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

lamivudine resistance.

# 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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**FIGURE**

***Fig.1***

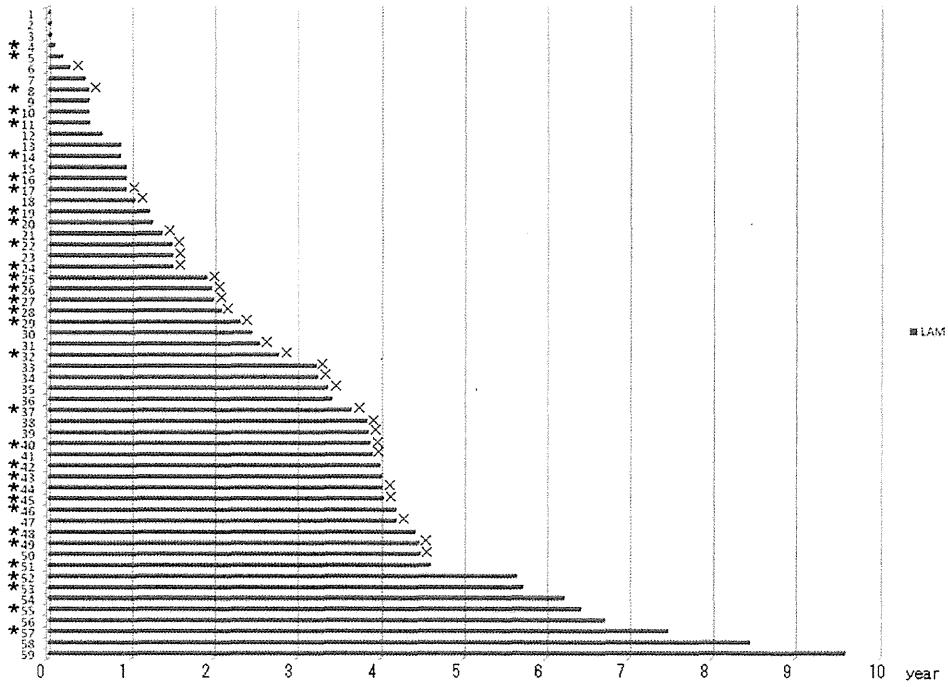

*Fig.2*

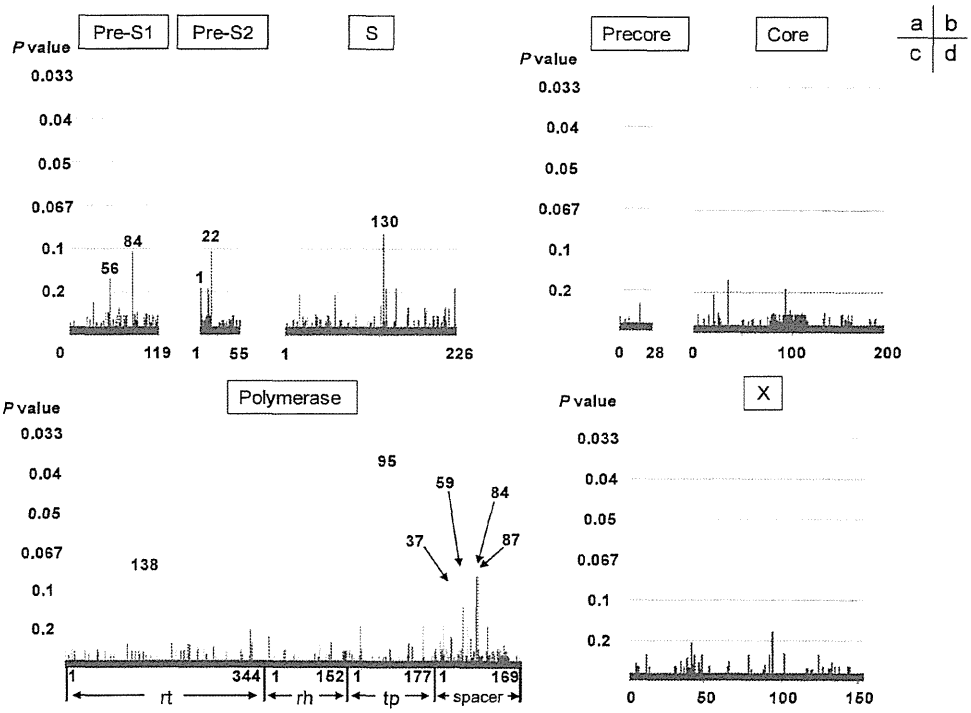
Pre-S1
Pre-S2
S
Precore
Core
a
b
c
d
P value
0.033
0.04
0.05
0.067
0.1
0.2
84
56
22
1
130
0
119
1
55
1
226
0
28
0
100
200
Polymerase
X
95
59
84
37
87
138
1
344
1
152
1
177
1
169
rt
rh
tp
spacer
0
50
100
150

