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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

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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.

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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

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of appearance of lamivudine-resistance, it is considered that the preexisting substitutions in those pre-S regions enabled the later mutation of rt 204V/I in the polymerase gene. On the other hand, although regions of the polymerase gene overlapping with pre-S1 84 and pre-S2 1, 22 genes were evaluated for their association with lamivudine resistance, the corresponding amino acid changes in the polymerase gene did not correlate with lamivudine resistance according to Kaplan-Meier analysis, demonstrating the importance of the pre-S regions in the development of resistance (data not shown). Interestingly, patients with a substitution in pre-S1 84 exhibited high viral loads and displayed longer times until HBV DNA became undetectable compared to patients without this substitution. In contrast, a substitution in pre-S2 22 correlated with increased age, and the substitution in polymerase tp 95 with advanced disease.

Although the study was focused on the viral amino acid substitutions, viral nucleotide differences also were compared between patients, according to their responses to lamivudine treatment (data not shown). In this analysis, pretreatment substitutions at nucleotide position 53 in the polymerase/pre-S1 region and at nucleotide position 2151 in the core region correlated significantly with the later appearance of lamivudine resistance. In fact, nucleotide position 53 corresponds to the pre-S1 84, and its substitution causes an amino acid change at pre-S1 84. On the other hand, the substitution at nucleotide position 2152 in the core region is synonymous and the role of this substitution should be investigated in a further study.

The pre-S1/pre-S2/S region encodes the small surface (S), middle (M), and large (L) proteins using alternative codons for the initiation of translation [Gao et al., 2007]. These proteins are considered to have crucial functional roles in the life cycle of HBV [Cooper et al., 2003; De Meyer et al., 1997; Kay and Zoulim, 2007; Lian et al., 2008; Ni et al., ; Watanabe et al., 2007]. Apart from the HBV life cycle, recent studies have shown that pre-S sequences significantly impact on the pathogenesis of liver disease [Fang et al., 2008; Sugauchi et al., 2003; Zhang et al., 2007]. The pre-S1 and pre-S2 regions serve as immune targets for T and B cells accumulating in the liver [Bauer et al., 2002], while mutant HBV pre-S epitopes stimulated a lower T cell response than wild-type HBV. HBV with pre-S substitutions leads to cellular retention of viral proteins and a dramatic reduction of virion production [Ni et al.]. The appearance of pre-S substitutions inhibits apoptosis of infected hepatocytes

[Ni et al.]. Patients with progressive liver disease or HCC experience a higher frequency of pre-S substitutions or deletions than patients with stable disease [Chaudhuri et al., 2004]. In association with nucleoside analog therapy, Ohkawa et al. showed the possibility that pre-S2 substitutions might support the replication capacity of lamivudine-resistant HBV [Ohkawa et al., 2008].

On the other hand, there have been no previous studies reporting the correlation between pretreatment pre-S substitutions and the development of lamivudine resistance to date. While the mechanisms need further clarification, it is possible to hypothesize a model explaining the correlation, considering these previous findings. Because those previous reports indicate that HBVs with pre-S substitutions function as immune escape mutants, it is possible that HBVs with pre-S substitutions are advantageous for viral survival and replication in hepatocytes, despite that virion production is reduced. In addition, those infected cells are themselves protected from apoptosis. In those circumstances of persistent viral replication, the chances of acquiring the essential substitutions in the polymerase gene conferring lamivudine resistance might increase.

Before these findings can be applied confidently in clinical settings, some caveats must be considered. First, the number of patients analyzed in the study was quite small, and therefore the potential role of the substitutions detected in drug susceptibility must be evaluated by studies of larger populations. Second, because HBV sequences were determined directly, as opposed to first cloning multiple genomes, the dynamics of minor HBV populations and their contribution to the appearance of resistance are not known. Subcloning analysis or deep sequencing might help further to establish the clinical importance and role of these substitutions in drug resistance. The utility of these viral substitutions for designing HBV therapies with the second-generation nucleoside analogs requires additional research. As for the stability of these predictive viral regions during the treatment period, five patients were available for the analysis of the complete HBV genome sequence after the acquisition of lamivudine resistance. Interestingly, the predictive positions of Pre-S1 84 and Pre-S2 1 changed after the acquisition of lamivudine resistance in some patients. However, the role of those changes needs to be further clarified by larger sample sizes.

