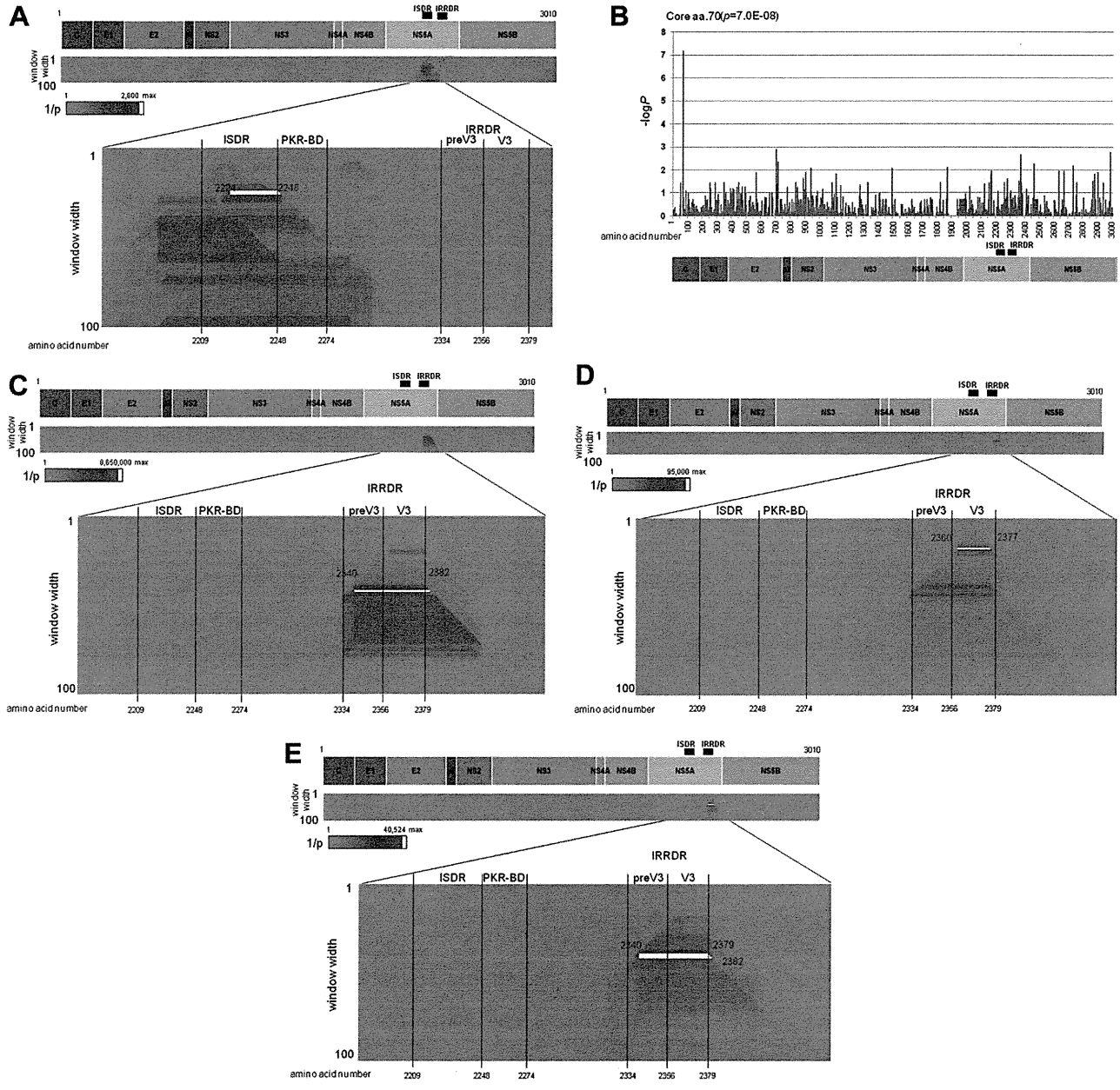

Fig. 1. The contribution of viral sequences and IL28B SNPs in the treatment response to Peg-IFN/RBV was studied. (A) Sliding-window analysis for RVR versus the remainder (n = 103). (B) Single aa analysis for nEVR versus the remainder (n = 103). (C) Sliding-window analysis for SVR versus non-SVR (n = 76). (D) Sliding-window analysis for relapsers versus nonrelapsers among ETR (n = 57). (E) Sliding-window analysis for SVR versus non-SVR in IL28B TT patients with standard therapy (n = 47).

in the HCV ORF, including core aa.70 and NS5B (data not shown). However, a sliding-window analysis disclosed that NS5A region aa.2340 to aa.2382, the region almost coinciding with IRRDR, was extracted as the most clearly related to the final outcome (P = 1.2E-07; Fig. 1C).

HCV Sequences Related to Relapse. To identify the viral regions related to relapse, we compared SVR patients and non-SVR patients among 57 patients with standard therapy achieving ETR (40 nonrelapsers and 17 relapsers). A sliding-window analysis disclosed that the NS5A region aa.2360 to aa.2377, the region almost coinciding with the V3 region in the IRRDR, could be extracted as the most strongly related to relapse (P = 1.1E-05; Fig. 1D).