*Fig. 3*

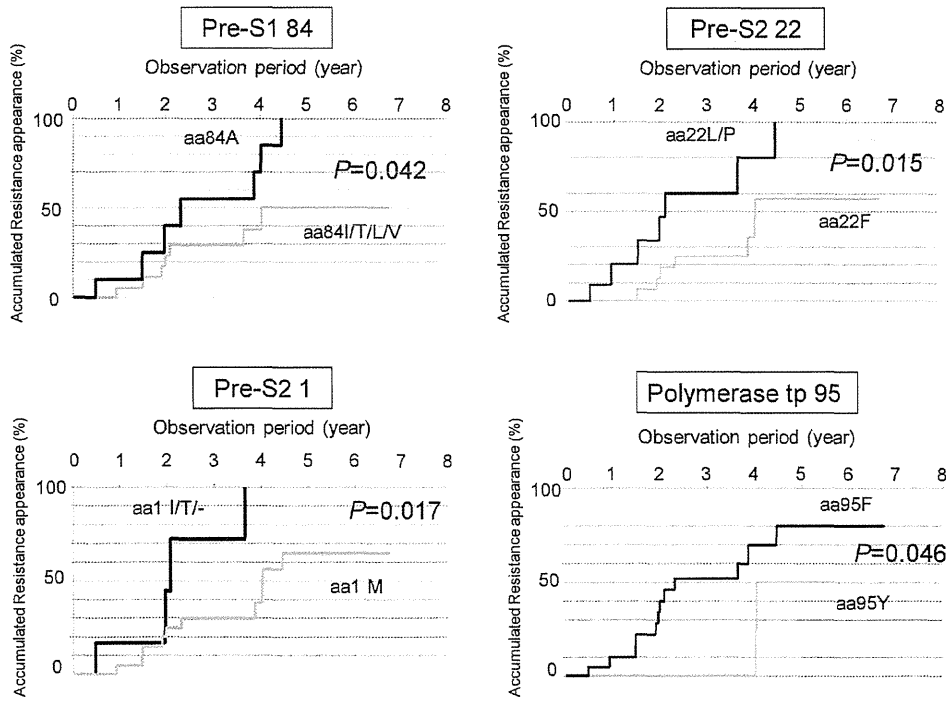
Pre-S1 84
Observation period (year)
Accumulated Resistance appearance (%)
aa84A
P=0.042
aa84I/T/L/V
Pre-S2 22
aa22L/P
P=0.015
aa22F
Pre-S2 1
aa1 I/T/-
P=0.017
aa1 M
Polymerase tp 95
aa95F
P=0.046
aa95Y

