counts. The lack of a significant association may reflect an indirect, rather than a direct, relationship between *ITPA* genotype and platelet physiology.

Tanaka et al. [12] showed that a *DDRGK1* SNP near the *ITPA* gene, like the *ITPA* SNP, was associated with treatment-induced anemia; moreover, the *DDRGK1* SNP was associated with treatment-induced thrombocytopenia during PEG-IFN/RBV combination therapy. Because a protective *DDRGK1* allele showed linkage with a protective *ITPA* allele in all the patients enrolled in the present study, the association between *DDRGK1* SNP and changes in platelet counts could not be further examined.

In conclusion, an association was found between *ITPA* SNP genotype and treatment-induced anemia during a 4-week course of RBV monotherapy. This RBV-induced anemia may have led to increases in endogenous serum EPO that, in turn, resulted in the stimulation of platelet production. However, the sample size in this study was small; therefore, further investigations are needed to elucidate the effects of RBV on hematopoietic parameters.

Acknowledgments We appreciate the technical advice given by ProfessorYasuhito Tanaka.

Conflict of interest Shuhei Hige has received a research grant from MSD. The other authors have declared that no conflict of interest exists.

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Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.

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Abstract

Combination therapy with ribavirin, interferon, and viral protease inhibitors could be expected to elicit a high level of sustained virologic response in patients infected with hepatitis C virus (HCV). However, several severe side effects of this combination therapy have been encountered in clinical trials. In order to develop more effective and safer anti-HCV compounds, we employed the replicon systems derived from several strains of HCV to screen 84 extracts from 54 organisms that were gathered from the sea surrounding Okinawa Prefecture, Japan. The ethyl acetate-soluble extract that was prepared from marine sponge *Amphimedon* sp. showed the highest inhibitory effect on viral replication, with EC_{50} values of 1.5 and 24.9 μg/ml in sub-genomic replicon cell lines derived from genotypes 1b and 2a, respectively. But the extract had no effect on interferon-inducing signaling or cytotoxicity. Treatment with the extract inhibited virus production by 30% relative to the control in the JFH1-Huh7 cell culture system. The *in vitro* enzymological assays revealed that treatment with the extract suppressed both helicase and protease activities of NS3 with IC_{50} values of 18.9 and 10.9 μg/ml, respectively. Treatment with the extract of *Amphimedon* sp. inhibited RNA-binding ability but not ATPase activity. These results suggest that the novel compound(s) included in *Amphimedon* sp. can target the protease and helicase activities of HCV NS3.

Citation: Fujimoto Y, Salam KA, Furuta A, Matsuda Y, Fujita O, et al. (2012) Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.. PLoS ONE 7(11): e48685. doi:10.1371/journal.pone.0048685

Editor: Tetsuo Takehara, Osaka University Graduate School of Medicine, Japan

Received June 16, 2012; **Accepted** October 1, 2012; **Published** November 7, 2012

Funding: This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare (http://www.mhlw.go.jp/) and from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (http://www.mext.go.jp/). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus of the genus *Hepacivirus* of the *Flaviviridae* family. More than 170 million patients persistently infected with HCV have been reported worldwide, leading to liver diseases including steatosis, cirrhosis, and hepatocellular carcinoma [1,2]. The genome of HCV is characterized as a single positive-strand RNA with a nucleotide length of 9.6 kb, flanked by 5′ and 3′-untranslated regions (UTRs). The genomic RNA encodes a large polyprotein consisting of approximately 3,000 amino acids [3], which is translated under the control of an internal ribosome entry site (IRES) located within the 5′-UTR of the genomic RNA [4]. The translated polyprotein is cleaved by host and viral proteases, resulting in 10 mature viral proteins [3]. The structural proteins, consisting of core, E1, and E2, are located in the N-terminal quarter of the polyprotein, followed by viroporin p7, which has not yet been classified into a structural or nonstructural protein. Further cleavage of the remaining portion by viral proteases produces six nonstructural proteins–NS2, NS3, NS4A, NS4B, NS5A, and NS5B–which form a viral replication complex with various host factors. The viral protease NS2 cleaves its own C-terminal between NS2 and NS3. After that, NS3 cleaves the C-terminal ends of NS3 and NS4A and then forms a complex with NS4A. The NS3/4A complex becomes a fully active form to cleave the C-terminal parts of the polyprotein, including nonstructural proteins. NS3 also possesses

RNA helicase activity to unwind the double-stranded RNA during the synthesis of genomic RNA [5,6].

Although the previous standard therapy, combining pegylated interferon with ribavirin, was effective in only about half of patients infected with genotype 1, the most common genotype worldwide [7–9], recent biotechnological advances have led to the development of a novel therapy using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication and have improved the sustained virologic response (SVR) [10–12]. 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 [13,14]. The triple combination therapy with pegylated interferon, ribavirin, and telaprevir improved SVR by 77% in patients infected with genotype 1 [15]. However, this therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7,16,17]. Furthermore, the possibility of the emergence of drug-resistant viruses is a serious problem with therapies that use antiviral compounds [18,19].

Recent technical advances in the determination of molecular structures and the synthesis of chemical compounds have led to the development of various drugs based on natural products, especially drugs identified from terrestrial plants and microbes [20–22]. Marine organisms, including plants and animals, were recently established as representative of a natural resource library for drug development. Potent biological activity is often found in products isolated from marine organisms because of their novel molecular structures [23,24]. Trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven), which are known as antitumor drugs, were developed from compounds found in marine organisms [25].

In this study, we screened 84 extracts prepared from 54 marine organisms by using replicon cell lines derived from HCV genotype 1b and attempted to identify the extract that inhibits HCV RNA replication. A marine organism may produce anti-HCV agent(s) that could inhibit the protease and helicase activities of NS3.

Results

Effect of the Extract from Marine Sponge and Tunicate on HCV Replication

We prepared methanol (MeOH)- and ethyl acetate (EtOAc)-soluble extracts from 54 marine organisms in order to test which of these extracts could best suppress HCV replication. Each extract was added at 25 μg/ml to the culture supernatant of HCV replicon cell lines derived from O and Con1 strains of genotype 1b, which produce the luciferase/neomycin hybrid protein depending on RNA replication. Luciferase activity and cell viability were measured 72 h after treatment with the extracts (Table 1). The extracts exhibiting more than 85% cell viability and lower than 15% luciferase activity were selected as arbitrary candidates for the extract including anti-HCV compounds. The EtOAc-extract prepared from sample C-29 (C-29EA) was selected as a candidate in both cell lines. Thus, the anti-HCV activity of extract C-29EA was tested.

The EtOAc-soluble extract C-29EA was prepared from the marine sponge *Amphimedon* sp. (Fig. 1A), which inhabits the sea surrounding Okinawa Prefecture, Japan. HCV replication was inhibited in a dose-dependent manner but did not exhibit cytotoxicity when replicon cells were treated with C-29EA (Fig. 1 B). The extract C-29EA exhibited EC_{50} values of 1.5 μg/ml (Table 2). Furthermore, treatment with C-29EA suppressed the HCV replication derived from the genotype 2a strain JFH1 with an EC_{50} of 24.9 μg/ml, irrespective of cell viability (Fig. 2A and Table 2). Extract C-29EA also inhibited the production of infectious viral particles, viral RNA, and core protein from JFH1-infected cells in the supernatant (Fig. 2B and C). These results suggest that the marine sponge *Amphimedon* sp. possesses anti-HCV agents.

Effect of Extract C-29EA on IRES-dependent Translation

Extract C-29EA had the most potent inhibitory activity against HCV replication. The viral replication (Fig. 1 B and 2 A) and viral proteins (Fig. 3 A and B) in replicon cell lines derived from genotype 1b strain Con1 and 2a strain JFH1 were decreased 72 h after treatment in a dose-dependent manner. HCV protein has been translated based on the positive-sense viral RNA in an IRES-dependent manner. The replicon RNA of HCV is composed of the 5′-UTR of HCV, indicator genes (a luciferase-fused drug-resistant gene), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3′-UTR of HCV, in that order [26]. 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 (Fig. 3C). When the mRNA expression was transcribed by an EF promoter of the transfected plasmid in the presence of C-29EA, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed (Fig. 3C). This suggested that treatment with C-29EA exhibited no effect on EMCV-IRES-dependent translation. Furthermore, treatment with C-29EA did not significantly affect the activity of HCV IRES that was used instead of EMCV IRES in the system described above (Fig. 3D). Thus, these results suggest that treatment with C-29EA exhibits no effect on EMCV- or HCV-IRES-dependent translation.

Effect of C-29EA on the Interferon Signaling Pathway

It has been well known that HCV replication in cultured cells is potently inhibited by interferon [27,28]. We examined whether or not treatment with C-29EA elicits an interferon-inducible gene from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 15 μg of C-29EA per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible gene 2′, 5′-OAS, was induced with IFN-alpha 2b but not with a 10-times EC_{50} concentration of C-29EA (Fig. 4). These results suggest that the inhibitory effect of C-29EA on the replication of the HCV replicon is independent of the IFN signaling pathway.

Effect of C-29EA on the NS3 Helicase Activity

We previously established an assay system for unwinding HCV activity based on photoinduced electron transfer (PET) [29,30]. 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 PET. When helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits a 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. Treatment with C-29EA inhibited the helicase activity in a dose-dependent manner, with an IC_{50} value of 18.9 μg/ml (Fig. 5A). We confirmed the effect of C-29EA on NS3 helicase unwinding activity by the RNA helicase assay using ^{32}P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment

Table 1. Effect of marine organism extracts on HCV replication and cell viability.

