

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease, and persistent HCV infection may result in liver cirrhosis and hepatocellular carcinoma over the course of 20–30 years [1–3]. Antiviral treatment has been shown to improve liver histology and decrease the incidence of hepatocellular carcinoma in chronic hepatitis C (CHC) patients [4, 5]. A combination of ribavirin plus pegylated interferon (PEG-IFN)-alpha [6, 7] is effective, but less than 50 % of patients infected with HCV genotype 1 treated in this way achieved a sustained virological response (SVR) or a cure of the infection [6, 8]. In particular, failure of treatment is due to either a lack of virological response or relapse after the completion of therapy, despite an initial virological response. Hematologic abnormalities and ribavirin-induced hemolytic anemia necessitate dose reduction and premature withdrawal from therapy in 10–14 % of patients [6, 9–12]. Although new drugs and therapeutic approaches for CHC are being developed actively and several candidates are in early phase trials [13, 14], ribavirin remains mandatory for improving clinical anti-HCV chemotherapeutic responses [15–17].

Given this background, several recent studies have demonstrated that genetic variation leading to inosine triphosphatase (*ITPA*) deficiency, a condition not thought to be clinically important, protects CHC patients receiving ribavirin against hemolytic anemia [18]. However, factors other than *ITPA* gene polymorphism also contribute to the risk of severe anemia and consequent ribavirin dose reduction, and the impact of the *ITPA* genotype on treatment outcome has been studied with conflicting results [19–23]. The aims of this study were to analyze the relationship between *ITPA* gene variation and serum ribavirin concentration associated with reduction in blood cell concentrations.

Patients and methods

Patients

In this retrospective, cross-sectional case–control study, 300 patients with chronic HCV infection who were treated at Tokyo Medical and Dental University Hospital and associated hospitals, part of the Ochanomizu-Liver Conference Study Group, were enrolled from December 2004 to November 2010. Each patient was treated with combination therapy comprising PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 µg/kg subcutaneously and ribavirin (Rebetol; Schering-Plough Nordic Biotech) (b.w. < 60 kg: 600 mg po daily; b.w.:

60–80 kg: 800 mg po daily; b.w. > 80 kg: 1,000 mg po daily; in two divided doses). The treatment duration was set at a standard 48 weeks for patients infected with genotype 1b with high viral loads (≥ 5 log copies/ml) and 24 weeks for patients with genotype 1 with low viral loads (≤ 5 log copies/ml) or with genotype 2. On-treatment dose reduction and discontinuation of PEG-IFN or ribavirin were decided based on the recommendations of package inserts or the clinical situations of individual patients to avoid possible side effects. The amounts of PEG-IFN and ribavirin administered were expressed as percentages of the target standard total dose over 48 or 24 weeks, according to body weight before therapy. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by RT-PCR. Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was carried out by a central laboratory.

Informed consent was obtained from each patient who participated in the study. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of this hospital and of all the participating hospitals.

Patient evaluation

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age, gender, body mass index (BMI), grade of inflammation and stage of fibrosis on liver biopsy, pretreatment biochemical parameters, such as white blood cells (WBC), hemoglobin (Hb), platelet (PLT) count, alanine transaminase (ALT) level, gamma-glutamyl transpeptidase (γ -GTP) level, serum creatinine, and serum HCV RNA level (log IU/ml). Liver biopsy specimens were evaluated blindly, to determine the grade of inflammation and stage of fibrosis, by an independent interpreter who was not aware of the clinical data. The activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows mild fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

Single nucleotide polymorphism genotyping

Human genomic DNA was extracted from whole blood from each patient. Genetic polymorphisms, rs1127354 in *ITPA* and rs8099917 around the *IL28B* gene, were determined by real-time detection PCR with the TaqMan probe or DigiTag2 assay, typing one tag SNP located within each locus [24]. Another functional SNP, rs727010 within the *ITPA* gene, was excluded because it does not vary in the Asian population, as reported in the International HapMap Project database. Preliminary genotyping of 100 patients from the study population did not reveal variation in that SNP.

Measurement of the ribavirin concentration

The ribavirin concentration was measured using high-performance liquid chromatography (HPLC; SRL, Tokyo, Japan); the detection limit was 50 ng/ml.

Outcomes

The primary end point showed a decline in the blood cell concentration and dose reduction in PEG-IFN or ribavirin at week 4; the secondary end point was an SVR. SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Adverse events and drug adherence were recorded.

Statistical analyses

The association between the individual *ITPA* SNP and the occurrence of a significant decline in the Hb concentration

was evaluated by a basic allelic test and calculated using the Chi-square test. Multivariate logistic regression analysis with stepwise forward selection was performed with *p* values of less than 0.05 as the criteria for model inclusion. These statistical analyses were conducted using the SPSS software package (SPSS 18 J; SPSS, Chicago, IL, USA). Discrete variables were evaluated by Fisher's exact probability test. The *p* values were calculated by two-tailed Student's *t*-test for continuous data and the Chi-square test for categorical data; values less than 0.05 were considered as statistically significant.

Results

Association between *ITPA* rs1127354 genotypes and decline in blood cells

The clinical characteristics of the 300 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, 79 (41 %) of the 195 patients infected with HCV genotype 1 achieved SVRs and 85 (81 %) of the 105 patients infected with HCV genotype 2 achieved SVR.

The quantitative reduction in blood cells from the baseline according to the *ITPA* rs1127354 genotypes is shown in Fig. 1. Patients with the *ITPA-CA/AA* genotypes showed a lower degree of Hb reduction throughout the therapy than those with the *ITPA-CC* genotype (Fig. 1a), and a marked difference in the mean Hb reduction was found at week 4 (*AA/CA* -1.0 vs. *CC* -2.8 , $p < 0.001$). These results show that the *ITPA-CA/AA* genotypes are

Table 1 Baseline characteristics of participating patients

Total number	300
HCV genotype (1/2)	195/105
<i>ITPA</i> gene (rs1127354); <i>AA/CA/CC</i>	2/80/218
<i>IL28B</i> gene (rs8099917); <i>TT/non-TT</i>	225/75
Age (years) ^a	57 (20–78)
Gender (male/female)	153/147
Body mass index (kg/m ²) ^a	23.5 (15.3–33.7)
Histology at biopsy	
Grade of inflammation; A0–1/A2–3/ND	96/109/95
Stage of fibrosis; F0–2/F3–4/ND	165/40/95
Baseline white blood cells (/μl) ^a	5270 (2000–10,300)
Baseline hemoglobin (g/dl) ^a	14.2 (9.7–17.5)
Baseline platelet count ($\times 10^3/\mu\text{l}$) ^a	170 (61–458)
Baseline ALT (IU/l) ^a	85 (9–541)
Baseline γ -GTP (IU/l) ^a	67 (10–731)
Baseline serum creatinine (mg/dl) ^a	0.72 (0.4–1.4)
Serum HCV RNA level (log ₁₀ IU/ml) ^{a, b}	6.1 (2.9–7.6)
Initial RBV dose (mg/kg) ^a	11.2 (6.0–15.7)
Serum RBV concentration at week 4 (μg/ml) ^a	2.3 (0.4–5.2)

HCV hepatitis C virus, ALT alanine transaminase

^a Data are shown as median (range) values

^b Data are shown as log (IU/ml)