In conclusion, it was demonstrated that the presence of pre-S1 and pre-S2 substitutions in the HBV genome prior to treatment might play an important role in the subsequent evolution of

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lamivudine resistance.

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FIGURE LEGENDS

Fig. 1 Clinical course of HBV-infected patients treated with lamivudine. "X" indicates the emergence of lamivudine resistance. Asterisks indicate patients selected for HBV nucleotide sequence analysis.

Fig. 2 Codon differences in each viral ORF between lamivudine sensitive and resistant groups. The differences are indicated by a vertical line representing the inverse of the P value. (a) pre-S1/S2, and S ORF, (b) polymerase ORF, (c) precore and core ORFs, (d) X ORF.

Although a few genotype A and B viruses were included in the analysis, for convenience, the sequences are numbered according to the system for genotype C HBV. Viral amino acids are numbered according to the adopted standardized numbering system for the HBV polymerase [Stuyver et al., 2001].

Fig. 3 Kaplan-Meier analysis of relationship of substitutions with the emergence of lamivudine resistance.

The sequences are numbered according to the system for genotype C HBV.

Fig. 4 Amino acid sequence alignment of the pre-S1, pre-S2, and polymerase ORFs associated with the lamivudine resistance. Duration of the LAM administration indicates the period for HBV to become LAM resistant in the resistant group, while it indicates the overall observation period in the non-resistant group. Above the sequences observed in each patient, representative viral sequences of genotype A, B, and C around those areas also are shown to indicate genotype-specific viral amino acids.

(a) Part of pre-S1 ORF.

(b) Part of pre-S2 ORF.

(c) Part of polymerase ORF.

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REFERENCES

Andersson KL, Chung RT. 2009. Monitoring during and after antiviral therapy for hepatitis B. Hepatology 49:S166-173.

Bauer T, Weinberger K, Jilg W. 2002. Variants of two major T cell epitopes within the hepatitis B surface antigen are not recognized by specific T helper cells of vaccinated individuals. Hepatology 35:455-465.

Carey I, Harrison PM. 2009. Monotherapy versus combination therapy for the treatment of chronic hepatitis B. Expert Opin Investig Drugs 18:1655-1666.

Chaudhuri V, Tayal R, Nayak B, Acharya SK, Panda SK. 2004. Occult hepatitis B virus infection in chronic liver disease: full-length genome and analysis of mutant surface promoter. Gastroenterology 127:1356-1371.

Chen CH, Lee CM, Lu SN, Changchien CS, Wang JC, Wang JH, Hung CH, Hu TH. 2006a. Comparison of sequence changes of precore and core promoter regions in HBeAg-positive chronic hepatitis B patients with and without HBeAg clearance in lamivudine therapy. J Hepatol 44:76-82.

Chen CJ, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. 2006b. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. JAMA 295:65-73.

Chen EQ, Wang LC, Lei J, Xu L, Tang H. 2009. Meta-analysis: adefovir dipivoxil in combination with lamivudine in patients with lamivudine-resistant hepatitis B virus. Virol J 6:163.

Cooper A, Paran N, Shaul Y. 2003. The earliest steps in hepatitis B virus infection. Biochim Biophys Acta 1614:89-96.

De Meyer S, Gong ZJ, Suwandhi W, van Pelt J, Soumillion A, Yap SH. 1997. Organ and species specificity of hepatitis B virus (HBV) infection: a review of literature with a special reference to preferential attachment of HBV to human hepatocytes. J Viral Hepat 4:145-153.

