 20 μM or 40 μM griseofulvin. After 72 h of culture, luciferase assays were performed using the luciferase assay system and the Luminescencer-JNR AB-2100. Luciferase activity was normalized by the protein concentration, measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

The Huh7 cells stably transfected with the 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 h after exposure to griseofulvin using the dual luciferase reporter assay system and the Luminescencer-JNR AB-2100.

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

RNA analysis

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

Quantitative real-time polymerase chain reaction (PCR) was carried out using ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The forward and reverse primers for the 5′ UTR of HCV-RNA were 5′-TGCGGAACCGGTGAGTACA-3′ and 5′-CTTAAGG TTTAGGATTCGTGCTCAT-3′, respectively. The fluorogenic probe used for the quantification of HCV-RNA was 5′-(FAM)-CACCCTATCAGGCAGTA-CCACAAGG CC-(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′-GATGT GGTAGCCGTTTCTCAGG-3′, respectively. The fluorogenic probe used for quantification of human 18S ribo-

somal RNA was 5′-(FAM)-CCATTCGAACGTCTGCCC TATCAACTTT-(TAMRA)-3′. The method has been described elsewhere.[17]

The primers used for reverse transcription (RT)–PCR were as follows: human 2′,5′-oligoadenylate synthetase (2′,5′-OAS): forward primer, 5′-CAATCAGCGAGGCC AGTAATC-3′ and reverse primer, 5′-TGGTGAGAAGTGC TGGGGTC-3′; human myxovirus resistance protein A (MxA): forward primer, 5′-GTCAGGAGT-TGCCCTT CCCA-3′ and reverse primer, 5′-GGCCCCTTCCTT ACCCTTA-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′-GAAG GTGAAGGTCGGAGTC-3′ and reverse primer, 5′-CTT TAGGGTAGTGGTAGAAG-3′, respectively. Each reaction mixture contained cDNA (3 μL), 1.5 mM $MgCl_2$, 200 μM dNTP, 1 μM each primer, and 1.25 U AmpliTaq Gold (Applied Biosystems, USA) with 1× 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 for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min. For 2′,5′-OAS, the conditions were 32 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 1 min. For GAPDH, the conditions were 30 cycles consisting of denaturation at 95°C for 30 s, annealing at 53°C for 1 min, and extension at 72°C for 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 the anti-NS3 antibody (Santa Cruz Biotechnology, USA) anti-NS5A antibody (Virogen, Watertown, MA, USA) and anti-β-actin antibody (Cell Signaling, Danvers, MA, USA). Alkaline phosphatase-conjugated secondary antibodies and CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA, USA) 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 h. After an additional wash, the cells were treated with 250 μg/mL RNase A at 37°C for 1 h and subsequently stained with 50 μg/mL propidium iodide at 4°C for 1 h. The DNA content was then analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) with ModFit LT software (Verity Software House, Topsham, ME, USA).

Analyses of drug synergy

The effects of the treatment of Huh7/Rep-Feo cells with griseofulvin and IFNα, 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). The CI 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, USA) by PCR using a pair of primers (5′-GAATTCATGGAAGAC GCCAAAAACATAAA-3′ [*Eco*RI site] and 5′-GCGGC CGCTTACACGGCGATCTTTCCGCCC-3′ [*Not*I site]). The PCR product was cloned into the pGEM-T Easy vector (Promega, USA). The EMCV IRES Neo fragment was excised from the pMXs-IN vector by *Not*I and *Sal*I digestion.[20] The *Eco*RI–*Sal*I fragment of the pCHO vector was excised from the pGag-pol-IRES-bs' 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, USA) by PCR using a pair of primers (5′-GAATTCATGGCT TCCAAGGTGTACGACCC-3′ [*Eco*RI site] and 5′-GGAT CCTTACTGCTCGTTCTTCAGCACGC-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′-GGATCCGCCAGCCCCCGATTGGGGGCG AC-3′ [*Bam*HI site] and 5′-GTCGACTCAGAAGAAC TCGTCAAGAAGGC-3′ [*Sal*I site]). Each PCR product was cloned into the pGEM-T Easy vector. 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 Effectene transfection reagent (QIAGEN, Hilden, Germany), according to the manufacturer's recommendation. Two days after trans-

fection, the Huh7 cells were selected in a medium containing 250 μg/mL G418.

