virus following transfection of its synthesized RNA into Huh7 cells (28). It is thus clear that JFH-1 is not the only HCV strain that can be propagated in cultured cells. However, it is also important to understand why JFH-1 is the only strain that replicates efficiently in cultured cells in the absence of adaptive mutations. In order to understand this characteristic of JFH-1, we analyzed the mechanisms that underlie the efficient replication of the JFH-1 strain (29, 30) and identified important sequence differences in the JFH-1 strain compared to the J6CF strain. However, introduction of mutations that would abrogate such sequence differences did not enable efficient replication of genotype 1b HCV strains. These data suggested that it is necessary to identify different mutations that would increase the virus production efficiency of genotype 1b HCV. Further analysis of JFH-1 genomic replication mechanisms may help to determine other differences between the replication of JFH-1 and other strains.

Previous reports have indicated that adaptive mutations enhance viral RNA replication at the expense of virus particle formation efficiency (31). A highly cell culture-adapted Con1 strain can replicate in cultured cells, but it cannot produce infectious virus particles. In the present study, we also attempted to establish a replication-competent genotype 1b HCV strain from a patient with acute severe hepatitis. We identified several adaptive mutations in colony-forming experiments using a subgenomic replicon of this NC1 strain. Notably, S2197Y and S2204G mutations exhibited an adaptive effect for both virus replication and virus particle secretion. The adaptive mutations at amino acid positions 2197 and 2204 were previously reported in genotype 1a and 1b replicon studies (reviewed in ref 32). Although the S2197Y mutation enabled the NC1 strain to produce infectious virus, the infection efficiency of this virus was very weak. We therefore hypothesized that it may be necessary to increase replication efficiency by introducing additional mutations that would facilitate efficient virus production. For this purpose, we introduced and tested the effect of introduction of the previously reported mutations, E1202G in NS3 (25) and K1846T in NS4B (26), into the pNC1/SY construct. Interestingly, virus core protein secretion of NC1/SY was enhanced by introduction of the E1202G mutation but not by introduction of the K2864T mutation. This result suggested that the selection and combination of adaptive mutations is very important for establishment of an infectious genotype 1b HCV strain. Although NC1/EGSY RNA-transfected cells produced high levels of HCV core protein into the culture medium with infectivity, not all of the cells were infected with the virus and viral infection did not continue as the cells were passaged. After several cell passages, only a small number of the transfected cells remained infected (data not shown). This result may be due to a low degree of infection efficiency that was not sufficient for productive infection or may be due to some other unknown mechanisms.

The *in vivo* infectivity of the NC1/SY virus was tested using the human hepatocyte-transplanted uPA/SCID mouse system. The NC1/SY virus showed only transient viremia in one out of two inoculated mice. Interestingly, a highly adapted Con1 strain was previously shown not to be infectious for chimpanzees, whereas a moderately adapted Con1 was infectious. However, the virus recovered from the infected animal was wild-type Con1 virus (33). This result clearly suggests that HCV strains with low replication efficiency are favorable for *in vivo* infection. A wild-type virus with low replication capacity is likely to evade the host immune surveillance system and thereby survive in an *in vivo* situation. However, the JFH-1 virus was infectious not only for cultured cells but also for chimpanzees and human liver-transplanted mice (12, 34, 35). It is therefore important to analyze how HCV can evade host defense mechanisms to understand the mechanism of persistent viral infection. Further detailed studies are necessary for such analysis.

In the present study, we established the cell culture-adapted genotype 1b HCV strain, NC1. Infectious virus was produced from RNA-transfected cells. However, virus infection could not be continuously passaged in Huh7.5.1 cells. Novel antiviral drugs targeting genotype 1b HCV are under development and some of these drugs will be used in a clinical setting. It is likely that such new therapy will be accompanied by the appearance of significant adverse effects and by the emergence of drug-resistant virus. It is therefore important to further develop improved genotype 1b infectious HCV culture systems for future studies so that these problems can be circumvented.

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DISCLOSURE

None of the authors have any conflicts of interest associated with this study.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

S-Table 1: Primers used to amplify and sequence the NC1 HCV strain.

S-Table 2: Primers used to introduce mutations into pNC1 and pSGR-NC1.

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PLOS ONE

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.

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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.