Fang ZL, Sabin CA, Dong BQ, Wei SC, Chen QY, Fang KX, Yang JY, Huang J, Wang XY, Harrison TJ. 2008. Hepatitis B virus pre-S deletion mutations are a risk factor for hepatocellular carcinoma: a matched nested case-control study. J Gen Virol 89:2882-2890.

Gao ZY, Li T, Wang J, Du JM, Li YJ, Li J, Lu FM, Zhuang H. 2007. Mutations in preS genes of genotype C hepatitis B virus in patients with chronic hepatitis B and hepatocellular carcinoma. J Gastroenterol 42:761-768.

Ghany MG, Doo EC. 2009. Antiviral resistance and hepatitis B therapy. Hepatology 49:S174-184.

Iloeje UH, Yang HI, Su J, Jen CL, You SL, Chen CJ. 2006. Predicting cirrhosis risk based on the level of circulating hepatitis B viral load. Gastroenterology 130:678-686.

Kay A, Zoulim F. 2007. Hepatitis B virus genetic variability and evolution. Virus Res 127:164-176.

Kobayashi M, Suzuki F, Akuta N, Yatsuji H, Hosaka T, Sezaki H, Kawamura Y, Suzuki Y, Arase Y, Ikeda K, Mineta R, Iwasaki S, Watahiki S, Kumada H. 2009. Correlation of YMDD mutation and breakthrough hepatitis with hepatitis B virus DNA and serum ALT during lamivudine treatment. Hepatol Res 40:125-34.

Leung N. 2000. Liver disease-significant improvement with lamivudine. J Med Virol 61:380-385.

Leung NW, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Condreay LD, Chien RN. 2001. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 33:1527-1532.

Lian M, Zhou X, Chen B, Li C, Gu X, Luo M, Zheng X. 2008. Identification of the critical regions in hepatitis B virus preS required for its stability. J Pept Sci 14:307-312.

Liang TJ. 2009. Hepatitis B: the virus and disease. Hepatology 49:S13-21.

Liaw YF, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. 2000. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. Gastroenterology 119:172-180.

Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. 2004. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med 351:1521-1531.

Lim SG, Mohammed R, Yuen MF, Kao JH. 2009. Prevention of hepatocellular carcinoma in hepatitis B virus infection. J Gastroenterol Hepatol 24:1352-1357.

Ling R, Mutimer D, Ahmed M, Boxall EH, Elias E, Dusheiko GM, Harrison TJ. 1996. Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. Hepatology 24:711-713.

Lok AS, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, Dienstag JL, Heathcote EJ, Little NR, Griffiths DA, Gardner SD, Castiglia M. 2003. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. Gastroenterology 125:1714-1722.

Ni Y, Sonnabend J, Seitz S, Urban S. 2010.The pre-s2 domain of the hepatitis B virus is dispensable for infectivity but serves a spacer function for L-protein-connected virus assembly. J Virol 84:3879-3888.

Ohkawa K, Takehara T, Kato M, Deguchi M, Kagita M, Hikita H, Sasakawa A, Kohga K, Uemura A, Sakamori R, Yamaguchi S, Miyagi T, Ishida H, Tatsumi T, Hayashi N. 2008. Supportive role played by precore and preS2 genomic changes in the establishment of lamivudine-resistant hepatitis B virus. J Infect Dis 198:1150-1158.

Rizzetto M, Tassopoulos NC, Goldin RD, Esteban R, Santantonio T, Heathcote EJ, Lagget M, Taak NK, Woessner MA, Gardner SD. 2005. Extended lamivudine treatment in patients with HBeAg-negative chronic hepatitis B. J Hepatol 42:173-179.

Stuyver LJ, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF. 2001. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. Hepatology 33:751-757.

Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 82:883-892.

Sugauchi F, Ohno T, Orito E, Sakugawa H, Ichida T, Komatsu M, Kuramitsu T, Ueda R, Miyakawa Y, Mizokami M. 2003. Influence of hepatitis B virus genotypes on the development of preS deletions and advanced liver disease. J Med Virol 70:537-544.