No.	Sample	Luciferase activity (% of control)		Cell viability (% of control)		Phylum	Specimen	Extract	Site
		O	Con1	O	Con1				
1	A-1	10	111	105	104	Sponge	*Unidentified*	MeOH	A
2	A-2	82	209	91	132	Soft coral	*Briareum*	MeOH	A
3	A-3	87	177	54	110	Tunicate	*unidentified*	MeOH	A
4	A-4	82	186	84	100	Sponge	*Liosina*	MeOH	A
5	B-5	110	165	86	110	Sponge	*unidentified*	MeOH	B
6	B-6	70	149	103	119	Sponge	*Xestospongia*	MeOH	B
7	B-7	89	191	111	144	Sponge	*Epipolasis*	MeOH	B
8	B-8	89	182	115	132	Sponge	*unidentified*	MeOH	B
9	B-9	57	72	92	124	Sponge	*Strongylophora*	MeOH	B
10	B-10	106	182	73	96	Sponge	*Stylotella aurantium*	MeOH	B
11	C-12	96	162	114	98	Sponge	*Epipolasis*	MeOH	B
12	C-13	123	141	91	103	Sponge	*unidentified*	MeOH	B
13	C-14	89	175	77	100	Sponge	*Hippospongia*	MeOH	B
14	C-16	80	177	108	88	Sponge	*unidentified*	MeOH	B
15	C-18	119	170	93	94	Sponge	*unidentified*	MeOH	B
16	C-19	0	0	0	4	Sponge	*unidentified*	MeOH	B
17	C-20	101	158	61	106	Sponge	*Xestospongia testudinaria*	MeOH	B
18	C-21	85	161	83	102	Sponge	*unidentified*	MeOH	B
19	C-22	109	88	38	89	Sponge	*unidentified*	MeOH	B
20	C-23	94	156	32	90	Sponge	*unidentified*	MeOH	B
21	C-24	118	86	42	94	Sponge	*Theonella*	MeOH	B
22	C-25	82	111	91	106	Sponge	*unidentified*	MeOH	B
23	C-27	0	0	15	2	Sponge	*unidentified*	MeOH	B
24	C-28	90	166	30	90	Sponge	*Petrosia*	MeOH	B
25	C-29	65	151	29	101	Sponge	*Amphimedon*	MeOH	B
26	D-31	81	127	55	91	Tunicate	*unidentified*	MeOH	C
27	D-32	80	141	47	93	Sponge	*unidentified*	MeOH	C
28	D-33	88	153	72	90	Gorgonian	*Junceella fragilis*	MeOH	C
29	E-35	114	156	40	118	Sponge	*Phyllospongia sp.*	MeOH	C
30	E-36	80	125	69	116	Tunicate	*Didemnum molle*	MeOH	C
31	E-37	88	129	54	108	Sponge	*Xestospongia sp.*	MeOH	C
32	E-38	70	153	35	112	Sponge	*unidentified*	MeOH	C
33	F-40	119	170	38	104	Sponge	*unidentified*	MeOH	C
34	F-41	88	166	48	101	Soft coral	*unidentified*	MeOH	C
35	G-42	113	157	31	126	Sponge	*unidentified*	MeOH	D
36	H-43	83	0	39	5	Sponge	*unidentified*	MeOH	D
37	J-44	62	183	27	105	Sponge	*Cinachyra*	MeOH	D
38	J-45	96	140	47	103	Sponge	*Liosina*	MeOH	D
39	J-46	83	149	77	102	Sponge	*unidentified*	MeOH	D
40	J-47	94	37	40	111	Sponge	*unidentified*	MeOH	D
41	J-48	24	16	53	70	Sponge	*Stylotella*	MeOH	D
42	J-49	78	123	55	105	Sponge	*unidentified*	MeOH	D
43	J-50	93	138	51	108	Sponge	*unidentified*	MeOH	D
44	J-51	103	73	41	115	Sponge	*unidentified*	MeOH	D
45	J-52	162	237	113	131	Sponge	*unidentified*	MeOH	D
46	J-53	51	90	93	122	Tunicate	*Didemnum*	MeOH	D
47	J-54	42	90	113	124	Sponge	*unidentified*	MeOH	D

Table 1. Cont.

No.	Sample	Luciferase activity (% of control)		Cell viability (% of control)		Phylum	Specimen	Extract	Site
		O	Con1	O	Con1				
48	J-55	88	133	131	110	Jellyfish	*unidentified*	MeOH	D
49	J-56	28	51	113	103	Sponge	*unidentified*	MeOH	D
50	J-57	8	63	94	85	Tunicate	*Pseudodistoma kanoko*	MeOH	D
51	J-58	0	2	48	65	Sponge	*unidentified*	MeOH	D
52	J-59	0	2	45	71	Sponge	*unidentified*	MeOH	D
53	J-60	98	134	122	95	Annelid	*unidentified*	MeOH	D
54	A-2	0	1	6	15	Soft coral	*Briareum*	EtOAc	A
55	A-3	0	0	6	9	Tunicate	*unidentified*	EtOAc	A
56	A-4	22	36	74	76	Sponge	*Liosina*	EtOAc	A
57	B-5	33	107	69	93	Sponge	*unidentified*	EtOAc	B
58	B-6	0	0	5	8	Sponge	*Xestospongia*	EtOAc	B
59	B-7	0	0	5	9	Sponge	*Epipolasis*	EtOAc	B
60	B-8	0	0	2	46	Sponge	*unidentified*	EtOAc	B
61	B-9	0	0	8	14	Sponge	*Strongylophora*	EtOAc	B
62	B-10	0	0	3	8	Sponge	*Stylotella aurantium*	EtOAc	B
63	C-12	0	0	4	14	Sponge	*Epipolasis*	EtOAc	B
64	C-13	0	0	4	5	Sponge	*unidentified*	EtOAc	B
65	C-14	48	119	82	102	Sponge	*Hippospongia*	EtOAc	B
66	C-15	0	0	8	11	Sponge	*unidentified*	EtOAc	B
67	C-18	0	0	4	3	Sponge	*unidentified*	EtOAc	B
68	C-19	23	76	63	109	Sponge	*unidentified*	EtOAc	B
69	C-20	34	32	63	112	Sponge	*Xestospongia testudinaria*	EtOAc	B
70	C-21	1	0	52	12	Sponge	*unidentified*	EtOAc	B
71	C-22	76	34	74	110	Sponge	*unidentified*	EtOAc	B
72	C-24	0	0	20	7	Sponge	*Theonella*	EtOAc	B
73	C-26	41	43	80	110	Sponge	*unidentified*	EtOAc	B
74	C-27	1	0	35	40	Sponge	*unidentified*	EtOAc	B
75	C-28	68	62	82	115	Sponge	*Petrosia*	EtOAc	B
76	C-29	10	11	93	88	Sponge	*Amphimedon*	EtOAc	B
77	D-31	20	71	85	120	Tunicate	*Eudistoma*	EtOAc	C
78	D-33	0	0	5	7	Gorgonian	*Junceella fragilis*	EtOAc	C
79	E-35	0	0	4	5	Sponge	*Phyllospongia sp.*	EtOAc	C
80	E-36	71	83	75	100	Tunicate	*Didemnum molle*	EtOAc	C
81	F-40	72	110	87	130	Sponge	*unidentified*	EtOAc	C
82	F-41	8	33	73	104	Soft coral	*unidentified*	EtOAc	C
83	H-43	0	197	4	119	Sponge	*unidentified*	EtOAc	D
84	J-46	113	58	103	126	Sponge	*unidentified*	EtOAc	D

There are a total of 54 marine organisms, while 84 extracts were prepared from them with ethyl acetate and/or methanol. Aragusuku, Iriomote, Kohama, and Ishigaki islands are indicated by A, B, C, and D, respectively, in the collection-site column?(right end). EtOAc: Ethyl acetate; MeOH: Methanol.
doi:10.1371/journal.pone.0048685.t001

with C-29EA inhibited dsRNA dissociation at a concentration of 16 μg/ml and above (Fig. 5B).

The unwinding ability of HCV helicase depends on ATP binding, ATP hydrolysis, and RNA binding [30,31]. We examined the effect of C-29EA on the ATPase activity of NS3. The ratio of free phosphate (^{32}P-Pi) to ATP (^{32}P-ATP) was determined in the presence of C-29EA. The reaction was carried out between 16 and 250 μg of C-29EA per milliliter. The ATPase activity of NS3 helicase was not inhibited (Fig. 6A), although the helicase activity was decreased to less than 20% in the presence of 50 μg of C-29EA per milliliter (Fig. 5A). Next, we examined the effect of C-29EA 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 the 21-mer of ssRNA. The binding of NS3 to ssRNA was inhibited by C-29EA in a dose-dependent manner (Fig. 6 B and C). These results suggest that treatment with C-29EA inhibits the helicase activity of NS3 by suppressing RNA binding.

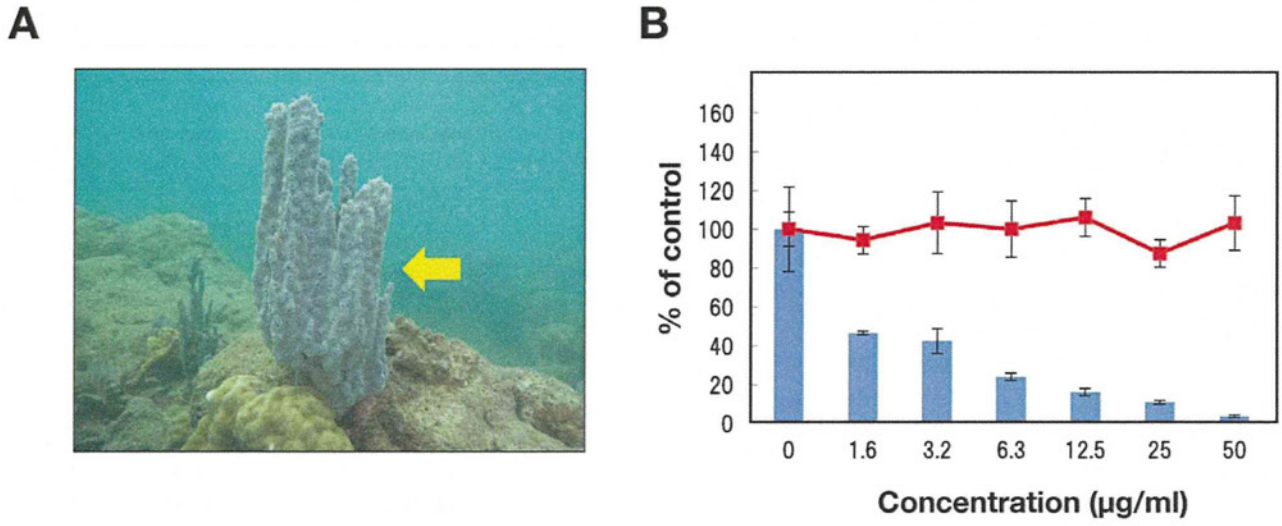

Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b. (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments.
doi:10.1371/journal.pone.0048685.g001