Uni- and Multivariate Analyses. We performed further analyses to extract the factors associated with RVR, nEVR, SVR, and relapse by univariate, as well as multivariate, analyses. For achieving RVR, ISDR aa.2224-2248 and HCV-RNA were extracted as independent variables (Table 3). Because all the RVR patients possessed IL28B TT alleles and OR

Table 3. Factors Associated With RVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	0.7	0.18-2.59	0.57			
Gender	Male	1.5	0.36-6.07	0.59			
ISDR 2224-2248	1≤	24.6	4.70-129	8.5E-07†	14.7	1.10-198	0.04‡
IRRDR 2340-2382	4≤	6.2	0.76-51.1	0.06			
Core 70	Arg	0.7	0.18-3.07	0.68			
Fibrosis	<2	3.6	0.72-17.8	0.10			
HCV RNA	<600 k/Ul/mL	74.7	8.55-653	8.3E-10†	51.2	3.97-662	0.003†
BMI	<23	1.3	0.34-4.87	0.71			
ALB	4.1 g/dL≤	1.1	0.30-4.28	0.85			
γ-GTP	50 IU/mL≤	0.9	0.24-3.49	0.91			
ALT	60 IU/mL<	0.9	0.25-3.59	0.94			
T-Cho	<170 mg/dL	1.2	0.33-4.67	0.76			
WBC	4,700/μL≤	1.9	0.47-7.89	0.36			
Hb	14 g/dL≤	1.5	0.37-6.35	0.55			
PLT	150,000/μL≤	1.8	0.48-6.88	0.37			
AFP	10 ng/mL≤	0.3	0.03-2.37	0.22			
Peg-IFN dose (%)	80≤	1.3	0.33-5.55	0.68			
RBV dose (%)	80≤	3.0	0.79-11.4	0.09			

Because all RVR patients possessed IL28B TT alleles and OR calculation was impossible, IL28B SNPs were secluded from analysis.
Abbreviation: WBC, white blood cell count.
*$n = 103$.
†$P < 0.01$.
‡$P < 0.05$.

calculation was impossible, IL28B SNPs were excluded from the analysis. Likewise, core aa.70 and IL28B were extracted as independent variables associated with nEVR (Table 4). In performing the analysis for SVR and relapse, we excluded patients with extended length of therapy to standardize the treatment periods. Because this restriction reduced the number of available patients for the analysis, we included 30 additional patients (Supporting Table 1) with available clinical information, including HCV core, NS5A, and

Table 4. Factors Associated with nEVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	1.18	0.42-3.30	0.75			
Gender	Male	0.86	0.31-2.38	0.77			
ISDR 2224-2248	1≤	0.97	0.29-3.28	0.96			
IRRDR 2340-2382	4≤	0.25	0.09-0.69	5.0E-03‡	0.21	0.03-1.33	0.1
Core 70	Arg	0.03	0.01-0.16	2.0E-08‡	0.04	0.00-0.04	0.008‡
IL28B†	Major allele	0.05	0.01-0.17	5.4E-08‡	0.1	0.01-0.57	0.011§
Fibrosis	<2	0.28	0.08-1.0	0.04§	0.5	0.03-0.57	0.55
HCV RNA	<600 k/Ul/mL	0.19	0.02-1.5	0.08			
BMI	<23	0.97	0.36-2.58	0.95			
ALB	4.1 g/dL≤	0.69	0.26-1.85	0.46			
γ-GTP	50 IU/mL≤	1.95	0.73-5.22	0.18			
ALT	60 IU/mL<	0.38	0.14-1.03	0.05			
T-Cho	<170 mg/dL	0.34	0.11-1.03	0.06			
WBC	4,700/μL≤	0.64	0.23-1.76	0.38			
Hb	14 g/dL≤	0.82	0.29-2.26	0.70			
PLT	150,000/μL≤	0.42	0.15-1.19	0.10			
AFP	10 ng/mL≤	5.12	1.82-14.4	0.001‡	3.5	0.52-23.2	0.20
Peg-IFN dose (%)	80≤	0.37	0.14-1.01	0.048§	0.9	0.13-5.93	0.89
RBV dose (%)	80≤	0.38	0.12-1.23	0.10			

Abbreviation: WBC, white blood cell count.
*n = 103.
†n = 89.
‡$P < 0.01$.
§$P < 0.05$.

Table 5. Factors Associated With SVR Analyzed by Uni- and Multivariate Logistic Regression Analysis*

		Univariate			Multivariate		
		OR	95% CI	*P* Value	OR	95% CI	*P* Value
Age, years	60≤	0.8	0.34-1.78	0.55			
Gender	Male	1.4	0.61-3.22	0.43			
ISDR 2224-2248	1≤	6.3	1.98-20.26	0.001†	13.4	1.86-96.5	0.010†
IRRDR 2340-2382	4≤	11.1	4.07-30.54	4.08E-07‡	13.8	3.31-57.4	0.0003‡
Core 70	Arg	3.2	1.37-7.59	0.007‡	2.2	0.43-11.7	0.34
IL28B	Major allele	9.6	2.92-31.34	0.00003‡	16.8	2.04-139	0.009‡
Fibrosis	<2	3.1	1.33-7.23	0.008‡	1.4	0.31-6.64	0.65
HCV RNA	<600 k/UI/mL	3.5	1.39-9.02	0.007‡	3.5	0.72-17.3	0.12
BMI	<23	1.0	0.44-2.20	0.97			
ALB	4.1 g/dL≤	0.9	0.39-1.96	0.75			
γ-GTP	<50 IU/mL	2.6	1.13-5.88	0.02†	3.5	0.90-13.47	0.07
ALT	≤60 IU/mL	0.8	0.35-1.77	0.57			
T-Cho	<170 mg/dL	1.7	0.71-3.94	0.24			
WBC	<4,700/μL	0.8	0.36-1.87	0.64			
Hb	<14 g/dL	0.9	0.35-2.13	0.75			
PLT	150,000/μL≤	2.6	1.06-6.56	0.03†	3.5	0.71-16.8	0.20
AFP	<10 ng/mL	3.7	1.49-9.29	0.004‡	3.4	0.54-21.2	0.20
Peg-IFN dose (%)	80≤	2.2	0.96-5.13	0.06			
RBV dose (%)	80≤	0.8	0.37-1.92	0.68			

Abbreviation: WBC, white blood cell count.
*n = 97.
†$P < 0.05$.
‡$P < 0.01$.