Tipples GA, Ma MM, Fischer KP, Bain VG, Kneteman NM, Tyrrell DL. 1996. Mutation in HBV RNA-dependent DNA polymerase confers resistance to lamivudine in vivo. Hepatology 24:714-717.

Villeneuve JP, Condreay LD, Willems B, Pomier-Layrargues G, Fenyves D, Bilodeau M, Leduc R, Peltekian K, Wong F, Margulies M, Heathcote EJ. 2000. Lamivudine treatment for decompensated cirrhosis resulting from chronic hepatitis B. Hepatology 31:207-210.

Watanabe T, Sorensen EM, Naito A, Schott M, Kim S, Ahlquist P. 2007. Involvement of host cellular multivesicular body functions in hepatitis B virus budding. Proc Natl Acad Sci U S A 104:10205-10210.

Zhang KY, Imazeki F, Fukai K, Arai M, Kanda T, Mikata R, Yokosuka O. 2007. Analysis of the complete hepatitis B virus genome in patients with genotype C chronic hepatitis and hepatocellular carcinoma. Cancer Sci 98:1921-1929.

Zhou HJ, Li SG, Wen FY, Yang XY, Wu JL, Tan B, Fu J. 2009. [Factors associated with response to lamivudine: retrospective study of 233 patients with chronic hepatitis B]. Zhonghua Gan Zang Bing Za Zhi 17:564-568.

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Sueki et al.