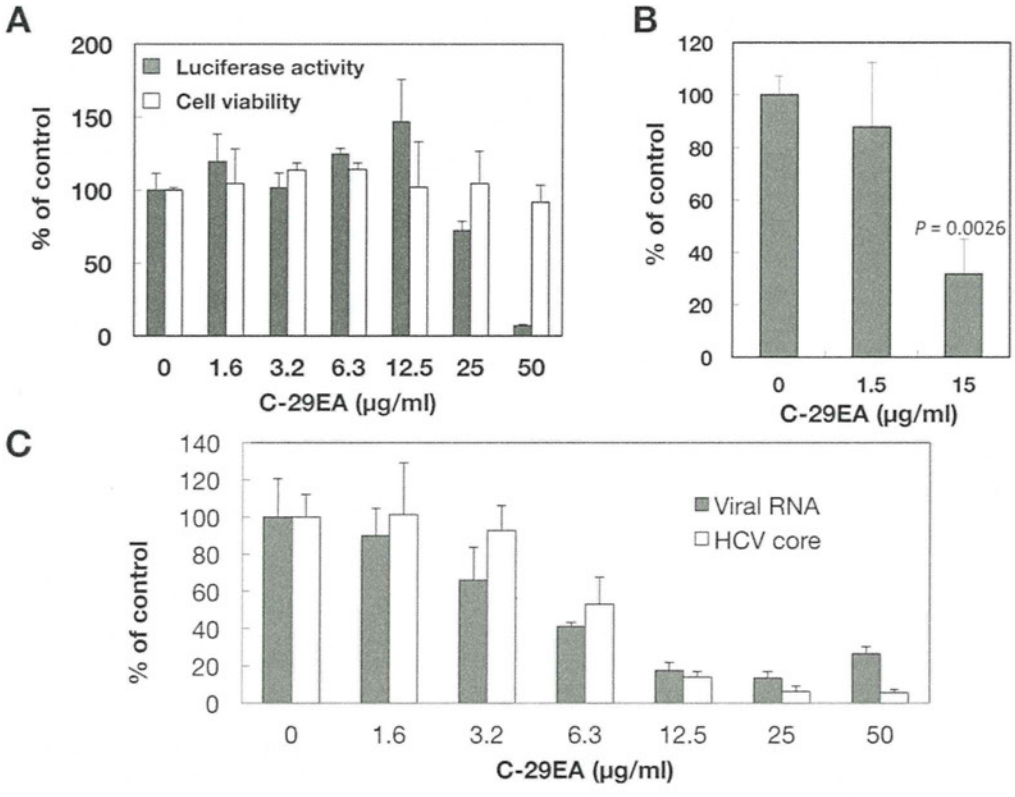

Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. (B) The Huh7 OK1 cell line infected with HCVcc JFH1 was incubated with various concentrations of C-29EA or DMSO (0). The virus titers were determined by a focus-forming assay. The significance of differences in the means was determined by Student's *t*-test. (C) Amounts of viral RNA and core protein were estimated by qRT-PCR and ELISA, respectively. Error bars indicate standard deviation. The data represent three independent experiments. Treatment with DMSO corresponds to '0'.
doi:10.1371/journal.pone.0048685.g002

Table 2. Effect of C29EA on HCV replication.

HCV strain (genotype)	EC_{50} (μg/ml)[a]	CC_{50} (μg/ml)[b]	SI[c]
Con 1 (1b)	1.5	>50	>33.3
JFH1 (2a)	24.9	>50	>2.3

[a]: Fifty percent effective concentration based on the inhibition of HCV replication.
[b]: Fifty percent cytotoxicity concentration based on the reduction of cell viability.
[c]: SI, selectivity index (CC_{50}/EC_{50}).
doi:10.1371/journal.pone.0048685.t002

Effect of C-29EA on NS3 Protease Activity

Serine protease and helicase domains are respectively located on the N-terminal and C-terminal portions of NS3 [32]. Thus, we examined the effect of C-29EA on NS3 protease activity by using an NS3 protease assay based on FRET. NS3/4A serine protease was mixed with various concentrations of C-29EA. The initial velocity at each concentration of C-29EA was calculated during a 120 min reaction. The initial velocity in the absence of C-29EA represented 100% of relative protease activity. C-29EA decreased the serine protease activity in a dose-dependent manner (Fig. 7). The IC_{50} of C-29EA was 10.9 μg/ml, which is similar to the value estimated by helicase assay. These results suggest that C-29EA includes the compound(s) inhibiting the protease activity of NS3 in addition to the helicase activity.

Combination Antiviral Activity of C-29EA and Interferon-alpha

Treatment with C-29EA may potentiate inhibitory action of interferon-alpha, since it inhibited the protease and helicase activities of NS3 but not induce the interferon response as described above. Then, we examined effect of treatment using both interferon and C-29EA on HCV replication. The replication

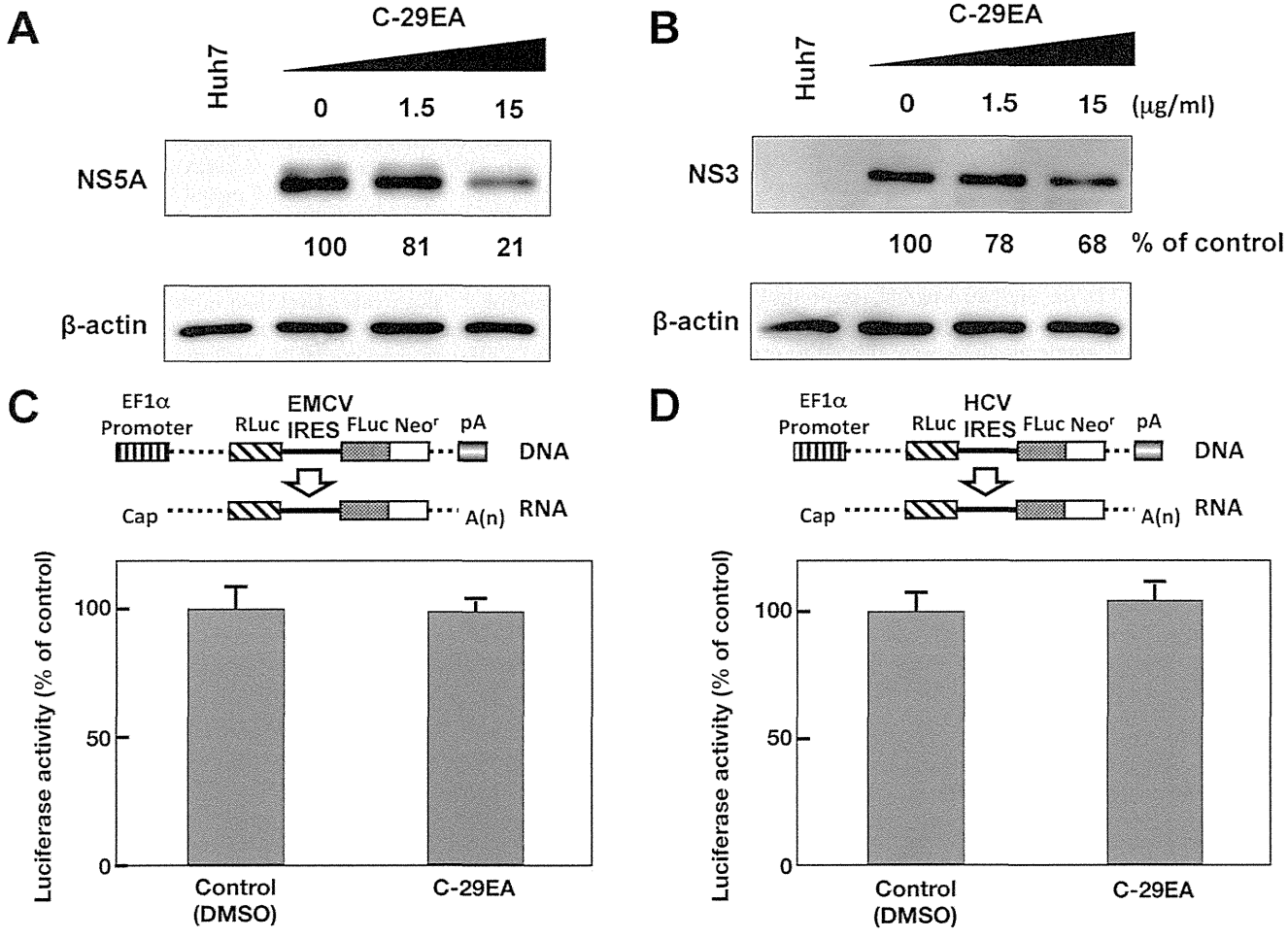

Figure 3. Effect of C-29EA on expression of viral proteins in replicon cell lines. The Huh7 replicon cell lines derived from genotype 1b (A) and 2a (B) were incubated with C-29EA at 37°C for 72 h. The treated cells were harvested and then subjected to Western blotting. Treatment with DMSO corresponds to '0'. The bicistronic gene is transcribed under the control of the 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 (C) or HCV IRES (D). The Huh7 cell line transfected with the plasmid (each above the panel in C and D) was established in the presence of G418. The cells were incubated for 72 h without (control) and with 15 μg/ml of C-29EA. 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.
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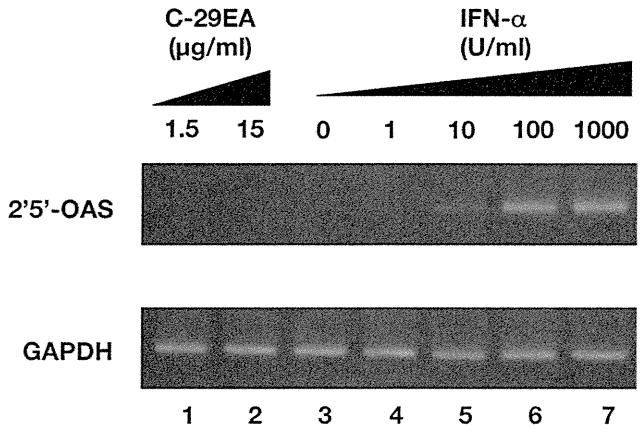

Figure 4. Effect of C-29EA on interferon signaling pathway. The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 μg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2', 5'-OAS, and GAPDH as an internal control were detected by RT-PCR. Error bars indicate standard deviation. The data represent three independent experiments.
doi:10.1371/journal.pone.0048685.g004

of replicon was decreased in the presence of C-29EA or interferon-alpha and further decreased by combination treatment using interferon-alpha and C-29EA (Fig. 8A). Furthermore, we employed the isobologram method [33] to determine whether antiviral effect of the combination treatment exhibits additive or synergistic. EC_{90} values of interferon-alpha and C-29EA were estimated at 10.7 U/ml and 26.4 μg/ml, respectively, in the absence of each other. EC_{90} values of C-29EA in the presence of 0, 2.5 and 5 U/ml interferon-alpha were plotted to generate an isobole. Figure 8B shows that the isobole exhibits concave curvilinear, representing synergy but not additivity. These results suggest that combination treatment of interferon-alpha and C-29EA exhibits synergistic inhibition of HCV replication.