***Fig. 4***

| genotype | | aa | (a) Pre-S1 84 | | | | | (b) Pre-S2 1 | | | | | | | | | | | | | | | | | | | | (c) Polymerase tp 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 |
| | AY233290 | | - | - | I | - | A | - | - | - | - | - | - | A | - | - | - | - | - | Q | - | - | - | - | R | - | - | - | - | - | - | - | - | - | - |
| A | AY128092 | | - | - | I | - | - | - | - | - | - | - | - | A | - | - | - | - | - | Q | - | - | - | - | R | - | - | - | L | - | - | - | - | - | - |
| | AB194950 | | - | - | T | - | - | I | K | - | - | - | P | A | - | - | - | - | - | Q | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | AB033554 | | - | - | I | - | - | - | - | - | - | - | - | - | - | - | - | T | - | Q | - | - | - | - | - | A | - | - | - | - | K | - | - | - | - |
| B | AB031267 | | - | - | I | - | - | - | - | - | - | - | A | - | - | - | - | T | - | Q | - | - | - | - | - | A | - | - | L | - | K | - | - | - | - |
| | AB219429 | | - | - | I | - | - | - | - | - | - | - | - | - | - | - | - | T | - | Q | - | - | - | - | - | A | - | - | - | - | K | - | - | - | - |
| | AB112063 | | - | - | I | - | - | - | - | - | - | - | S | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | L | - | - | - | Y | - | - |
| C | AF241410 | | - | - | I | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | R | - | - | - | - | - | - | - | - | - | - | - | - |
| | X52939 | | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | R | - | - | - | - | - | - | - | - | - | Y | V | - |

| | Pt No. | Duration of LAM administration (Months) | aa | (a) Pre-S1 84 | | | | | (b) Pre-S2 1 | | | | | | | | | | | | | | | | | | | | (c) Polymerase tp 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 |
| | 1 | 0.9 | | - | - | I | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | K | - | - | - | - | - | L | - | - | - | Y | - | - |
| | 2 | 2.0 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 3 | 5.8 | | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 4 | 6.0 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | - |
| | 5 | 10.4 | | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | L | - | - | - | - | - | - |
| | 6 | 11.2 | | - | - | - | - | - | I | - | - | - | - | - | - | - | - | K | - | - | - | - | - | - | - | - | - | S | - | - | L | - | - | Y | - | - |
| Lamivudine | 7 | 14.7 | | - | - | I | - | - | - | / | / | H | - | - | A | - | - | - | - | - | Q | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| non-resistant | 8 | 15.1 | | - | - | T | - | - | I | H | W | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | E | - | Y | - | - |
| | 9 | 33.3 | | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| | 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 | - | - |
| | 4 | 18.0 | | - | - | T | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | S | T | T | F | H | Q | A | L | - | H | Q | Y | L | - |
| | 5 | 23.0 | | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | - |
| | 6 | 23.6 | | - | - | V | - | - | / | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | L | - | - | - | Y | L | - |
| Lamivudine | 7 | 23.9 | | - | - | I | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | Y | - | - |
| resistant | 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 | - |

## 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

**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

**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

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*Article*

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

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**Abstract:** Hepatitis C virus (HCV) is a causative agent of acute and chronic hepatitis, leading to the development of hepatic cirrhosis and hepatocellular carcinoma. We prepared extracts from 61 marine organisms and screened them by an *in vitro* fluorescence assay targeting the viral helicase (NS3), which plays an important role in HCV replication, to identify effective candidates for anti-HCV agents. An ethyl acetate-soluble fraction of the feather star *Alloeocomatella polycladia* exhibited the strongest inhibition of NS3 helicase activity, with an $IC_{50}$ of 11.7 μg/mL. The extract of *A. polycladia* inhibited interaction between NS3 and RNA but not ATPase of NS3. Furthermore, the replication of the replicons derived from three HCV strains of genotype 1b in cultured cells was suppressed by the extract with an $EC_{50}$ value of 23 to 44 μg/mL, which is similar to the $IC_{50}$ value of the NS3 helicase assay. The extract did not induce interferon or inhibit cell growth. These results suggest that the unknown compound(s) included in *A. polycladia* can inhibit HCV replication by suppressing the helicase activity of HCV NS3. This study may present a new approach toward the development of a novel therapy for chronic hepatitis C.

**Keywords:** marine organism; *Alloeocomatella polycladia*; hepatitis C virus; NS3 helicase

## 1. Introduction

Hepatitis C virus (HCV) is an etiological agent of liver disease including steatosis, cirrhosis, and hepatocellular carcinoma, and has infected over 170 million individuals worldwide [1,2]. HCV belongs to the genus *Hepacivirus* of the *Flaviviridae* family. The genome of HCV is a single positive-strand RNA composed of 9.6 kb flanked by 5' and 3'-untranscribed regions (UTRs) and encodes a polyprotein consisting of approximately 3000 amino acids [3]. The polyprotein is translated from a viral genome by an internal ribosome entry site (IRES), which is localized in 5'-UTR [4]. The translated polyprotein is cleaved by host and viral proteases into 10 proteins. The structural proteins consisting of core, E1, and E2 and a viroporin p7, which has not yet been classified as either a structural or nonstructural protein, are located in the *N*-terminal quarter of the polyprotein. The nonstructural proteins including

NS2, NS3, NS4A, NS4B, NS5A, and NS5B occupy the remaining portion of the polyprotein and form a replication complex with several host factors.