FIGURE

Fig.1

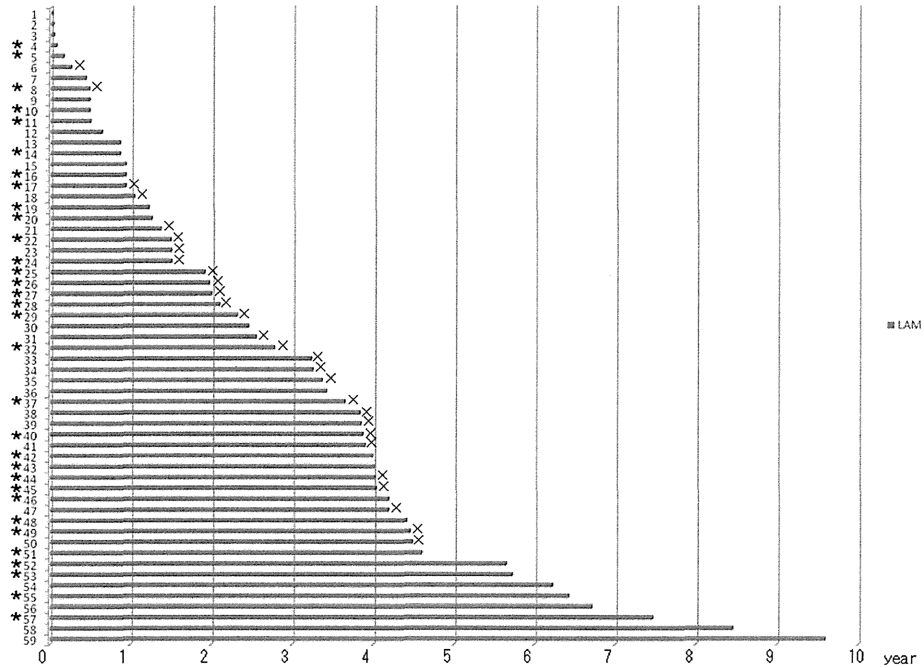

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Fig.2

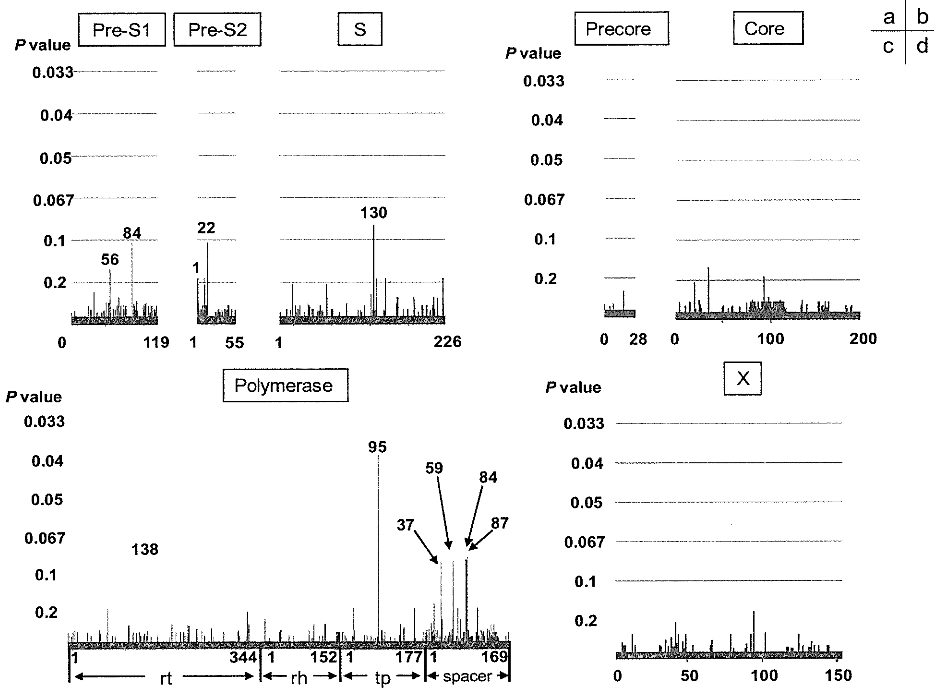

Fig. 3

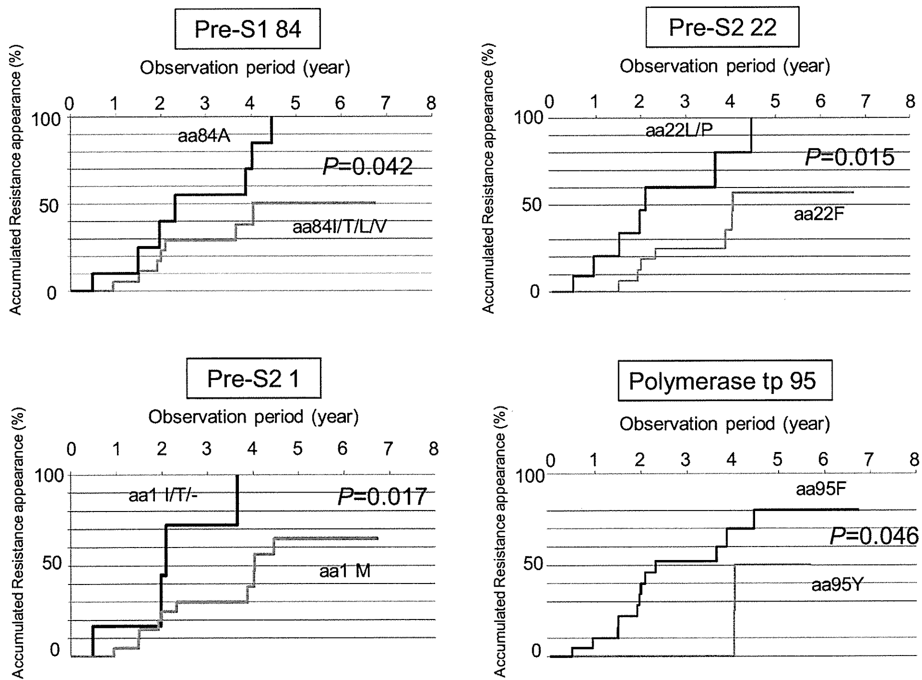

Fig. 4

genotype		(a) Pre-S1					(b) Pre-S2																							(c) Polymerase tp				
	aa			84			1																					22				95		
		Q	G	A	L	T	M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	Q	Q	F	V	G
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	AB033554	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	A	-	-	-	-	K	-	-	-	-
B	AB031267	-	-	I	-	-	-	-	-	-	-	A	-	-	-	-	T	-	Q	-	-	-	-	-	A	-	-	L	-	K	-	-	-	-
	AB219429	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	A	-	-	-	-	K	-	-	-	-
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			(a) Pre-S1					(b) Pre-S2																							(c) Polymerase tp				
		aa			84			1																					22				95		
	Pt No.	Duration of LAM administration (Months)	Q	G	A	L	T	M	Q	W	N	S	T	T	F	H	Q	A	L	L	D	P	R	V	R	G	L	Y	F	P	Q	Q	F	V	G
	1	0.9	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	K	-	-	-	-	-	L	-	-	-	Y	-	-
	2	2.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	5.8	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	6.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	5	10.4	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	-	-	-
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Lamivudine non-resistant	7	14.7	-	-	I	-	-	-	/	/	H	-	-	A	-	-	-	-	-	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	10	47.9	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	T	-	Q	-	-	-	-	-	A	-	-	L	-	K	-	-	-	-
	11	48.1	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	12	50.2	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	13	52.9	-	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	55.1	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	15	67.7	-	-	L	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H	-	-	-
	16	68.6	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	17	77.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	18	89.6	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	1	5.8	-	-	-	-	-	I	-	-	-	-	-	-	-	-	K	-	-	-	-	-	-	-	-	S	-	-	L	-	S	-	-	L	-
