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.

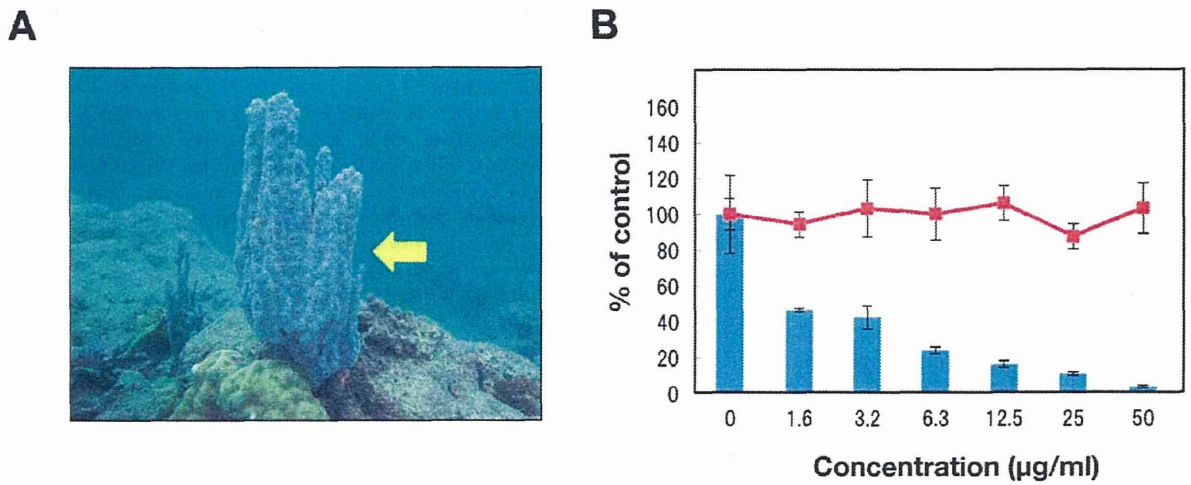

Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b. (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7 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

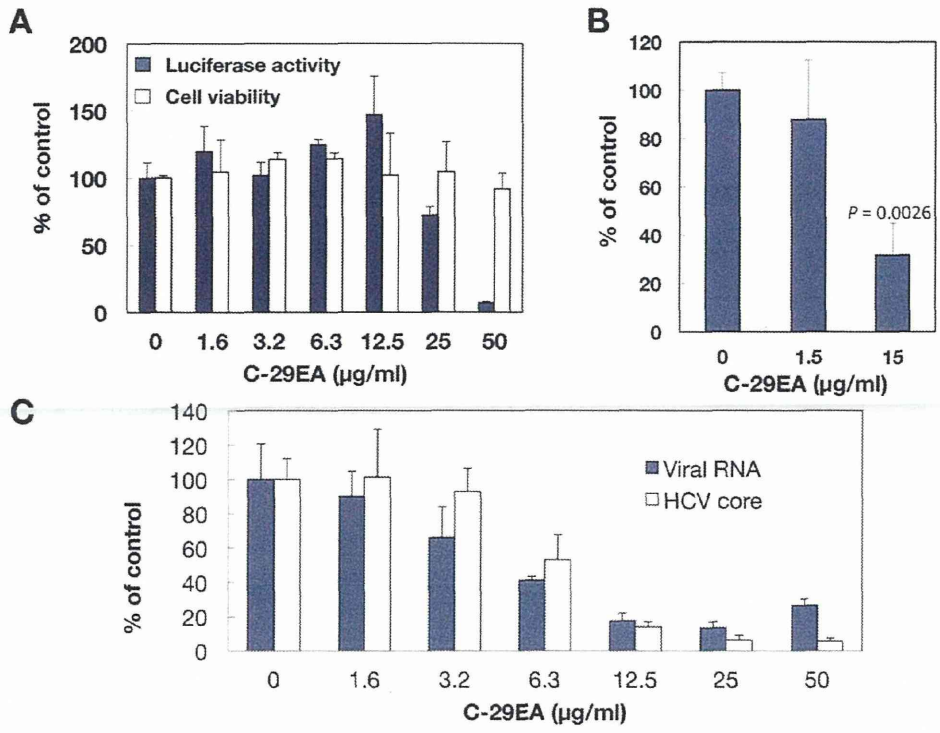

Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity 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