IL28B SNPs. Those 30 patients were consecutively introduced the Peg-IFN/RBV therapy at Yamanashi University Hospital in succession to the initial 103 patients. As a result, 97 patients were available for SVR analysis, and 78 patients were available for relapse analysis. ISDR aa.2224-2248, IRRDR aa.2340-2382, and IL28B SNPs were extracted as the independent variables affecting SVR (Table 5). On the other hand, IRRDR-V3 aa.2360-2377 was extracted as an independent factor for relapse (Supporting Table 2).

Contribution of IL28B SNPs and NS5A aa.2340-2382 in Determining Treatment Response. Because multivariate analysis finally extracted IL28B SNPs and IRRDR aa.2340-2382 as the two most-significant variables determining final outcome, the correlation of IL28B SNPs and IRRDR aa.2340-2382 in association with final outcome was further investigated. Alignment of IRRDR aa.2340-2382 in association with SVR was demonstrated (Fig. 2). By this analysis, it was evident that three or more mutations in IRRDR aa.2340-2382 were significantly associated with SVR. Last, to disclose the viral sequence contribution in the determination of final outcome in IL28B TT haplotype patients with the standard therapy (n = 47), sliding-window analysis was performed (Fig. 1E). As demonstrated here, NS5A IRRDR aa.2340-2379 (~2382) was finally extracted as the most-significant viral region contributing to final outcome ($P = 2.47E-05$).

The contribution of these three viral regions in the phase-specific treatment responses is schematically illustrated (Fig. 3).

Discussion

In this study, we determined 103 complete HCV ORF sequences in consecutive Japanese patients, infected with genotype 1b HCV and given PEG-IFN/RBV therapy, and systematically searched and investigated the contribution of viral regions associated with the phase-specific treatment responses with IL28B SNP haplotypes. To our knowledge, this study is most comprehensive in the following aspects: (1) complete HCV ORF studied with the largest analyzed number of patients; (2) analyzed according to viral kinetics closely related to outcome; (3) unified to a single genotype (1b); (4) unified background of patients; (5) introduction of a sliding-window method to screen the responsible viral regions systematically; and (6) analysis of IL28B SNPs.

In a recent randomized, controlled study of Peg-IFN/RBV combination therapy, the status of patients according to response to Peg-IFN/RBV therapy at 12 weeks showed a marked correlation with final outcome, and viral response at week 12 has been considered as a useful predictor in early-response–guided therapy.[26] In agreement with the previous study, virological responses to Peg-IFN/RBV at week 12 had a

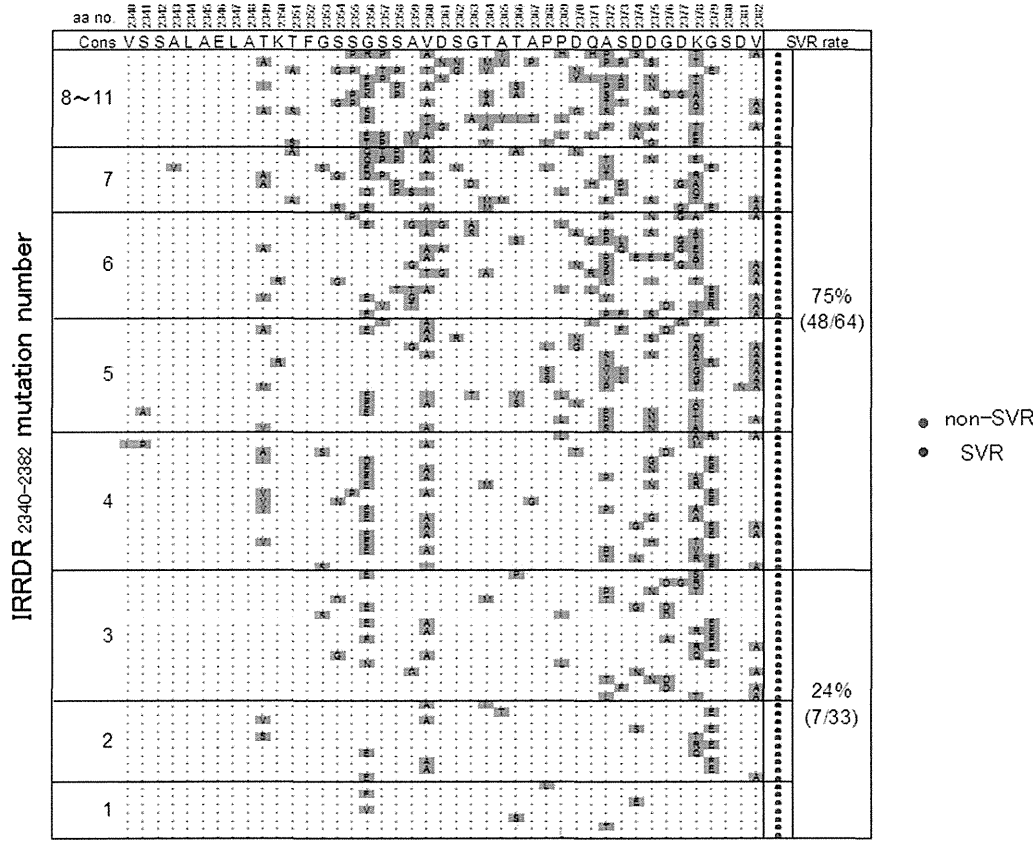

Fig. 2. Alignment of NS5A region around IRRDR aa.2340-2382, along with SVR.

distinct correlation with the final outcomes in our study group (SVR rate: 100%, 80%, 20%, and 0% for RVR, cEVR, pEVR, and nEVR in standard therapy). These results demonstrated that classification by viral response at week 12 provides distinct groups with different characteristics.