	2	11.2	-	-	-	-	-	-	H	G	T	-	-	A	-	P	-	-	-	I	-	-	-	-	-	-	-	-	L	-	-	-	Y	-	-
	3	17.9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
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	5	23.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
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	8	25.1	-	-	I	-	-	T	-	-	-	-	-	R	-	Q	K	-	-	-	-	-	-	-	-	-	-	-	P	-	-	-	Y	P	-
	9	27.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	10	43.8	-	-	T	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	L	-	-	-	Y	L	-
	11	46.4	-	-	L	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	12	48.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Y	-	-
	13	48.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	14	53.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	S	-	-	L	-	S	-	Y	L	-

For Peer Review

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

TABLES

TABLE I . Baseline Clinical Charactaristics

Clinical factor	LAM non-resistant n=18	LAM resistant n=14	*P*-value
Demographic Characteristics			
Age, years *1	53.9 (±13.2)	55.6 (±7.7)	0.662
Sex, no. male/female	13/5	9/5	0.712
CH/LC	5/13	3/11	0.261
HCC (+/-)	11/7	7/7	0.721
Biochemical Characteristics			
Alanine aminotransferase level, IU/l *2	91 (13-1780)	70.5 (17-2739)	0.805
Platelets count, $\times 10^{-4}$/ml *1	11.8 (±5.8)	12.1 (±5.3)	0.900
Total bilirubin, mg/dl *2	0.95 (0.3-19.7)	1.1 (0.4-5.0)	0.634
Albumin, g/dl *2	3.2 (±0.6)	3.5 (±0.9)	0.270
ChE, IU/l *1	196.4 (±105.0)	207.1 (±92.4)	0.566
T-chol, mg/dl *1	156.1 (±39.6)	163.6 (±37.4)	0.590
Prothrombin time, % *1	64.5 (±16.1)	69.9 (±15.9)	0.358
α-fetoprotein, ng/ml *2	16.1 (1.9-35194)	11.5 (1.6-611.5)	0.506
Virological Characteristics			
HBV Genotype (A/B/C)	1/1/16	0/1/13	0.662
HBV DNA level Log10copies/ml*1	5.80 (±1.45)	6.61 (±0.97)	0.078
HBeAg, positive/negative	6/12	8/6	0.283
Precore mutation ratio (%)	38.9	28.6	0.712
Core promotor mutation	4/14	3/11	0.880
Duration of LAM administration until HBV PCR negative (Month)*2	2.1 (0.4-7.7)	3.7 (1.4-69.0)	0.024