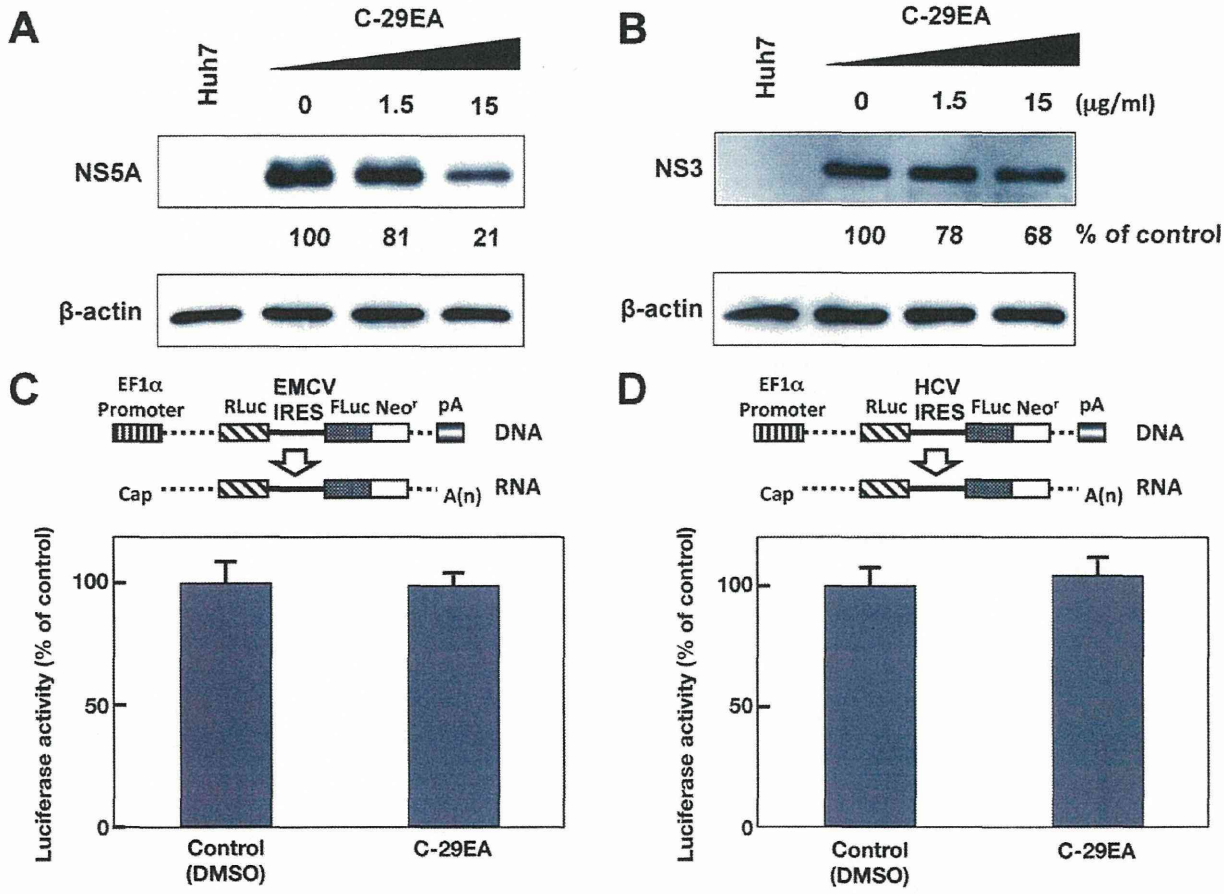

Figure 3. Effect of C-29EA on expression of viral proteins in replicon cell lines. The Huh7 replicon cell lines derived from genotype 1b (A) and 2a (B) were incubated with C-29EA at 37°C for 72 h. The treated cells were harvested and then subjected to Western blotting. Treatment with DMSO corresponds 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.
doi:10.1371/journal.pone.0048685.g003

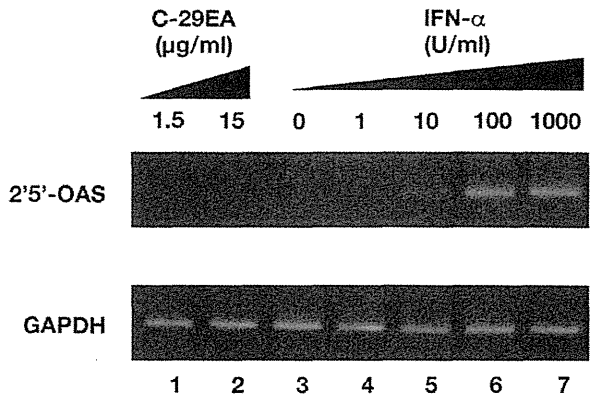

Figure 4. Effect of C-29EA on interferon signaling pathway. The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 μg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2', 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.

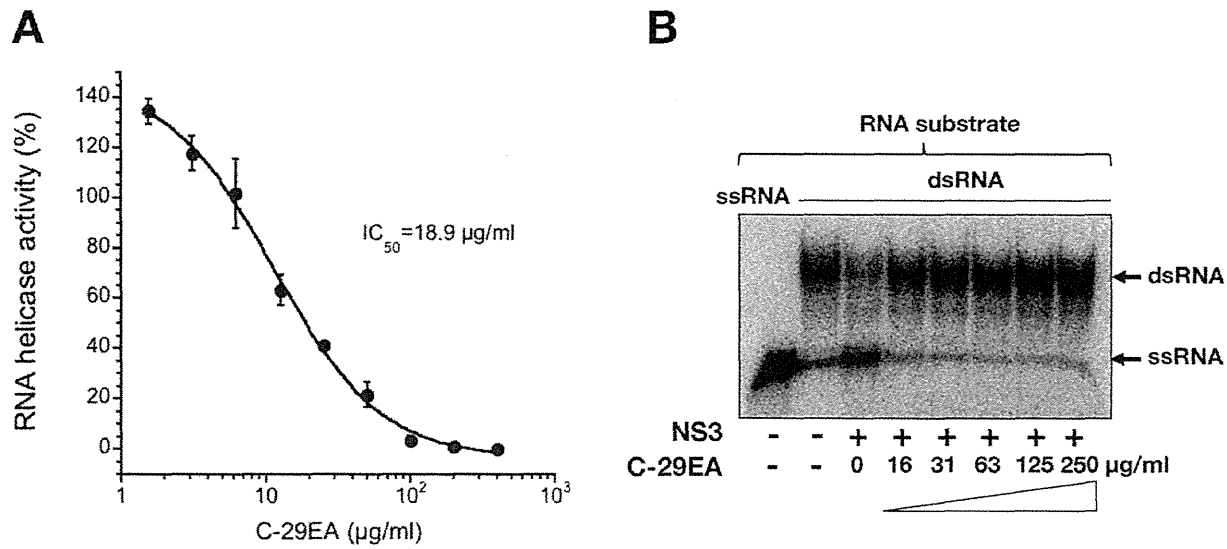

Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds 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

