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

Takako Watanabe,† Naoya Sakamoto,‡ Mina Nakagawa,‡ Sei Kaiinuma,‡ Yasuhiro Itsui,‡ Yuki Nishimura-Sakurai,‡ Mayumi Ueyama,‡ Yusuke Funoka,‡ Akiko Kitazume,‡ Sayuri Nitta,‡ Kei Kiyohashi,‡ Miyako Murakawa,‡ Seishin Azuma,‡ Kichiro Tsuchiya,‡ Shinya Oooka,‡ and Mamoru Watanabe‡

Department of Gastroenterology and Hepatology† and Department for Hepatitis Control‡ Tokyo Medical and Dental University, Tokyo, Japan, and Department of Internal Medicine, Soka Municipal Hospital, Saitama, Japan‡

Received 19 December 2010/Returned for modification 11 January 2011/Accepted 14 March 2011

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 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; C30H38O11; 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 ascarsis (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...
concentration (EC50) values were calculated using the probit method (2, 33). The

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.

Reporters 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

pSRE-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 × 10³ 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 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% CO2. To maintain cell lines carrying an HCV subgenomic replicon (Huh7/Rep-Feo), G418 (Nakai Tesque, Kyoto, Japan) was added to the culture medium at a final concentration of 500 µg/ml. The HCV subgenomic replicon 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.

Cell viability. To evaluate cell viability, dimethylthiazol carboxymethoxyphenyl sulphone (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 × 10³ cells/well. After treatment, the MTS 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 (CC50). The drug selectivity index was calculated as CC50/EC50. 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, EC50 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 EC50 for each 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.
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 EC50 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 CC50 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 increases ISRE reporter activity with α-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 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
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 /H9251-IFN on the replicon. We next assessed the effects of TSN combination with /H9251-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 g/ml) and /H9251-IFN (0 to 100 IU/ml). Replication of the HCV replicon was suppressed by pretreatment with TSN, followed by treatment with /H9251-IFN, in a dose-dependent manner (Fig. 4A; see Fig. S1 in the supplemental material). The EC50 of /H9251-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 /H9251-IFN treatment is more effective in inhibiting HCV replication than treatment with /H9251-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 /H9251-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 EC50 ratio of 1:0, 2:3, 1:4, or 0:1, and the dose-effect plots were drawn (Fig. 4E). The fractional EC50s for /α-IFN and TSN were plotted on the x and y axes, respectively, to generate an isobologram (Fig. 4F). Each plot showing
the fractional EC50 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 α-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 EC50 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 EC50 for each drug alone. All of the fractional EC50 plots for the TSN and α-IFN combinations fell below the line of additivity, indicating synergy.

TSN upregulates ISGF3 in combination with α-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
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 H9251-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 H9251-IFN and TSN than by H9251-IFN treatment alone (Fig. 7A and B). In addition, IRF9 mRNA expression was significantly higher following pretreatment with TSN prior to H9251-IFN therapy than by H9251-IFN monotherapy (Fig. 8). These findings are consistent with the hypothesis that TSN activate ISGF3 components in combination with H9251-IFN.

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 H9251-IFN augmented H9251-IFN receptor-mediated, ISRE-regulated gene expression (Fig. 3). Consistent with these findings, TSN pretreatment significantly enhanced the suppressive effects of H9251-IFN on the HCV replicon and HCV cell culture (Fig. 4 and 5). Finally, we demonstrated that the H9251-IFN-enhancing effects of TSN are through increased transcriptional activation of a component of ISGF3 (Fig. 7 and 8). Taken together, our results demonstrate that TSN is potentially an effective antiviral agent when it is used alone and especially when it is used in combination with H9251-IFN and that screening for such H9251-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...
may cancel the suppressive effect of HCV gene products on the
\(/H9251\)-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 \(/H9251\)-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 \(/H9251\)-IFN effect.

Other than the canonical Jak/STAT-mediated \(/H9251\)-IFN signaling pathway, several alternative \(/H9251\)-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 evidence for antiviral drugs against HCV. It would be important to assess drug resistance after continuous treatment with TSN. There is no evidence for antiviral drugs against HCV. 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.

ACKNOWLEDGMENTS

We thank Frank Chisari for providing Huh7.5.1 cells, Charles Rice for providing plasmid pJ6/JFH1full, and Takaji Wakita for providing plasmid pJFH1full. This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Japan Society for the Promotion of Science, the Ministry of Health, Labor and Welfare of Japan, the Japan Health Sciences Foundation, the National Institute of Biomedical Innovation, and the Miyakawa Memorial Research Foundation.

REFERENCES