Discussion

Several natural products have been reported as anti-viral agents against HCV replication. Silbinin, epigallocatechin 3-gallate, and proanthocyanidins, which were prepared from milk thistle, green tea, and blueberry leaves, respectively, have exhibited inhibitory activity against HCV replication in cultured cells [34–37]. In our previous report, we identified manoalide as an anti-HCV agent from a marine sponge extract by high-throughput screening targeting NS3 helicase activity [38]. Manoalide inhibited ATPase, RNA binding, and NS3 helicase activity in enzymological assays. The EtOAc extract of the marine feather star also suppressed HCV replication in HCV replicon cell lines derived from genotype 1b, and it inhibited the RNA-binding activity but not the ATPase activity of NS3 helicase [30]. In this study, we screened 84 extracts of marine organisms for their ability to inhibit HCV replication in replicon cell lines and HCV cell culture system. Among these extracts, C-29EA, which was extracted from *Amphimedon* sp., most strongly inhibited HCV replication regardless of cytotoxicity. We previously reported that the EtOAc extract (SG1-23-1) of the feather star *Alloeocomatella polycladia* inhibited HCV replication with an EC_{50} of 22.9 to 44.2 μg/ml in HCV replicon cells derived from genotype 1b [30]. Treatment with C-29EA potently inhibited HCV replication with an EC_{50} of 1.5 μg/ml and with an SI of more than 33.3 in the replicon cell line derived from genotype 1b, regardless of cytotoxicity (Fig. 1B and Table 2). However, C-29EA exhibited an EC_{50} of 24.9 μg/ml in a replicon cell line derived from genotype 2a at a weaker level than in the replicon cell line derived from genotype 1b (Figs. 1 and 2), suggesting that the ability of C-29EA to suppress HCV replication is dependent on the viral genotype or strain.

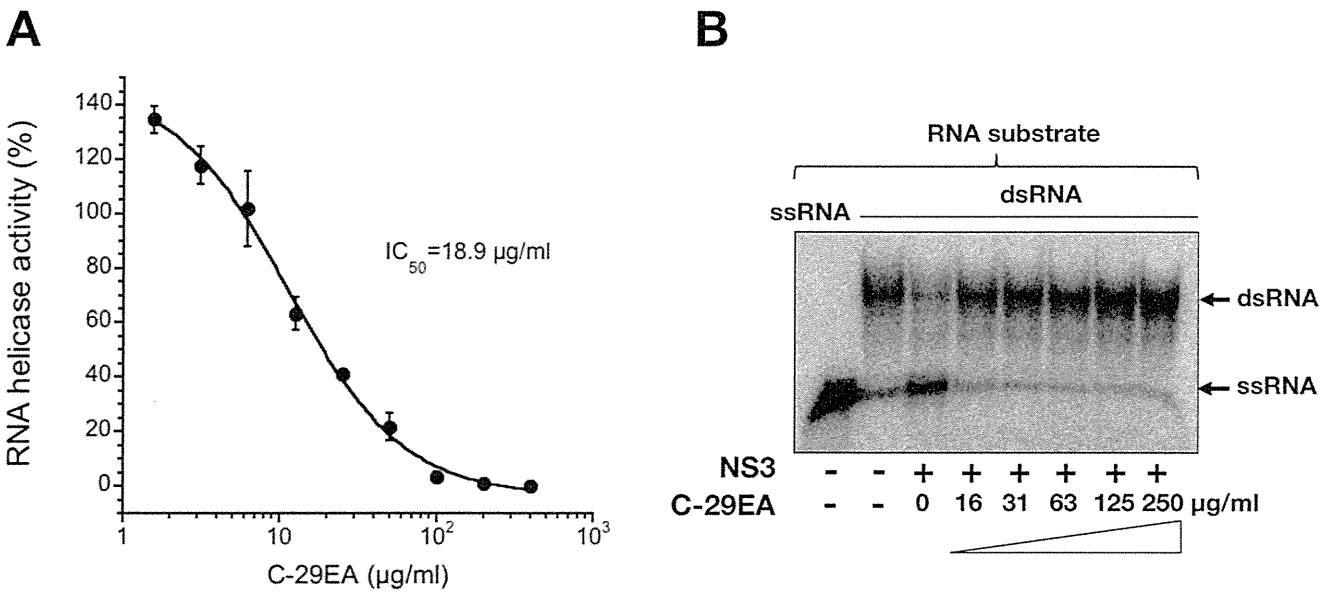

Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds to '0'. The data are presented as the mean ± standard deviation for three replicates. (B) The unwinding activity of NS3 helicase was measured by an 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 C-29EA (lanes 4–9, 16–250 μg/ml). The resulting samples were subjected to native polyacrylamide gel electrophoresis. Treatment with DMSO corresponds to '0'.
doi:10.1371/journal.pone.0048685.g005

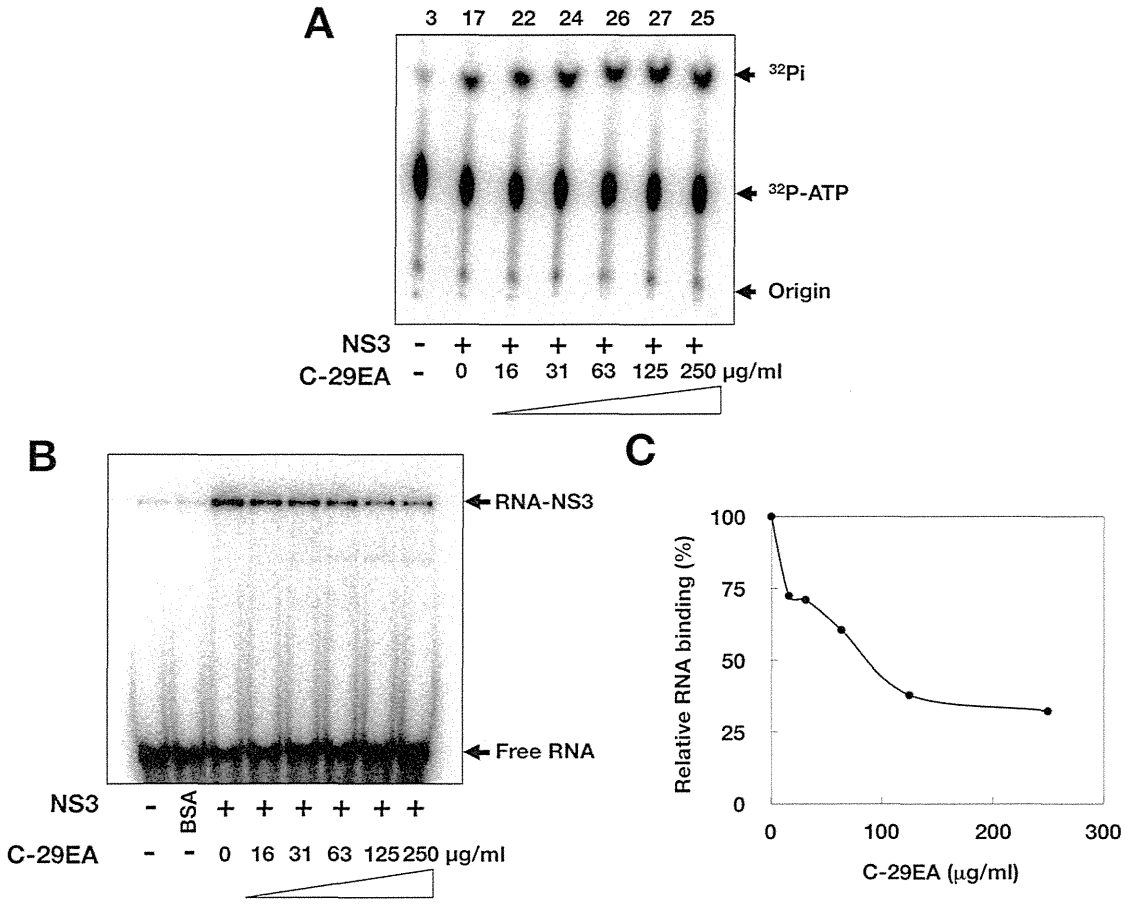

Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as 'Origin', '^{32}P-ATP', and '^{32}P-Pi', respectively, on the right side of the figure. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. (B) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out with 0.5 nM labeled ssRNA at the indicated concentrations of C-29EA or DMSO. The reaction mixture was subjected to gel mobility shift assay. (C) The relative RNA-binding ability was calculated with band densities in each lane and presented as a percentage of RNA-NS3 in the total density. The data represent three independent experiments. Treatment with DMSO corresponds to '0'.
doi:10.1371/journal.pone.0048685.g006

HCV NS3 is well known to play a crucial role in viral replication through helicase and protease activities [5,39]. The N-terminal third of NS3 is responsible for serine protease activity in order to process the C-terminal portion of polyprotein containing viral nonstructural proteins [32]. The remaining portion of NS3 exhibits ATPase and RNA-binding activities responsible for helicase activity, which is involved in unwinding double-stranded RNA during replication of genomic viral RNA [40–42]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after viral particles in the infected cells are uncoated, and is then used itself as a template to synthesize a positive-stranded RNA, which is translated or packaged into viral particles. Thus, both helicase and protease activities of NS3 are critical for HCV replication and could be targeted for the development of antiviral agents against HCV.

NS3 helicase activity was inhibited by treatment with C-29EA in a dose-dependent manner with an IC_{50} of 18.9 μg/ml (Fig. 5A). RNA-binding activity, but not ATPase activity, was inhibited by treatment with C-29EA (Fig. 6). Treatment with C-29EA did not significantly affect the HCV-IRES activity and did not induce interferon-stimulated gene 2′,5′-OAS (Figs. 3 and 4). Furthermore, the serine protease activity of NS3 was inhibited by using C-29EA with an IC_{50} of 10.9 μg/ml (Fig. 7). These results suggest that *Amphimedon* sp. includes the unknown compound(s) that could suppress NS3 enzymatic activity to inhibit HCV replication. Although the mechanism by which treatment with C-29EA could inhibit HCV replication has not yet been revealed, the unknown compound(s) may be associated with the inhibition of NS3 protease and helicase, leading to the suppression of HCV replication. However, other effects of extract C-29EA on HCV replication could not be excluded in this study.