HCV NS3 is well known to play a crucial role in viral replication because it possesses helicase and protease activities [5,6]. The *N*-terminal third of NS3 forms a complex with the NS4A protein and exhibits serine protease activity (NS3-4A protease) to cleave the viral polyprotein for the maturation of viral proteins [7]. The remaining portion of NS3 occupies the RNA helicase domain, characterized by the activities of ATPase and RNA binding, both of which contribute to the unwinding of duplex RNA [8,9]. The helicase activity is needed to separate duplex RNA during viral RNA replication [10]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after the uncoating of a viral particle in the infected cells and then is itself used as a template to synthesize a positive-strand RNA packaged into the viral particle. Thus, helicase as well as protease activities of NS3 can be targeted for use in the development of antiviral agents against HCV.

The current therapy, which combines pegylated interferon with ribavirin, is effective in only about half of patients infected with the most common genotype worldwide, genotype 1 [11–13]. However, this therapy has side effects including influenza-like symptoms, cytopenias, and depression [11]. Furthermore, no effective vaccines for HCV have been developed yet. Biotechnological advances of the past decade have led to the development of novel therapies using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication. This approach has been named either "specifically targeted antiviral therapy for hepatitis C" (STAT-C) or "directed-acting antiviral agents" (DAA) [14–16]. Several compounds of STAT-C or DAA have proceeded to clinical trials. Telaprevir and boceprevir, which are categorized as advanced NS3/4A protease inhibitors, were recently approved for the treatment of chronic hepatitis C patients infected with genotype 1 in the US, EU, Canada, and Japan [17,18]. However, the emergence of drug-resistant viruses is the major problem for therapies using antiviral compounds [19,20]. Accordingly, several kinds of drugs targeting various molecules or positions will be required for the complete eradication of the virus from hepatitis C patients.

The helicase activity of NS3 could be targeted by development of anti-HCV compound in addition to its protease activity. Belon *et al.* reported that 1-*N*,4-*N*-*bis*[4-(1*H*-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamine, designated as $(BIP)_2B$, is a potent and selective inhibitor of HCV NS3 helicase [21]. $(BIP)_2B$ could not affect ATP hydrolysis without RNA or at a saturated concentration of RNA. QU663 inhibits the unwinding activity of NS3 helicase by binding to the RNA-binding groove irrespective of its own ATPase activity [22]. Compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity.

Various drugs have been generated from natural products, especially those from terrestrial plants and microbes. The development of drugs from natural products has declined in the past two decades by the emergence of high-throughput screening of synthetic chemical libraries. However, recent technical advances in the determination of molecular structures and in the synthesis of chemical compounds have raised awareness about natural products as a resource for drug development [23–25]. Several groups recently reported natural products that inhibit HCV replication *in vitro*. For instance, silbinin, which is identified from the milk thistle [26,27], epigallocatechin 3-gallate, which is from green tea [28], and proanthocyanidins, which are from blueberry leaves [29], can inhibit HCV replication in cultured cells. Marine organisms including plants and animals were recently established as a representative natural resource library for drug development, since there are estimated to be more than 300,000

species of marine organisms. The products isolated from the marine organisms often possess potent biological activities corresponding to the organisms' own novel molecular structures. Thus, marine natural products are considered to include highly significant lead compounds for drug development [30,31]. For example, trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven) are approved anticancer drugs developed from marine organisms [32]. However, marine organisms have not yet been screened for development into anti-HCV agents.