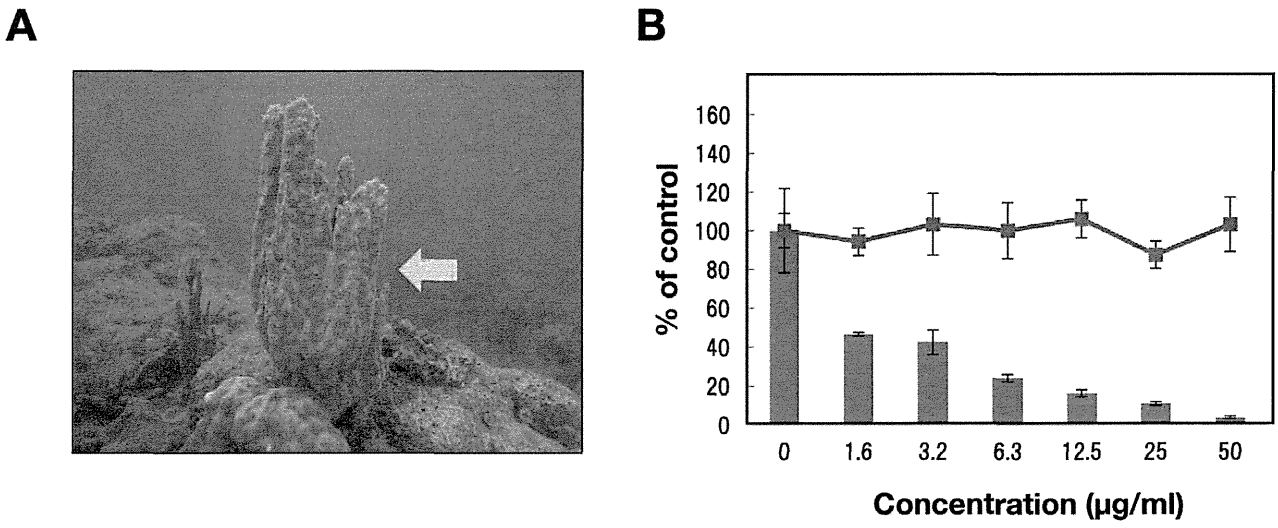

Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b. (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7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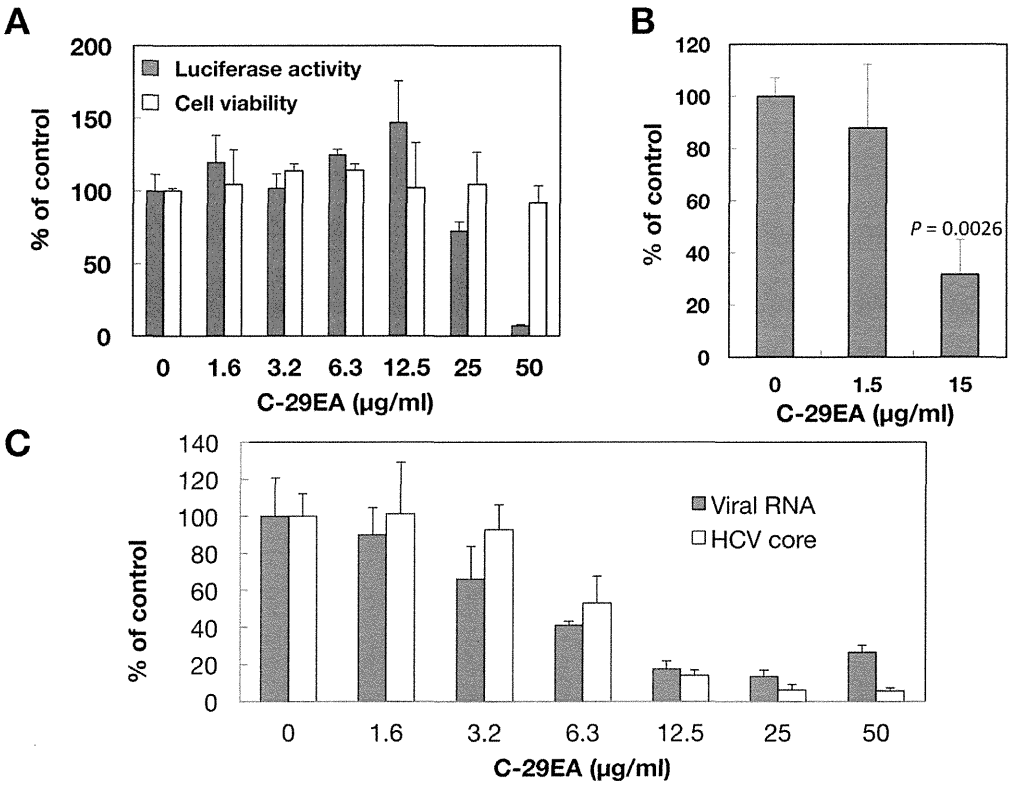

Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity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

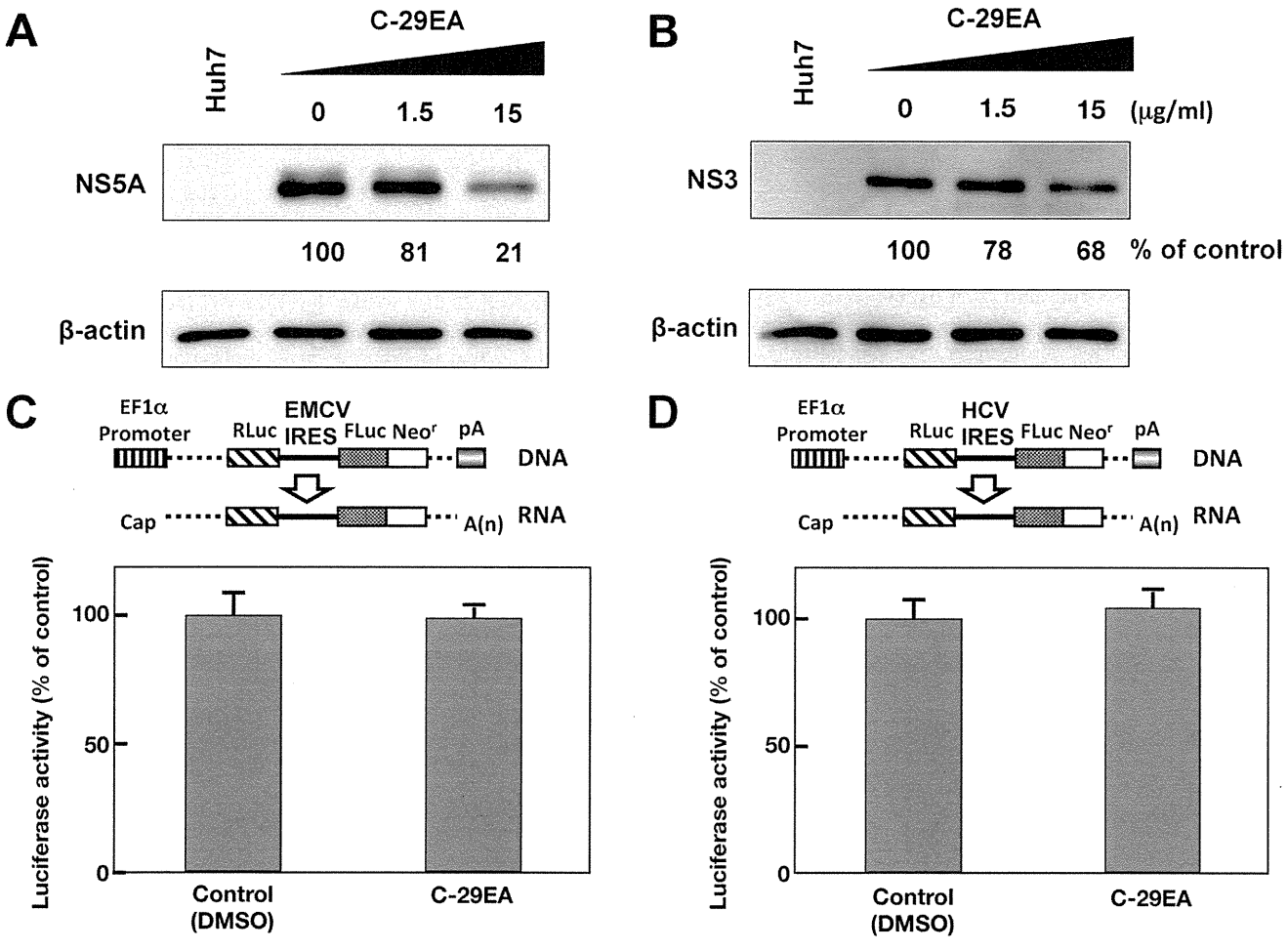

Figure 3. Effect of C-29EA on expression of viral proteins in replicon cell lines. The Huh7 replicon cell lines derived from genotype 1b (A) and 2a (B) were incubated with C-29EA at 37°C for 72 h. The treated cells were harvested and then subjected to Western blotting. Treatment with DMSO corresponds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

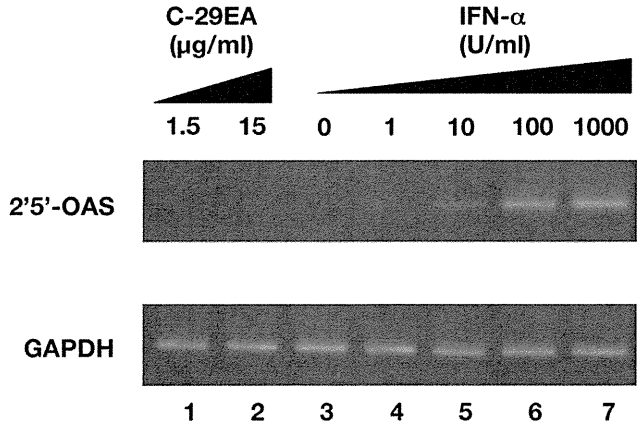

Figure 4. Effect of C-29EA on interferon signaling pathway. The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 μg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2',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