Immunofluorescent staining

After treatment with griseofulvin for 72 h, HCV IFH-1-infected cells were fixed with cold methanol and blocked using Blocking One (Nacalai Tesque, Kyoto, Japan). For the detection of the NS3 protein, the cells were incubated with the anti-NS3 antibody (Virogen, USA) for 1 h at room temperature. After washing with PBS, the cells were incubated with an Alexa Fluor 488 goat antimouse immunoglobulin G antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing with PBS, the cells were stained with 7-aminoactinomycin D 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. The treatment with griseofulvin had little effect on cellular viability at this range of concentration, as revealed by the 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 or not treated with griseofulvin 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 non-structural proteins NS3 and NS5A. The Western blot analysis demon-

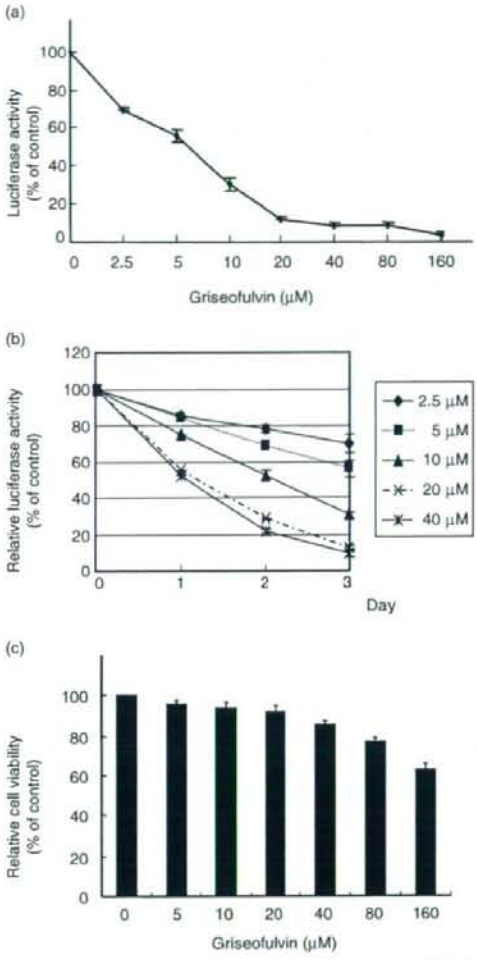

Figure 1 Inhibition of hepatitis C virus replication in Huh7/Rep-Feo cells by griseofulvin. (a) Huh7/Rep-Feo cells were cultured with various concentrations of griseofulvin in the medium and luciferase assays were performed after 72 h of culture. Luciferase assays were performed in triplicate. Error bars indicate mean ± standard deviation. (b) Huh7/Rep-Feo cells were treated with various concentrations of griseofulvin (2.5–40.0 μM). Luciferase activity was measured at the time points indicated after exposure to griseofulvin. (C) 5-(3-Carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt of Huh7/Rep-Feo cells cultured with the concentration of griseofulvin indicated.

Table 1 Antihepatitis C virus activities of oral antifungal agents in Huh7/Rep-Feo cells†

	EC_{50} (μM)	CC_{50} (μM)	SI
Griseofulvin	6.13 ± 0.17	217.93 ± 3.49	35.5
Fluconazole	135.6 ± 1.25	159.06 ± 1.07	1.2
Itraconazole	1.24 ± 0.21	3.35 ± 0.17	2.7

†All data represent means ± standard deviation for three separate experiments. CC_{50}, 50% cytotoxicity concentration based on the reduction of cell viability; EC_{50}, 50% effective concentration based on the inhibition of HCV replication; SI, selectivity index (CC_{50}/EC_{50}).

strated that griseofulvin treatment results in reduced levels of these viral proteins (Fig. 2b).

However, it remains 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. The 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.

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 the activation of IFN-stimulated gene responses, we analyzed the expression of IFN inducible genes in HCV replicon cells. The RT–PCR analysis showed that the messenger RNA for MxA and 2′,5′-OAS, which are both IFN inducible genes, were induced by IFNα-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 IFNα on HCV replicon

Whether a combination of griseofulvin and IFNα exhibits a synergistic, additive, or antagonistic effect was assessed using an isobologram method.[19] An isobologram analysis is an approach used in preclinical 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 IFNα 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