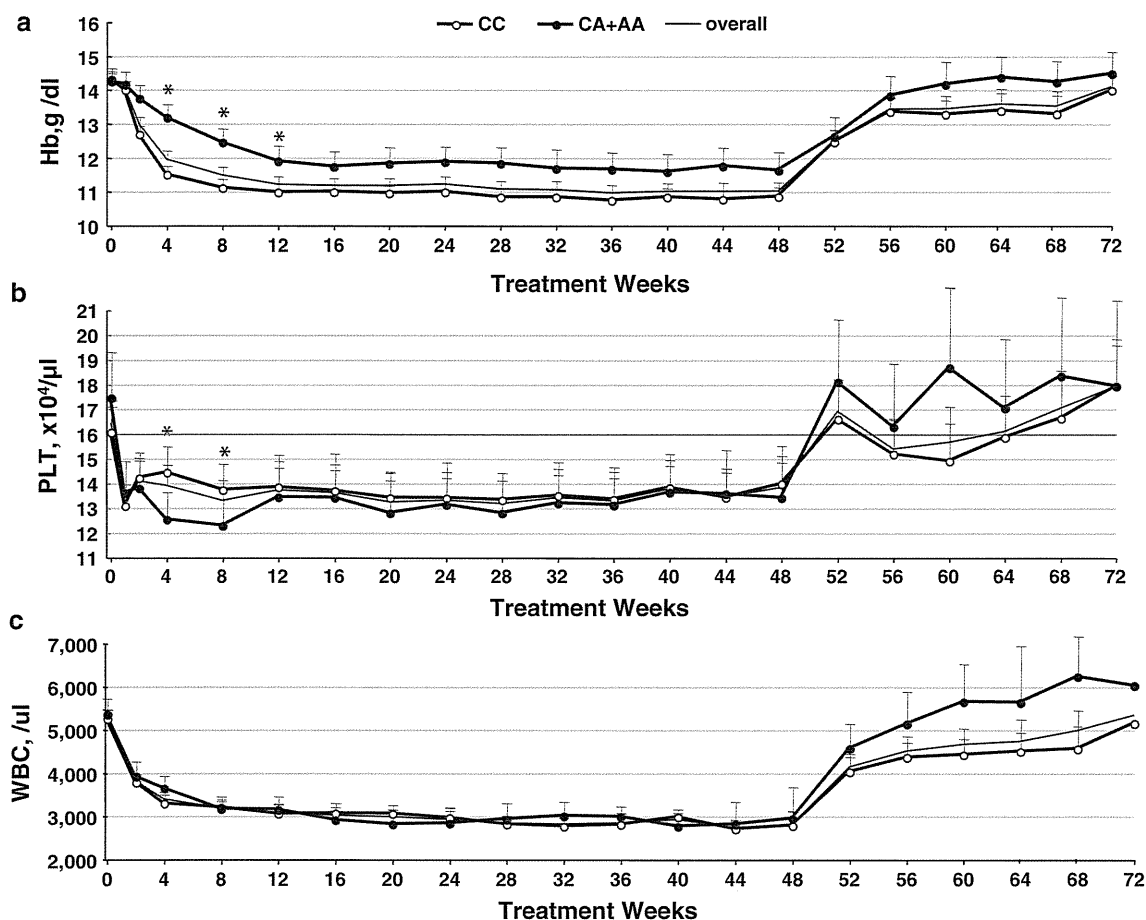


Fig. 1 The quantitative reduction of blood cells from the baseline according to the *ITPA* rs1127354 major and minor variants. Blood cell counts were determined every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy for Hb (a),

PLT (b), and WBC (c). Patients with *ITPA-CC* are indicated by open circles and those with *ITPA-CA/AA* by closed circles. Error bars indicate mean \pm SE. Asterisks indicate statistical significance. WBC white blood cells, Hb hemoglobin, PLT platelet

associated significantly with a lower reduction in Hb levels throughout the therapy and protect against the development of severe hemolytic anemia. Figure 1b shows that the *ITPA-CC* genotype is associated with a significantly lower reduction in the mean PLT count than the *ITPA-CA/AA* genotypes at the early stages of treatment ($p < 0.001$ for weeks 4 and 8), possibly due to a reactive increase in the PLT count. That is to say that a severe decline in the Hb concentration, which was associated particularly with the *ITPA-CC* genotype, was inversely correlated with platelet reduction. There were no differences in the WBC count between the *ITPA-CC* and *ITPA-CA/AA* variants (Fig. 1c).

These results show that the *ITPA* minor variant A has a protective phenotype against treatment-induced anemia, and the quantitative reduction of Hb in patients with *ITPA-CC* was greater, especially with a high concentration of ribavirin. In contrast, the patients with the *ITPA-CC* genotype had a lower reduction in the PLT count than the patients with the *ITPA-CA/AA* genotypes, as reported previously [25].

Relationship between *ITPA* rs1127354 variants and treatment outcome due to dose reduction of PEG-IFN or ribavirin

To evaluate the clinical relevance of the *ITPA* genotype and decline in the Hb concentration or PLT counts, the decline in Hb and PLT levels was analyzed at week 4 and compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA* (Fig. 2). Patients with the *ITPA-CA/AA* genotype were less likely to develop anemia regardless of the concentration of ribavirin.

The percentages of patients requiring PEG-IFN or ribavirin dose reduction at week 4 among those with the *ITPA* rs1127354 major and minor variants in accordance with the incidence of anemia or thrombocytopenia were investigated. There was a significant difference between the *ITPA-CC* and *ITPA-CA/AA* variants in terms of the need for ribavirin dose reduction. At week 4 of treatment, ribavirin

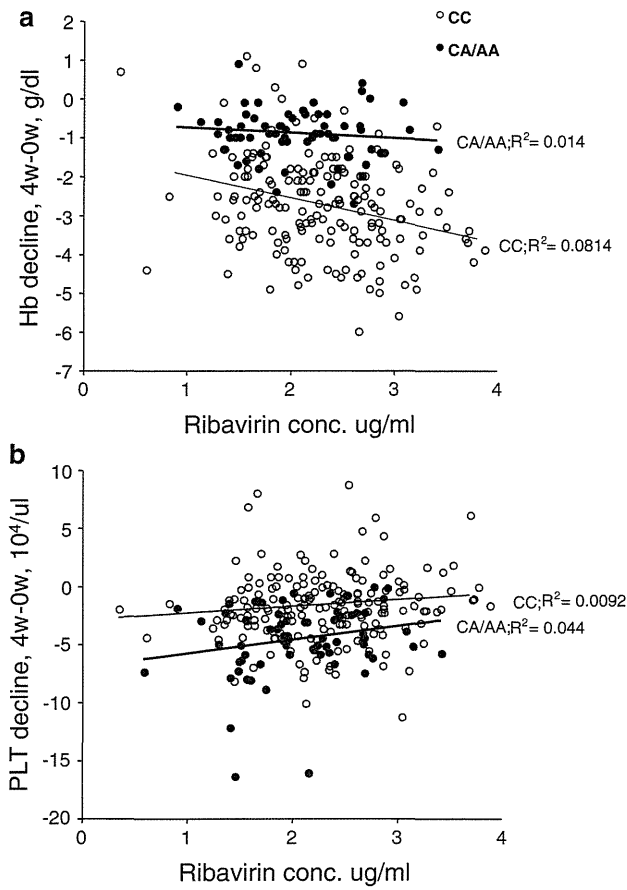


Fig. 2 ITPA genotype and decline in Hb and platelets at week 4 compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA*. The decline in Hb (a) and PLT (b) levels was analyzed at week 4 and compared with baseline according to ribavirin concentrations in patients with *ITPA-CC* and *ITPA-CA/AA*, to evaluate the clinical relevance of ITPA genotypes and decline in Hb or PLT. Patients with *ITPA-CC* are indicated by open circles and those with *ITPA-CA/AA* by closed circles. The Y axis indicates Hb or PLT concentrations (g/dl or 10,000/ μ l) and the X axis indicates ribavirin concentration (μ g/ml)

doses were reduced in 20.6 % of patients with *ITPA-CC*, but in only 4.9 % of patients with *ITPA-CA/AA* ($p = 0.001$; Fig. 3a). Similar to ribavirin, PEG-IFN dose reduction was apparently more common in patients with *ITPA-CC*, although this did not reach statistical significance.

The treatment outcome in patients with *ITPA-CC* and *ITPA-CA/AA* was analyzed according to baseline PLT counts because an inverse correlation was observed in the Hb and PLT decline between the *ITPA-CC* and *ITPA-CA/AA* variants. Figure 3b shows the percentages of SVR in the patients infected with genotype 1 according to the baseline PLT count. Patients with baseline PLT counts below $130 \times 10^3/\mu$ l had a significantly lower tendency to achieve SVR than patients with baseline PLT counts above $180 \times 10^3/\mu$ l ($p = 0.024$) and the difference was more

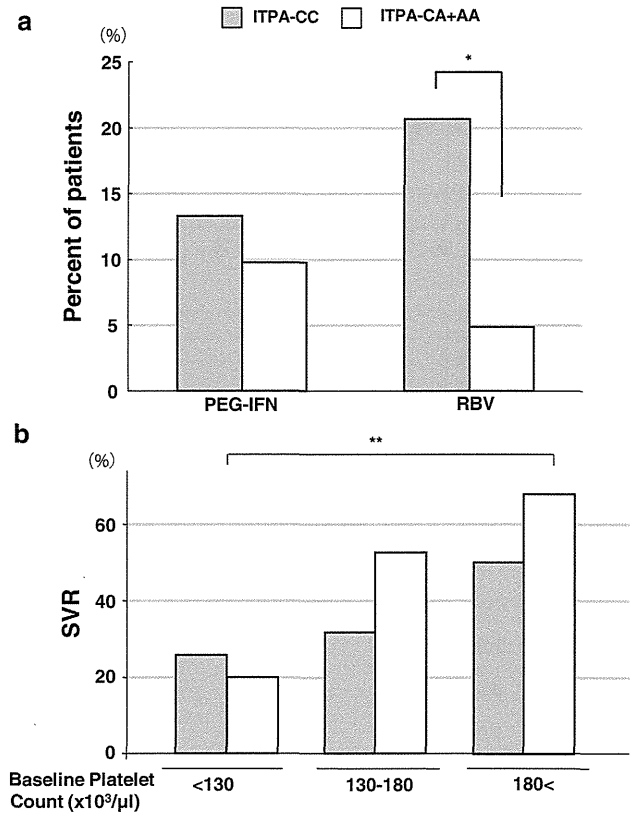


Fig. 3 Relationship between *ITPA* rs1127354 major and minor variants and treatment outcome due to dose reduction of PEG-IFN or ribavirin. **a** The percentages of patients requiring PEG-IFN or ribavirin dose reduction at week 4 among patients with *ITPA* rs1127354 major and minor variants. The Y axis indicates the percentage of patients who required dose reduction. Asterisk indicates statistical significance, $p = 0.001$. **b** Percentages of SVR in patients infected with genotype 1 according to the baseline platelet count. Asterisks indicate statistical significance, $p = 0.024$

pronounced in patients with the *ITPA-CA/AA* genotypes. No difference was observed between the *ITPA-CC* and *ITPA-CA/AA* variants according to the baseline PLT count in patients infected with genotype 2.