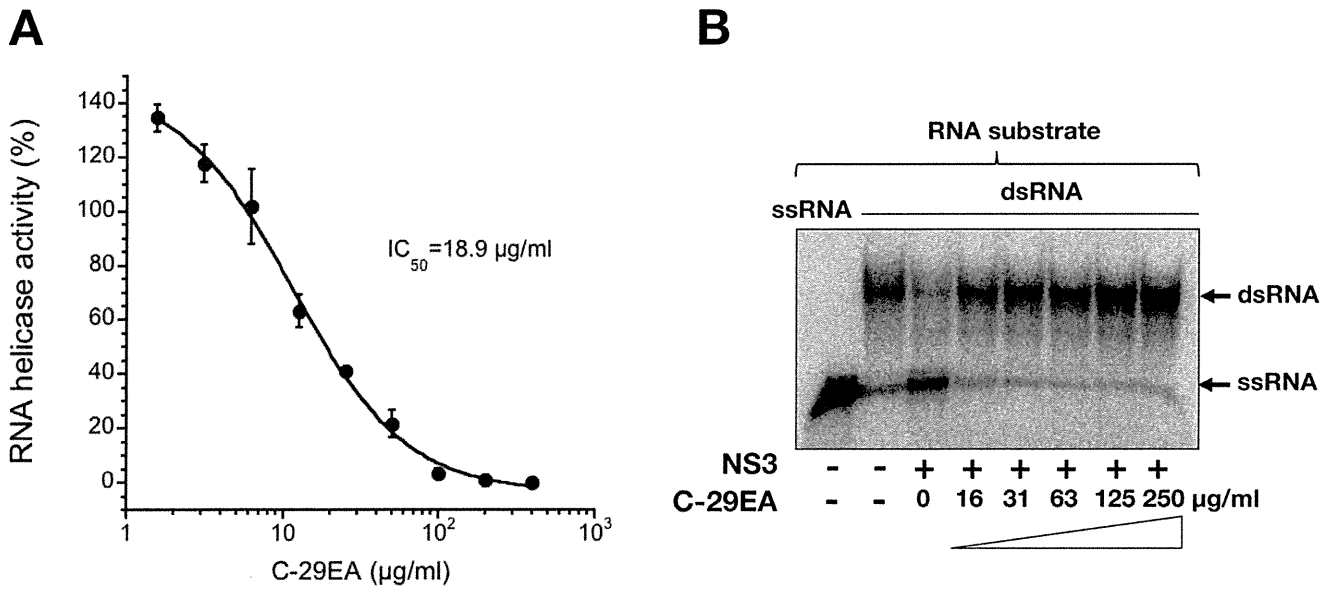

Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase. (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds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

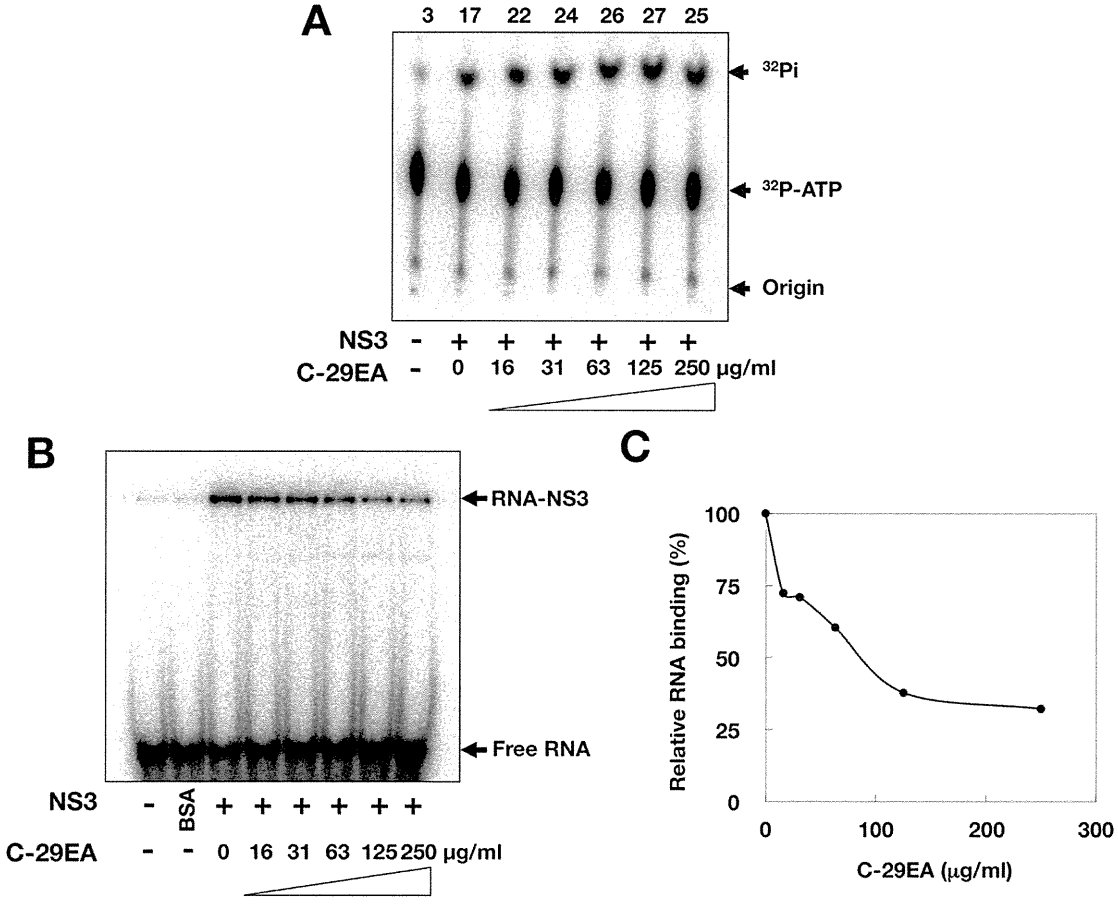

Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase. (A) The reaction mixtures were incubated with [γ-^{32}P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and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

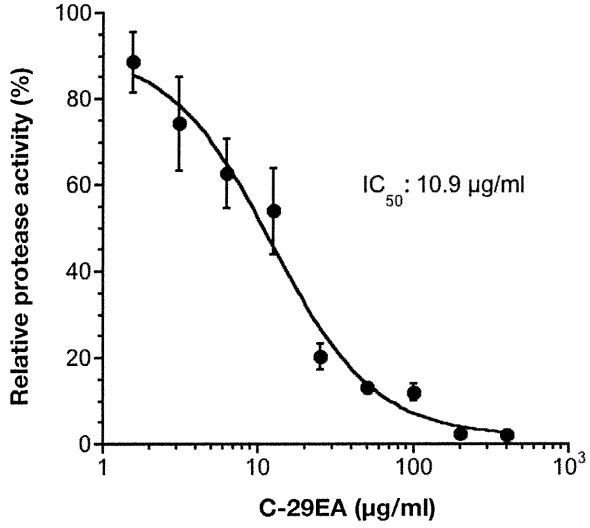

Figure 7. Effect of C-29EA on the activity of NS3 serine protease. NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction.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

