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High-throughput screening assay of hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon

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ABSTRACT

We have developed a novel high-throughput screening assay of hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase inhibitors using the fluorescence-quenching phenomenon via photoinduced electron transfer between fluorescent dyes and guanine bases. We prepared double-stranded DNA (dsDNA) with a 5′-fluorescent-dye (BODIPY FL)-labeled strand hybridized with a complementary strand, the 3′-end of which has guanine bases. When dsDNA is unwound by helicase, the dye emits fluorescence owing to its release from the guanine bases. Our results demonstrate that this assay is suitable for quantitative assay of HCV NS3 helicase activity and useful for high-throughput screening for inhibitors. Furthermore, we applied this assay to the screening for NS3 helicase inhibitors from cell extracts of microorganisms, and found several cell extracts containing potential inhibitors.

Hepatitis C virus (HCV) is the major etiological agent of non-A, non-B hepatitis with 3% of the world population infected. Most patients develop chronic hepatitis, and persistent infection often leads to liver cirrhosis or hepatocellular carcinoma [1]. To date, no vaccine against HCV has been developed despite numerous attempts [2], and current therapies based on pegylated interferon-α (IFN-α) and rebavirin have limited efficacy for the common HCV genotypes [3]; moreover, the combination therapies have serious side effects and are poorly tolerated [4]. Therefore, the development of anti-HCV drugs with greater safety and efficacy is desired.

HCV is a single-stranded (+) RNA virus belonging to the family *Flaviviridae* [5]. The viral genome of ~9600 nucleotides encodes a polyprotein of ~3010 amino acids that is cleaved into 10 mature proteins by cellular and viral proteases. These include structural proteins (C, E1, and E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The NS3 protein possesses multiple enzymatic activities; a serine protease activity at the N-terminus, and helicase and nucleotide triphosphatase (NTPase) activities at the C-terminus [6]. The NS3 helicase can unwind double-stranded RNA (dsRNA), double-stranded DNA (dsDNA), and DNA–RNA heteroduplexes in a 3′–5′ direction on a 3′ overhang region, using any nucleoside triphosphates (NTPs) or dNTPs as the energy source [7–10]. The NS3 protein has a higher affinity for dsDNA than for dsRNA and requires the NS4A cofactor for full activity on RNA [11]. Helicase activity is essential for viral replication, presumably unwinding double-stranded replication intermediates and secondary structures, allowing RNA amplification [12–14]. Therefore, the inhibition of helicase activity has the potential to terminate the proliferation of HCV. The development of a simple and high-throughput screening system for NS3 helicase inhibitors contributes to the discovery of anti-HCV drugs.

The current methods for the monitoring of helicase activity can be broadly classified into two categories: radioactive methods and nonradioactive methods. Radioactive methods use the double-stranded nucleic acid as a substrate, and one strand is radiolabeled with ^{32}P. In conventional gel-based assays [7–11], the double-stranded substrate and the unwound strand are separated by gel electrophoresis and visualized by autoradiography. These steps are laborious and time-consuming; therefore, these assays are not suitable for screening of multiple samples. Additionally, there are hazards involved in the use of radioisotopes.

As nonradioactive methods, enzyme-linked immunosorbent assay (ELISA)-based assays [15] and fluorescence resonance energy transfer (FRET)-based assays [16] were reported. In ELISA-based assays, a double-stranded substrate is used with a biotin-labeled template strand hybridized to a digoxigenin (DIG)-labeled release strand. The DIG-labeled release strand is detected using an anti-DIG

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antibody coupled to horseradish peroxidase. This assay does not require radioactivity, but involves complex and time-consuming steps. In FRET-based assays, a double-stranded substrate is used with a 5′ Cy3-labeled donor strand hybridized to a 3′ Black Hole Quencher (BHQ)-labeled quencher strand. Fluorescence is emitted by the separation of the strands from helicase activity. This assay can simply measure the real-time kinetics of helicase activity. However, this assay requires two different types of fluorescent dyes; therefore, the synthesis of the strands is relatively expensive.