Knowing that significantly less frequent ribavirin dose reduction was necessary in patients with the *ITPA-CA/AA* genotypes, it was determined whether the *ITPA* gene variation affected final treatment outcomes. The treatment outcomes were available for 195 patients infected with genotype 1 and 105 patients infected with genotype 2. On multivariable analysis of patients infected with genotype 1 (Table 2), the IL28B genotype was the most important predictor of SVR at baseline [adjusted odds ratio 16.949 (95 % confidence interval 0.014–0.248), $p < 0.0001$], along with baseline serum HCV RNA level [7.813 (0.046–0.359), $p = 0.003$]. *ITPA* gene variation was not significant in patients infected with genotype 1 (Table 2) or in those infected with genotype 2 (Table 3).

Table 2 Comparison of clinical and laboratory characteristics of patients infected with genotype 1 based on the therapeutic response

All patients	SVR (n = 79)	Non-SVR (n = 116)	Univariate analysis p value	Multivariate analysis		
				OR	95 % CI	p value
Age (years) ^a	57 (24–8)	61 (21–78)	0.027	1.024	0.972–1.080	0.373
Gender (male/female)	42/37	56/60	0.560	–		
BMI (kg/m ²) ^a	23.4 (16.9–3.7)	23.3 (15.3–30.8)	0.730	–		
Grade of inflammation (A0–1/2–3/ND)	27/36/16	28/44/44	0.043	3.658	0.533–25.086	0.187
Stage of fibrosis (F0–2/3–4/ND)	55/8/16	51/21/44	0.002	2.847	0.788–10.285	0.110
Baseline white blood cells (/μl) ^a	5,230 (2,600–9,650)	4,900 (2,000–9,700)	0.038	1.000	1.000–1.000	0.505
Baseline hemoglobin (g/dl) ^a	14.5 (11.9–7.5)	14.0 (9.7–17.0)	0.027	1.423	0.949–2.134	0.088
Baseline platelet count (× 10 ³ /μl) ^a	174 (73–458)	145 (61–309)	0.001	1.075	0.984–1.173	0.108
Baseline γ-GTP (IU/l) ^a	35 (10–731)	50 (10–618)	0.012	1.003	0.998–1.008	0.284
Serum HCV RNA level [log (IU/ml)] ^{a, b}	6.1 (2.9–7.3)	6.3 (5.1–7.4)	0.003	7.813	0.046–0.359	0.000
RBV concentration at week 4 (μg/ml) ^a	2.4 (1.4–5.2)	2.3 (0.4–4.8)	0.154	–		
Substitutions in the ISDR (≤ 1/ ≥ 2/ND)	52/18/9	91/7/18	0.004	2.469	0.105–1.555	0.188
Substitutions of core amino acid 70 (wild type/mutant/ND)	44/23/12	60/39/17	0.799	–		
IL28B SNPs (rs8099917; TT/non-TT)	71/8	68/48	0.000	16.949	0.014–0.248	0.000
ITPA gene (rs1127354; CC/non-CC)	51/28	89/27	0.064	–		

Medians (ranges) are shown

OR odds ratio, CI confidence interval, RBV ribavirin, SVR sustained virological response

^a Data are shown as median (range) values

^b Data are shown as log (IU/ml)

Table 3 Comparison of clinical and laboratory characteristics of patients infected with genotype 2 based on the therapeutic response

All patients	SVR (n = 85)	Non-SVR (n = 20)	Univariate analysis p value	Multivariate analysis		
				OR	95 % CI	p value
Age (years) ^a	56 (20–72)	61 (48–69)	0.019	1.091	0.838–1.004	0.061
Gender (male/female)	42/43	13/7	0.226	–		
BMI (kg/m ²) ^a	23.0 (16.9–33.5)	24.3 (19.4–27.7)	0.071	–		
Grade of inflammation (A0–1/2–3/ND)	36/22/27	5/7/8	0.356	–		
Stage of fibrosis (F0–2/3–4/ND)	50/8/27	9/3/8	0.506	–		
Baseline white blood cells (/μl) ^a	5,200 (2,600–10,300)	4,310 (2,380–7,900)	0.124	–		
Baseline hemoglobin (g/dl) ^a	14.2 (11.0–17.3)	14.0 (10.3–17.4)	0.967	–		
Baseline platelet count (× 10 ³ /μl) ^a	186 (68–340)	175 (80–284)	0.172	–		
Baseline γ-GTP (IU/l) ^a	31 (11–209)	74 (14–292)	0.017	1.014	0.972–1.000	0.050
Serum HCV RNA level [log (IU/ml)] ^{a, b}	6.1 (3.6–7.4)	6.2 (4.0–7.6)	0.601	–		
RBV concentration at week 4 (μg/ml) ^a	2.1 (0.6–4.1)	2.1 (1.3–3.5)	0.877	–		
Substitutions in the ISDR (≤ 1/ ≥ 2/ND)	46/26/13	11/4/5	0.466	–		
IL28B SNPs (rs8099917; TT/non-TT)	70/15	16/4	0.115	–		
ITPA gene (rs1127354; CC/non-CC)	60/25	18/2	0.092	–		

Medians (ranges) are shown

OR odds ratio, CI confidence interval, RBV ribavirin, SVR sustained virological response

^a Data are shown as median (range) values

^b Data are shown as log (IU/ml)

Discussion

This study confirmed the most recent report that the *ITPA* rs1127354 genotype is a useful marker for predicting hematological side effects of treatment with ribavirin [25, 26]. The results show that the severe Hb decline, which is found predominantly in patients with the *ITPA-CC* genotypes, was inversely correlated with platelet reduction and that the opposite correlation is observed in Hb and PLT decline, but not in WBC concentration, as reported previously [25]. While patients with the *ITPA-CA/AA* genotype were less likely to develop anemia, regardless of the concentration of ribavirin (Fig. 2), patients with baseline PLT counts below $130 \times 10^3/\mu\text{l}$ had a significantly lower tendency to achieve SVR, especially those with the *ITPA-CA/AA* genotype, possibly due to PEG-IFN dose reduction in response to the PLT decline. As a result, *ITPA* gene variation was not extracted as an important predictor of SVR in CHC patients with either genotype 1 or 2, which is consistent with a very recent report [27].

Ribavirin is directly toxic to erythrocytes and is associated with hemolysis, which is usually reversible and dose related [28, 29]. Ribavirin is incorporated into erythrocytes where it undergoes phosphorylation by adenosine kinase to its pharmacologically active forms. The ribavirin-phosphate conjugates are unable to cross the erythrocyte cell membrane and are, therefore, accumulated intracellularly and cleared slowly from red cells, with a half-life of ~40 days [30]. The possible mechanism of protection against ribavirin-induced hemolysis is that ITP deficiency or low-activity variants (*ITPA-CA/AA* groups) are associated with the accumulation of ITP in red blood cells [31, 32] and ITP confers protection against ribavirin-induced ATP reduction by substituting for erythrocyte GTP, which is depleted by ribavirin, in the biosynthesis of ATP [33]. In addition, the sequence homology of thrombopoietin (TPO) and erythropoietin (EPO) may explain the synergy of the physiological role of TPO and EPO in platelet production [25]. Ochi et al. [23] analyzed the genomes of Japanese patients, including the *ITPA* and *DDRGKI* loci, which are located together on chromosome 20. Their report indicates that the *ITPA* SNP, rs1127354, which was genotyped in this study, represents the dominant variant of *ITPA* deficiency that protects against ribavirin-induced anemia in Japanese/Asian populations. Ribavirin is a synthetic guanosine analog and has in vitro activity against a wide range of RNA and DNA viruses [34]. Possible antiviral mechanisms of ribavirin include immune modulation by switching the T-cell phenotype from type 2 to type 1 [35], antiproliferative effects by the inhibition of cellular GTP synthesis [34], and direct inhibition of virus replication [36]. Although monotherapy with ribavirin showed a minimal effect on the viral load and almost no effect on

viral clearance [37–40], the combinatory use of ribavirin with IFN elicits strong synergistic effects against HCV in vitro [41] and in vivo [28, 29]. Interestingly, Snoeck et al. [42] reported that the probability of SVR was not influenced by the ribavirin dose in patients with HCV genotype 2 or 3 infection, but increased as a function of ribavirin dose in patients with HCV genotype 1 infection (40–50 % increase in the probability of SVR for a ribavirin dose increase of 12–6 mg/kg¹). Indeed, while there are several directly acting antiviral (DAA) agents being tested for clinical efficacy against hepatitis C [13, 14], most experts believe that ribavirin remains mandatory for improving clinical anti-HCV chemotherapeutic effects when new drugs are approved to treat hepatitis C.