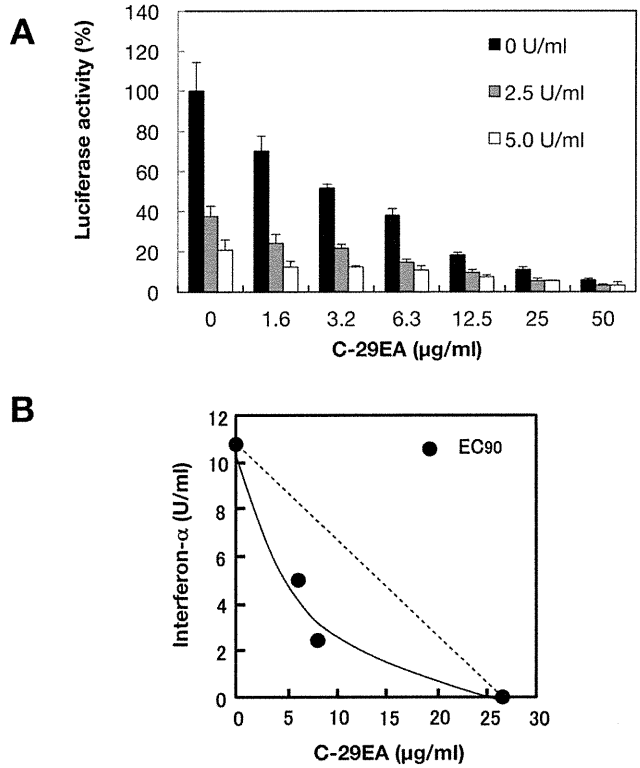

Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase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

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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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ENT1, a Ribavirin Transporter, Plays a Pivotal Role in Antiviral Efficacy of Ribavirin in a Hepatitis C Virus Replication Cell System

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We previously showed that equilibrative nucleoside transporter 1 (ENT1) is a primary ribavirin transporter in human hepatocytes. However, because the role of this transporter in the antiviral mechanism of the drug remains unclear, the present study aimed to elucidate the role of ENT1 in ribavirin antiviral action. OR6 cells, a hepatitis C virus (HCV) replication system, were used to evaluate both ribavirin uptake and efficacy. The ribavirin transporter in OR6 cells was identified by mRNA expression analyses and transport assays. Nitrobenzylmercaptopurine riboside (NBMPR) and micro-RNA targeted to ENT1 mRNA (miR-ENT1) were used to reduce the ribavirin uptake level in OR6 cells. Our results showed that ribavirin antiviral activity was associated with its accumulation in OR6 cells, which was also closely associated with the uptake of the drug. It was found that the primary ribavirin transporter in OR6 cells was ENT1 and that inhibition of ENT1-mediated ribavirin uptake by NBMPR significantly attenuated the antiviral activity of the drug as well as its accumulation in OR6 cells. The results also showed that even a small reduction in the ENT1-mediated ribavirin uptake, achieved in this case using miR-ENT1, caused a significant decrease in its antiviral activity, thus indicating that the ENT1-mediated ribavirin uptake level determined its antiviral activity level in OR6 cells. In conclusion, our results show that by facilitating its uptake and accumulation in OR6 cells, ENT1 plays a pivotal role in the antiviral effectiveness of ribavirin and therefore provides an important insight into the efficacy of the drug in anti-HCV therapy.

Chronic hepatitis C is a major cause of liver cirrhosis and hepatocellular carcinoma, and a combination of interferon-α (IFN-α) and ribavirin is a standard anti-hepatitis C virus (HCV) therapy. Since the addition of ribavirin to IFN-α significantly improves the rate of sustained virologic response (SVR) (40 to 60% in genotype 1 patients) (5), the drug plays a key role in current anti-HCV therapy.

Ribavirin, a purine nucleoside analog, is phosphorylated intracellularly to form mono-, di-, and tri-phosphates, which then accumulate within cells at high concentrations (4, 13). While the primary anti-HCV mechanisms of the drug are still under debate, it is considered likely that the important actions take place within the cells themselves, and several mechanisms have been proposed to explain what occurs there. These include inhibition of inosine monophosphate dehydrogenase (reviewed in references 4 and 7 and references therein). Additionally, a recent study revealed that ribavirin potentiates IFN-α action by augmenting IFN-stimulated induction of gene expression (16).

Taking into consideration the above-mentioned mechanisms, it is reasonable to assume that the uptake of ribavirin into hepatocytes is a prerequisite for its antiviral activity. Since ribavirin is a hydrophilic molecule, import of the drug into cells requires host nucleoside transporters, which are divided into two families: equilibrative nucleoside transporters (such as ENT1 to ENT4) and concentrative nucleoside transporters (such as CNT1 to CNT3) (9). ENTs are facilitated transporters, while CNTs are sodium-dependent active transporters. These transporters differ in tissue distribution, substrate preference, and inhibitor sensitivity. For example, sensitivities to inhibition by nitrobenzylmercaptopurine riboside (NBMPR) are different between ENT1 and ENT2 (20).

Our recent investigations into the ribavirin uptake system in human hepatocytes determined that ENT1 is a primary ribavirin uptake transporter (6). In addition, Morello et al. (12) reported the association of an intronic single nucleotide polymorphism (SNP) of the *SLC29A1* (ENT1) gene with rapid virologic response (RVR; defined as an undetectable serum HCV RNA level at week 4) of treatment of genotype-1 Caucasian patients. More recently, Tsubota and colleagues revealed that another intronic SNP in the *SLC29A1* gene is associated with SVR, as well as RVR, in genotype-1 Japanese patients (18). Based on these findings, it can be hypothesized that ENT1 plays an essential role in ribavirin anti-HCV activity.

In the present study, along with a detailed characterization of ribavirin uptake and its relationship to antiviral activity, we tested the above-mentioned hypothesis through the use of OR6 cells, which have been established as an efficient replication system for the HCV RNA genome. The HCV replication level was evaluated by monitoring the level of *Renilla* luciferase activity (8), which enabled us to simultaneously evaluate both ribavirin uptake and its antiviral activity.