The compound 1-N, 4-N-bis [4-(1H-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamide, which is designated as $(BIP)_2B$, was reported to be a potent and selective inhibitor of HCV NS3 helicase [43]. This compound competitively decreases the binding ability of HCV NS3 helicase to nucleic acids. The compound $(BIP)_2B$ inhibited RNA-induced stimulation of ATPase, although it did not directly affect the ATP hydrolysis activity of NS3 helicase. Thus, $(BIP)_2B$ could not affect ATPase activity without RNA or with a high concentration of RNA. Treatment with C-29EA inhibited helicase activity and viral replication but not ATPase activity (Figs. 1B, 2, 5, and 6). This extract suppressed the binding of RNA to helicase but exhibited no suppression of ATPase by NS3 helicase. Thus, the inhibitory action of extract C-29EA seems

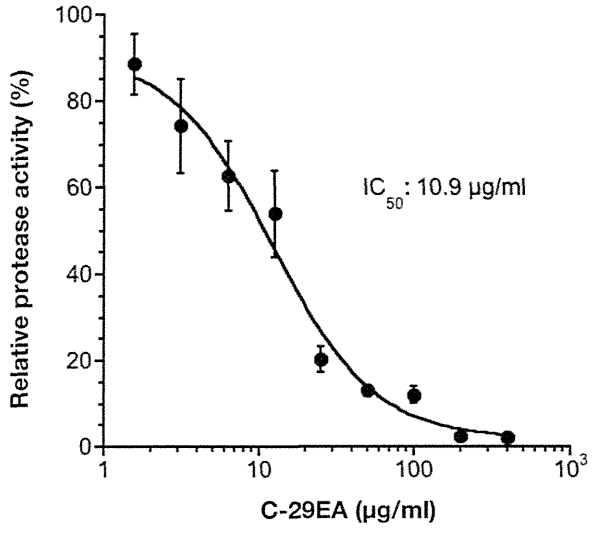

Figure 7. Effect of C-29EA on the activity of NS3 serine protease. NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction. The initial velocity in the absence of C-29EA was defined as 100% of relative protease activity. The data are presented as the mean ± standard deviation for three replicates.
doi:10.1371/journal.pone.0048685.g007

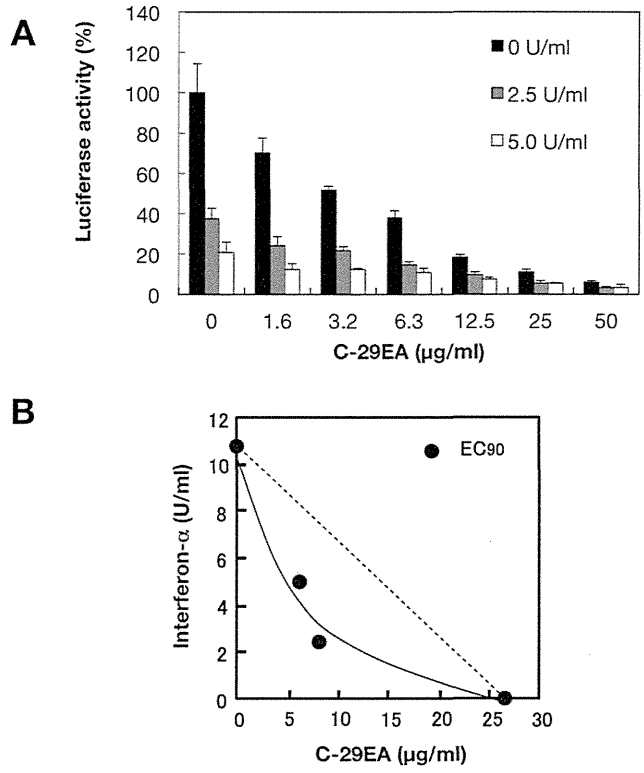

Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase assay were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (B) Isobole plots of 90% inhibition of HCV replication. The broken line indicates the additive effect in the isobologram.
doi:10.1371/journal.pone.0048685.g008

different from that of $(BIP)_2B$. The quinolone derivative QU663 was reported to inhibit the unwinding activity of NS3 helicase by binding to an RNA-binding groove irrespective of its own ATPase activity [44]. The compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity. In this study, treatment with C-29EA inhibited the RNA-binding activities of NS3 helicase but did not affect ATPase activity (Fig. 6). Furthermore, treatment with C-29EA suppressed the viral replication of HCV in an HCV cell culture system derived from several virus strains (Figs. 1 and 2, Table 2). The mechanism of C-29EA on the inhibition of NS3 helicase may be similar to that of compound QU663.

It is unknown whether one or several molecules included in C-29EA are critical for the inhibition of protease and helicase activities. The serine protease NS3/4A is one of the viral factors targeted for development into antiviral agents. Improvements in HCV therapy over the past several years have resulted in FDA approval of telaprevir (VX-950) [15,45] and boceprevir (SCH503034) [46,47]. Several studies suggest that the activities of NS3/4A protease and helicase in the full-length molecule enhance each other [48,49]. The NS3/4A protease has formed a complex with macrocyclic acylsulfonamide inhibitors [50,51]. Schiering et al. recently reported the structure of full-length NS3/4A in complex with a macrocyclic acylsulfonamide protease inhibitor [52], although the structure of full-length HCV NS3/4A in complex with a protease inhibitor has not been reported. The inhibitor binds to the active site of the protease, while the P4-capping and P2 moieties of the inhibitor are exposed toward the helicase interface and interact with both protease and helicase residues [52]. An unknown compound included in C-29EA might interact with both protease and helicase domains of NS3 to inhibit their activities. However, our data in this study have not excluded the possibility that several compounds included in C-29EA are related to the inhibition of protease and helicase of NS3/4A.

In conclusion, we showed that the EtOAc extract from *Amphimedon* sp. significantly inhibits HCV replication by suppressing viral helicase and protease activities. The purification of an inhibitory compound from the extract of *Amphimedon* sp. will be necessary in order to improve its efficacy by chemical modification.

Materials and Methods

Preparation of Extracts from Marine Organisms

All marine organisms used in this study were hand-collected by scuba diving off islands in Okinawa Prefecture, Japan. No specific permits were required for the described field studies. We do not have to obtain a local government permit to collect invertebrates except for stony corals and marine organisms for fisheries, which we did not collect in this study. The areas where we collected are not privately-owned or protected in any way. We did not collect any invertebrates listed in the red data book issued by Ministry of Environment, Japan. The sponges, tunicates, and soft corals used in this study are not listed at all. Hence, no specific permits are required for this collection in the same way as the previous report of Aratake et al. [53].

The sponge from which C-29EA was extracted was identified as *Amphimedon* sp. and deposited at Naturalis under the code RMNH POR 6100. Each specimen was soaked in acetone. The acetone-extract fraction prepared from each specimen was concentrated.

The resulting material was fractionated as an EtOAc- and water-soluble fraction. The water-soluble fraction was dried up and solubilized in MeOH. The EtOAc- and the MeOH-soluble fractions were used for screening. All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

Cell Lines and Virus

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/ml G418. The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was kindly provided by Ralf Bartenschlager [26]. Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) was reported previously [54] and used for screening in this study (Table 1). HCV replicon cell line derived from genotype 2a strain JFH1 was described previously [55]. The surviving cells were infected with the JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [56]. The infectivity of the JFH1 strain was determined by a focus-forming assay [56].

Quantitative Reverse-transcription PCR (qRT-PCR) and Estimation of Core Protein

The estimation of viral RNA genome was carried out by the method described previously [57] with slight modification. Total RNAs were prepared from cells and culture supernatants by using an RNeasy mini kit (QIAGEN, Tokyo, Japan) and QIAamp Viral RNA mini kit (QIAGEN), respectively. First-strand cDNA was synthesized by using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) with random primers. Each cDNA was estimated by using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Fluorescent signals of SYBR Green were analyzed by using an ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region was amplified using the primer pair 5′-GAGTGTCGTGCAGCCTCCA -3′ and 5′- CACTCGCAAG-CACCCTATCA -3′. Expression of HCV core protein was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [57].

Determination of Luciferase Activity and Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. C-29EA was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega, Madison, WI, USA) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon. Cell viability was measured by a dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Effects on Activities of Internal Ribosome Entry Site (IRES)

Huh7 cells were transfected with pEF.Rluc.HCV.IRES.Feo or pEF.Rluc.EMCV.IRES.Feo and then were established in medium containing 0.25 mg/ml G418, as described previously [58]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 15 μg/ml extract C-29EA, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

Western Blotting and Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Western blotting was carried out by a method described previously [30]. The antibodies to NS3 (clone 8G-2, mouse monoclonal, Abcam, Cambridge, UK), NS5A (clone 256-A, mouse monoclonal, ViroGen, Watertown, MA, USA), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study. RT-PCR was carried out by a method described previously [30,58].

Assays for RNA Helicase, ATPase, and RNA-binding Activities

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [29] and was slightly modified with regard to the reaction mixture [30]. The NS3 RNA unwinding assay was carried out by the method of Gallinari et al. [59] with slight modifications [30]. NS3 ATPase activity was determined by the method of Gallinari et al. [59] with slight modifications [30]. RNA binding to NS3 helicase was analyzed by a gel mobility shift assay [30,31]. The gene encoding NS3 helicase was amplified from the viral genome of genotype 1b and was introduced into a plasmid for the expression of a recombinant protein [38,60]. The radioactive band was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

NS3 Protease Assay

The fluorescence NS3 serine protease assay based on fluorescence resonance energy transfer (FRET) was carried out by the modified method using the SensoLyte™ 520 HCV protease assay kit (AnaSpec, Fremont, CA, USA). In brief, NS3 protein with a two-fold excess of NS4A cofactor peptide (Pep4AK) was prepared in 1× assay buffer provided with the kit. HCV NS3/4A protease was mixed with increasing concentrations of C-29EA and incubated at 37°C for 15 min. The reaction was started by adding the 5-FAM/QXL 520 substrate to the reaction mixture containing 180 nM HCV NS3/4A protease and various concentrations (0–400 μg/ml) of C-29EA. The resulting mixture (20 μl) was incubated at 37°C for 120 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). The fluorescence intensity was recorded every minute for 120 min. The NS3 serine protease activity was calculated as the initial reaction velocity and presented as a percentage of relative activity to that of the control examined with DMSO solvent but not C-29EA, in the same way as described in the fluorescence helicase assay [29].

Analysis of Drug-drug Interaction

The effects of drug combinations were evaluated using the isobologram method [33]. Various doses of C-29EA and interferon-alpha on 90% inhibition of HCV replication were combined to generate an isoeffect curve (isobole) to determine drug–drug interaction. Concave, linear, and convex curves exhibit synergy, additivity, and antagonism, respectively.

Statistical Analysis

The results are expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t*-test.

Acknowledgments

We thank T. Wakita and R. Bartenschlager for kindly providing the virus, cell lines, and plasmids; and H. Kasai and I. Katoh for their helpful comments and discussions.

Author Contributions

Conceived and designed the experiments: MN MT YS ST NA NN AY JT KM. Performed the experiments: YF KAS AF YM OF HT AY. Analyzed the data: MI NK NS SM NE. Wrote the paper: YF AY JT KM. Collected marine organisms: JT. Identified the sponge: NJdV.

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Cell. Mol. Life Sci.
DOI 10.1007/s00018-012-0940-6

Cellular and Molecular Life Sciences

RESEARCH ARTICLE

Let-7b is a novel regulator of hepatitis C virus replication

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Received: 29 September 2011 / Revised: 28 January 2012 / Accepted: 9 February 2012

Abstract The non-coding microRNA (miRNA) is involved in the regulation of hepatitis C virus (HCV) infection and offers an alternative target for developing anti-HCV agent. In this study, we aim to identify novel cellular miRNAs that directly target the HCV genome with anti-HCV therapeutic potential. Bioinformatic analyses were performed to unveil liver-abundant miRNAs with predicted target sequences on HCV genome. Various cell-based systems confirmed that let-7b plays a negative role in HCV expression. In particular, let-7b suppressed HCV replicon activity and down-regulated HCV accumulation leading to reduced infectivity of HCVcc. Mutational analysis identified let-7b binding sites at the coding sequences of NS5B and 5′-UTR of HCV genome that were conserved among various HCV genotypes. We further demonstrated that the underlying mechanism for let-7b-mediated suppression of HCV RNA accumulation was not dependent on inhibition of HCV translation. Let-7b and IFNα-2a also elicited a synergistic inhibitory effect on HCV infection. Together, let-7b represents a novel cellular miRNA that targets the HCV genome and elicits anti-HCV activity. This study thereby sheds new insight into understanding the role of host miRNAs in HCV pathogenesis and to developing a potential anti-HCV therapeutic strategy.