There are a number of ongoing trials registered with ClinicalTrials.gov. Although the limited results have been presented thus far, the addition of ribavirin without IFN was shown to accelerate the HCV RNA level decline and reduce the incidence of virological breakthroughs, at least in the short term [43]. New therapeutic approaches using combinations of DAA agents in the IFN-spared regimens with or without ribavirin are currently under study, but ribavirin appears to exert its own effect independently of IFN in some studies. While *ITPA* gene variation was not extracted as an important predictor of SVR in combination therapy with PEG-IFN as reported, including our data, the *ITPA* gene may play an important role as a significant and independent pre-treatment marker to predict SVR in IFN-free regimens.

In conclusion, the results presented here show that an inverse correlation is observed in the reduction in Hb and PLT count in patients with the *ITPA-CC* and *ITPA-CA/AA* genotypes. Despite the fact that *ITPA* variants were less likely to develop anemia, regardless of a high concentration of ribavirin, patients with baseline PLT counts below $130 \times 10^3/\mu\text{l}$ had a significantly lower tendency to achieve SVR, especially those with the *ITPA-CA/AA* genotype. These results may give a valuable pharmacogenetic diagnostic tool for the tailoring of ribavirin dosing to minimize drug-induced adverse events and for further optimization of the clinical anti-HCV treatment outcomes.

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Article

Inhibition of Hepatitis C Virus Replication and Viral Helicase by Ethyl Acetate Extract of the Marine Feather Star *Alloeocomatella polycladia*

Atsuya Yamashita ¹, Kazi Abdus Salam ^{2,†}, Atsushi Furuta ^{3,4,†}, Yasuyoshi Matsuda ^{3,4}, Osamu Fujita ^{3,4}, Hidenori Tani ², Yoshihisa Fujita ^{5,6}, Yuusuke Fujimoto ¹, Masanori Ikeda ⁷, Nobuyuki Kato ⁷, Naoya Sakamoto ⁸, Shinya Maekawa ⁹, Nobuyuki Enomoto ⁹, Masamichi Nakakoshi ¹⁰, Masayoshi Tsubuki ¹⁰, Yuji Sekiguchi ³, Satoshi Tsuneda ⁴, Nobuyoshi Akimitsu ², Naohiro Noda ³, Junichi Tanaka ^{11,*} and Kohji Moriishi ^{1,*}

¹ Department of Microbiology, Division of Medicine, Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan;

E-Mails: atsuyay@yamanashi.ac.jp (A.Y.); yfujimoto@yamanashi.ac.jp (Y.F.)

² Radioisotope Center, The University of Tokyo, 2-11-16 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan;

E-Mails: salam_bio26@yahoo.com (K.A.S); tani@ric.u-tokyo.ac.jp (H.T.);

akimitsu@ric.u-tokyo.ac.jp (N.A.)

³ Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology

(AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan; E-Mails: atsushi.furuta@aist.go.jp (A.F.);

yellow-3359@hotmail.co.jp (Y.M.); 036.fujita@gmail.com (O.F.); y.sekiguchi@aist.go.jp (Y.S.);

noda-naohiro@aist.go.jp (N.N.)

⁴ Department of Life Science and Medical Bio-Science, Waseda University, 2-2 Wakamatsu-cho,

Shinjuku-ku, Tokyo 162-8480, Japan; E-Mail: stsuneda@waseda.jp

⁵ University Education Center, University of the Ryukyus, Okinawa, 1 Senbaru, Nishihara,

Okinawa 903-0213, Japan; E-Mail: galatheids@yahoo.co.jp

⁶ Marine Learning Center, 2-95-101 Miyagi, Chatan, Okinawa 901-0113, Japan

⁷ Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and

Pharmaceutical Sciences, Okayama, 2-5-1 Shikata-cho, Okayama 700-8558, Japan;

E-Mails: maikeda@md.okayama-u.ac.jp (M.I.); nkato@md.okayama-u.ac.jp (N.K.)

⁸ Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University,

1-5-45 Yushima, Bunkyo-ku, Tokyo, Japan; E-Mail: nsakamoto.gast@tmd.ac.jp

⁹ First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi, Yamanashi,

1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan;

E-Mails: maekawa@yamanashi.ac.jp (S.M); enomoto@yamanashi.ac.jp (N.E)

¹⁰ Institute of Medical Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501,

Japan; E-Mails: mnakako@hoshi.ac.jp (M.N.); tsubuki@hoshi.ac.jp (M.T.)

¹¹ Department of Chemistry, Biology and Marine Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan

† These authors contributed equally to this work.

* Authors to whom correspondence should be addressed; E-Mails: jtanaka@sci.u-ryukyu.ac.jp (J.T.); kmoriishi@yamanashi.ac.jp (K.M.); Tel: +81-98-895-8560 (J.T.); +81-55-273-9537 (K.M.); Fax: +81-98-895-8565 (J.T.); Fax: +81-55-273-6728 (K.M.).

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Abstract: Hepatitis C virus (HCV) is a causative agent of acute and chronic hepatitis, leading to the development of hepatic cirrhosis and hepatocellular carcinoma. We prepared extracts from 61 marine organisms and screened them by an *in vitro* fluorescence assay targeting the viral helicase (NS3), which plays an important role in HCV replication, to identify effective candidates for anti-HCV agents. An ethyl acetate-soluble fraction of the feather star *Alloeocomatella polycladia* exhibited the strongest inhibition of NS3 helicase activity, with an IC₅₀ of 11.7 µg/mL. The extract of *A. polycladia* inhibited interaction between NS3 and RNA but not ATPase of NS3. Furthermore, the replication of the replicons derived from three HCV strains of genotype 1b in cultured cells was suppressed by the extract with an EC₅₀ value of 23 to 44 µg/mL, which is similar to the IC₅₀ value of the NS3 helicase assay. The extract did not induce interferon or inhibit cell growth. These results suggest that the unknown compound(s) included in *A. polycladia* can inhibit HCV replication by suppressing the helicase activity of HCV NS3. This study may present a new approach toward the development of a novel therapy for chronic hepatitis C.

Keywords: marine organism; *Alloeocomatella polycladia*; hepatitis C virus; NS3 helicase

1. Introduction

Hepatitis C virus (HCV) is an etiological agent of liver disease including steatosis, cirrhosis, and hepatocellular carcinoma, and has infected over 170 million individuals worldwide [1,2]. HCV belongs to the genus *Hepacivirus* of the *Flaviviridae* family. The genome of HCV is a single positive-strand RNA composed of 9.6 kb flanked by 5' and 3'-untranscribed regions (UTRs) and encodes a polyprotein consisting of approximately 3000 amino acids [3]. The polyprotein is translated from a viral genome by an internal ribosome entry site (IRES), which is localized in 5'-UTR [4]. The translated polyprotein is cleaved by host and viral proteases into 10 proteins. The structural proteins consisting of core, E1, and E2 and a viroporin p7, which has not yet been classified as either a structural or nonstructural protein, are located in the N-terminal quarter of the polyprotein. The nonstructural proteins including

NS2, NS3, NS4A, NS4B, NS5A, and NS5B occupy the remaining portion of the polyprotein and form a replication complex with several host factors.

HCV NS3 is well known to play a crucial role in viral replication because it possesses helicase and protease activities [5,6]. The *N*-terminal third of NS3 forms a complex with the NS4A protein and exhibits serine protease activity (NS3-4A protease) to cleave the viral polyprotein for the maturation of viral proteins [7]. The remaining portion of NS3 occupies the RNA helicase domain, characterized by the activities of ATPase and RNA binding, both of which contribute to the unwinding of duplex RNA [8,9]. The helicase activity is needed to separate duplex RNA during viral RNA replication [10]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after the uncoating of a viral particle in the infected cells and then is itself used as a template to synthesize a positive-strand RNA packaged into the viral particle. Thus, helicase as well as protease activities of NS3 can be targeted for use in the development of antiviral agents against HCV.