MATERIALS AND METHODS

Cell culture. OR6 cells were cloned from ORN/C-5B/KE cells (derived from Huh-7 cells) supporting genome-length HCV RNA (strain O of

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genotype 1b) containing the *Renilla* luciferase reporter gene, and the cells were cultured as described previously (8). Huh-7 cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). The Huh-7 cells were cultured at 37°C with 5% CO_2–95% air in RPMI 1640 medium (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Luciferase reporter assay. OR6 cells were plated 1 day prior to the assay on 24-well plates at 1.5×10^4 to 2.5×10^4 cells/well, followed by treatment with ribavirin (Wako, Osaka, Japan) in the absence of G418 and at the indicated concentrations for 24, 48, and 72 h. The cells were then subjected to the luciferase assay using a dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's protocol. For data normalization, the protein contents were determined with a Pierce 660-nm protein assay reagent (Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's protocol. The relative luciferase activity value of the untreated or vehicle treated cells (dimethyl sulfoxide [DMSO] for NBMPR and sterile water for others) was set to 100%. NBMPR (Sigma, St. Louis, MO), hypoxanthine (MP Biomedicals, Solon, OH), and formycin B (Berry & Associates, Ann Arbor, MI) were included in inhibition analyses at various concentrations.

Western blot analysis. OR6 cells treated with ribavirin at various concentrations in the absence of G418 for 24, 48, and 72 h were harvested and homogenized. The homogenates (60 μg/well) were resolved in a sodium dodecyl sulfate (SDS)–15% polyacrylamide gel and then transferred onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk and then incubated with either antibodies against the HCV core protein (2,000-fold dilution; Institute of immunology, Tokyo, Japan) or antibodies against β-actin (500-fold dilution; Sigma). Immunocomplexes were detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (GE Healthcare, Giles, United Kingdom).

Accumulation assay. OR6 cells were plated 1 day prior to the assay on 24-well plates, after which the cells were incubated with 0.5 μCi/ml [^{3}H]ribavirin (Moravek Biochemicals, Brea, CA) and nonradiolabeled ribavirin at various concentrations. NBMPR was included in inhibition analyses at concentrations of 0.1, 1, 3, 10, 31, and 100 μM. After treatment for 9.6, 24, 48, or 72 h, the cells were washed twice with ice-cold Na^+-free Krebs-Henseleit buffer (KHB) and lysed with 0.2% SDS. Radioactivity was measured using a liquid scintillation counter (LSC 5100; Aloka, Tokyo, Japan). The protein contents were determined as described above. To completely inhibit ENT-mediated ribavirin uptake, 30 μM dipyridamole (Wako) was used in the same experimental sets (20). The data were calculated by subtracting the accumulation values obtained with dipyridamole from those without dipyridamole at the same ribavirin concentrations. All assays were performed at 37°C.

Transport assays. Transport assays were performed using the previously described centrifugal filtration method (6). OR6 cells were collected and resuspended in ice-cold Na^+-containing KHB or Na^+-free KHB at 1.4×10^6 cells/ml. NBMPR, troglitazone (Wako), hypoxanthine, and formycin B were included in the inhibition analyses. Since the rate of ribavirin uptake by OR6 cells was linear for at least 60 s in the preliminary assays, the incubation time was set to 30 s. The radioactivity and protein contents of the cells used in the assay were measured as described above. The same experiments were also performed at 4°C, and the data were obtained by subtracting the uptake levels at 4°C from those at 37°C at the same ribavirin concentrations.

Total RNA preparation, cDNA synthesis, reverse transcription-PCR (RT-PCR), and quantitative real-time PCR (qPCR). Total RNA preparation, cDNA synthesis, RT-PCR, and qPCR were performed using previously described procedures (6). Among the nucleoside transporters, ENT1, ENT2, CNT2, and CNT3 mRNAs were examined by RT-PCR because they have been identified as ribavirin transporters (21). The primers for RT-PCR and qPCR are listed in Table S1 in the supplemental material. The UPL universal probes used were no. 9 (ENT1), no. 48 (ENT2), and no. 60 (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]).

Knockdown of ENT1 mRNA expression in OR6 cells. The BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen) was used to suppress ENT1 mRNA expression in OR6 cells. The oligonucleotide containing micro-RNA targeted to ENT1 mRNA (miR-ENT1) was cloned into the pcDNA6.2-GW/EmGFP-miR vector. The control plasmid pcDNA6.2-GW/EmGFP-miR-neg, carrying an insert that is not known to target any identified vertebrate genes (miR-Neg), was used as a negative control. The sequences of inserts are shown in Table S1 in the supplemental material. The plasmids were transfected into OR6 cells using Lipofectamine LTX (Invitrogen). Two days after transfection, the culture medium was replaced with fresh medium containing 4 μg/ml blasticidin to obtain cells stably expressing miR-ENT1 (OR6/miR-ENT1) and cells stably expressing miR-Neg (OR6/miR-Ng).

Data analysis. Statistical analysis was performed using Student's *t* test. The four-parameter logistic model was used to calculate the 50% effective concentration (EC_{50}).

RESULTS

Concentration- and time-dependent anti-HCV activity and accumulation of ribavirin in OR6 cells. The inhibitory effects of ribavirin (1 to 3,162 μM) on HCV replication in OR6 cells were analyzed by monitoring the luciferase activity and HCV core protein expression levels. It was found that the HCV replication activity and core protein levels decreased in a ribavirin concentration-dependent manner (Fig. 1A and B), while the level of ribavirin accumulation increased in a saturable manner (Fig. 1C). Next, the time course of anti-HCV activity of ribavirin at concentrations of 10, 100, and 1,000 μM was examined. The results of our examination showed that, similar to the concentration-dependent profile, the HCV replication activity and core protein amounts decreased over time at each of the ribavirin concentrations tested (Fig. 1D and E) and that the levels of ribavirin accumulation increased linearly or saturably over time (Fig. 1F). These results suggest that ribavirin exerts concentration- and time-dependent antiviral activity that could be associated with the concentration- and time-dependent intracellular accumulation of the drug.

Identification of the ribavirin uptake transporter in OR6 cells. To identify the ribavirin uptake transporter in OR6 cells, we characterized the uptake profile of the drug and the nucleoside transporters mRNA expression in the cells. The ribavirin (1 to 3,162 μM) uptake level in Na^+-plus KHB was found to increase linearly up to 3 mM (Fig. 2A), and the uptake activities of the drug (nmol/mg protein/30 s) at 10, 100 (data not shown), and 1,000 μM were recorded as 0.03 ± 0.01, 0.33 ± 0.02 and 3.2 ± 0.3, respectively (Fig. 2B). The removal of Na^+ from the transport medium did not affect the uptake activities at any of the ribavirin concentrations tested, indicating that all the uptake activities of the drug were sodium independent. These activities were mostly abolished by the addition of 100 μM NBMPR, an inhibitor of ENT1 and ENT2. Consistently, the results of RT-PCR showed that ENT1 and ENT2 mRNAs were abundantly expressed in OR6 cells, while hardly any CNT2 and CNT3 mRNAs were expressed (Fig. 2C). During the above-described experiments, we found that a low concentration of NBMPR (100 nM) failed to inhibit ribavirin uptake by OR6 cells (M. Iikura, unpublished data). Considering that ENT1-mediated nucleoside uptake is generally sensitive to NBMPR inhibition at 100 nM (20), it was hypothesized that ENT2 should have contributed to ribavirin uptake in OR6 cells. However, our previous results indicated that ENT2 cannot transport ribavirin (6). Therefore, to clearly distinguish between ENT1- and