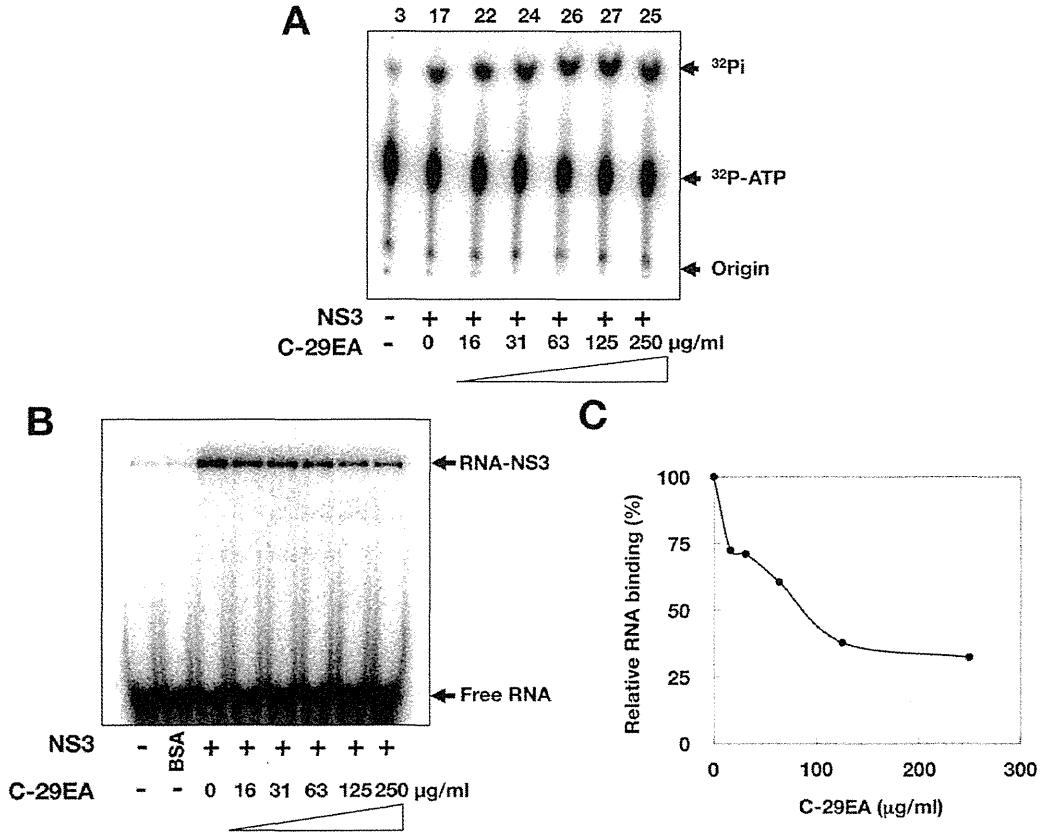

Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and 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

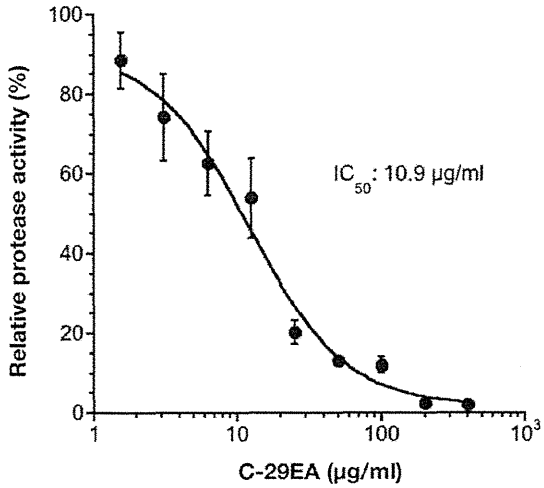

Figure 7. Effect of C-29EA on the activity of NS3 serine protease. NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction. 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

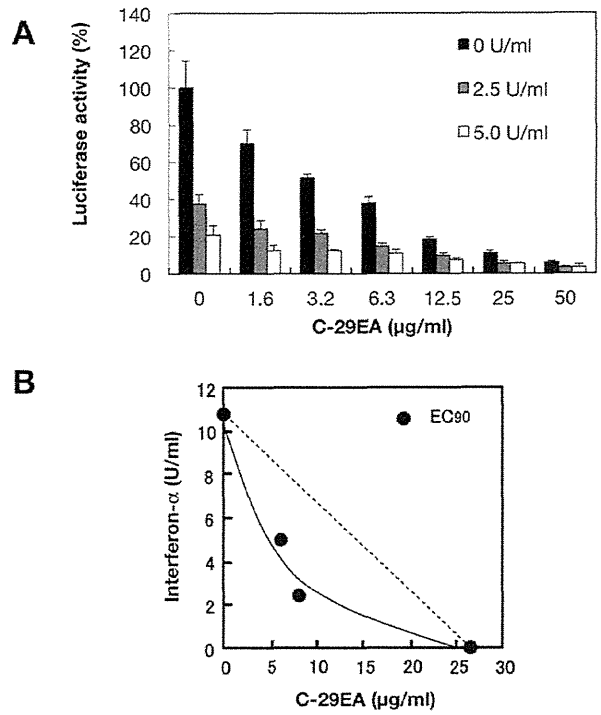

Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase 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^{TM} 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.