The current therapy, which combines pegylated interferon with ribavirin, is effective in only about half of patients infected with the most common genotype worldwide, genotype 1 [11–13]. However, this therapy has side effects including influenza-like symptoms, cytopenias, and depression [11]. Furthermore, no effective vaccines for HCV have been developed yet. Biotechnological advances of the past decade have led to the development of novel therapies using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication. This approach has been named either “specifically targeted antiviral therapy for hepatitis C” (STAT-C) or “directed-acting antiviral agents” (DAA) [14–16]. Several compounds of STAT-C or DAA have proceeded to clinical trials. Telaprevir and boceprevir, which are categorized as advanced NS3/4A protease inhibitors, were recently approved for the treatment of chronic hepatitis C patients infected with genotype 1 in the US, EU, Canada, and Japan [17,18]. However, the emergence of drug-resistant viruses is the major problem for therapies using antiviral compounds [19,20]. Accordingly, several kinds of drugs targeting various molecules or positions will be required for the complete eradication of the virus from hepatitis C patients.

The helicase activity of NS3 could be targeted by development of anti-HCV compound in addition to its protease activity. Belon *et al.* reported that 1-*N*,4-*N*-bis[4-(1*H*-benzimidazol-2-yl)phenyl]benzene-1,4-dicarboxamine, designated as (BIP)₂B, is a potent and selective inhibitor of HCV NS3 helicase [21]. (BIP)₂B could not affect ATP hydrolysis without RNA or at a saturated concentration of RNA. QU663 inhibits the unwinding activity of NS3 helicase by binding to the RNA-binding groove irrespective of its own ATPase activity [22]. Compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity.

Various drugs have been generated from natural products, especially those from terrestrial plants and microbes. The development of drugs from natural products has declined in the past two decades by the emergence of high-throughput screening of synthetic chemical libraries. However, recent technical advances in the determination of molecular structures and in the synthesis of chemical compounds have raised awareness about natural products as a resource for drug development [23–25]. Several groups recently reported natural products that inhibit HCV replication *in vitro*. For instance, silbinin, which is identified from the milk thistle [26,27], epigallocatechin 3-gallate, which is from green tea [28], and proanthocyanidins, which are from blueberry leaves [29], can inhibit HCV replication in cultured cells. Marine organisms including plants and animals were recently established as a representative natural resource library for drug development, since there are estimated to be more than 300,000

species of marine organisms. The products isolated from the marine organisms often possess potent biological activities corresponding to the organisms' own novel molecular structures. Thus, marine natural products are considered to include highly significant lead compounds for drug development [30,31]. For example, trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven) are approved anticancer drugs developed from marine organisms [32]. However, marine organisms have not yet been screened for development into anti-HCV agents.

In this study, we screened extracts of marine organisms by using an *in vitro* fluorescence NS3 helicase assay and HCV replicon system to find candidates for safe and effective anti-HCV agents. The marine feather star *Alloeocomatella polycladia* may produce anti-HCV helicase agents that suppress HCV replication.

2. Results and Discussion

2.1. Primary Screening of Marine Organism Extracts on HCV NS3 Helicase Activity

We employed high-throughput screening using a photoinduced electron transfer (PET) assay to identify inhibitors of HCV NS3 helicase activity from extracts of marine organisms (Figure 1). The EtOAc- and MeOH-soluble extracts were prepared from marine organisms obtained from the sea around Okinawa Prefecture, Japan. We identified 16 extracts possessing an arbitrary level of inhibitory activity, which is defined as below 60% of the control in this study (Table 1). Five extracts exhibited high inhibition levels (<30%), and eleven extracts exhibited intermediate inhibition levels (30% to 60%). The EtOAc extract prepared from the feather star *Alloeocomatella polycladia* (Figure 2) exhibited the strongest inhibitory activity among them, and was designated SG1-23-1 in this study. Treatment with SG1-23-1 inhibited the helicase activity in a dose-dependent manner (Figure 3A). The value of IC_{50} is calculated as $11.7 \pm 0.7 \mu\text{g/mL}$. We confirmed the effect of SG1-23-1 on NS3 helicase unwinding activity by the RNA helicase assay using ^{32}P -labeled double-stranded RNA (dsRNA) as a substrate. Treatment with SG1-23-1 inhibited dsRNA dissociation at concentrations of $16 \mu\text{g/mL}$ and above (Figure 3B). These results suggest that treatment with SG1-23-1 inhibits the unwinding ability of HCV NS3 helicase.

Table 1. Inhibitory effects of marine organism extracts on hepatitis C virus (HCV) NS3 helicase activity.

Sample	Helicase Activity		Phylum	Extract	Collection Site
	(% of control)	Specimen			
OK-99-2	78	<i>Agelas</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-3	73	<i>Plakortis</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-4	60	<i>Dysidea arenaria</i>	Porifera	EtOAc	Shimoji Island
OK-99-5	96	<i>Theonella cupola</i>	Porifera	EtOAc	Shimoji Island
OK-99-6	52	<i>Theonella conica</i>	Porifera	EtOAc	Shimoji Island
OK-99-7	85	<i>Epipolasis kushimotoensis</i>	Porifera	EtOAc	Shimoji Island
OK-99-9	51	<i>Hyrtios</i> sp.	Porifera	EtOAc	Shimoji Island

Table 1. Cont.

OK-99-10	75	<i>Theonella</i> sp.	Porifera	EtOAc	Shimoji Island
<u>OK-99-12</u>	53	<i>Isis hippuris</i>	Cnidaria	EtOAc	Shimoji Island
OK-99-13	68	<i>Acanthella</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-15	64	<i>Phyllospongia</i> sp.	Porifera	EtOAc	Shimoji Island
<u>OK-99-17</u>	59	<i>Petrosia</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-18	80	<i>Fasciospongia rimosa</i>	Porifera	EtOAc	Shimoji Island
OK-99-20	77	<i>Echinoclathria</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-21	68	<i>Strongylophora</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-23	74	<i>Dysidea herbacea</i>	Porifera	EtOAc	Shimoji Island
<u>OK-99-26</u>	55	<i>Dysidea</i> cf. <i>arenaria</i>	Porifera	EtOAc	Shimoji Island
OK-99-28	123	<i>Plakortis</i> sp.	Porifera	EtOAc	Shimoji Island
OK-99-31	118	<i>Spongia</i> sp.	Porifera	EtOAc	Okinawa Island
OK-99-34	119	<i>Theonella swinhoei</i>	Porifera	EtOAc	Okinawa Island
OK-99-35	108	<i>Petrosia</i> sp.	Porifera	EtOAc	Okinawa Island
OK-99-36	90	<i>Acanthella</i> sp.	Porifera	EtOAc	Okinawa Island
OK-99-37	102	<i>Luffariella</i> sp.	Porifera	EtOAc	Okinawa Island
OK-99-41	62	<i>Dysidea</i> cf. <i>arenaria</i>	Porifera	EtOAc	Okinawa Island
OK-99-43	85	<i>Xestospongia</i> sp.	Porifera	EtOAc	Okinawa Island
OK-99-44	61	<i>Dysidea arenaria</i>	Porifera	EtOAc	Okinawa Island
OK-99-47	108	<i>Dysidea</i> cf. <i>arenaria</i>	Porifera	EtOAc	Okinawa Island
OK-99-49	90	<i>Petrosia</i> sp.	Porifera	EtOAc	Chibishi
OK-99-51	69	<i>Isis hippuris</i>	Cnidaria	EtOAc	Chibishi
OK-99-52	78	<i>Petrosia</i> sp.	Porifera	EtOAc	Kuro Island
OK-99-55	65	<i>Acanthella</i> sp.	Porifera	EtOAc	Kuro Island
OK-99-57	84	<i>Theonella swinhoei</i>	Porifera	EtOAc	Kuro Island
OK-99-63	117	<i>Epipolasis kushimotoensis</i>	Porifera	EtOAc	Kuro Island
OK-99-64	98	<i>Xestospongia</i> sp.	Porifera	EtOAc	Kuro Island
SG1-1-2	77	<i>Comanthus gisleni</i>	Echinodermata	MeOH	Kume Island
SG1-2-2	112	<i>Stephanometra indica</i>	Echinodermata	MeOH	Kume Island
<u>SG1-5-2</u>	33	<i>Comantella</i> sp. cf. <i>maculata</i>	Echinodermata	MeOH	Kume Island
SG1-9-2	57	<i>Phanogenia gracilis</i>	Echinodermata	MeOH	Kume Island
<u>SG1-12-2</u>	39	<i>Comanthus parvicirrus</i>	Echinodermata	MeOH	Kume Island
SG1-14-2	117	<i>Comaster schlegelii</i>	Echinodermata	MeOH	Kume Island
<u>SG1-15-2</u>	26	Colobometridae sp.	Echinodermata	MeOH	Kume Island
SG1-16-2	66	<i>Cenometra bella</i>	Echinodermata	MeOH	Kume Island
SG1-19-2	78	<i>Comaster nobilis</i>	Echinodermata	MeOH	Kume Island
<u>SG1-21-2</u>	32	<i>Oxycomanthus</i> sp.	Echinodermata	MeOH	Kume Island
<u>SG1-23-1</u>	-3	<i>Alloeocomatella polycladia</i>	Echinodermata	EtOAc	Kume Island

Table 1. Cont.