We first tried to identify regions of the HCV ORF by showing a distinct linkage to RVR and nEVR. We found that HCV substitutions around the ISDR (aa.2224-2248 in RVR) were most significantly correlated with early viral clearance in Peg-IFN/RBV therapy. In contrast, core aa.70 substitution was most significantly correlated with nEVR, demonstrating the association with treatment resistance. According to the results shown here, early HCV dynamics in Peg-IFN/RBV therapy are significantly regulated by the specific viral sequences in core and NS5A (Fig. 1A,B).

Next, we determined that HCV genomic region correlated with SVR of patients with standard therapy. We excluded patients with extended therapy to unify treatment duration. Considering the length of treatment, we first suspected that multiple factors might affect the final outcome of 48 weeks of standard therapy, and that determining viral regions reflecting pure

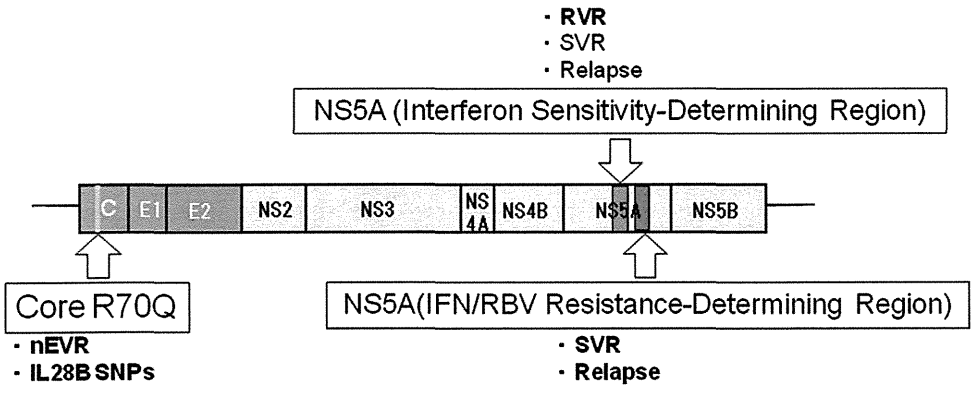

Fig. 3. Roles of three HCV-1b viral regions in the determination of time-dependent treatment response to Peg-IFN/RBV therapy.

biological response would be difficult. Contrary to our prediction, a region almost identical to the IRRDR (aa.2340-2382) was extracted by systematic sliding analysis as correlated with outcome, with a significantly high *P* value, demonstrating the remarkable influence of the IRRDR aa.2340-2382 in determining final outcome (Fig. 1C). Importantly, in addition to final outcome, when relapser and nonrelapser in the ETR were compared, aa.2360-2377, the region almost coinciding with the V3 region of the IRRDR, was extracted as the region discriminating these two groups (Fig. 1D).

In the analysis of IL28B SNPs (rs8099917), we observed a significant correlation between IL28B SNP and viral dynamics at week 12; patients with minor/minor or minor/major alleles showed significantly poor responses, as demonstrated in Table 2. On the other hand, because poor response was significantly associated with the substitution of the core aa.70 (as shown in Fig. 1B) in our study, we next tried to unveil the correlation between HCV ORF and IL28B SNPs. The significant link with the single core aa.70 substitution was observed through searching for the complete HCV ORFs (Supporting Fig. 1). The result coincides with recent studies[27-29] and, moreover, confirms that this single spot is extraordinarily linked to the initial poor response among the complete 3,010 HCV aa residues. Though the underlying mechanism for the association of IL28B and core aa.70 is unclear, the association would be a reflection of an interaction between the IL28B SNPs and HCV sequences in the development of chronic HCV infection, as discussed by Kurosaki et al.[29] Namely, it is possible that HCV sequences within the patient might have been selected during the course of chronic infection, depending on the IL28B SNPs, by selective pressures of unknown mechanism.

By multivariate analysis, IL28B SNP, IRRDR aa.2340-2382, and ISDR aa.2224-2248 were extracted as independent variables related to final outcome in patients with standard length of therapy with the inclusion of an additional 30 patients (Table 5). Among these, IL28B SNPs and IRRDR aa.2340-2382 were the two most-significant variables determining final outcome. Moreover, NS5A IRRDR aa.2340-2379 (~2382) was the most-significant viral region contributing to final outcome in patients with IL28B TT haplotype ($P = 2.47E-05$), demonstrating that combined information of the IL28B and IRRDR is significantly important in predicting viral kinetics and treatment outcome (Fig. 1D).