References

1. Baldo V, Baldovin T, Trivello R, Floreani A (2008) Epidemiology of HCV infection. Curr Pharm Des 14: 1646–1654.
2. Seeff LB (2002) Natural history of chronic hepatitis C. Hepatology 36: S35–46.
3. Moriishi K, Matsuura Y (2012) Exploitation of lipid components by viral and host proteins for hepatitis C virus infection. Front Microbiol 3: 54.
4. Tsukiyama-Kohara K, Iizuka N, Kohara M, Nomoto A (1992) Internal ribosome entry site within hepatitis C virus RNA. J Virol 66: 1476–1483.
5. Kim DW, Gwack Y, Han JH, Choe J (1995) C-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. Biochem Biophys Res Commun 215: 160–166.
6. Kanai A, Tanabe K, Kohara M (1995) Poly(U) binding activity of hepatitis C virus NS3 protein, a putative RNA helicase. FEBS Lett 376: 221–224.
7. Manns MP, Wedemeyer H, Cornberg M (2006) Treating viral hepatitis C: efficacy, side effects, and complications. Gut 55: 1350–1359.
8. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, et al. (2009) Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. N Engl J Med 360: 1827–1838.
9. Zeuzem S, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, et al. (2004) Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. J Hepatol 40: 993–999.
10. Asselah T, Marcellin P (2011) New direct-acting antivirals' combination for the treatment of chronic hepatitis C. Liver Int 31 Suppl 1: 68–77.
11. Jazwinski AB, Muir AJ (2011) Direct-acting antiviral medications for chronic hepatitis C virus infection. Gastroenterol Hepatol (N Y) 7: 154–162.
12. Lange CM, Sarrazin C, Zeuzem S (2010) Review article: specifically targeted anti-viral therapy for hepatitis C - a new era in therapy. Aliment Pharmacol Ther 32: 14–28.
13. Hofmann WP, Zeuzem S (2011) A new standard of care for the treatment of chronic HCV infection. Nat Rev Gastroenterol Hepatol 8: 257–264.
14. Kwong AD, Kauffman RS, Hurter P, Mueller P (2011) Discovery and development of telaprevir: an NS3-4A protease inhibitor for treating genotype 1 chronic hepatitis C virus. Nat Biotechnol 29: 993–1003.
15. Jacobson IM, McHutchison JG, Dusheiko G, Di Bisceglie AM, Reddy KR, et al. (2011) Telaprevir for previously untreated chronic hepatitis C virus infection. N Engl J Med 364: 2405–2416.
16. Sarrazin C, Hezode C, Zeuzem S, Pawlotsky JM (2012) Antiviral strategies in hepatitis C virus infection. J Hepatol 56 Suppl 1: S88–100.
17. Chen ST, Wu PA (2012) Severe Cutaneous Eruptions on Telaprevir. J Hepatol 57: 470–472.
18. Kieffer TL, Kwong AD, Picchio GR (2010) Viral resistance to specifically targeted antiviral therapies for hepatitis C (STAT-Cs). J Antimicrob Chemother 65: 202–212.
19. Thompson AJ, McHutchison JG (2009) Antiviral resistance and specifically targeted therapy for HCV (STAT-C). J Viral Hepat 16: 377–387.
20. Chin YW, Balunas MJ, Chai HB, Kinghorn AD (2006) Drug discovery from natural sources. AAPS J 8: E239–253.
21. Koehn FE, Carter GT (2005) The evolving role of natural products in drug discovery. Nat Rev Drug Discov 4: 206–220.
22. Li JW, Vederas JC (2009) Drug discovery and natural products: end of an era or an endless frontier? Science 325: 161–165.
23. Donia M, Hamann MT (2003) Marine natural products and their potential applications as anti-infective agents. Lancet Infect Dis 3: 338–348.
24. Molinski TF, Dalisay DS, Lievens SL, Saludes JP (2009) Drug development from marine natural products. Nat Rev Drug Discov 8: 69–85.
25. Mayer AM, Glaser KB, Cuevas C, Jacobs RS, Kem W, et al. (2010) The odyssey of marine pharmaceuticals: a current pipeline perspective. Trends Pharmacol Sci 31: 255–265.
26. Frese M, Barth K, Kaul A, Lohmann V, Schwarzle V, et al. (2003) Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. J Gen Virol 84: 1253–1259.
27. Blight KJ, Kolykhalov AA, Rice CM (2000) Efficient initiation of HCV RNA replication in cell culture. Science 290: 1972–1974.
28. Guo JT, Bichko VV, Seeger C (2001) Effect of alpha interferon on the hepatitis C virus replicon. J Virol 75: 8516–8523.
29. Tani H, Akimitsu N, Fujita O, Matsuda Y, Miyata R, et al. (2009) High-throughput screening assay of hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon. Biochem Biophys Res Commun 379: 1054–1059.