<u>SG1-24-1</u>	24	<i>Comanthus</i> sp.	Echinodermata	EtOAc	Kume Island
<u>SG1-26-2</u>	51	<i>Oxycomanthus benetti</i>	Echinodermata	MeOH	Kume Island
<u>SG1-28-2</u>	38	<i>Lamprometra palmata</i>	Echinodermata	MeOH	Kume Island
<u>SG1-30-1</u>	25	<i>Colobometra perspinosa</i>	Echinodermata	EtOAc	Kume Island
<u>SG1-31-1</u>	26	<i>Comanthus</i> sp.	Echinodermata	EtOAc	Kume Island
<u>SG1-33-1</u>	32	<i>Basilometra boschmai</i>	Echinodermata	EtOAc	Kume Island
SG3-1	82	<i>Stereonephthya</i> sp.	Cnidaria	EtOAc	Tokashiki Island
SG3-4	73	<i>Dysidea</i> cf. <i>arenaria</i>	Porifera	EtOAc	Tokashiki Island
SG3-6	74	<i>Stylotella</i> sp.	Porifera	EtOAc	Tokashiki Island
SG3-10	139	<i>Epipolasis</i> sp.	Porifera	EtOAc	Tokashiki Island
SG3-11	97	<i>Nephtea</i> sp.	Cnidaria	EtOAc	Tokashiki Island
SG3-21	106	<i>Myrmekioderma</i> sp.	Porifera	EtOAc	Tokashiki Island
SG3-25	111	<i>Pseudoceratina purpurea</i>	Porifera	EtOAc	Tokashiki Island
SG3-26	95	<i>Leucetta</i> sp.	Porifera	EtOAc	Tokashiki Island
SG3-28	65	<i>Lyngbya</i> sp.	Cyanobacteria	EtOAc	Tokashiki Island
SG3-29	61	<i>Dysidea</i> sp.	Porifera	EtOAc	Tokashiki Island

Total number of marine organisms: 61; Marine organisms that strongly inhibit NS3 helicase activity (<30%) (boldface and underlined): 5; Extracts of organisms that exhibit intermediate inhibition of NS3 helicase activity (30%–60%) (underlined): 11; EtOAc: Ethyl acetate; MeOH: Methanol.

Figure 1. Schematic representation of the PET assay system for unwinding activity of HCV NS3 helicase. The fluorescent dye (BODIPY FL) is attached to the cytosine at the 5'-end of the fluorescent strand and quenched by the guanine base at the 3'-end of the complementary strand via photoinduced electron transfer. When the helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits bright light upon the release of the dye from the guanine base. The capture strand, which is complementary to the complementary strand, prevents the reannealing of the unwound duplex.

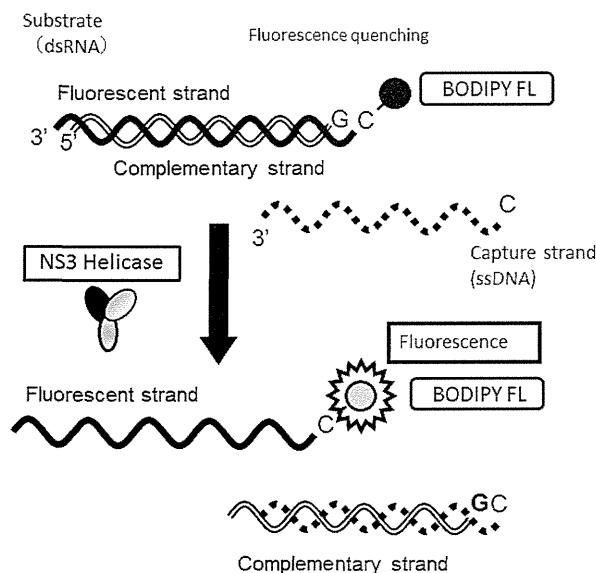


Figure 2. *Alloeocomatella polycladia* belongs to a class of feather star (Echinodermata, Crinoidea). The ethyl acetate fraction prepared from the marine organism was designated SG1-23-1 in this study.

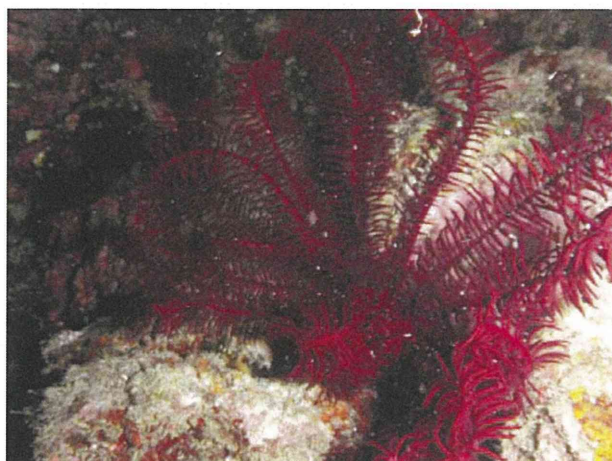
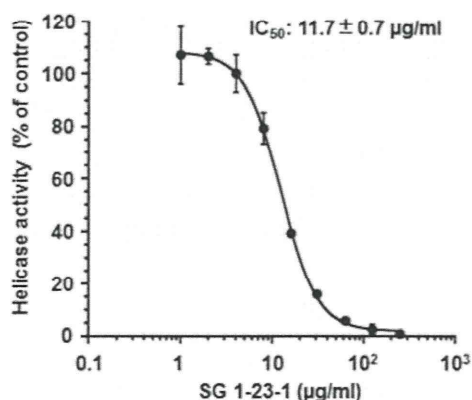
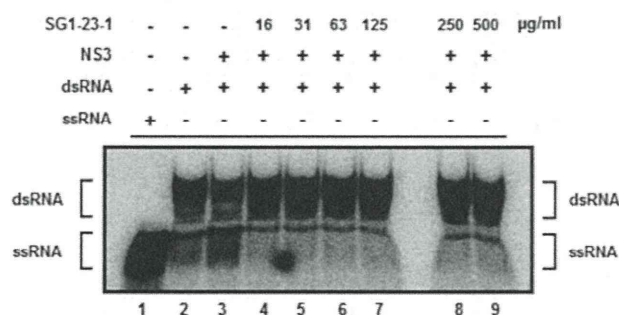


Figure 3. Effect of SG1-23-1 on the unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of SG1-23-1. Helicase activity in the absence of SG1-23-1 was defined as 100% helicase activity. Each value represents the mean of three independent reactions. Error bars indicate standard deviation. The data represent three independent experiments. (B) The unwinding activity of NS3 helicase was measured by RNA unwinding assay using radioisotope-labeled RNA. The heat-denatured single-strand RNA (26-mer) and the partial duplex RNA substrate were applied to lanes 1 and 2, respectively. The duplex RNA was reacted with NS3 (300 nM) in the presence of SG1-23-1 (lanes 4 to 9, 16 to 500 µg/mL). The resulting samples were subjected to native polyacrylamide gel electrophoresis.