Keywords microRNA · Let-7b · HCV

Ju-Chien Cheng and Yung-Ju Yeh contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s00018-012-0940-6) contains supplementary material, which is available to authorized users.

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Abbreviations

miRNA	microRNA
HCV	Hepatitis C virus
MRE	MicroRNA responsive element
IFNα-2a	Peginterferon alpha-2a
IFN	Interferon
LF2000	Lipofectamine 2000
DMEM	Dulbecco's modified Eagle's medium
FITC	Fluorescein isothiocyanate
DAPI	4′,6-diamidino-2-phenylindole

Introduction

Hepatitis C virus (HCV) frequently causes chronic infection, leading to hepatic fibrosis and hepatocellular carcinoma [1]. Due to the lack of viral vaccine, the population affected by HCV infection is increased substantially [2]. With the strong side-effects and the moderate successful rate associated with the first-line interferon (IFN)-based

Published online: 06 March 2012

treatment [3], development of effective therapeutic regimens is still an emerging focus in the control of HCV infection.

Small molecules such as telaprevir have been developed to alleviate disease progression. However, the infidelity of HCV RNA polymerase constantly causes mutation and genome instability that result in the generation of drug-resistant viral strain [4, 5]. Targeting the host factors with important roles in viral infection offers an alternative strategy for development of anti-HCV regimen [6]. Apart from host proteins, a new class of small non-coding endogenous RNA molecule microRNA (miRNA) has been recently unveiled [7]. Although it is not yet fully clarified, miRNA is involved in various biological functions, including the response to HCV infection [7–10]. For example, miR-122 enhances whereas mir-199a* suppresses HCV replication and viral production [11–13]; interferon β (IFN-β)-mediated attenuation of viral replication is associated with an increase in miRNAs that have predicted target sequences within the HCV genome [14]. In addition, miRNA effectors including Argonaute 2 (Ago2) and DDX6 were found to positively regulate HCV replication [15, 16]. These findings suggest that cellular miRNAs regulate HCV gene expression and play roles in the host response against HCV infection.

In this study, bioinformatic analyses were performed to identify liver miRNAs targeting the HCV genome. Various cellular and viral systems were used to confirm bioinformatic prediction and to investigate the functional effects of the selected miRNAs. Our data reveal for the first time that let-7b is a negative regulator of HCV replication with the effective target sequences located on the 5′-untranslation region (UTR) and NS5B coding region of the HCV genome. The suppressive effect of let-7b on HCV RNA is not through translation inhibition. Besides, let-7b and Peginterferon alpha-2a (IFNα-2a) elicit a synergistic inhibitory effect on HCV infection. The roles of let-7b in the regulation of HCV pathogenesis and in the development of novel anti-HCV therapeutic strategy are discussed.

Materials and methods

Materials

The plasmid pRep-Feo and the replicon cells Huh7/Rep-Feo were obtained from Dr. Naoya Sakamoto (Tokyo Medical and Dental University). The plasmids pFL-J6/JFH, pJ6/JFH(p7-Rlu2A), and pJ6/JFH(p7-Rlu2A)GNN, the ConI replicon cells and Huh7.5 [17, 18], were kindly provided by Professor Charles Rice (The Rockefeller University, NY). The plasmid JC1-Luc2A, which replaced the Rluc gene of pJ6/JFH(p7-Rlu2A) to firefly luciferase (Luc) gene was kindly provided by Professor Robert T. Schooley (University of California San Diego, CA). Pre-miRNA, miRNA inhibitors and negative control for miRNA and miRNA inhibitors were purchased from Ambion (Austin, TX). The pLKO.1-shGFP control plasmid (clone ID: TRCN0000072197) and the two pLKO.1-shHMGA2 plasmids (clone ID: TRCN0000021965 and TRCN0000021968) were purchased from National RNAi Core Facility (Academia Sinica, Taiwan). The Lipofectamine 2000 (LF2000) and RNAiMAX transfection reagents were purchased from Invitrogen (Carlsbad, CA). The anti-HCV NS5A antibody was purchased from Bio-Design (Carmel, NY). The anti-HCV Core antibody was purchased from Affinity BioReagents (Golden, CO). The anti-β-actin antibody was purchased from Sigma (St. Louis, MO). The IFNα-2a was purchased from Roche (Mannheim, Germany). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium (MTS) reduction assay and the luciferase assay reagents were purchased from Promega (Madison, WI).

Identification of liver miRNAs targeting the HCV genome

Bioinformatic strategy for the identification of liver miRNAs targeting the HCV genome is presented in Fig. 1. Briefly, two published miRNAs expression profiles [19, 20] were used to select for liver-abundant miRNAs. The miRNA is defined as liver abundance when the expression level of a specific liver miRNA divided by the average expression level of the total liver miRNAs is greater than one. The 23 miRNAs (Supplementary Table 1) that were identified as liver abundant in the two profiling databases were subject to bioinformatic analyses using miRanda, RNAhybrid, TargetScan, and PITA [21–24] to predict their target sequences on all six HCV genotypes (Supplementary Table 2). According to the calculation of miRanda, a filter was set to select for miRNAs of which the prediction scores for at least four genotypes were higher than 155. These miRNAs were considered as the candidate liver miRNAs targeting the HCV genome.

Cell culture and viability assay

Human hepatoma Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The Huh7.5 cells that represent a Huh7 subline and are highly permissive for HCV replication were maintained in DMEM with 1% non-essential amino acid (NEAA). The ConI cells were cultured in DMEM supplemented with 10% FBS and 750 μg/ml G418. The Huh7/Rep-Feo subgenome replicon cells were maintained in the same medium except that 1%

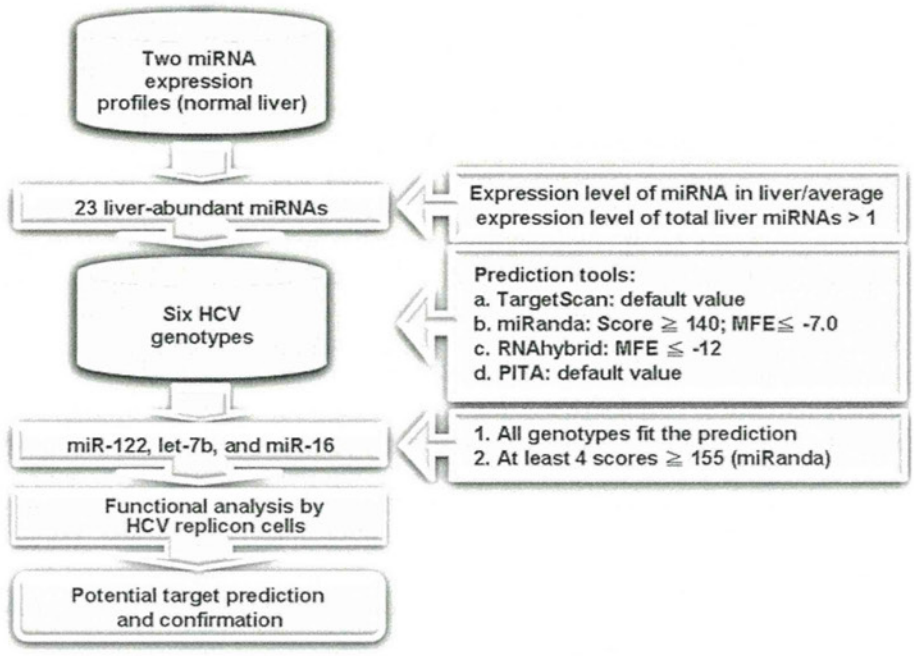

Fig. 1 Bioinformatic strategy for identifying liver miRNAs with target sites on HCV genome. Two published normal liver tissue miRNA expression profiles were used to select liver-abundant miRNAs for bioinformatics analyses as described in "Materials and methods"

NEAA was added and only 250 μg/ml G418 was used [25]. Viable cells were determined by the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit.

Plasmid construction

For luciferase (luc) reporter plasmids, the predicted miRNA responsive element (MRE) for let-7b, as listed in Table 1, was inserted into the *Eco*RI/*Xba*I site downstream of the luciferase gene in phDab2-luc [26] to generate pluc-c-let-7b, pluc-MRE1, pluc-MRE2, pluc-MRE3, and pluc-MRE4. For generation of HCV subgenome mutants with nucleotide mismatch at the ''seed region'' of let-7b or "S1 binding site" of miR-122, site-directed mutagenesis was performed by QuickChange (Stratagene, CA) using pRep-Feo as the template [25] and the primer sets for mMRE1, mMRE2, mMRE3, and miR-122-mut as listed in Table 1. To generate a capped RNA transcript encoding firefly luciferase (FLuc) for use as an internal control in the transient translation assay, the FLuc gene from the plasmid pGL3-Promoter (Madison, WI) was first digested by the restriction enzyme *Nco*I. The nucleotides at the sticky end were filled up as blunt end by Klenow DNA polymerase and the FLuc gene was then excised by the restriction enzyme *Xba*I. On the other hand, pRL-TK plasmid was digested by *Nhe*I and the sticky end was filled up by Klenow DNA polymerase followed by *Xba*I restriction enzyme digestion to remove the Renilla luciferase gene (RLuc). The Fluc gene fragment was then cloned into pRL-TK to replace RLuc to generate pLUC-TK. This plasmid contains a T7 promoter and can transcribe mRNA after linearization by *Bam*HI.

Transient transfection and luciferase activity assay

For transient transfection, Huh7/Rep-Feo cells were seeded at a density of 1×10^4 cells/well for 24 h and the miRNA precursor or inhibitor was transfected into cells by LF2000. At 72 h after transfection, the luciferase activity was quantified using the Bright-Glo luciferase assay reagent. On the other hand, 293T cells were seeded at a density of 2×10^4 cells/well and the reporter plasmid, pRL-TK and miRNA precursor (100 nM) were cotransfected into cells by LF2000. At 24 h after transfection, the firefly and Renilla luciferase activities were quantified using the Dual-Glo luciferase assay reagent.

For RNA transfection, the *Xba*I-digested wild-type pRep-Feo or the mutant subgenome plasmid was subject to in vitro transcription for RNA synthesis. The Huh7.5 cells were transfected with 10 μg HCV RNA, 100 pmol miRNA, and 10 μg pRL-TK by electroporation using Gene Pulser II (Bio-Rad) at 260 V and 950 μF.