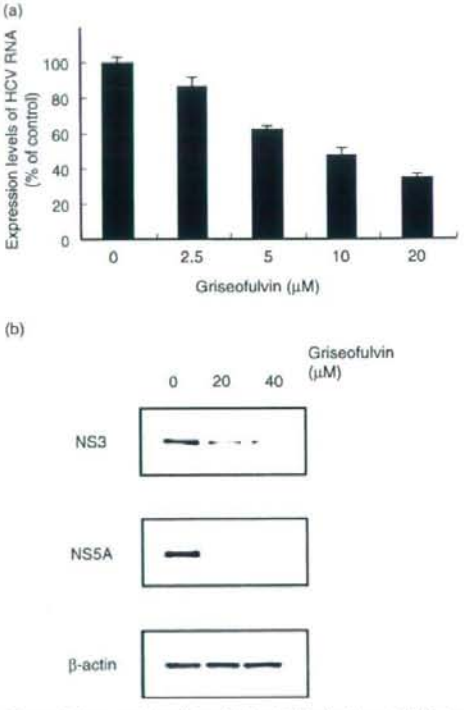

Figure 2 Suppressive effect of griseofulvin for hepatitis C virus (HCV) replicon was confirmed by real-time reverse transcription–polymerase chain reaction (RT–PCR) and Western blot analysis. (a) Incubation of Huh7/Rep-Feo cells with griseofulvin for 72 h resulted in dose-dependent antiviral effects. Real-time RT–PCR was performed on the extracted RNA. HCV-RNA levels are shown as relative percentages of untreated control. Error bars indicate mean ± SD. (b) Western blot analyses of NS3 and NS5A protein expressions were performed on protein extracts from cells that were treated for 72 h with varying dose of griseofulvin. β-Actin was used as a loading control.

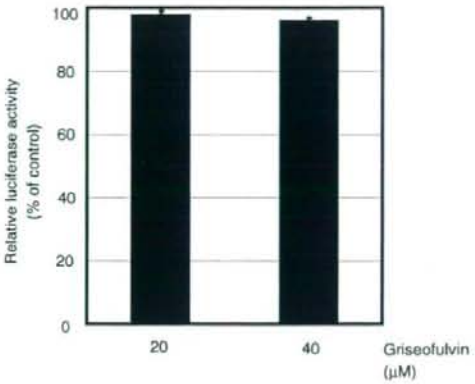

Figure 3 No inhibition of firefly luciferase activity by griseofulvin. pEF Fluc IN vector was stably transfected into Huh7 cells. Cells were cultured without (control) and with 20 μM or 40 μM griseofulvin for 72 h. Firefly luciferase assay was performed. Luciferase activity was normalized by the protein concentration. Error bars indicate mean ± SD.

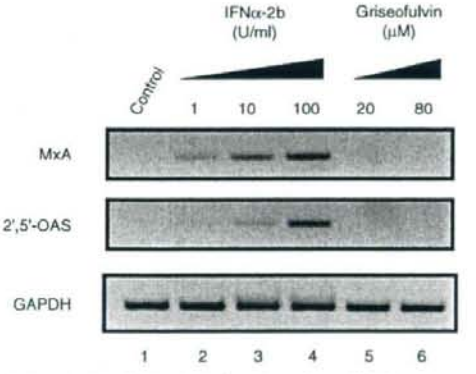

Figure 4 Griseofulvin elicited an interferon (IFN) response. Huh7/Rep-Feo cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 20 (lane 5) or 80 μM griseofulvin (lane 6) for 72 h. Messenger RNA of human myxovirus resistance protein A (MxA), 2′,5′-oligoadenylate synthetase (2′,5′-OAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse transcription–polymerase chain reaction analysis.