A



B

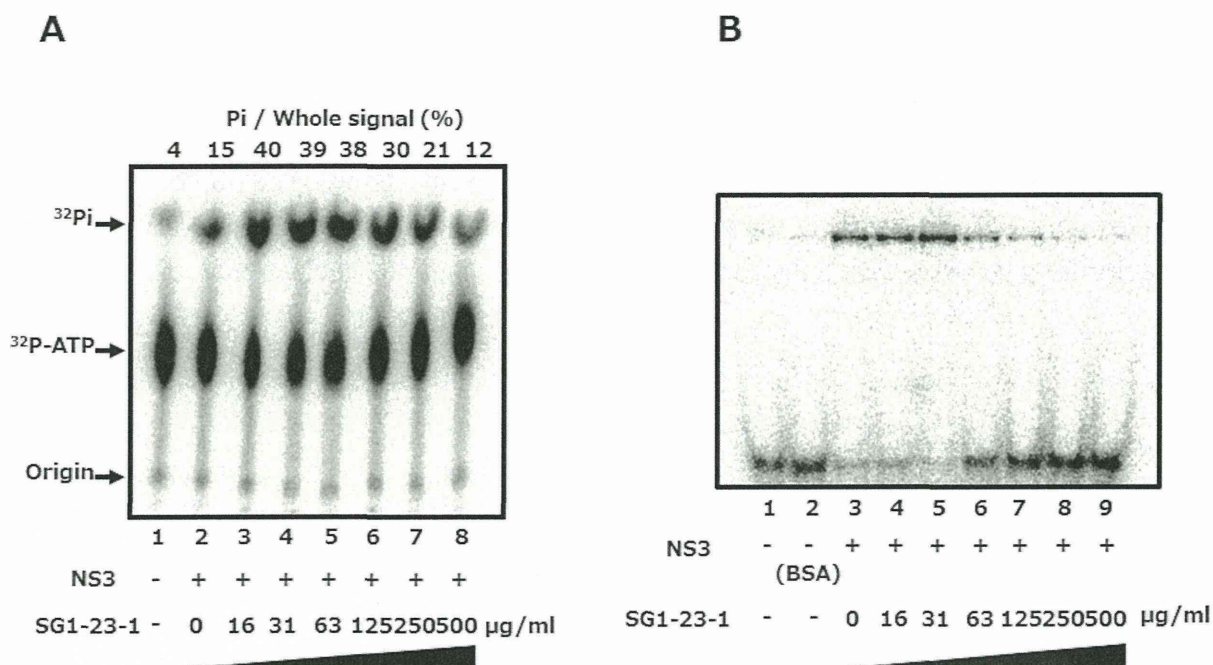


2.2. Effect of SG1-23-1 on HCV NS3 ATPase and RNA Binding Activities

The unwinding ability of HCV helicase is dependent on ATP binding, ATP hydrolysis, and RNA binding [8,9]. We examined the effect of SG1-23-1 on the ATPase activity of NS3 helicase. The ratio of free phosphate ($^{32}\text{P-Pi}$) in ATP ($^{32}\text{P-ATP}$) was measured in the presence of SG1-23-1. The reaction

was carried out between 16 and 500 μg of SG1-23-1 per milliliter. ATPase activity was slightly increased at 16 μg SG1-23-1 per milliliter and slightly decreased at 500 μg SG1-23-1 per milliliter (Figure 4A). However, the helicase activity was decreased to less than 10% in the presence of 50 μg of SG1-23-1 per milliliter (Figure 3A,B). Next, we examined the effect of SG1-23-1 on the binding of NS3 helicase to single-strand RNA (ssRNA). A gel-mobility shift assay was employed to estimate the binding activity of NS3 to 21 mer of ssRNA. The binding of NS3 to ssRNA was inhibited with SG1-23-1 in a dose-dependent manner (Figure 4B). These results suggest that SG1-23-1 contains the compound that inhibits RNA binding to NS3 helicase.

Figure 4. Effect of SG1-23-1 on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ - ^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as “Origin”, “ ^{32}P -ATP”, and “ ^{32}P -Pi”, respectively, on the left side of this figure. The data represent three independent experiments. (B) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out at the indicated concentration of SG1-23-1. The reaction mixture was subjected to gel mobility shift assay. The data represent three independent experiments.



2.3. Effect of SG1-23-1 on HCV RNA Replication in HCV 1b Replicon Cells

We investigated the effect of SG1-23-1 on both viral replication and growth of the replicon cell lines. The cell lines possess viral subgenomic RNAs derived from three genotype 1b strains (strains N [33], Con1 [34], and O [35]) or a full genomic RNA derived from the O strain [35]. Each cell line was treated with various concentrations of SG1-23-1. The treated cells were harvested 72 h post-treatment. Treatment with SG1-23-1 suppressed HCV RNA replications of all cell lines in a dose-dependent manner irrespectively of full- and sub-genome replicons; it exhibited no effect below 25 $\mu\text{g}/\text{mL}$ and

little effect on cellular viability at the highest concentration, 50 $\mu\text{g}/\text{mL}$ (Figure 5C,D). Both HCV NS3 and NS5A were decreased at the protein level in a dose-dependent manner, corresponding to the viral replication, but beta-actin was not changed in the cell line harboring subgenome replicon RNA of the Con1 strain (Figure 5E).

The inhibitory effect of SG1-23-1 on HCV replication is summarized in Table 2. The inhibitory effects on the HCV replication of the subgenome replicon derived from Con1, O, and N strains were 22.9 ± 0.4 , 19.9 ± 1.8 , and 44.2 ± 1.5 $\mu\text{g}/\text{mL}$, respectively, as EC_{50} ; and 48.1 ± 1.5 , 48.5 ± 0.3 , and >50 $\mu\text{g}/\text{mL}$, respectively, as EC_{90} . Treatment with SG1-23-1 inhibited the replication of the subgenome replicon of the O strain (EC_{50} : 19.9 ± 1.8 $\mu\text{g}/\text{mL}$; EC_{90} : 48.5 ± 0.3 $\mu\text{g}/\text{mL}$) at a more potent level than the replication of the full genomic replicon of the O strain (EC_{50} : 39.5 ± 0.8 $\mu\text{g}/\text{mL}$; EC_{90} : >50 $\mu\text{g}/\text{mL}$). When luciferase of firefly or *Renilla* was expressed under the control of the EF promoter, neither showed a significant change in activity in the presence of SG1-23-1 (Figure 5F). The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (luciferase and drug-resistant genes), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV in that order [33–35]. The replicon RNA replicated autonomously in several HCV replication-permissive cell lines derived from several hepatoma cell lines. Nonstructural proteins in replicon cells were polycistronically translated through EMCV IRES. The cap-dependent translated mRNA, including *Renilla* luciferase, EMCV IRES, and the firefly luciferase/neomycin-resistant gene in that order, was constructed to examine the effect of the extract on EMCV-IRES-dependent translation (Figure 5G). When the expression of the mRNA was transcribed by an EF promoter of the transfected plasmid in the presence of SG1-23-1, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed, suggesting that treatment with SG1-23-1 exhibited no effect on EMCV-IRES-dependent translation (Figure 5H). Thus, the inhibitory effect of SG1-23-1 on the luciferase activity must correspond to the replication efficiency of the replicon RNA but not to the inhibition of luciferase activity or the inhibition of EMCV-IRES-dependent translation. The inhibitory effect of the extract on the viral replication is similar to that of the extract on the helicase activity with respect to the values of IC_{50} and EC_{50} (Figure 3A and Table 2). These results suggest that treatment with SG1-23-1 inhibits HCV replication in a manner similar to that of the inhibitory effect on NS3 helicase activity.

Figure 5. Effect of SG1-23-1 on viral replication in replicon cell lines. (A–D) Huh7 Lunet/Con1 LUN Sb #26 (A), Huh7 rep Feo (B), Huh7#94/ORN3-5B#24 (C), and OR6 (D) cell lines were incubated in medium containing various concentrations of SG1-23-1. Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (E) Protein extract was prepared from Huh7 Lunet/Con1 LUN Sb #26 cells treated for 72 h with an indicated concentration of SG1-23-1 and then was subjected to Western blotting using antibodies to NS3, NS5A, and beta-actin. (F) Huh7 cell line transfected with pEF Fluc IN vector or pEF Rluc IN was established in the presence of G418. Both cell lines were incubated without (control) and with 50 $\mu\text{g}/\text{mL}$ SG1-23-1. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data represent three

independent experiments. (G) Schematic structure of the plasmid, pEF RLuc EMCV IRES Feo. The bicistronic gene is transcribed under the control of elongation factor 1 α (EF1 α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (FLuc) and neomycin phosphotransferase (Neo^r), and is translated under the control of the EMCV IRES. (H) Huh7 cell line transfected with pEF RLuc EMCV IRES Feo was established in the presence of G418. The cells were incubated for 72 h without (control) and with 50 μ g/mL of SG1-23-1. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments.