*1 average (±SD) student's t test
*2 median (range) Mann-Whitney U test

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TABLE II. Amino acid substitution number in each region of the HBV ganome

HBV protein	LAM non-resisitant	LAM resisitant	*P*-value
Pre-S1, median (range)	2.0 (0-6)	2.0 (0-11)	0.460
Pre-S2, median (range)	0 (0-4)	2.0 (0-8)	0.060
S, median (range)	3.0 (1-9)	4.0 (2-8)	0.372
Pre-S1/Pre-S2/S, median (range)	7.0 (3-15)	7.0 (4-23)	0.206
Polymerase, median (range)	15.5 (9-30)	17.0 (8-35)	0.448
Precore, median (range)	0.5 (0-1)	0 (0-1)	0.144
Core, median (range)	3.5 (0-9)	5.0 (0-35)	0.859
X, median (range)	4.0 (1-7)	3.0 (1-9)	0.706

Mann-Whitney U test

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60

TABLE Ⅲ. Baseline Clinical Charactaristics classified by the mutation at codon 84 in pre-S1

Clinical factor	Pre-S1 84I/T/L/V n=20	Pre-S1 84A n=12	*P*-value
HBV DNA level Log10 copies/ml*[1]	5.75 (±1.38)	6.83 (±0.86)	0.022
Duration of LAM administration until HBV PCR negative (Months)*[2]	2.1 (0.4-7.6)	4.0 (1.9-69.0)	0.005

*1 average (±SD) student's t test

*2 median (range) Mann-Whitney U test

TABLE IV. Baseline Clinical Charactaristics classified by the mutation at codon 22 in pre-S2

Clinical factor	Pre-S2 22F n=21	Pre-S2 22L/P n=11	*P*-value
Age, years *[1]	50.7 (±9.6)	62.3 (±9.7)	0.003

*1 average (±SD) student's t test

TABLE V . Baseline Clinical Charactaristics classified by the mutation at tp aa95 in polymerase

Clinical factor	Polymerase tp 95Y n=21	Polymerase tp 95F n=11	*P*-value
Alanine aminotransferase level, IU/l *[1]	52 (13-810)	133 (23-2739)	0.0495
Total bilirubin, mg/dl *[1]	0.9 (0.3-5.0)	1.2 (0.5-19.7)	0.049
α-fetoprotein, ng/ml *[1]	8 (1.6-35194)	81 (4-214.3)	0.034

*2 median (range) Mann-Whitney U test

TABLE VI. Factors asociated with LAM resistance identified by multivariate analysis

Variable	Hazard Ratio (95% CI)	*P*-value
Duration of LAM administration until HBV PCR negative	1.1 (1.0 - 1.1)	0.700
Albumin	1.2 (0.6 - 2.4)	0.682
Pre-S1 84	8.5 (1.5 - 49.3)	0.017
Pre-S2 1	12.4 (1.1 - 139.7)	0.041
Pre-S2 22	1.2 (0.2 - 5.9)	0.833
Polymerase tp 95	0.3 (0.4 - 32.2)	0.275

CI = confidence interval
Cox proportional-hazards regression

For Peer Review

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.
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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.