For permissive assay, Huh7 cells were seeded at a density of 2×10^5 cells/well and were transfected with the miRNA precursor (100 pmol) by RNAiMAX (Invitrogen) for 24 h. The transfected cells were subsequently infected with HCVcc (6×10^6 copies/ml) for 4 h. After washing away the virus, the cells were cultured for 72 h and the HCV RNA was detected from the infected cells by real-time reverse transcription-PCR (RT-PCR).

Table 1 The sequences for the primers used in this study

Primer name	Primer sequences	Size (mer)
For luciferase reporter constructs[a]		
c-let-7b	S:5′-CTAGA**AACCACACAACCTACTACCTCA**G-3′	22
	AS:5′-AATTC**TGAGGTAGTAGGTTGTGTGGTT**T-3′	
MRE1	S:5′-CTAGA**CACCATGAGCACGAATCCTAAACCTCA**G-3′	27
	AS: 5′-AATTC**TGAGGTTTAGGATTCGTGCTCATGGTG**T-3′	
MRE2	S: 5′-CTAGA**GGCAAAAGGGTGTACTACCTCA**G-3′	22
	AS: 5′-AATTC**TGAGGTAGTACACCCTTTTGCC**T-3′	
MRE3	S: 5′-CTAGA**AGCCACTTGACCTACCTCA**G-3′	19
	AS: 5′-AATTC**TGAGGTAGGTCAAGTGGCT**T-3′	
MRE4	S:5′-CTA**GAGCCGCATGACTGCAGAGAGTGCTGATACTGGCCTCT**G-3′	38
	AS: 5′-AATTC**AGAGGCCAGTATCAGCACTCTCTGCAGTCATGCGGCT-**3′	
For in vitro mutagenesis[b]		
mMRE1	F: 5′-GAGCACGAATCCTAA**TGGAGT**AAGAAAAACCAAAGG-3′	36
	R: 5′-CCTTTGGTTTTTCTT**ACTCCA**TTAGGATTCGTGCTC-3′	
mMRE2	F: 5′-CTGGCAAAAGGGTGTA**T**TA**T**CTCACTCGCGATCCCAC-3′	47
	R: 5′-GTGGGATCGCGAGUGAGA**T**AA**T**ACACCCTTTTGCCAG-3′	
mMRE3	F: 5′-CATTGAGCCACTTGACCT**T**CC**G**CAGATCATTGAACGACTC-3′	40
	R: 5′-GAGTCGTTCAATGATCTG**C**GGA**A**AGGTCAAGTGGCTCAATG-3′	
miR-122 mut	F: 5′-CCCGATTGGGGGCGACAC**AG**CACCATAGATCACTCCCC-3′	38
	R: 5′-GGGGAGTGATCTATGGTG**CT**GTGTCGCCCCCAATCGGG-3′	
For synthesis of mature miRNA[c]		
7b	S: 5′UGAGGUAGUAGGUUGUGUGGUU 3′ AS:	22
	5′UUCCACACAACCUACUACCUCA 3′	
m7b	S: 5′UGA**AC**UAA**U**AGGUUGUGUGGUU 3′ AS:	22
	5′UUCCACACAACCUA**U**UA**GU**UCA 3′	

S sense strand, *AS* antisense strand *F* forward primer, *R* reverse primer

[a] The *bold letters* indicate the predicted sequence while the other sequence was generated for cloning into *EcoRI*/*Xba*I restriction enzyme site

[b,c] The *bold letters* indicate the mutated nucleotides

Ribonucleoprotein immunoprecipitation (RIP) assay

The RIP assay was performed using the miRNA isolation kit (Wako Laboratory Chemicals, Osaka, Japan) according to the manufacturer's instruction. Briefly, 10 μg of Rep-Feo subgenomic RNA was obtained by in vitro transcription and was co-transfected with 100 pmol of the indicated miRNA into Huh7.5 cells by electroporation. At 6 h after transfection, the cells were lysed in 1 ml of cell lysis solution (20 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 200 mM NaCl, and 0.05% NP40). After centrifugation, the supernatant was collected and mixed with anti-human Ago2 monoclonal antibody-conjugated agarose beads for 2 h at 4°C. After several washes with cell lysis solution, the HCV RNAs associated with Ago2-containing miRNA ribonucleoprotein (miRNP) complexes were eluted and were quantified by real-time RT-PCR. For knockdown of endogenous let-7b, the let-7b inhibitor (100 pmol) was transfected into Huh7.5 cells for 24 h followed by electroporation of the cells with the Rep-Feo HCV subgenomic RNA mutated at the miR-122 binding site (10 μg) and 100 pmol of let-7b inhibitor.

Western-blot analysis

The cell lysates were harvested and separated by 10% SDS-PAGE. The expression of HCV viral protein was detected using ECL kit (Perkin-Elmer) as described previously [27].

Production of HCVcc infectious particles and infectivity inhibition assay

The HCVcc infectious particle was produced as described previously [28]. Briefly, in vitro transcribed J6/JFH-based HCV genomic RNA was electroporated into Huh7.5 cells. The virus-containing supernatant was clarified by low-speed centrifugation, passed through a 0.45-μm filter, and concentrated by ultracentrifugation.

For infectivity inhibition assay, Huh7.5 cells were seeded in a six-swell plate at a density of 2×10^5 cells/well. At 24 h after plating, 100 nM miRNA was transfected into the cells using LF2000. HCVcc (0.1 MOI) was then added to each well for 4 h and the transfection complex was replaced with 2% FBS-containing medium for 72 h. The cells were fixed and stained by anti-Core antibody following by FITC-conjugated second antibody, counterstained with 4′,6-diamidino-2-phenylindole (DAPI), and the infectious foci were counted using fluorescence microscopy.

For JC1-Luc2A HCV reporter virus, Huh7.5 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well. At 24 h after plating, HCV reporter virus (0.01 MOI) was added to each well for 4 h. Then 100 nM of the indicated miRNA was transfected into the infected cells using RNAiMax and the transfection complex was replaced with 2% FBS-containing medium for 72 h. The cell lysates were collected for luciferase activity and MTS assay.

RNA isolation and real-time quantitative RT-PCR

Total RNAs were extracted using ReZol method and were quantified using a NanoDrop spectrophotometer. For quantification of HCV RNA expression, total cellular RNA (100 ng) was subject to one-step RT-PCR (25 μl) containing 2× TaqMan master mix and the primer/probe set for HCV (HCV-F: 5′-TGCGGAACCGGTGAGTACA-3′, HCV-R: 5′-CTTAAGGTTTAGGATTCGTGCTCAT-3′, and probe: 5′-CACCCTATCAGGCAGTACCACAAGG CC-3′). The reaction condition was one cycle of 48°C for 30 min, one cycle of 95°C for 10 min, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min using the ABI Prism 7000 Sequence Detection System. The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a normalization control. HCV RNA expression was quantified by the $\Delta\Delta C_t$ method, where C_t represented the threshold cycle.

The TaqMan® microRNA Assay System was used for miRNA detection and quantification. Briefly, the RT reaction was performed in a final volume of 15 μl containing 1.5 μl of 10× RT buffer, 2.5 μl of total RNA (25 ng), 3 μl of 5× miRNA-specific RT primer, 0.15 μl of 100 mM dNTP, 0.2 μl of 40 U/μl RNase inhibitor, and 1 μl of MultiScribe reverse transcriptase (50 U/μl). The reaction condition was 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was then performed in a 20-μl PCR containing 1.33 μl of RT product, 10 μl of 10× TaqMan Universal PCR master mix, and 1 μl of the primer and probe mix from the TaqMan® MicroRNA Assay Kit. The reaction condition was 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The expression of RNU6B gene was used as the internal control.

HCV translation assay

Huh7.5 cells were seeded into a six-well plate at a density of 4×10^5 cells/well. At 24 h after transfection of let-7b miRNA (100 nM), the replication-deficient J6/JFH (p7-Rlu2A) GNN mutant RNA (1.25 μg/well) was transfected together with the capped and polyadenylated FLuc mRNA (125 ng/well) by LF2000. After 4 h, cells were harvested and dual luciferase activity assays were performed.

Statistical analysis

Statistical analysis was performed by Student's t test. $p < 0.05$ was considered as statistically significant.

Results

Identification and functional characterization of liver miRNAs with potential recognition sequences on HCV genome

A bioinformatic strategy as described in the "Materials and methods" section was developed to search for novel miRNAs with potential recognition sequences on HCV genome (Fig. 1). Three miRNAs including miR-122, let-7b, and miR-16 were uncovered. To elucidate whether these miRNAs have any functional effect on HCV infection, Huh7/Rep-Feo replicon cells (genotype 1b) were transfected with the indicated miRNAs and the luciferase activity was determined (Fig. 2a, b). In accord with a previous report [11], miR-122 enhanced HCV expression ($p < 0.05$). Notably, let-7b significantly suppressed HCV expression ($p < 0.01$) while miR-16 had only a moderate effect ($p = 0.343$).

To further confirm that let-7b can regulate HCV RNA accumulation, mutated let-7b (m7b) was designed to change three nucleotides on the wild-type let-7b sequences (Fig. 2b; Table 1). As calculated and predicted by miRanda, no m7b target sequence was found on HCV genome (data not shown). After transfection into Huh7/Rep-Feo replicon cells, m7b abrogated the inhibitory effect of let-7b on the luciferase activity (Fig. 2b) thereby demonstrating that let-7b is a negative regulator of HCV expression.