30. Yamashita A, Salam KA, Furuta A, Matsuda Y, Fujita O, et al. (2012) Inhibition of hepatitis C virus replication and NS3 helicase by the extract of the feather star *Alloeocomatella polycladia*. Mar Drugs 10: 744–761.
31. Huang Y, Liu ZR (2002) The ATPase, RNA unwinding, and RNA binding activities of recombinant p68 RNA helicase. J Biol Chem 277: 12810–12815.
32. Failla C, Tomei L, De Francesco R (1994) Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. J Virol 68: 3753–3760.
33. Leu GZ, Lin TY, Hsu JT (2004) Anti-HCV activities of selective polyunsaturated fatty acids. Biochem Biophys Res Commun 318: 275–280.
34. Ahmed-Belkacem A, Ahnou N, Barbotte L, Wychowski C, Pallier C, et al. (2010) Silibinin and related compounds are direct inhibitors of hepatitis C virus RNA-dependent RNA polymerase. Gastroenterology 138: 1112–1122.
35. Ciesek S, von Hahn T, Colpitts CC, Schang LM, Friesland M, et al. (2011) The green tea polyphenol, epigallocatechin-3-gallate, inhibits hepatitis C virus entry. Hepatology 54: 1947–1955.
36. Takeshita M, Ishida Y, Akamatsu E, Ohmori Y, Sudoh M, et al. (2009) Proanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. J Biol Chem 284: 21165–21176.
37. Wagoner J, Negash A, Kane OJ, Martinez LE, Nahmias Y, et al. (2010) Multiple effects of silymarin on the hepatitis C virus lifecycle. Hepatology 51: 1912–1921.
38. Salam KA, Furuta A, Noda N, Tsuneda S, Sekiguchi Y, et al. (2012) Inhibition of Hepatitis C Virus NS3 Helicase by Manoalide. J Nat Prod 75: 650–654.
39. Bartenschlager R, Ahlborn-Laake L, Mous J, Jacobsen H (1993) Nonstructural protein 3 of the hepatitis C virus encodes a serine-type proteinase required for cleavage at the NS3/4 and NS4/5 junctions. J Virol 67: 3835–3844.
40. Belon CA, Frick DN (2009) Helicase inhibitors as specifically targeted antiviral therapy for hepatitis C. Future Virol 4: 277–293.
41. Frick DN (2007) The hepatitis C virus NS3 protein: a model RNA helicase and potential drug target. Curr Issues Mol Biol 9: 1–20.
42. Kwong AD, Rao BG, Jeang KT (2005) Viral and cellular RNA helicases as antiviral targets. Nat Rev Drug Discov 4: 845–853.
43. Belon CA, High YD, Lin TI, Pauwels F, Frick DN (2010) Mechanism and specificity of a symmetrical benzimidazolephenylcarboxamide helicase inhibitor. Biochemistry 49: 1822–1832.
44. Maga G, Gemma S, Fattorusso C, Locatelli GA, Butini S, et al. (2005) Specific targeting of hepatitis C virus NS3 RNA helicase. Discovery of the potent and selective competitive nucleotide-mimicking inhibitor QU663. Biochemistry 44: 9637–9644.
45. Reesink HW, Zeuzem S, Weegink CJ, Forestier N, van Vliet A, et al. (2006) Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. Gastroenterology 131: 997–1002.
46. Malcolm BA, Liu R, Lahser F, Agrawal S, Belanger B, et al. (2006) SCH 503034, a mechanism-based inhibitor of hepatitis C virus NS3 protease, suppresses polyprotein maturation and enhances the antiviral activity of alpha interferon in replicon cells. Antimicrob Agents Chemother 50: 1013–1020.
47. Njoroge FG, Chen KX, Shih NY, Piwinski JJ (2008) Challenges in modern drug discovery: a case study of boceprevir, an HCV protease inhibitor for the treatment of hepatitis C virus infection. Acc Chem Res 41: 50–59.
48. Beran RK, Pyle AM (2008) Hepatitis C viral NS3-4A protease activity is enhanced by the NS3 helicase. J Biol Chem 283: 29929–29937.
49. Beran RK, Serebrov V, Pyle AM (2007) The serine protease domain of hepatitis C viral NS3 activates RNA helicase activity by promoting the binding of RNA substrate. J Biol Chem 282: 34913–34920.
50. Cummings MD, Lindberg J, Lin TI, de Kock H, Lenz O, et al. (2010) Induced-fit binding of the macrocyclic noncovalent inhibitor TMC435 to its HCV NS3/NS4A protease target. Angew Chem Int Ed Engl 49: 1652–1655.
51. Romano KP, Ali A, Royer WE, Schiffer CA (2010) Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. Proc Natl Acad Sci U S A 107: 20986–20991.
52. Schiering N, D'Arcy A, Villard F, Simic O, Kamke M, et al. (2011) A macrocyclic HCV NS3/4A protease inhibitor interacts with protease and helicase residues in the complex with its full-length target. Proc Natl Acad Sci U S A 108: 21052–21056.