In this study, we screened extracts of marine organisms by using an *in vitro* fluorescence NS3 helicase assay and HCV replicon system to find candidates for safe and effective anti-HCV agents. The marine feather star *Alloeocomatella polycladia* may produce anti-HCV helicase agents that suppress HCV replication.

## 2. Results and Discussion

### *2.1. Primary Screening of Marine Organism Extracts on HCV NS3 Helicase Activity*

We employed high-throughput screening using a photoinduced electron transfer (PET) assay to identify inhibitors of HCV NS3 helicase activity from extracts of marine organisms (Figure 1). The EtOAc- and MeOH-soluble extracts were prepared from marine organisms obtained from the sea around Okinawa Prefecture, Japan. We identified 16 extracts possessing an arbitrary level of inhibitory activity, which is defined as below 60% of the control in this study (Table 1). Five extracts exhibited high inhibition levels (<30%), and eleven extracts exhibited intermediate inhibition levels (30% to 60%). The EtOAc extract prepared from the feather star *Alloeocomatella polycladia* (Figure 2) exhibited the strongest inhibitory activity among them, and was designated SG1-23-1 in this study. Treatment with SG1-23-1 inhibited the helicase activity in a dose-dependent manner (Figure 3A). The value of $IC_{50}$ is calculated as 11.7 ± 0.7 μg/mL. We confirmed the effect of SG1-23-1 on NS3 helicase unwinding activity by the RNA helicase assay using $^{32}P$-labeled double-stranded RNA (dsRNA) as a substrate. Treatment with SG1-23-1 inhibited dsRNA dissociation at concentrations of 16 μg/mL and above (Figure 3B). These results suggest that treatment with SG1-23-1 inhibits the unwinding ability of HCV NS3 helicase.

**Table 1.** Inhibitory effects of marine organism extracts on hepatitis C virus (HCV) NS3 helicase activity.

| Sample | Helicase Activity (% of control) | Specimen | Phylum | Extract | Collection Site |
|---|---|---|---|---|---|
| OK-99-2 | 78 | *Agelas* sp. | Porifera | EtOAc | Shimoji Island |
| OK-99-3 | 73 | *Plakortis* sp. | Porifera | EtOAc | Shimoji Island |
| OK-99-4 | 60 | *Dysidea arenaria* | Porifera | EtOAc | Shimoji Island |
| OK-99-5 | 96 | *Theonella cupola* | Porifera | EtOAc | Shimoji Island |
| OK-99-6 | 52 | *Theonella conica* | Porifera | EtOAc | Shimoji Island |
| OK-99-7 | 85 | *Epipolasis kushimotoensis* | Porifera | EtOAc | Shimoji Island |
| OK-99-9 | 51 | *Hyrtios* sp. | Porifera | EtOAc | Shimoji Island |

**Table 1.** *Cont.*

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

**Table 1.** *Cont.*

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

Total number of marine organisms: 61; Marine organisms that strongly inhibit NS3 helicase activity (<30%) (boldface and underlined): 5; Extracts of organisms that exhibit intermediate inhibition of NS3 helicase activity (30%–60%) (underlined): 11; EtOAc: Ethyl acetate; MeOH: Methanol.

**Figure 1.** Schematic representation of the PET assay system for unwinding activity of HCV NS3 helicase. The fluorescent dye (BODIPY FL) is attached to the cytosine at the 5'-end of the fluorescent strand and quenched by the guanine base at the 3'-end of the complementary strand via photoinduced electron transfer. When the helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits bright light upon the release of the dye from the guanine base. The capture strand, which is complementary to the complementary strand, prevents the reannealing of the unwound duplex.