To overcome these limitations, we have developed a novel high-throughput screening assay that is based on a fluorescence-quenching phenomenon via photoinduced electron transfer between fluorescent dyes and guanine bases [17,18]. The fluorescence-quenching phenomenon has been applied to quantitative methods for specific nucleic acid sequences [19–21]. We prepared a double-stranded substrate with a 5′ fluorescent dye (e.g., BODIPY FL)-labeled strand hybridized to a complementary strand consisting of guanine bases at the 3′-end. This assay requires only one type of fluorescent dye; therefore, it is more cost-effective than FRET-based assays. We optimized this new fluorescent helicase assay using the dsDNA substrate, and demonstrated the usefulness of the assay using known inhibitors of NS3 helicase activity, such as KCl [8], NaCl [6], and adenosine 5′-O-(3-thio) triphosphate (ATP-γ-S) [10]. Furthermore, we applied this assay to the screening for NS3 helicase inhibitors from cell extracts of microorganisms, and found several cell extracts containing potential inhibitors.

Materials and methods

Purification of recombinant NS3 protein. The expression plasmid (pT7/His-NS3) containing an N-terminal His-tagged full-length HCV NS3 gene derived from the HCV 1b genotype [22] was transformed in *Escherichia coli* BL21 (DE3) Codon Plus RIL, and the recombinant NS3 protein was induced with 0.02 mM IPTG at 25 °C for 4 h. The cells were harvested and resuspended in buffer AG [50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, 5% glycerol, 3 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulphonylfluoride and 5 mM imidazole]. After sonication in the presence of 0.1 mg/ml lysozyme, the lysate containing the NS3 protein was suspended with Ni-NTA agarose (Qiagen). The Ni-NTA agarose was washed with washing buffer [50 mM sodium phosphate buffer (pH 7.4), 300 mM NaCl, 5% glycerol, 3 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulphonylfluoride and 40 mM imidazole], and the bound proteins were eluted with washing buffer containing 200 mM imidazol. The obtained fractions were dialyzed against dialysis buffer [25 mM Tris–HCl (pH 7.4), 50 mM NaCl, 2.5% glycerol and 2 mM 2-mercaptoethanol]. The purity of the recombinant NS3 protein was >90%, as determined from the CBB staining pattern of SDS–PAGE gel. The ATPase activity of recombinant NS3 was assayed as described previously with modifications [23]. Briefly, a 10 μl reaction mixture contained 50 mM MOPS (pH 6.5), 3 mM $MgCl_2$, 2 mM DTT, 0.1 mM ATP, 5 μCi of [γ-^{32}P] ATP (Amersham), and 80 ng of recombinant NS3. The mixture was incubated at 37 °C for 30 min, spotted on a polyethylene-imine (PEI) cellulose (Merck), and developed in 0.75 M LiCl/1 M formic acid solution for 20 min. Visualization and quantitation of radioactive signals were performed using BAS2500 (Fuji film).

Oligonucleotides. A fluorescent-dye-labeled oligonucleotide was purchased from the J-Bio 21 Corporation, and unlabeled oligonucleotides were purchased from Hokkaido System Science Co., Ltd. BODIPY FL was attached to the 5′-end via an aminohexylphosphate linker having a six-carbon spacer.

Preparation of cell extracts from microorganisms. Various strains of *Streptomyces* species were cultured at 30 °C for 2 days in a liquid medium containing 2% glycerol, 2% dextrin, 1% soypepton (DIFCO), 0.3% yeast extract, 0.2% $(NH_4)_2SO_4$, and 0.2% $CaCO_3$. Then, 5% of grown cultures were inoculated on a solid medium composed of 20 g of barley and 20 mL of H_2O. The cultures were incubated at 30 °C for 2 weeks. Then, colonies on the medium were harvested, fixed in 40 mL of ethanol at room temperature for 24 h, and dried to obtain cell extracts. The dried cell extracts from microorganisms were dissolved in 100 μL of DMSO and stored at −20 °C in the dark until use.