53. Aratake S, Tomura T, Saitoh S, Yokokura R, Kawanishi Y, et al. (2012) Soft coral Sarcophyton (Cnidaria: Anthozoa: Octocorallia) species diversity and chemotypes. PLoS One 7: e30410.
54. Ikeda M, Abe K, Dansako H, Nakamura T, Naka K, et al. (2005) Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem Biophys Res Commun 329: 1350–1359.
55. Nishimura-Sakurai Y, Sakamoto N, Mogushi K, Nagaie S, Nakagawa M, et al. (2010) Comparison of HCV-associated gene expression and cell signaling pathways in cells with or without HCV replicon and in replicon-cured cells. J Gastroenterol 45: 523–536.
56. Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, et al. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 11: 791–796.
57. Moriishi K, Shoji I, Mori Y, Suzuki R, Suzuki T, et al. (2010) Involvement of PA28gamma in the propagation of hepatitis C virus. Hepatology 52: 411–420.
58. Jin H, Yamashita A, Maekawa S, Yang P, He L, et al. (2008) Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication in vitro. Hepatol Res 38: 909–918.
59. Gallinari P, Brennan D, Nardi C, Brunetti M, Tomei L, et al. (1998) Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. J Virol 72: 6758–6769.
60. Nishikawa F, Funaji K, Fukuda K, Nishikawa S (2004) In vitro selection of RNA aptamers against the HCV NS3 helicase domain. Oligonucleotides 14: 114–129.

Correlation between Pretreatment Viral Sequences and the Emergence of Lamivudine Resistance in Hepatitis B Virus Infection.

Ryota Sueki[‡], Shinya Maekawa[‡], Mika Miura, Makoto Kadokura, Kazuki Komase, Hiroko Shindo, Asuka Kanayama, Takako Ohmori, Kuniaki Shindo, Fumitake Amemiya, Yasuhiro Nakayama, Tomoyoshi Uetake, Taisuke Inoue, Minoru Sakamoto, Nobuyuki Enomoto

Sueki[‡] and Maekawa[‡] contributed equally to the work.

First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi,
1110 Shimokato, Chuo, Yamanashi 409-3898, JAPAN

Key words: *HBV, lamivudine resistance, pre-S substitution, ORF sequence analysis*

Running title: *Prediction of HBV lamivudine resistance*

Corresponding author:
Shinya Maekawa, M.D., Ph.D.
First Department of Internal Medicine, Faculty of Medicine, University of Yamanashi,
1110 Shimokato, Chuo, Yamanashi 409-3898, JAPAN
Tel: +81-55-273-9584
Fax: +81-5-5273-6748
E-mail: maekawa@yamanashi.ac.jp

The emergence of amino acid or nucleotide substitutions leads to lamivudine resistance in Hepatitis B virus (HBV) infected patients. The aim of this study was to investigate whether viral sequences help predict the emergence of lamivudine resistance. The study subjects comprised 59 consecutive patients infected with HBV treated with daily therapy of 100 mg lamivudine. Among those, 32 patients with adequate pretreatment serum preservation were investigated for the correlation between viral amino acid substitutions and the appearance of lamivudine resistance with consideration of clinical background by determining dominant HBV full open reading frames. Viral resistance to lamivudine emerged in 28 of 59 patients (47%) in a median period of 2.45 years. Sequence comparisons of HBV genomes between patients who later developed lamivudine resistance and patients who did not revealed the existence of significant differences between the two groups in the pre-S1 84 (P=0.042), pre-S2 1 (P = 0.017) and 22 (P=0.015), and polymerase tp 95 (P=0.046), judged by a log-rank test. Viral sequence analyses revealed the presence of amino acid substitutions in HBV pre-S1 and pre-S2 that may be associated with the emergence of lamivudine resistance during chronic HBV infection.

INTRODUCTION

Hepatitis B virus (HBV) infects persistently more than 350 million people worldwide [Liang, 2009], and increases their risk of developing liver cirrhosis, hepatic decompensation, and hepatocellular carcinoma (HCC) over the typically long disease course. High serum virus titers have been shown recently to promote significantly disease progression, as well as the development of HCC [Chen et al., 2006b; Iloeje et al., 2006]. Therefore, effective suppression of the serum viral load by an antiviral agent might inhibit disease progression [Lim et al., 2009].

Lamivudine was introduced clinically as one of the first-generation nucleoside analogs to inhibit HBV replication [Liaw et al., 2004]. Lamivudine is safe [Lok et al., 2003], effectively decreases serum viral load, improves alanine aminotransferase (ALT) levels and liver fibrosis [Leung, 2000; Villeneuve et al., 2000], and enhances hepatitis B e antigen (HBeAg) seroconversion rates [Chen et al., 2006a; Leung et al., 2001; Liaw et al., 2000], which lead to the suppression of HCC development [Liaw et al., 2004]. In contrast, prolonged use of lamivudine may lead to the emergence of drug-resistant HBV mutants in a substantial percentage of patients. When resistance emerges, patients should be treated with a different nucleoside analog, which does not show cross-resistance, alone or in combination with lamivudine [Carey and Harrison, 2009; Chen et al., 2009; Rizzetto et al., 2005]. Newly introduced second-generation nucleoside analogs, such as entecavir and tenofovir, have been shown to be superior in suppressing viral load and preventing the emergence of drug-resistant viruses. However, because of its high economical efficacy compared to other, newer-generation nucleoside analogs, the appropriate selection of patients suitable for lamivudine therapy by accurate prediction of the emergence of resistance would benefit economically-challenged patients worldwide. On the other hand, prediction of the eventual emergence of resistance to lamivudine has been difficult.