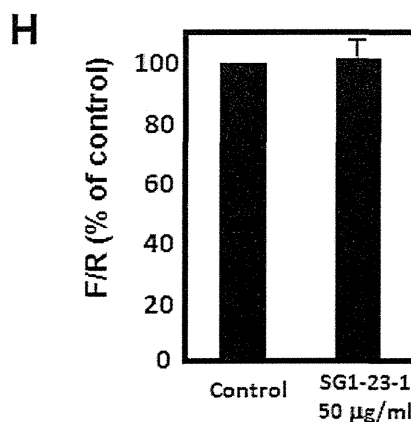
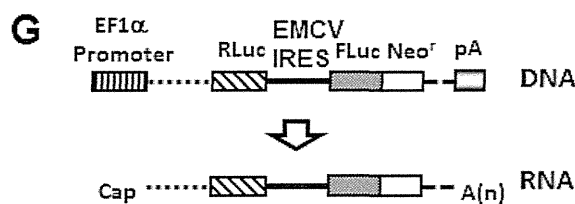
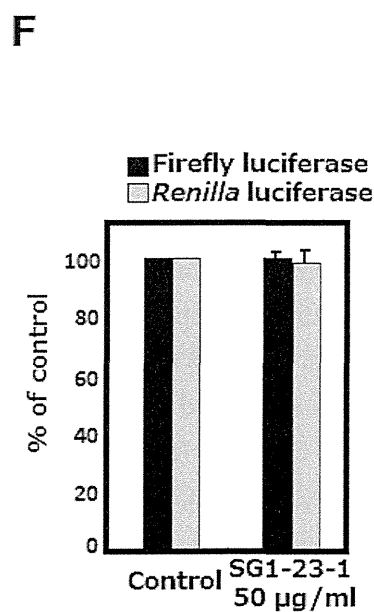
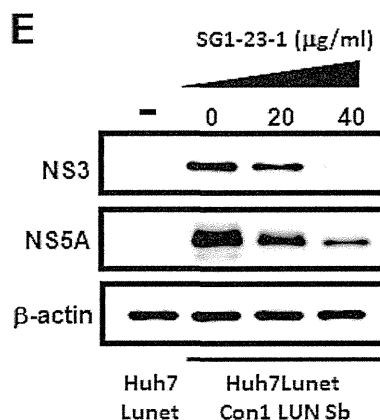
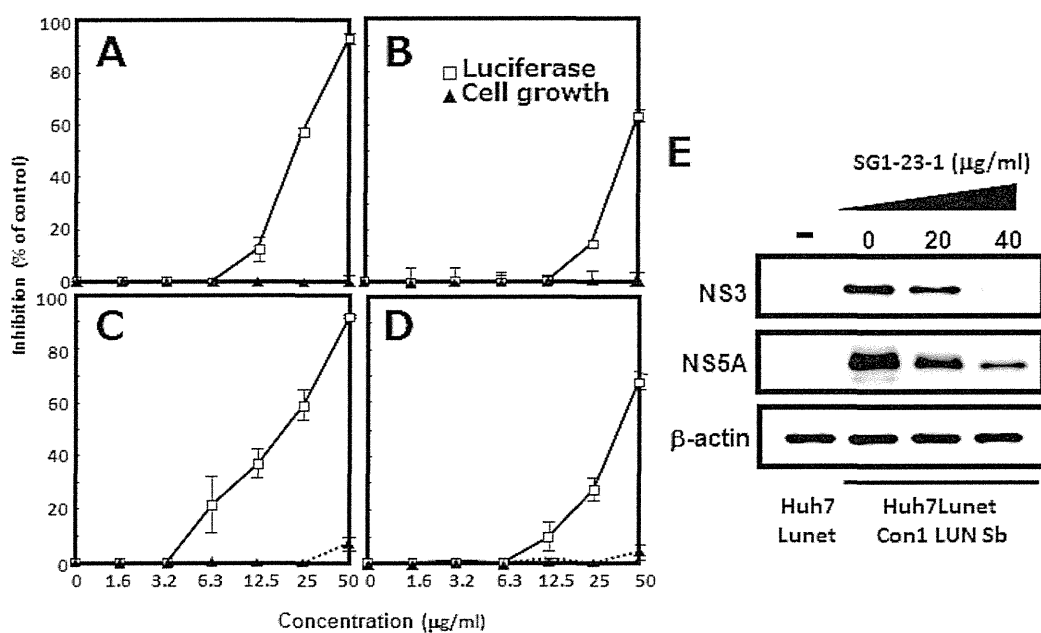


Table 2. Anti-HCV activity of SG1-23-1 in different replicon cell lines of genotype 1b.

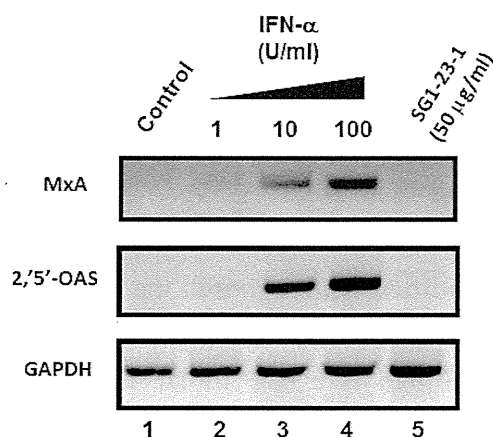
Replicon Cell Line	Virus Strain (Genotype 1b)	EC ₅₀ ^a (µg/mL)	EC ₉₀ ^b (µg/mL)	CC ₅₀ ^c (µg/mL)
<u>Subgenome</u>				
Huh7 Lunet/ Con1 LUN Sb #26	Con1	22.9 ± 0.4	48.1 ± 1.5	>50
Huh7 rep Feo	N	44.2 ± 1.5	>50	>50
Hu7#94/ORN3-5B#24	O	19.9 ± 1.8	48.8 ± 0.3	>50
<u>Full genome</u>				
OR6	O	39.5 ± 0.8	>50	>50

All data represent means ± standard deviation for three independent experiments; ^a Fifty percent effective concentration based on the inhibition of HCV replication; ^b Ninety percent effective concentration based on the inhibition of HCV replication; ^c Fifty percent cytotoxicity concentration based on the reduction of cell viability.

2.4. Effect of SG1-23-1 on the Interferon (IFN) Signaling Pathway

It has been reported that the HCV replication in cultured cells is potently inhibited by interferon (IFN) [36,37]. We examined whether or not treatment with SG1-23-1 induces interferon from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 50 µg of SG1-23-1 per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible genes, MxA and 2',5'-OAS, were induced with IFN-alpha 2b but not with SG1-23-1 (Figure 6). These results suggest that the inhibitory effect of SG1-23-1 on the replication of the HCV replicon is independent of the IFN signaling pathway.

Figure 6. Effect of SG1-23-1 on interferon signaling pathway. Huh7 Lunet/Con1 LUN Sb #26 cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 50 µg/mL SG1-23-1 (lane 5) for 48 h. The mRNAs of MxA, 2',5'-OAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse-transcription polymerase chain reaction (RT-PCR). Error bars indicate standard deviation. The data represent three independent experiments.



Treatment with SG1-23-1 suppressed the helicase activity of NS3 in a dose-dependent manner and exhibited an IC_{50} of 11.7 $\mu\text{g/mL}$. Interestingly, treatment with SG1-23-1 inhibited the RNA binding activity of the helicase but not the ATPase activity of NS3. Treatment with SG1-23-1 inhibited the luciferase activity corresponding to the HCV replication in the replicon cell lines, but not the enzymatic activity of luciferase or the translational activity of EMCV IRES, suggesting that treatment with SG1-23-1 decreases HCV replication. Figure 4 shows that the viral proteins NS3 and NS5A in replicon cells were decreased by treatment with SG1-23-1, supporting the notion that SG1-23-1 inhibits HCV replication but not the enzymatic activity of luciferase. The inhibition of cell growth would not contribute to the inhibition of HCV replication by SG1-23-1 (Figure 3 and Table 2). Treatment with SG1-23-1 did not induce the interferon-stimulated genes in the replicon cell lines (Figure 6), suggesting that inhibition of HCV replication by treatment with SG1-23-1 is not due to interferon induction or interferon signaling. The extract SG1-23-1 inhibited the HCV replicon with an EC_{50} of 22 to 44 $\mu\text{g/mL}$, which is similar to the value of IC_{50} . These results suggest that the anti-HCV compound(s) included in *A. polycladia* can suppress viral replication by inhibiting NS3 helicase activity.

3. Experimental Section

3.1. Preparation of Extracts from Marine Organisms

All marine organisms used in this study were collected by hand during scuba diving off Shimoji, Okinawa, Chibishi, Kuro, Kume, and Tokashiki Islands in Okinawa Prefecture, Japan. In the case of OK-99-tagged extract, a specimen was soaked in ethanol. The ethanol-soluble fraction was concentrated, and the resulting aqueous material was suspended in ethyl acetate (EtOAc). The organic fraction was used for screening.

Each specimen from Kume was soaked in ethanol. The ethanol-soluble fraction was concentrated. The resulting material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG1 and the last digit of “1”. The water layer was concentrated to dryness and suspended in methanol (MeOH). The MeOH-soluble fraction was used for screening and tagged with SG1 and the last digit of “2”.

Each specimen from Tokashiki was extracted three times with acetone. After removal of acetone from the solution, the residual material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG3.

All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

3.2. High-Throughput Screening of NS3 Helicase Inhibitors

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [38] and was slightly modified with regard to the reaction mixture. A schematic diagram of the PET assay for HCV NS3 helicase activity is shown in Figure 1. The double-strand RNA was prepared as a substrate by annealing, at a 1:2 molar ratio, a 5' BODIPY FL-labeled 37-mer (5'-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUUU-3') to a 23-mer (GGUUAUGAG GGUGGAGGUAUAG). When unwound by HCV NS3 helicase, the unlabeled ssRNA was captured by a DNA capture strand (5'-CTATTACCTCCACCCTCATAACC-3'). A fluorescent-dye-labeled