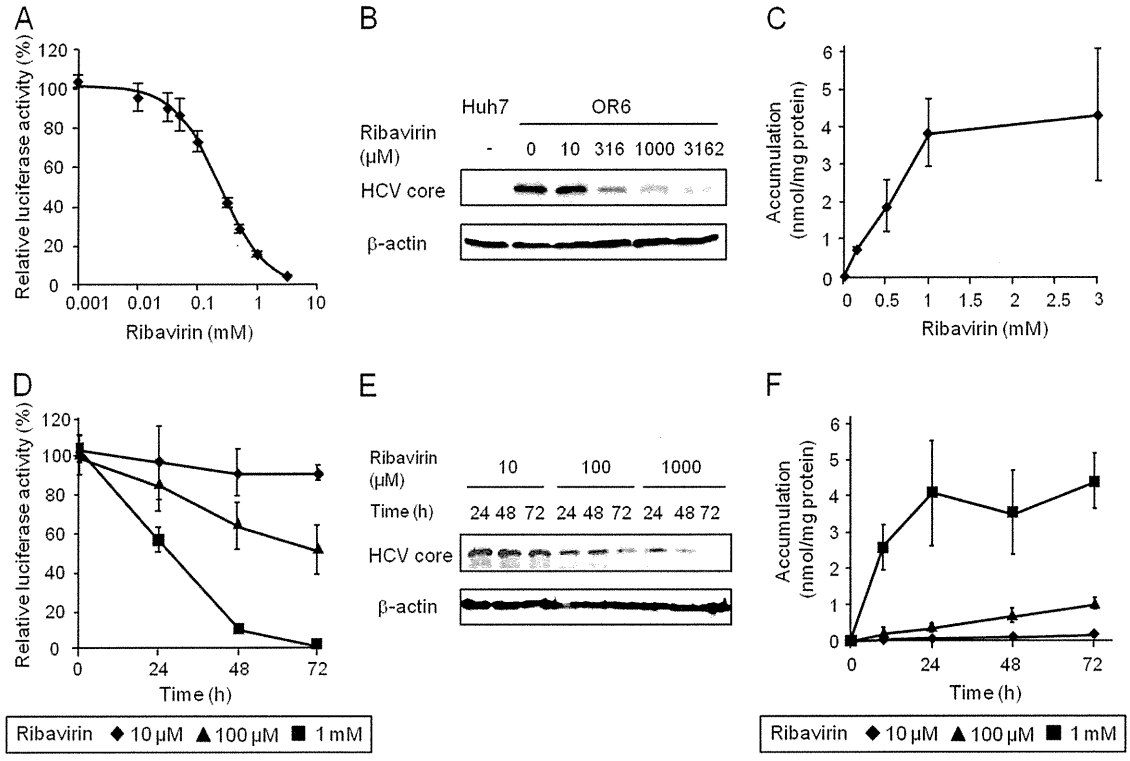

FIG 1 Concentration- and time-dependent profiles of anti-HCV activity and accumulation of ribavirin in OR6 cells. (A) OR6 cells were treated with ribavirin at concentrations of 0, 1, 10, 31, 50, 100, 316, 500, 1,000 and 3,162 μM for 48 h. The value of relative luciferase activity in the absence of ribavirin was set to 100%. (B) Expression levels of HCV core protein in OR6 cells treated with ribavirin for 48 h were examined by Western blot analysis. β-Actin was used as a loading control. Huh-7 cells were used as a negative control. (C) OR6 cells were treated with ribavirin at concentrations of 0.1, 0.5, 1, and 3 mM for 48 h, after which the radioactivity within the cells was determined. (D) OR6 cells were treated with ribavirin. The value of relative luciferase activity in the absence of ribavirin at each time point was set to 100%. (E) Expression levels of HCV core protein in OR6 cells treated with ribavirin were examined by Western blot analysis. (F) OR6 cells were treated with ribavirin, after which the radioactivity within the cells was determined. Values are means and standard deviations (SD) of the relative luciferase activity or the accumulation for three independent experiments. Each experiment was performed in duplicate. For Western blotting, the representative result for three independent assays was shown.

ENT2-mediated ribavirin uptake, inhibition analysis was performed using troglitazone (60 μM), hypoxanthine (5 mM), and formycin B (50 μM). Troglitazone has been reported to specifically inhibit ENT1 activity (10). Hypoxanthine and formycin B, at the indicated concentrations, were previously reported to preferentially inhibit ENT2 activity (3, 22), and we confirmed the inhibitory effects of these compounds on ENT2 activity by using HeLa cells (see Fig. S1 in the supplemental material). The results of the inhibition analysis showed that troglitazone completely inhibited the ribavirin uptake activity, while neither hypoxanthine nor formycin B inhibited uptake of the drug in OR6 cells (Fig. 2D). Taken together, the results indicated that, even though the affinity of ENT1 of OR6 cells for NBMPR was somehow reduced, ENT1 was exclusively responsible for the ribavirin uptake in OR6 cells.

Effect of inhibition of ribavirin uptake on its anti-HCV activity. After it was determined that ENT1 was responsible for ribavirin uptake in OR6 cells, the role of ENT1 in the anti-HCV activity of the drug (100 μM and 1 mM) was examined by chemical inhibition of ENT1-mediated ribavirin uptake in OR6 cells. Since troglitazone itself somewhat repressed HCV replication in OR6 cells (Iikura, unpublished), NBMPR was used as an ENT1 inhibitor. As shown in Fig. 3A, NBMPR decreased the level of ribavirin uptake in a dose-dependent manner and, accordingly, decreased the accumulation level of the drug in a dose-dependent manner (Fig. 3B). In association with these decreases, it was determined that the ribavirin antiviral effect was weakened by NBMPR in a concentration-dependent manner (Fig. 3C). We confirmed that ENT1 protein expression was not changed in the cells treated with the highest ribavirin and NBMPR concentrations for 48 h (see Fig. S2 in the supplemental material). To further clarify the importance of ENT1-mediated ribavirin uptake in its antiviral effects, the concentration and time dependencies of the antiviral effects of the drug were examined in cells treated with NBMPR or its vehicle (0.1% DMSO). The concentration of NBMPR was set to 7 μM, which is near the EC_{50} against ENT1 activity calculated from the results of Fig. 3A, indicating that the ENT1 activity level of NBMPR-treated cells was approximately half that of the vehicle-treated cells. As shown in Fig. 3D, the EC_{50} of ribavirin in the NBMPR-treated cells was 399 $\pm$ 22 μM, which was significantly higher than that of the vehicle-treated cells (203 $\pm$ 47 μM, P = 0.0005) (The results of the individual experiments are shown in Fig. S3 in the supplemental material.) In addition, the response to ribavirin in the NBMPR-treated cells was significantly delayed in comparison to that in the vehicle-treated cells (Fig. 3E). We also examined the constraining effects of ENT2 inhibitors on ribavirin antiviral activity but found that hypoxanthine (5 mM) and formycin B (50 μM) had no effect (see Fig. S4 in the supplemental material). Furthermore, NBMPR, hypoxanthine and formycin B