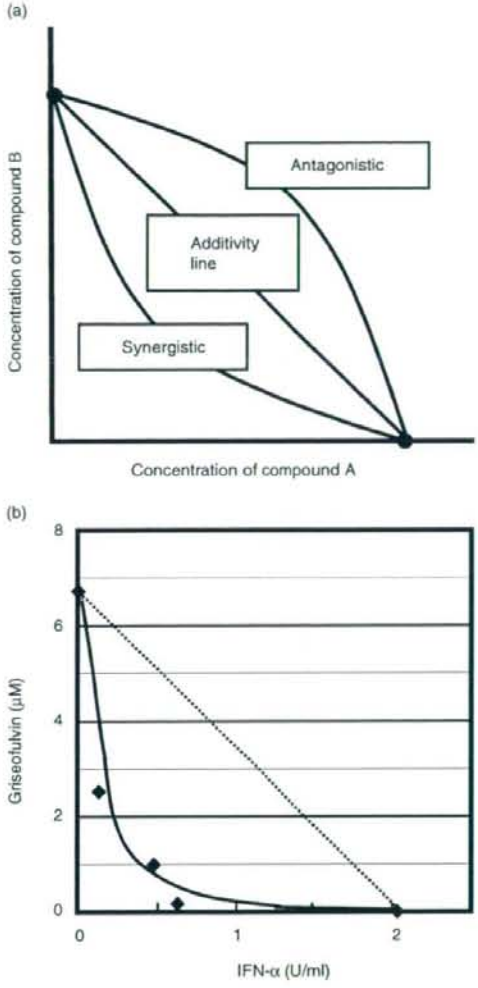

Figure 5 Effect of a combination of griseofulvin and interferon (IFN)α on intracellular hepatitis C virus (HCV)-RNA replication. (a) Representative isobologram for analyzing the interaction between two drugs. (b) Isobole plot of 50% inhibition of HCV-RNA replication. Huh7/Rep-Feo cells were treated with griseofulvin in combination with IFNα, and a luciferase assay was performed after 72 h of culture to obtain each isobole plot. Dotted line indicates an additive effect in the isobologram method used.

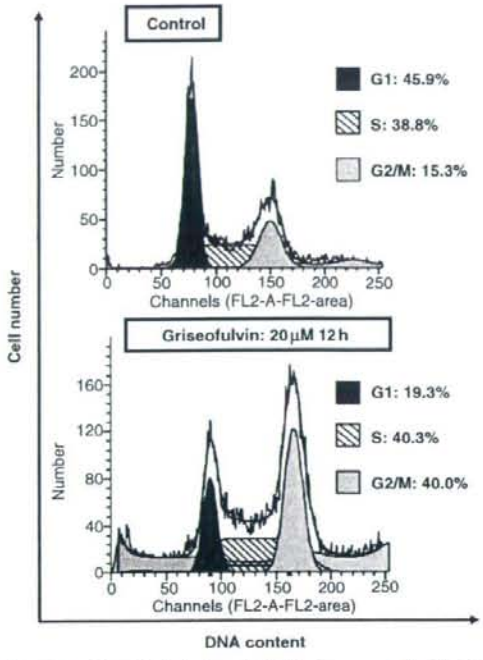

Figure 6 Griseofulvin induced G_2/M phase arrest in Huh7/Rep-Feo cells. Flow cytometry analysis of DNA content of untreated Huh7/Rep-Feo cells (control) and cells treated for 12 h with 20 μM of griseofulvin. [■ G_1: 45.9%, ▨ S: 38.8%, □ G_2/M: 15.3%; ■ G_1: 19.3%, ▨ S: 40.3%, □ G_2/M: 40.0%.]

(data not shown). The ratios of griseofulvin and IFNα were 9:1, 1:1, and 1:9. Each concentration of griseofulvin and IFNα at 50% inhibition was 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 IFNα combination on HCV-RNA replication is strongly synergistic.

Griseofulvin induces G_2/M cell cycle arrest in HCV replicon cells

As described previously, griseofulvin blocks cell cycle progression at the G_2/M phase 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 the G_2/M phase at 12 h was 40%, 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 the G_2/M phase.

As described earlier, the treatment of Huh7/Rep-Feo cells with 20 μM griseofulvin for 12 h results in G_2/M arrest (Fig. 6), while treatment for 72 h 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 h after treatment with 20 μM griseofulvin, but increased from 48 h to 72 h (Fig. 7). These data indicate that treatment with 20 μM griseofulvin arrests Huh7/Rep-Feo cells in the G_2/M phase, but does 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 G_2/M, inhibit

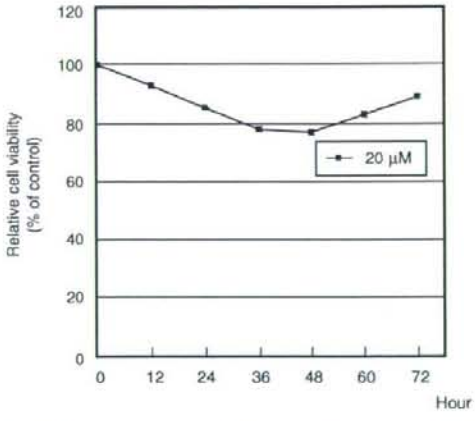

Figure 7 Growth kinetics of griseofulvin treatment of Huh7/Rep-Feo cells. Cells were cultured with [–•–] 20 μM griseofulvin, and cell viability was monitored by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt assay at the times indicated. Error bars indicate mean ± SD.