Many previous studies have shown a correlation between lamivudine resistance and the HBV mutations that appear with viral acquisition of lamivudine resistance. These mutations lead to amino acid mutations in the HBV polymerase, including rt M204I/V in the C domain and rt V173L and rt L180M in the B domain [Ghany and Doo, 2009; Ling et al., 1996; Tipples et al., 1996]. However, it is not known whether any specific sequences of viral genomes not exposed to lamivudine might predict

the development of resistance following the commencement of lamivudine treatment. Typically, the emergence of lamivudine resistance has been predicted by pretreatment or in-treatment clinical variables, such as HBeAg positivity, higher baseline HBV DNA levels, female sex, lower ALT levels and a poor early viral response to lamivudine [Andersson and Chung, 2009; Zhou et al., 2009].

The present study was conducted to clarify and characterize pretreatment HBV sequences associated with the subsequent emergence of lamivudine resistance by determining the complete sequences of HBV ORFs by direct nucleotide sequencing, using patients' sera as the source of HBV DNA.

PATIENTS AND METHODS

Patients

Fifty-nine patients with chronic hepatitis or liver cirrhosis, infected with HBV and who underwent lamivudine therapy at Yamanashi University Hospital from May 2001 to June 2010 were enrolled initially in the study. All patients received lamivudine orally, initially at a dosage of 100 mg per day. Although all 59 patients responded initially to lamivudine therapy and HBV DNA became undetectable, lamivudine resistance was diagnosed in 28 patients (47%) because HBV DNA reappeared during the observation period, while in the other 31 patients it did not (Fig. 1). Because pretreatment serum from 32 of the patients had been preserved adequately for determination of the complete HBV nucleotide sequence, the final analysis was based on these 32 patients. All patients included were positive for hepatitis B surface antigen (HBsAg) and were tested for HBV DNA by the Quantiplex HBV DNA assay (Bayer Diagnostics, Emeryville, CA, USA), transcription-mediated amplification assay (Chugai Diagnostics Science Co., Ltd., Tokyo, Japan), or COBAS® Amplicor HBV Monitor Test v2.0 (Roche Diagnostics, Indianapolis, IN, USA). Patients with co-existing autoimmune hepatitis, alcoholic liver disease, drug-induced liver injury, chronic hepatitis C, or human immunodeficiency virus infection were excluded from the study. For patients with emerging drug resistance, adefovir dipivoxil was started at a dosage of 10 mg per day, in addition to lamivudine, according to the guideline established by a hepatitis study group of the Ministry of Health, Labour, and Welfare, Japan. A signed consent was obtained for the study protocol that had been approved by Human Ethics Review Committee of Yamanashi University Hospital.

DNA extraction, PCR, and direct sequencing

Full-length HBV DNA was amplified by two-step PCR from patients' sera and sequenced directly as described elsewhere [Sugauchi et al., 2001]. Sequence reads were assembled using Vector NTI software (Invitrogen, Tokyo, Japan) and base-calling errors were corrected following inspection of the chromatogram. For ambiguous reads, only the dominant base was assigned after evaluation of all overlapping fragments. Full-length HBV genome sequences were assembled using this information

and translated *in silico* and the ORFs of drug-resistant and sensitive genomes were compared.

Statistical analysis

Statistical differences in the parameters, including all available demographic, biochemical, hematological, and virological statuses, were determined for the different patient groups by Student's t test for numerical variables and Fisher's exact probability test for categorical variables. The odds ratio and 95% confidence intervals were calculated. P values of <0.05 by the two-tailed test were considered to indicate statistical significance. In order to evaluate the contribution of pretreatment viral amino acid sequences to the development of lamivudine resistance, Kaplan-Meier analysis and Cox proportional hazards model was performed.

RESULTS

Patients' clinical parameters

The pretreatment clinical and virological characteristics of the 32 patients, prior to starting lamivudine therapy, are shown in Table I, sorted according to the subsequent emergence of lamivudine resistance. Although HBV DNA became undetectable initially after the commencement of lamivudine therapy, drug resistance was diagnosed in 14 patients because of reappearance of HBV DNA during the observation period. No statistical difference was observed in age, sex, ALT, total bilirubin, choline esterase, total cholesterol, prothrombin time, platelets, alpha-fetoprotein, HBeAg/anti-HBe positivity, viral genotypes, liver disease (chronic hepatitis or liver cirrhosis), or pretreatment HBV DNA level. Genotype C was most prevalent in both groups (16/18 in the non-resistant group and 13/14 in the resistant group). In contrast, the time for HBV DNA to become undetectable was longer in this group, compared to that in the susceptible group (P=0.024). Figure 1 shows the length of therapy for all 59 patients; "x" denotes the time of lamivudine resistance onset. Lamivudine resistance was diagnosed in 28 (47%) of 59 patients during a median observation period of 2.45 years.