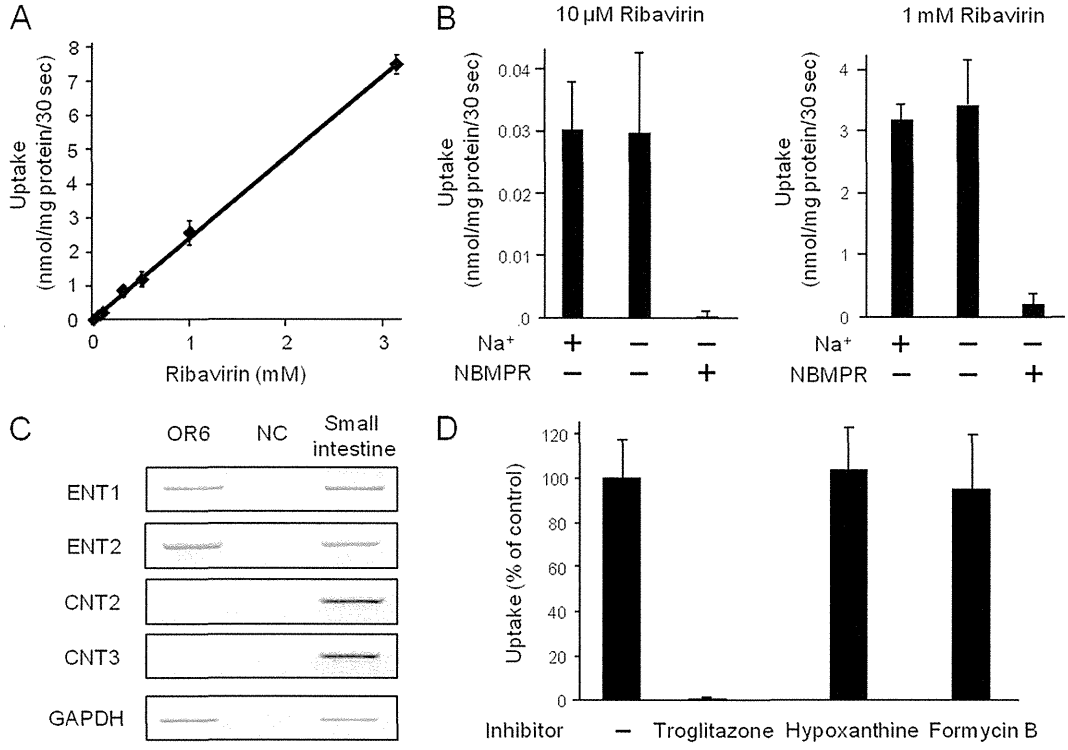

FIG 2 Identification of the ribavirin uptake transporter in OR6 cells. (A) The concentration dependence of ribavirin uptake (concentrations are given in the legend to Fig. 1A) by OR6 cells was analyzed in Na^+-containing KHB. (B) Ribavirin uptake by OR6 cells was analyzed in Na^+-containing KHB and Na^+-free KHB. In inhibition assays, the effect of 100 μM NBMPR on ribavirin uptake was analyzed in Na^+-free KHB. (C) ENT1, ENT2, CNT2, CNT3 and GAPDH mRNA expression was examined by RT-PCR. Small intestine cDNA was used as a PCR control. NC, nontemplate control. Representative results from one of three independent analyses are shown. (D) To clearly distinguish between ENT1- and ENT2-mediated ribavirin uptake, inhibition analysis of ribavirin (100 μM) uptake by OR6 cells was performed in Na^+-free KHB in the absence of inhibitor (−) or the presence of troglitazone (ENT1 inhibitor, 60 μM), hypoxanthine (ENT2 inhibitor, 5 mM), or formycin B (ENT2 inhibitor, 50 μM). The value of the transport activity of the control (no inhibitor) was set to 100%. In the above-described experiments, each value is the mean plus SD from three independent experiments, each performed in duplicate.

were found to have no effect on HCV replication activity in the above-described experiments (see Fig. S4 in the supplemental material), and NBMPR (7 μM) failed to affect telaprevir antiviral activity (see Fig. S5 in the supplemental material).

These results clearly show that inhibition of ENT1-mediated ribavirin uptake significantly attenuates ribavirin antiviral effectiveness by reducing the accumulation level of the drug in the cells.

Effect of ENT1 mRNA knockdown on ribavirin anti-HCV activity. The above-mentioned results prompted us to investigate whether a small change in ENT1 activity would similarly affect ribavirin antiviral effectiveness. miRNA targeted to ENT1 mRNA was used in this examination. We found that when stably expressed in OR6 cells (OR6/miR-ENT1), miR-ENT1 reduced the ENT1 mRNA expression level to 72.5 ± 3.4% of that of the control cells (OR6/miR-Ng) without affecting the ENT2 mRNA expression level (Fig. 4A). Accordingly, the ribavirin uptake level in OR6/miR-ENT1 cells was about 66.7 ± 14.0% of that in OR6/miR-Ng cells (Fig. 4B). To determine the degree to which this ENT1 mRNA knockdown affected ribavirin antiviral action, concentration dependencies of ribavirin action in OR6/miR-ENT1 and OR6/miR-Ng cells were characterized. We found that the EC_{50} of ribavirin in OR6/miR-ENT1 cells was 212 ± 11 μM, which was significantly higher than the EC_{50} in OR6/miR-Ng cells (143 ± 33 μM; $P = 0.013$) (The results of the individual experiments are shown in Fig. S3 in the supplemental material.) These results showed that even a small reduction in the ENT1 mRNA expression level could decrease the ribavirin uptake level, thus causing a reduction in the antiviral efficacy of the drug.

Toxicological analyses. Concurrent with the above-described experiments, the cytotoxic effects of ribavirin and other reagents on OR6 cells were examined independently and/or simultaneously (see the supplemental methods in the supplemental material). As shown in Table S2 and Fig. S6 of the supplemental materials, the lactate dehydrogenase (LDH) release assay results showed that no severe cytotoxicity in OR6 cells occurred in any treatments (less than 10%). Microscopic observation also showed that the cells were viable upon treatment with ribavirin (3,162 μM) together with NBMPR (100 μM) for 48 h (see Fig. S2 in the supplemental material). We further performed the MTS assay, which can detect different types of toxicity, to confirm the results of the LDH assay. The results showed that even though marginal toxicity was observed at the highest ribavirin and NBMPR concentrations tested (at most 25%), most treatments did not display severe cytotoxicity for OR6 cells (less than 10%; see Table S2 in the supplemental material).