Most of the viral genomic regions identified in this study have already been reported on in previous, independent studies. However, the importance of our study is shown in the result that these specific viral regions of core, ISDR, and IRRDR were extracted all at once through systematic full HCV ORF sequence screening. What is unique in our study is the introduction of the sliding-window analysis; through this analysis, we could effectively confine viral regions of ISDR and IRRDR that were not identified in other previous HCV ORF studies.[21,22] Furthermore, our study also disclosed that the importance of these viral regions was different according to each treatment-phase; RVR, nEVR, SVR, and relapse were mostly related to the ISDR, core aa.70, the IRRDR, and IRRDR, respectively. The ISDR was the first region identified as being related to SVR in the era of IFN monotherapy in Japanese patients, such that multiple mutations in the ISDR were associated with favorable IFN responses.[10,30] The contribution of the core region in treatment response in IFN/RBV therapy was first reported on by Akuta et al., in that the polymorphisms of core aa.70 and 91 were closely related to final outcome.[20] The further significance of core polymorphism was reported on in hepatocarcinogenesis as well.[31,32] Our analysis also confirmed the recent studies reporting on the close correlation between viral core and IL28B SNPs.[11,29,32] The present finding that the core aa.70 is correlated with nEVR independently of IL28B seems to reflect the recent report that core aa.70 is an independent determinant of poor response to the triple therapy of Peg-IFN/RBV and telaprevir in patients with the IL28B minor allele.[27] On the other hand, the IRRDR was originally reported on by El-Shamy et al. as being related to the result of Peg-IFN/RBV therapy.[15] Importantly, our study revealed that final SVR and relapse were significantly correlated with mutations around the IRRDR. The result indicates its significant role in late-phase viral responses in Peg-IFN/RBV therapy.

Core is a main-component protein of viral nucleocapsid, and it has recently been found that the core located on the surface of lipid droplets associates with NS5A to facilitate virion formation.[33] HCV-JFH1 with core R70Q/H and L91M was reported to impair virion formation resulting in the accumulation of intracellular core protein, which causes endoplasmic reticulum stress leading to IFN resistance through suppressor of cytokine signaling 3 up-regulation induced by IL-6.[34] NS5A is a phosphoprotein and is considered to play a pivotal role both in viral replication and virion production, depending on its phosphorylation state.[35-37] Mutations in centrally located serine residues required for NS5A hyperphosphorylation as well as in

its adjacently located ISDR work as adaptive mutations in the HCV replicon, possibly through decreasing the hyperphosphorylated form of NS5A,[37-40] which seems to control HCV replication. The conservation of c-terminal serine residual cluster of NS5A, downstream to IRRDR, is required for NS5A basal phosphorylation, interaction with the core protein on the lipid droplet, and thus virion formation.[41,42] Taken together, it can be speculated that the structural changes in core and NS5A protein can coordinately modify HCV replication, especially through virion formation around lipid droplets. However, the precise mechanism through which these modulations of viral proteins lead to the different treatment response should be further investigated.

In conclusion, we have found that polymorphic viral sequences in core aa.70, NS5A-ISDR aa.2224-2248, and NS5A-IRRDR aa.2340-2382 in genotype 1b HCV infection are correlated significantly with the treatment phase-specific viral responses to Peg-IFN/RBV therapy. In addition, these viral responses were also significantly correlated with the polymorphism in IL28B SNP, and this polymorphism was significantly correlated with the polymorphism in the core. More important, combined information of IL28B and IRRDR aa.2340-2382 is significantly important in predicting viral kinetics and treatment outcome. We consider that our comprehensive study provides a new basis for introducing Peg-IFN/RBV therapy as well as a new generation of anti-HCV therapies.

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Mar. Drugs **2012**, *10*, 744-761; doi:10.3390/md10040744

OPEN ACCESS

Marine Drugs

ISSN 1660-3397

www.mdpi.com/journal/marinedrugs

Article

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

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Received: 9 February 2012; in revised form: 17 March 2012 / Accepted: 19 March 2012 / Published: 28 March 2012

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 IC_{50} 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 EC_{50} value of 23 to 44 μg/mL, which is similar to the IC_{50} 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 ± 0.7 μg/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 μg/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 (% of control)	Specimen	Phylum	Extract	Collection Site
OK-99-2	78	*Agelas* sp.	Porifera	EtOAc	Shimoji Island
OK-99-3	73	*Plakortis* sp.	Porifera	EtOAc	Shimoji Island
OK-99-4	60	*Dysidea arenaria*	Porifera	EtOAc	Shimoji Island
OK-99-5	96	*Theonella cupola*	Porifera	EtOAc	Shimoji Island
OK-99-6	52	*Theonella conica*	Porifera	EtOAc	Shimoji Island
OK-99-7	85	*Epipolasis kushimotoensis*	Porifera	EtOAc	Shimoji Island
OK-99-9	51	*Hyrtios* sp.	Porifera	EtOAc	Shimoji Island

Table 1. *Cont.*

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

Table 1. *Cont.*

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

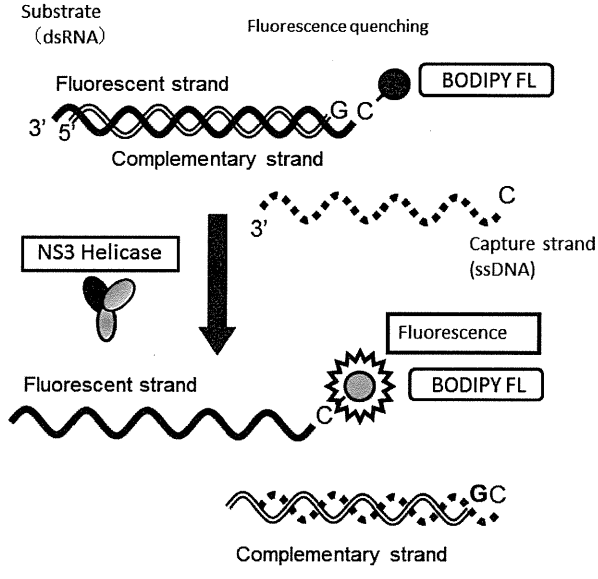


Figure 2. *Alloeocomatella polycladia* belongs to a class of feather star (Echinodermata, Crinoidea). The ethyl acetate fraction prepared from the marine organism was designated SG1-23-1 in this study.