Comparison of the HBV ORFs of the lamivudine resistant and non-resistant groups

Full-length HBV genomic sequences from the 32 patients were determined by direct nucleotide sequencing. Conceptual *in silico* translation of the dominant pretreatment HBV DNA sequences allowed correlation of the amino acid substitution numbers in each viral ORF with the drug resistance of the virus. Table II shows that the number of amino acid changes in each viral ORF did not differ significantly between the two groups. However, although not significant, there was a tendency that amino acid substitutions in the pre-S2 region were more frequent in patients with eventual development of lamivudine resistance (the median numbers of non-synonymous mutations were 0 and 2 in the sensitive and resistant groups, respectively; P=0.06).

Next, the amino acid residues differing between the two groups at each position in each viral protein were compared. The vertical line representing the P value for each HBV ORF (Figs. 2a–d) indicates the difference between the two groups. Comparison of the two groups revealed amino acid

differences at the residues indicated as follows: pre-S1 56, 84, pre-S2 1 and 22, S 130 (Fig. 2a), and polymerase rt 138, tp 95, spacer 37, 59, 84, and 87(Fig. 2c). The polymerase was numbered according to the standardized numbering system [Stuyver et al., 2001]. The most significant difference was observed at polymerase tp 95 in the (Fig. 2c). In contrast, only a slight difference was observed in the precore and core and X (Figs. 2b and d). In particular, the changes at pre-S1 84 and polymerase spacer 87 were seen to be coexistent because the pre-S1 and polymerase ORFs overlap. In contrast, the coding changes at polymerase rt M204I/V, rt L180M, rt 173L, rt A181V, and rt N236T, and at S I195M, S W196L, and S W196 (stop), previously reported to result from mutations associated with viral acquisition of resistance to lamivudine or adefovir, were not observed prior to lamivudine therapy in any patients in this study.

Thereafter, Kaplan-Meier curves were constructed to understand better the potential influence of the amino acid changes, as revealed above, on the emergence of lamivudine resistance (Fig. 3, log-rank test). When the time of emergence of resistance was considered, a significant difference was observed with respect to the substitutions of pre-S1 84 (P=0.042), pre-S2 1 (P=0.017) and 22 (P = 0.015), and polymerase tp 95 (P=0.046). Figure 4 shows a multiple alignment of amino acid sequences within the pre-S1, pre-S2, and polymerase ORFs.

Patient characteristics related to HBV ORF substitutions

As shown in Table III, patients with isoleucine, threonine, leucine or valine at pre-S1 84 had significantly lower HBV DNA levels, which became undetectable earlier than in patients with alanine at pre-S1 84. There were no evident differences between the characteristics of patients with and without substitutions at pre-S2 1 of. Patients with substitutions at pre-S2 22 were older (P=0.003, Table IV). On the other hand, patients with substitutions in the polymerase tp 95 had increased total bilirubin (P=0.049), ALT values (P=0.495) and alpha-fetoprotein values (P=0.034, Table V).

Multivariate analysis to reveal independent factors predicting lamivudine resistance

In an attempt to define independent factors that might predict the emergence of lamivudine

resistance, a multivariate analysis using the Cox proportional hazards model was performed. As shown in Table VI, the duration of lamivudine treatment until HBV DNA became undetectable, serum albumin level, pre-S1 84 substitutions or pre-S2 1 and 22 substitutions, and polymerase tp 95 substitution were entered into the analysis. As a result, the pre-S1 84 and pre-S2 1 substitution could be identified as independent variables.

DISCUSSION

In this study, the correlation between pretreatment HBV genomic sequences and the emergence of resistance in patients administered lamivudine to treat chronic HBV infection were investigated. Investigation was focused on determining whether a correlation exists between the viral genome diversity and emergence of lamivudine resistance. This was accomplished by determining the complete nucleotide sequences of HBV genomes amplified from the patients' pretreatment sera. Sequence comparisons revealed that substitutions in the pre-S1 and pre-S2 ORFs serve as predictors of emergence of lamivudine resistance.

In previous studies reporting the correlation between drug resistance and mutations in the HBV genome, the focus was confined to the HBV polymerase [Ghany and Doo, 2009]. Because the polymerase protein is the direct target of nucleoside analogues, amino acid residue changes in the HBV polymerase are considered to result from selective and mutational pressure exerted by those agents. Therefore, prominent amino acid sequence changes are generally considered to appear during therapy [Kobayashi et al., 2009]. However, the emergence of resistance obviously cannot be predicted by these mutations, and the emergence of resistance usually is predicted by studying clinical factors. Among these conventional pretreatment and in-treatment predictors of lamivudine resistance, it was observed that longer periods of HBV persistence (determined by DNA detection) after commencing lamivudine therapy correlated with the appearance of resistance, an observation that was consistent with most previous studies.[Andersson and Chung, 2009; Zhou et al., 2009]. This demonstrates that studied patients did not represent outliers from random populations studied previously.

Here, amino acid differences between patients were compared, according to their responses to lamivudine treatment, at each position in each viral ORF, and showed that patients who developed resistance accumulated more substitutions within specific regions of the pre-S1, pre-S2, and polymerase ORFs. Thereafter, a statistical analysis was conducted to investigate whether these substitutions correlated with the emergence of drug resistance. It was found that preexisting substitutions in pre-S1 84 and pre-S2 1 correlated significantly and independently with lamivudine resistance. Because the HBV polymerase genes evaluated all encoded rt 204V/I mutations at the time