DISCUSSION

In this paper, we provide results supporting our hypothesis that ENT1 plays an essential role in the anti-HCV activity of ribavirin

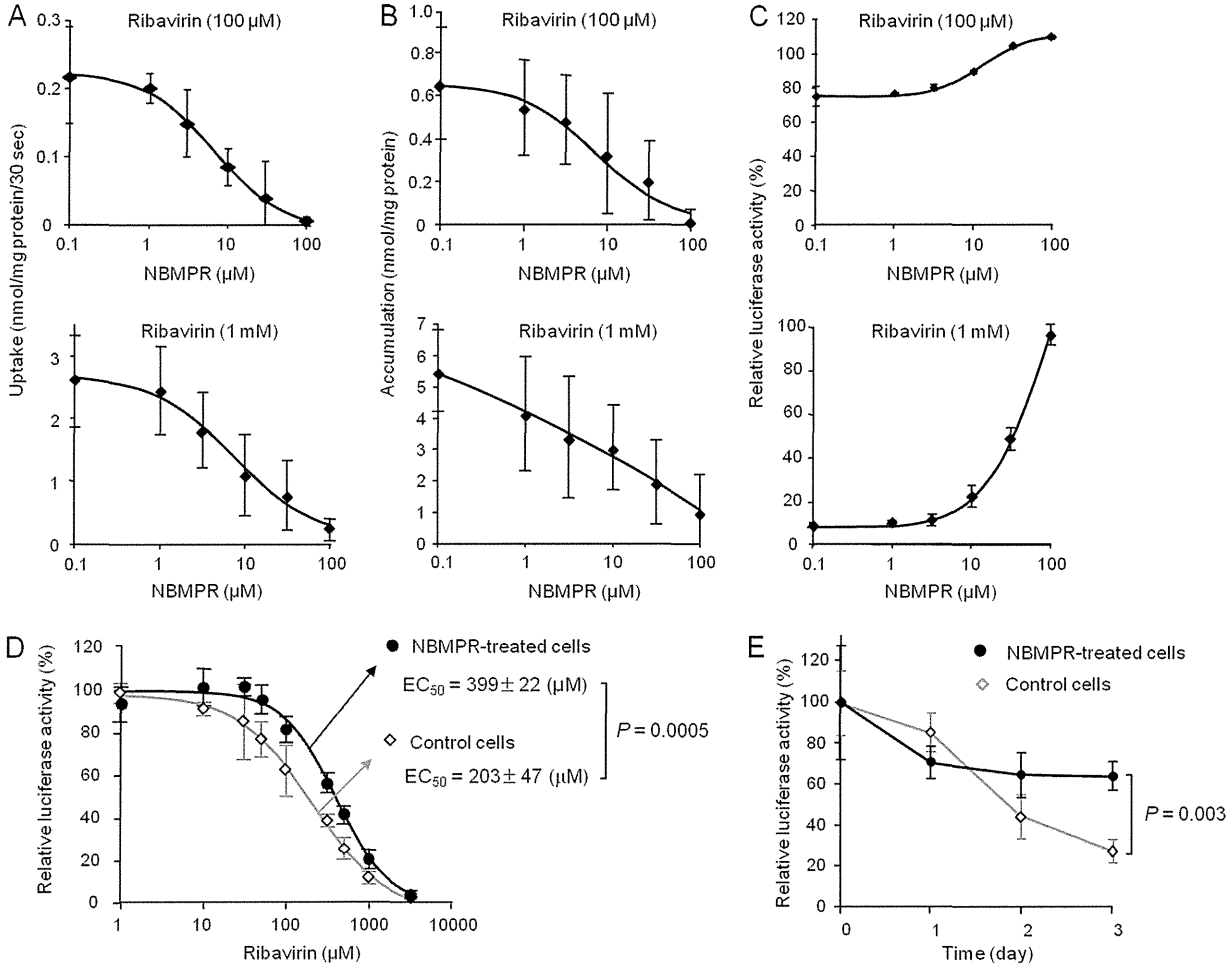

FIG 3 Inhibitory effect of NBMPR on ribavirin uptake, accumulation, and anti-HCV activity. The ribavirin concentration used in these experiments (A to C) was 100 μM or 1 mM, while the NBMPR concentrations used were 0.1, 1, 3, 10, 31, and 100 μM. (A) The effect of NBMPR on ribavirin uptake by OR6 cells was analyzed in Na^+-free KHB with NBMPR. Each value is the mean ± SD from five independent experiments, each performed in duplicate. (B) The effect of NBMPR on ribavirin accumulation in OR6 cells was analyzed by measuring the level of the drug within the cells, in the presence of NBMPR, for 48 h. Each value is the mean ± SD from three independent experiments, each performed in duplicate. (C) The effect of NBMPR on the anti-HCV activity of ribavirin in OR6 cells was analyzed by measuring the level of the luciferase activity, in the presence of NBMPR, for 48 h. The value of relative luciferase activity without ribavirin and NBMPR was set to 100%. Each value is the mean ± SD from three independent experiments, each performed in triplicate. (D) The concentration dependency of ribavirin antiviral action in the presence of NBMPR was examined. The ribavirin concentrations used are shown in the legend to Fig. 1A. The NBMPR concentration was set to 7 μM, which is near the EC_{50} of NBMPR calculated from the results in panel A. The value of relative luciferase activity in the absence of ribavirin was set to 100%. (E) The time dependency of ribavirin antiviral action in the presence of NBMPR was then examined. The ribavirin concentration was set to 150 μM, while the NBMPR concentration was still 7 μM. The value of relative luciferase activity in the absence of ribavirin at each time point was set to 100%.

through detailed characterization of the antiviral activity of the drug and its association with ENT1-mediated uptake in OR6 cells.

Our results showed that the concentration and time dependency of ribavirin antiviral activity was closely associated with its accumulation in OR6 cells. This association is supported by several reports. For example, it has been reported that larger ribavirin accumulations were associated with significant decreases in the intracellular GTP pool (13) or with higher antiviral potency against the Hantaan virus (14). Therefore, it is considered likely that continuous ribavirin accumulation in hepatic cells at the higher levels, which are achieved by the sustained and higher ribavirin extracellular concentrations, is critical to the antiviral efficacy of the drug.

Due to its hydrophilicity, ribavirin requires a "gate" to penetrate the plasma membrane of cells prior to its accumulation. Our results clearly show that ENT1 provides this gate, thus facilitating the drug's import into and accumulation in OR6 cells. Since we recently showed that ENT1 is also exclusively involved in ribavirin uptake in human hepatocytes, which has a ribavirin uptake profile similar to that of OR6 cells (6), it is considered likely that this ENT1 role can probably be extended to human hepatocytes as well. The mode of ENT1-mediated ribavirin uptake in OR6 cells, as well as human hepatocytes, was represented by a linear increase in the uptake level along with an increase in extracellular ribavirin concentration (6; also this study). This uptake feature was the most probable reason why the higher extracellular ribavirin concentration resulted in a stronger antiviral effect in OR6 cells but might also explain why clinical findings show that a higher exposure to ribavirin leads to the better virologic response in HCV genotype-1 patients (11, 17). Therefore, our results, together with