The effects of the three selected miRNAs on HCV expression were also evaluated using ConI replicon cells (Fig. 2c). These miRNAs were transfected into the replicon cells and the expression of viral proteins was determined at 72 h after transfection. Western-blot analysis revealed that miR-122 increased NS5A expression, while let-7b but not miR-16 caused a decrease in NS5A (Fig. 2c, left panel). Furthermore, the let-7b mutant form m7b lost its inhibitory effect on HCV and did not alter NS5A expression (Fig. 2c,

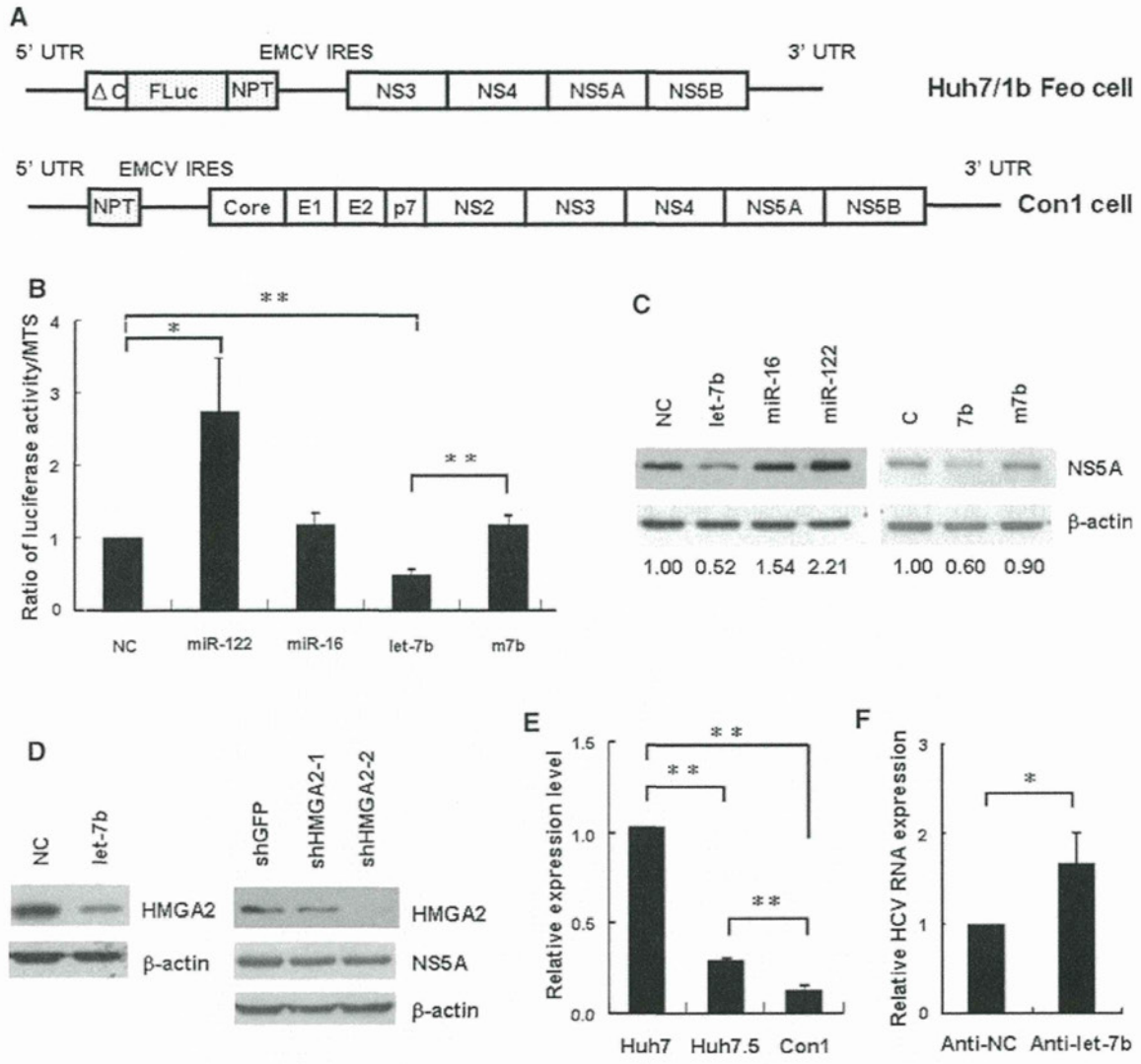

Fig. 2 Characterization of miRNAs with putative target sites on HCV genome. **a** Genomic structures of HCV maintaining in Huh7/Rep-Feo and ConI cells. **b** The miRNA precursors or mutant let-7b (m7b) were transfected into Huh7/Rep-Feo cells. MTS and luciferase activity assays were then performed at 72 h post-transfection. **c** The miRNA precursors (*left panel*) or the mutant form of let-7b (*right panel*) were transfected into the ConI cells. Western-blot analysis was then performed using the anti-NS5A and anti-β-actin antibody at 72 h post-transfection. The ratios for the relative band intensities of NS5A after normalization with β-actin were shown. *NC* negative control miRNA. **d** The precursors of let-7b (*left panel*) or shHMGA2 (*right panel*) were transfected into ConI replicon cells. Western-blot analysis was then performed using the anti-NS5A and anti-β-actin antibody at 72 h post-transfection. *NC* negative control miRNA. **e** Real-time RT-PCR of let-7b was performed using the total RNAs from the indicated cells. RNU6B was used as an internal control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). **f** The let-7b inhibitor (Anti-let-7b) or control inhibitor (Anti-NC) was transfected into Huh7 cells, respectively. HCV RNA expression was quantified by real-time RT-PCR using the total RNAs from the indicated transfected cells. The expression of GAPDH was used as a control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$)

right panel). These data thereby implicate that let-7b elicits suppressive activity in HCV protein expression.

HMGA2 is one of the major let-7b target genes and is down-regulated in let-7b-transfected ConI cells as previous reported (Fig. 2d, left panel) [29]. To rule out down-regulation of host transcripts accounts for the inhibitory effect of let-7b on HCV expression, HMGA2 was knockdown by two independent shHMGA2 plasmids. Although HMGA2 was significantly down-regulated in the shHMGA2 expressing cells, no effect was observed for the expression of the viral protein NS5A (Fig. 2d, right panel). These data indicate that down-regulation of HMGA2 does not contribute to the effect of let-7b on HCV expression.

To further delineate the association between let-7b and HCV infectivity, let-7b expression in various HCV-associated cell lines were determined. As shown in Fig. 2e, let-7b expression in the HCV permissive Huh7.5 cells was less than its expression in the parental Huh7 cells ($p < 0.01$).

Consistent with these observations, ConI cells bearing replicated HCV genome also had much lower let-7b (Fig. 2e, $p < 0.01$). Furthermore, Huh7 cells were more permissive for HCVcc infection when let-7b was inactivated by the let-7b inhibitor (Fig. 2f, $p < 0.05$). These data indicate that the cells capable of persistent HCV replication are usually associated with a low level of let-7b expression.

Let-7b reduces HCVcc infectivity

The HCVcc system was used to elucidate the role of let-7b in HCV infectivity. Let-7b was transfected into Huh7.5 cells followed by infection with HCVcc derived from J6/JFH-1 (genotype 2a). Hepatitis C virus expression was then monitored by fluorescent staining using the anti-HCV Core antibody (Fig. 3a). Our data revealed that let-7b reduced HCV infectivity for 42% ($p < 0.05$) while miR-122 enhanced the infectivity for 63% ($p < 0.05$) when compared to the cells expressing negative control miRNA (Fig. 3b). The HCV RNA was also decreased in let-7b-transfected cells (Fig. 3c, $p < 0.05$) indicating that let-7b suppresses HCV RNA level leading to a decrease in viral production.

To further confirm the negative regulatory effect of let-7b on HCVcc production, Huh7.5 cells were infected with the JC1-Luc2A HCV reporter virus and the luciferase activity was used to evaluate HCV viral production. Our data revealed that let-7b reduced 75% of the HCV reporter virus luciferase activity (Fig. 3d, $p < 0.001$) when compared to the cells expressing negative control miRNA. As a control, miR-122 increased 87% of the HCV reporter virus luciferase activity ($p < 0.05$). In contrast, mutation of

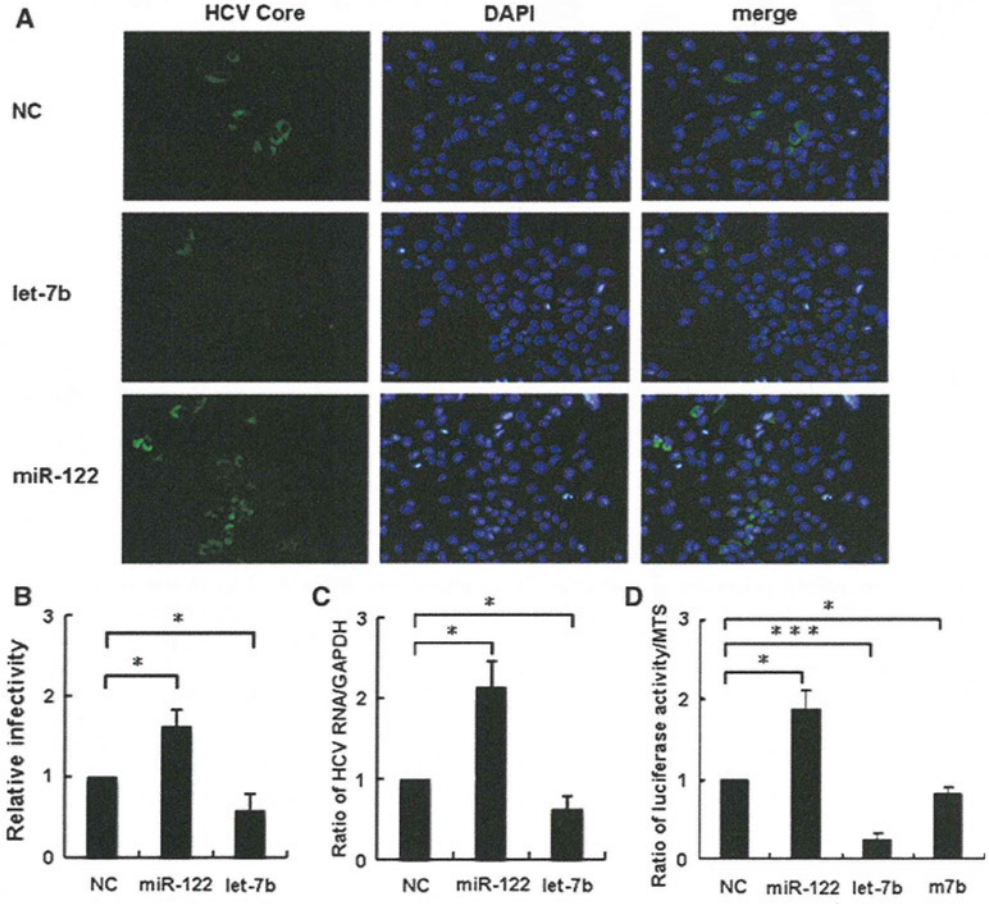

Fig. 3 Let-7b reduces HCVcc infectivity. **a** The indicated miRNAs were transfected into Huh7.5 cells for 24 h followed by infection with J6/JFH-based HCVcc. After 72 h, the cells were stained by anti-Core antibody. Nuclei were visualized by DAPI staining. *NC* negative control. **b** The infectious foci were counted by fluorescence microscopy. The infectivity for the cells transfected with negative control miRNA (NC) was set as one. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$). **c** HCV RNA expression was quantified by real-time RT-PCR using the total RNAs from the indicated transfected cells. The expression of GAPDH was used as a control for normalization. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$). **d** The miRNA precursors or the mutant let-7b (m7b) were transfected into Huh7.5 cells for 24 h followed by infection with JC1-luc2A HCV reporter virus. After 72 h, cell viability was determined by MTS assay and the cell extracts were collected for luciferase activity assay. The relative firefly luciferase versus MTS activity was shown and the negative control miRNA was arbitrarily denoted as one. The data represented the mean ± SD ($n = 3$; $^*p < 0.05$; $^{***}p < 0.001$)