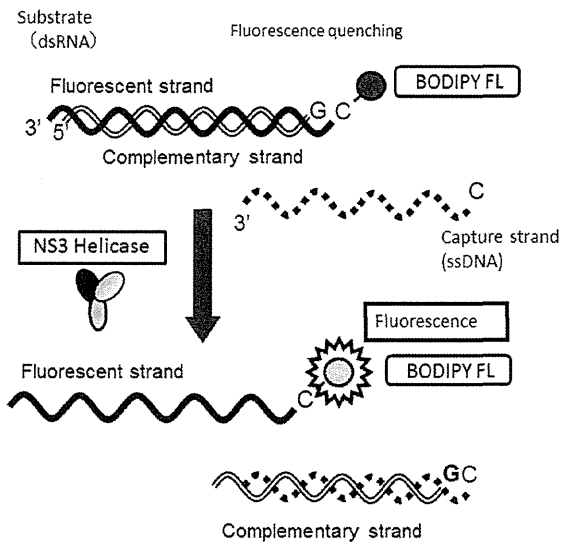


**Figure 2.** *Alloeocomatella polycladia* belongs to a class of feather star (Echinodermata, Crinoidea). The ethyl acetate fraction prepared from the marine organism was designated SG1-23-1 in this study.

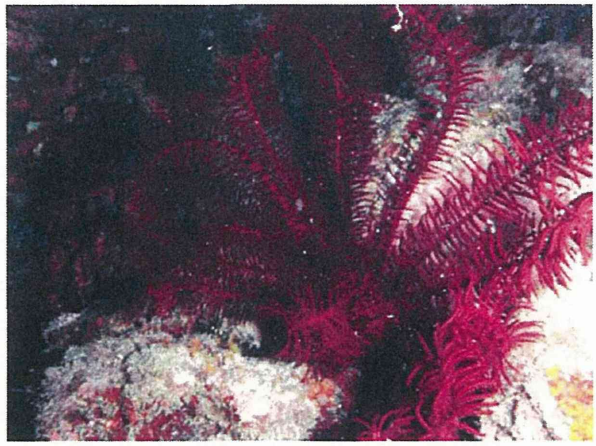
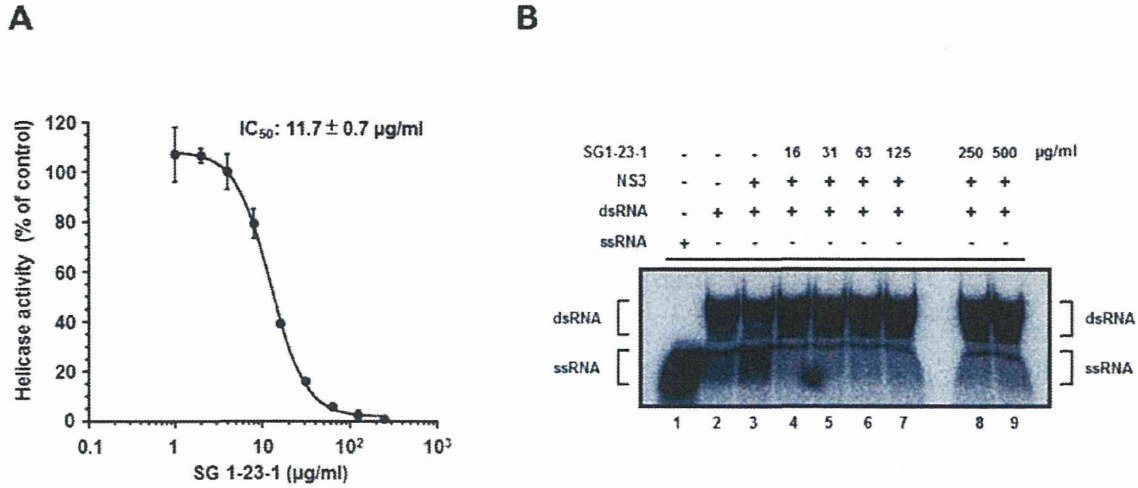


**Figure 3.** Effect of SG1-23-1 on the unwinding activity of NS3 helicase. (**A**) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of SG1-23-1. Helicase activity in the absence of SG1-23-1 was defined as 100% helicase activity. Each value represents the mean of three independent reactions. Error bars indicate standard deviation. The data represent three independent experiments. (**B**) The unwinding activity of NS3 helicase was measured by RNA unwinding assay using radioisotope-labeled RNA. The heat-denatured single-strand RNA (26-mer) and the partial duplex RNA substrate were applied to lanes 1 and 2, respectively. The duplex RNA was reacted with NS3 (300 nM) in the presence of SG1-23-1 (lanes 4 to 9, 16 to 500 μg/mL). The resulting samples were subjected to native polyacrylamide gel electrophoresis.