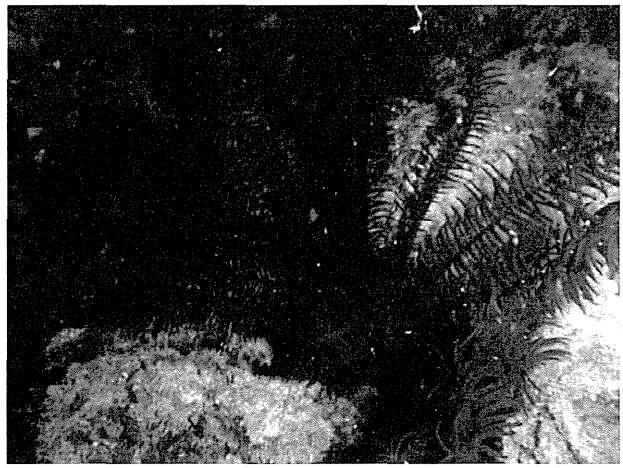
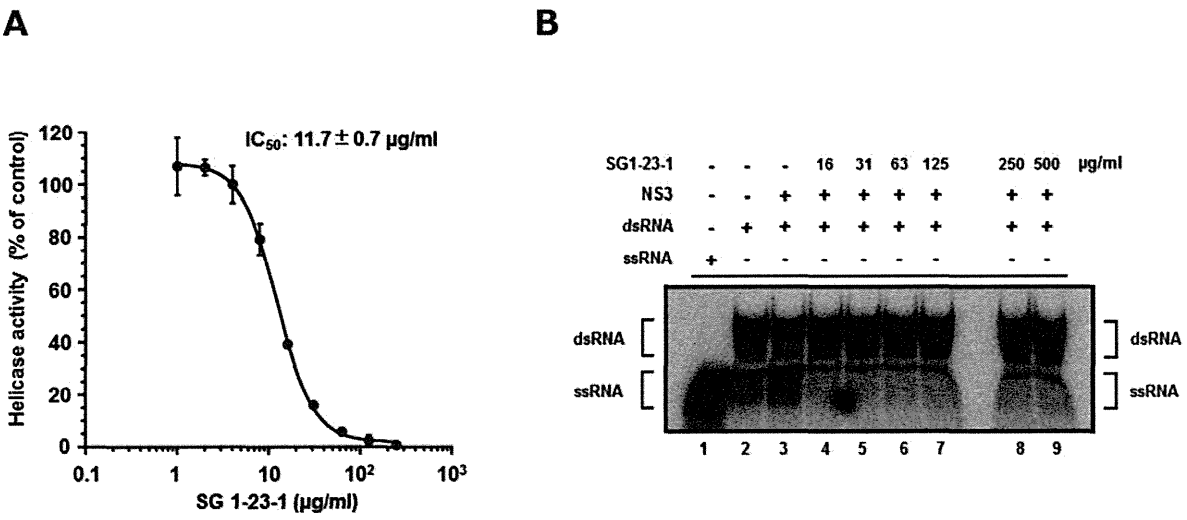


Figure 3. Effect of SG1-23-1 on the unwinding activity of NS3 helicase. (**A**) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of SG1-23-1. Helicase activity in the absence of SG1-23-1 was defined as 100% helicase activity. Each value represents 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.