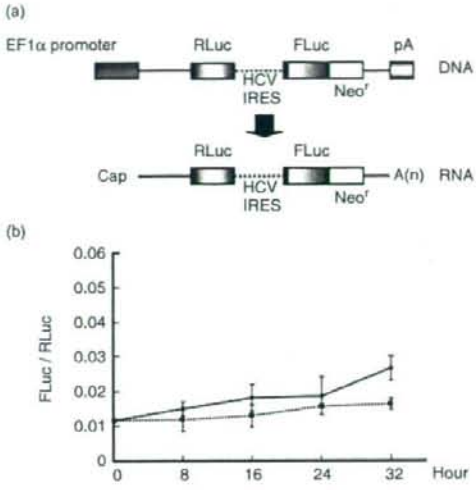

Figure 8 Griseofulvin does not influence hepatitis C virus (HCV) internal ribosomal entry site (IRES)-mediated translation. (a) Structure of the plasmid, pEF-Rluc-HCV IRES Feo. Transcription is initiated under the control of a composite elongation factor 1α (EF1α) promoter. Upstream cistron encodes Renilla luciferase (RLuc) and is translated by a cap-dependent mechanism in transfected cells, while the downstream cistron encodes a fusion (Feo) of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo') genes, translated under the control of the HCV IRES. (b) pEF-Rluc-HCV IRES Feo was stably transfected into Huh7 cells. Cells were treated without (control →) and with 20 μM griseofulvin →. Dual luciferase activities were measured at the indicated time points after exposure to griseofulvin. Values are displayed as ratios of Fluc to Rluc. Error bars indicate mean ± SD.

HCV replication, but not HCV IRES-dependent translation.[25] Therefore, we determined whether G_2/M cell cycle arrest by griseofulvin affects HCV IRES-dependent translation using Huh7 cells transfected with pEF Rluc-HCV IRES Feo (Fig. 8a). The treatment of these cells with 20 μM griseofulvin resulted in no significant change of the internal luciferase activities, a concentration that suppressed the expression of the HCV replicon and arrested the HCV replicon cells in the G_2/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 a virus that can be efficiently propagated in cell culture (HCVcc).[10] Therefore, we examined the effect of griseofulvin using the JFH-1 system. The Huh7.5.1/JFH-1 cells (cells persistently infected with HCV JFH-1) were cultured with 10 μM or 20 μM griseofulvin for 72 h. We detected the HCV NS3 protein in Huh7.5.1/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 suppresses 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 the 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. The 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 the combination treatment of griseofulvin and IFNα 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 G_2/M arrest in several human cell lines.[24] Here, we show that griseofulvin arrested the Huh7/Rep-Feo cells in the G_2/M phase. 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 the G_2/M phase.[26] In contrast, Venkatesan *et al.* reported that the HCV IRES activity was lowest in the G_2/M,[27] while other studies

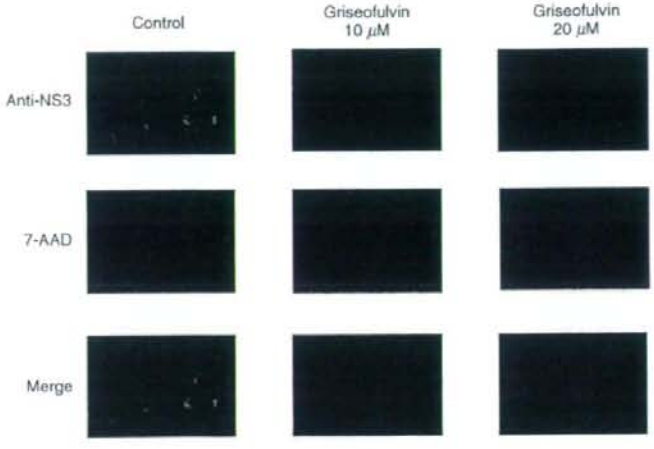

Figure 9 Griseofulvin suppresses IFH-1 replication. Immunofluorescent staining of Huh7.5.1/IFH-1 cells treated with various concentrations of griseofulvin. Hepatitis C virus NS3 protein is stained green and nuclei are stained with 7-aminoactinomycin D (7-AAD; red).

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 indicate that microtubule polymerization is required for the formation of the HCV replication complex. Griseofulvin has been shown to arrest human cells in the G_2/M phase by acting on microtubule polymerization.[30] Thus it is speculated that the inhibition of microtubule polymerization by griseofulvin may influence 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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