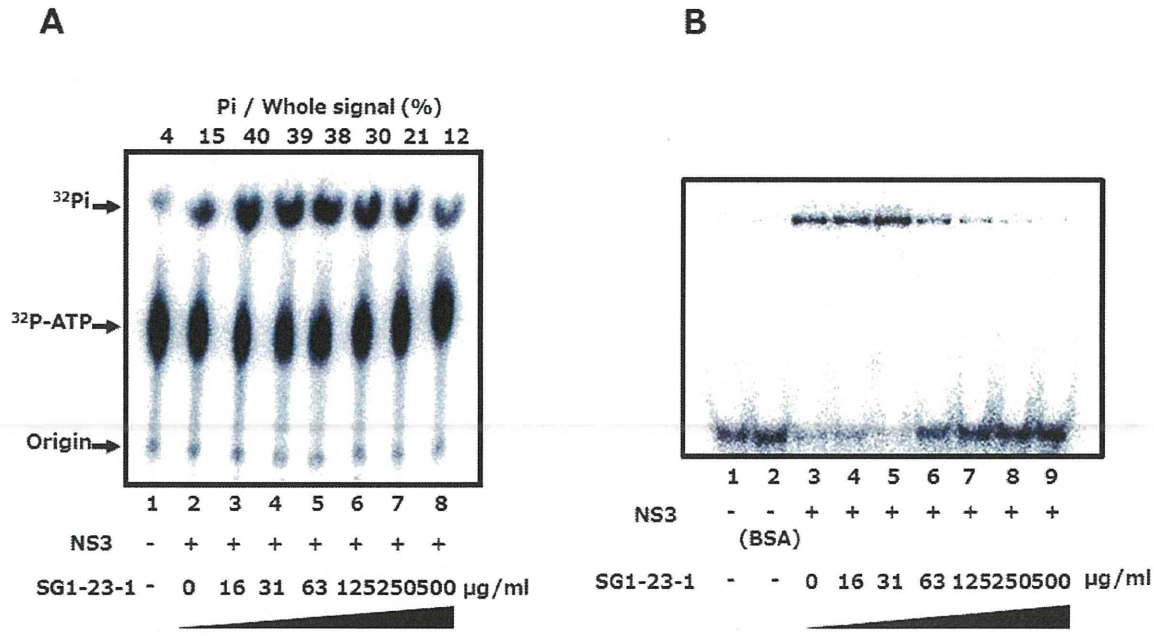



*2.2. Effect of SG1-23-1 on HCV NS3 ATPase and RNA Binding Activities*

The unwinding ability of HCV helicase is dependent on ATP binding, ATP hydrolysis, and RNA binding [8,9]. We examined the effect of SG1-23-1 on the ATPase activity of NS3 helicase. The ratio of free phosphate ($^{32}$P-Pi) in ATP ($^{32}$P-ATP) was measured in the presence of SG1-23-1. The reaction

was carried out between 16 and 500 μg of SG1-23-1 per milliliter. ATPase activity was slightly increased at 16 μg SG1-23-1 per milliliter and slightly decreased at 500 μg SG1-23-1 per milliliter (Figure 4A). However, the helicase activity was decreased to less than 10% in the presence of 50 μg of SG1-23-1 per milliliter (Figure 3A,B). Next, we examined the effect of SG1-23-1 on the binding of NS3 helicase to single-strand RNA (ssRNA). A gel-mobility shift assay was employed to estimate the binding activity of NS3 to 21 mer of ssRNA. The binding of NS3 to ssRNA was inhibited with SG1-23-1 in a dose-dependent manner (Figure 4B). These results suggest that SG1-23-1 contains the compound that inhibits RNA binding to NS3 helicase.

**Figure 4.** Effect of SG1-23-1 on ATPase and RNA-binding activities of NS3 helicase. (**A**) The reaction mixtures were incubated with [γ-$^{32}$P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as "Origin", "$^{32}$P-ATP", and "$^{32}$P-Pi", respectively, on the left side of this figure. The data represent three independent experiments. (**B**) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out at the indicated concentration of SG1-23-1. The reaction mixture was subjected to gel mobility shift assay. The data represent three independent experiments.