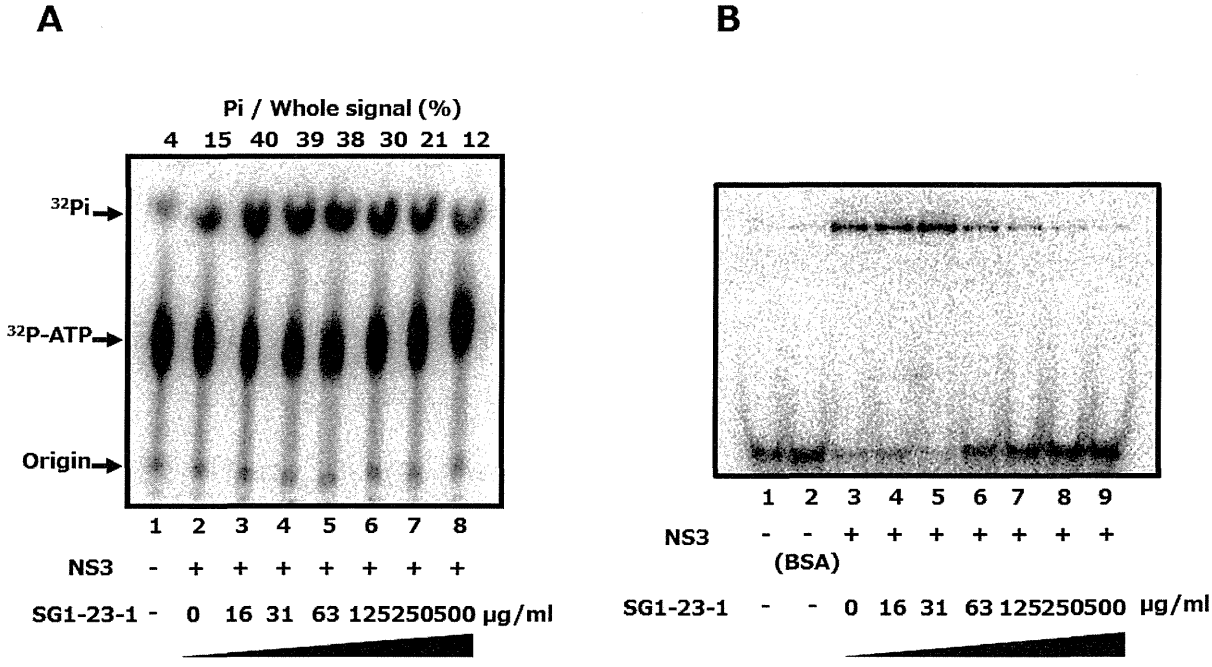


2.2. Effect of SG1-23-1 on HCV NS3 ATPase and RNA Binding Activities

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}P-Pi) in ATP (^{32}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 μg/mL and

little effect on cellular viability at the highest concentration, 50 μg/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 μg/mL, respectively, as EC_{50}; and 48.1 ± 1.5, 48.5 ± 0.3, and >50 μg/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 μg/mL; EC_{90}: 48.5 ± 0.3 μg/mL) at a more potent level than the replication of the full genomic replicon of the O strain (EC_{50}: 39.5 ± 0.8 μg/mL; EC_{90}: >50 μg/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 μg/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 1α (EF1α) 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 μg/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.

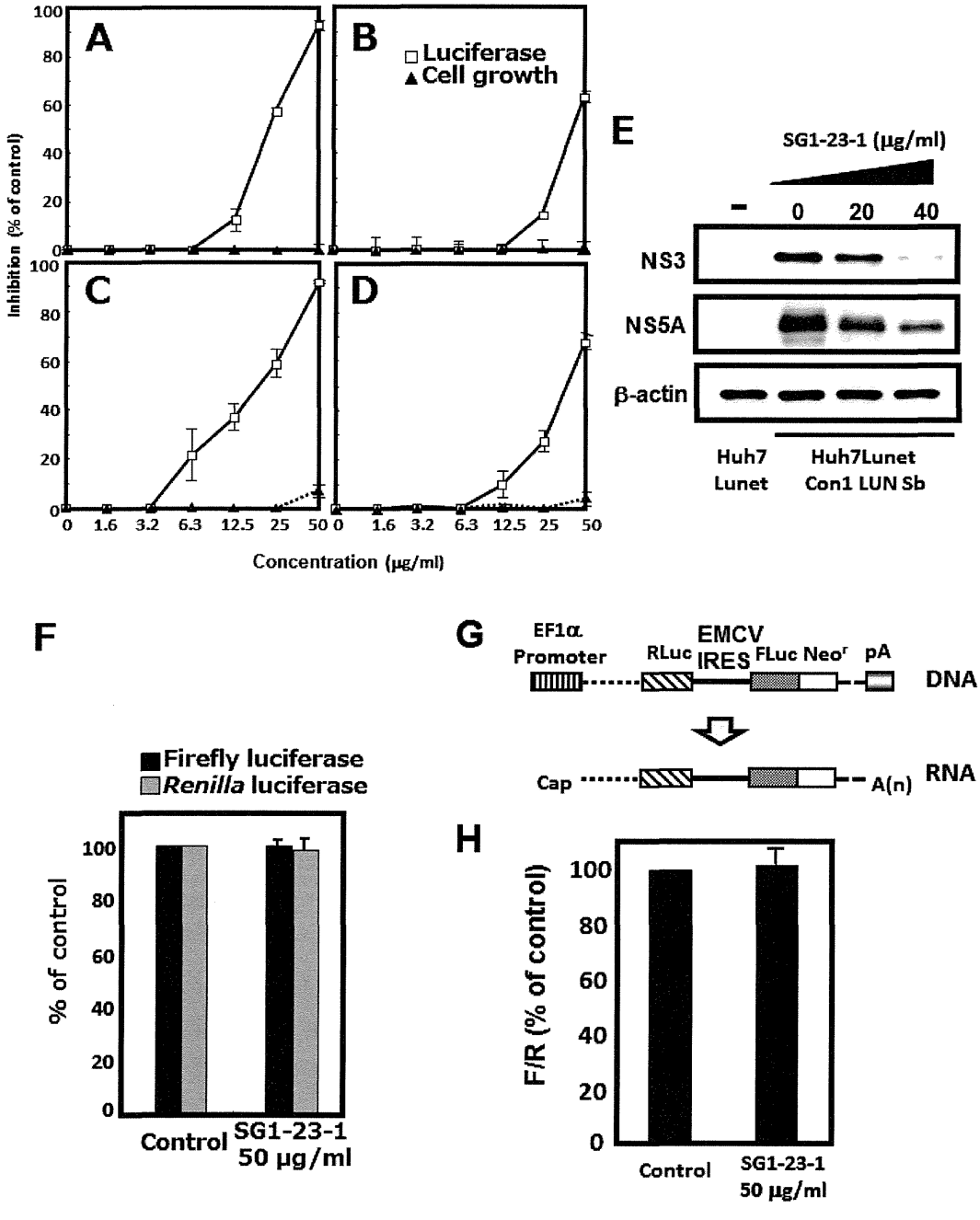


Table 2. Anti-HCV activity of SG1-23-1 in different replicon cell lines of genotype 1b.