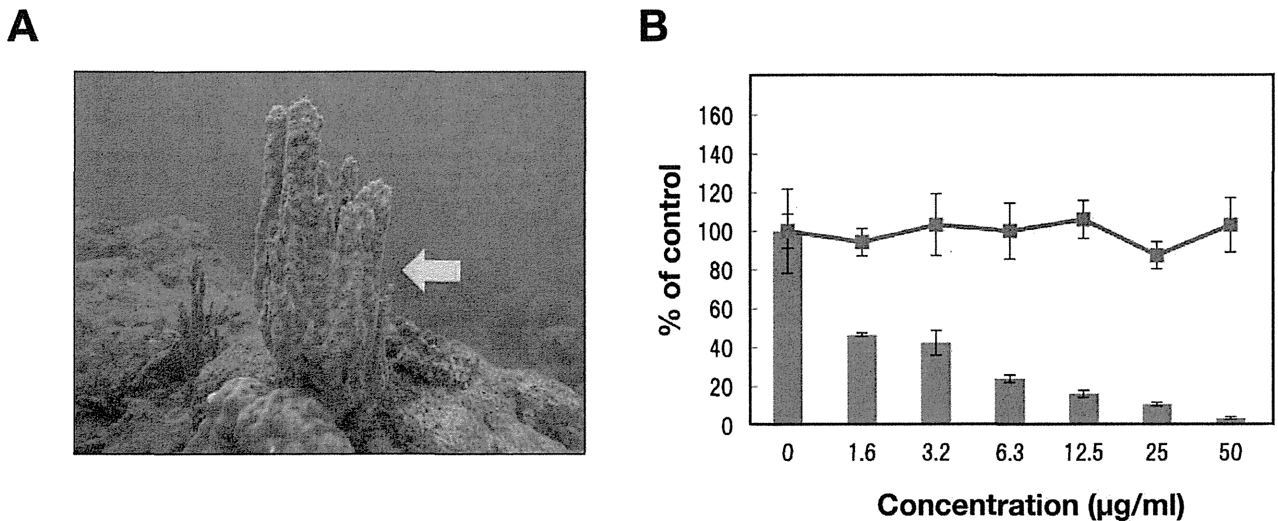

Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b. (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7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

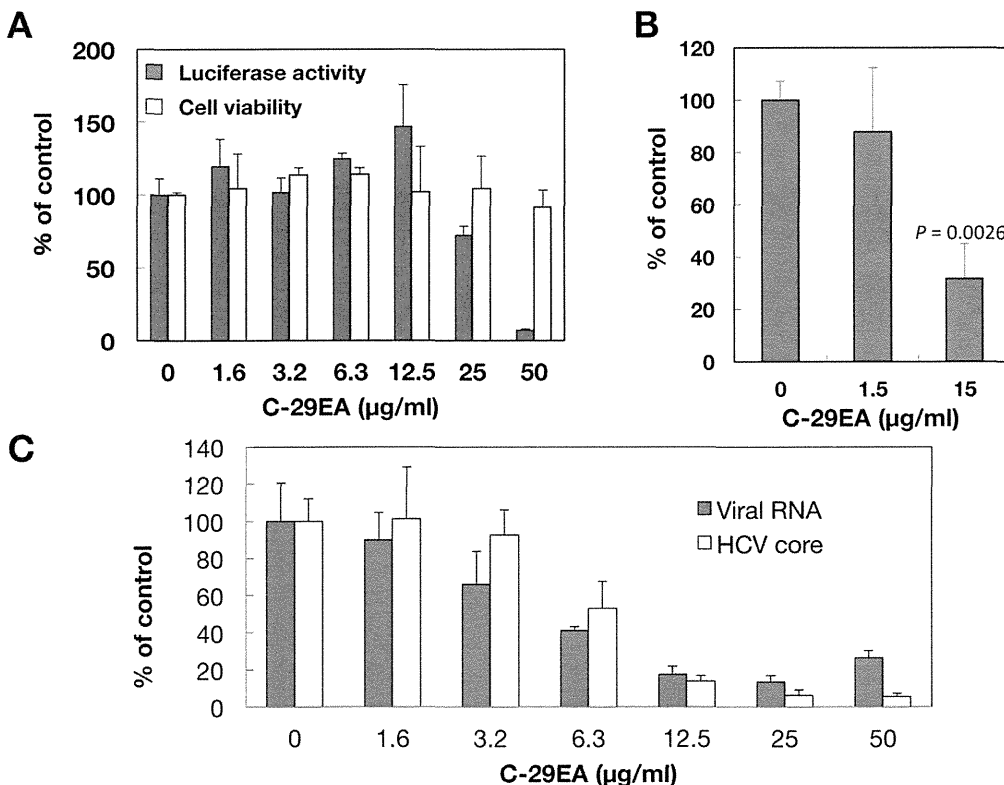

Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a. (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity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

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