*2.3. Effect of SG1-23-1 on HCV RNA Replication in HCV 1b Replicon Cells*

We investigated the effect of SG1-23-1 on both viral replication and growth of the replicon cell lines. The cell lines possess viral subgenomic RNAs derived from three genotype 1b strains (strains N [33], Con1 [34], and O [35]) or a full genomic RNA derived from the O strain [35]. Each cell line was treated with various concentrations of SG1-23-1. The treated cells were harvested 72 h post-treatment. Treatment with SG1-23-1 suppressed HCV RNA replications of all cell lines in a dose-dependent manner irrespectively of full- and sub-genome replicons; it exhibited no effect below 25 μg/mL and

little effect on cellular viability at the highest concentration, 50 μg/mL (Figure 5C,D). Both HCV NS3 and NS5A were decreased at the protein level in a dose-dependent manner, corresponding to the viral replication, but beta-actin was not changed in the cell line harboring subgenome replicon RNA of the Con1 strain (Figure 5E).

The inhibitory effect of SG1-23-1 on HCV replication is summarized in Table 2. The inhibitory effects on the HCV replication of the subgenome replicon derived from Con1, O, and N strains were 22.9 ± 0.4, 19.9 ± 1.8, and 44.2 ± 1.5 μg/mL, respectively, as $EC_{50}$; and 48.1 ± 1.5, 48.5 ± 0.3, and >50 μg/mL, respectively, as $EC_{90}$. Treatment with SG1-23-1 inhibited the replication of the subgenome replicon of the O strain ($EC_{50}$: 19.9 ± 1.8 μg/mL; $EC_{90}$: 48.5 ± 0.3 μg/mL) at a more potent level than the replication of the full genomic replicon of the O strain ($EC_{50}$: 39.5 ± 0.8 μg/mL; $EC_{90}$: >50 μg/mL). When luciferase of firefly or *Renilla* was expressed under the control of the EF promoter, neither showed a significant change in activity in the presence of SG1-23-1 (Figure 5F). The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (luciferase and drug-resistant genes), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV in that order [33–35]. The replicon RNA replicated autonomously in several HCV replication-permissive cell lines derived from several hepatoma cell lines. Nonstructural proteins in replicon cells were polycistronically translated through EMCV IRES. The cap-dependent translated mRNA, including *Renilla* luciferase, EMCV IRES, and the firefly luciferase/neomycin-resistant gene in that order, was constructed to examine the effect of the extract on EMCV-IRES-dependent translation (Figure 5G). When the expression of the mRNA was transcribed by an EF promoter of the transfected plasmid in the presence of SG1-23-1, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed, suggesting that treatment with SG1-23-1 exhibited no effect on EMCV-IRES-dependent translation (Figure 5H). Thus, the inhibitory effect of SG1-23-1 on the luciferase activity must correspond to the replication efficiency of the replicon RNA but not to the inhibition of luciferase activity or the inhibition of EMCV-IRES-dependent translation. The inhibitory effect of the extract on the viral replication is similar to that of the extract on the helicase activity with respect to the values of $IC_{50}$ and $EC_{50}$ (Figure 3A and Table 2). These results suggest that treatment with SG1-23-1 inhibits HCV replication in a manner similar to that of the inhibitory effect on NS3 helicase activity.

**Figure 5.** Effect of SG1-23-1 on viral replication in replicon cell lines. (**A–D**) Huh7 Lunet/Con1 LUN Sb #26 (**A**), Huh7 rep Feo (**B**), Huh7#94/ORN3-5B#24 (**C**), and OR6 (**D**) cell lines were incubated in medium containing various concentrations of SG1-23-1. Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (**E**) Protein extract was prepared from Huh7 Lunet/Con1 LUN Sb #26 cells treated for 72 h with an indicated concentration of SG1-23-1 and then was subjected to Western blotting using antibodies to NS3, NS5A, and beta-actin. (**F**) Huh7 cell line transfected with pEF Fluc IN vector or pEF Rluc IN was established in the presence of G418. Both cell lines were incubated without (control) and with 50 μg/mL SG1-23-1. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data represent three