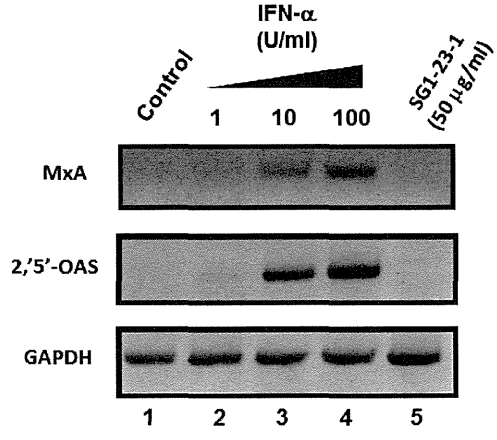
Replicon Cell Line	Virus Strain (Genotype 1b)	EC_{50} [a] (μg/mL)	EC_{90} [b] (μg/mL)	CC_{50} [c] (μg/mL)
Subgenome				
Huh7 Lunet/ Con1 LUN Sb #26	Con1	22.9 ± 0.4	48.1 ± 1.5	>50
Huh7 rep Feo	N	44.2 ± 1.5	>50	>50
Hu7#94/ORN3-5B#24	O	19.9 ± 1.8	48.8 ± 0.3	>50
Full genome				
OR6	O	39.5 ± 0.8	>50	>50

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 IFNa-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 μg/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 μg/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'-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUU-3') to a 23-mer (GGUUAUGAG GGUGGAGGUAAUAG). When unwound by HCV NS3 helicase, the unlabeled ssRNA was captured by a DNA capture strand (5'-CTATTACCTCCACCCTCATAACC-3'). A fluorescent-dye-labeled

oligonucleotide was purchased from J-Bio 21 Corporation. BODIPY FL was attached to the 5'-end via an aminohexylphosphate linker with a six-carbon spacer. Unlabeled oligonucleotides were purchased from Japan Bio Services Co., Ltd. The PET NS3 helicase assay was carried out in 22 μL of 25 mM MOPS-NaOH (pH 6.5) containing 3 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 4 U RNasin, 50 nM of the double-strand RNA described above, 100 nM DNA capture strand, 5 mM ATP, and the extract (25 μg/mL) and 240 nM HCV NS3 helicase. The reaction was started by the addition of HCV NS3 helicase. The reaction mixture was incubated at 37 °C for 30 min. The fluorescence intensity was recorded every 5 s until 5 min post-reaction, and then every 30 s between 5 and 30 min post-reaction by using a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The initial reaction velocity was calculated and represented as NS3 helicase activity.

3.3. ATPase Assay

NS3 ATPase activity was determined by the method of Gallinari *et al.* [39] with slight modifications. The reaction was carried out at 37 °C for 10 min in 10 μL of the reaction mixture containing 25 mM MOPS-NaOH (pH 7.0), 1 mM DTT, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM [γ-^{32}P] ATP (Muromachi, Tokyo, Japan), 300 nM NS3, and 0.1 μg poly (U) per microliter and an indicated concentration of SG1-23-1, and then was terminated by the addition of 15 microliters of 10 mM EDTA. Two microliters of the reaction mixture were spotted onto a polyethyleneimine cellulose sheet (Merck, Darmstadt, Germany) and then developed in 0.75 M LiCl/1 M formic acid solution at room temperature for 20 min. The sheet was air-dried completely and then exposed to an image plate. Radioactive bands were visualized with an Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software (version 3.11; Fujifilm: Tokyo, Japan, 2008).

3.4. RNA Helicase Assay

NS3 RNA helicase assay was carried out by the method of Gallinari *et al.* [39] with slight modifications. The substrate for annealing two complementary RNA oligonucleotides, 5'-AGAGAGAGAGGUUGAGAGAGAGAGUUUGAGAGAGAGAG-3' (40-mer, template strand) and 5'-CAAACUCUCUCUCUCAACAAAAAA-3' (26-mer, release strand) was purchased from Shanghai GenePharma Co., Ltd. The release strand was labeled at the 5'-end with [γ-^{32}P] ATP (Muromachi, Tokyo, Japan) using the T4 polynucleotide kinase (Toyobo, Osaka, Japan) at 37 °C for 60 min and then purified by phenol chloroform extraction. The template and the labeled release strands were annealed at a molar ratio of 3:1 (template: release), denatured at 80 °C for 5 min, and slowly renatured at 23 °C for 30 min in an annealing buffer consisting of 20 mM Tris-HCl (pH 8), 0.5 M NaCl, and 1 mM EDTA. The partial duplex RNA substrate was purified on a G-50 micro column (GE Healthcare, Uppsala, Sweden) and stored at −20 °C in H_2O containing 0.25 U of RNasin Plus (Promega, Madison, WI, USA) per microliter.

SG1-23-1 extract was added at various concentrations to a helicase reaction mixture consisting of 25 mM MOPS-NaOH (pH 7.0), 2.5 mM DTT, 2.5 U of RNasin Plus (Promega), 100 μg of BSA per milliliter, and 3 mM $MgCl_2$. The mixture was supplemented with 300 nM NS3 protein and 5 fM ^{32}P-labeled partial duplex RNA substrate. It was then preincubated at 23 °C for 15 min. After adding ATP at a final concentration of 5 mM, the reaction mixture (20 μL) was incubated at 37 °C for 30 min