Fluorescence quenching efficiency. dsDNA was prepared with the fluorescent strand (5′-CTACTACCCCCACCCTCACAACCTTTTTTTTTTTTTT-3′ with BODIPY FL coupled to the 5′-end) and the complementary strand (5′-GGTTGTGAGGGTGGGGGTAGTAG$_n$-3′ where n = 1–5) at a 1:3 molar ratio. dsDNA was formed in 20 mM Tris–HCl (pH 7.5) by brief heating at 90 °C, followed by slow cooling to room temperature. Then, fluorescence intensity was measured using LightCycler 1.5 (Roche). The reaction mixture contained 10 nM dsDNA, 30 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.075% Triton X-100, and 1 mM ATP in 20 μL reaction volume. The fluorescence intensity of each aliquot was measured at 37 and 95 °C. The fluorescence intensity at 37 °C was divided by that at 95 °C to normalize well-to-well variations in fluorescence measurement.

Fluorescent helicase assay. Fluorescent helicase assay was performed in 30 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 0.075% Triton X-100, 10 nM dsDNA substrate, 1 mM ATP, and 125 nM capture strand (5′-CTACTACCCCCACCCTCACAACC-3′) in 20 μL reaction volume. The unwinding reaction was started by adding NS3 helicase (40–200 nM) and was carried out at 37 °C for 90 min using LightCycler 1.5 (Roche). Fluorescence intensity was recorded every 30 s. The kinetics data of the fluorescence signal (DNA unwinding activity) up to 20 min were fitted to Eq. (1):

$$F(t) = C + A[1 - \exp(-k_{obs}t)], \quad (1)$$

where $F(t)$ is the fluorescence intensity at time t; C is the initial fluorescence intensity; A is the amplitude; and k_{obs} is the observed rate constant. Then, helicase activity was calculated as the initial reaction velocity by multiplying A with k_{obs} using Eq. (2).

$$F'(0) = Ak_{obs} \quad (2)$$

The Z' value [24] was determined using Eq. (3):

$$Z'\text{value} = 1 - (3\sigma_p + 3\sigma_n)/|\mu_p - \mu_m|, \quad (3)$$

where σ_p and σ_n are the standard deviations of the positive and negative controls, respectively, and μ_p and μ_n are the means of the positive and negative controls.

High-throughput screening for inhibition of NS3 helicase activity. The high-throughput screening assay of NS3 helicase inhibitors was carried out similarly to the fluorescent helicase assay described above with slight modifications, i.e., NS3 helicase (120 nM) was preincubated with the cell extracts of microorganisms (dissolved in DMSO) without ATP for 10 min at room temperature. The unwinding reaction was started by adding ATP and was carried out at 37 °C for 90 min using LightCycler 480 (Roche) with a 384-well plate format.

Results and discussion

Fluorescence quenching efficiency

A scheme of the proposed assay is shown in Fig. 1. The dsDNA substrate with a 3′ single-strand overhang was prepared by annealing two oligonucletides, a 5′ BODIPY FL-labeled nucleotide and a nonlabeled nucleotide that consists of guanine bases at the 3′-end. The fluorescence of the dye is quenched by the guanine bases, and is recovered by separation of the dsDNA substrate with the helicase. Thus, a high fluorescence quenching efficiency is crit-

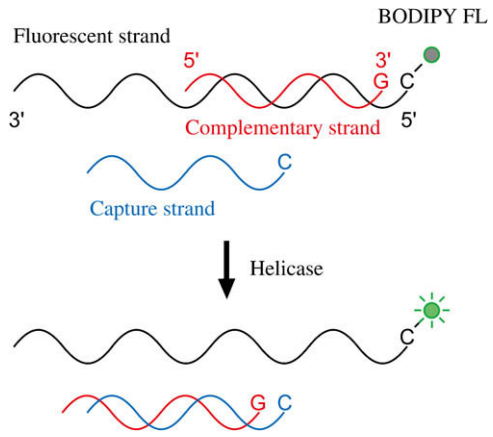

Fig. 1. Schematic representation of fluorescent helicase assay based on fluorescence quenching. The fluorescent dye (BODIPY FL) is attached to the cytosine at the 5′-end of the fluorescent strand and quenched by the guanine base at the 3′-end of the complementary strand via photoinduced electron transfer. When the dsDNA substrate is unwound by the helicase, 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.

ical for sensitive monitoring of helicase activity. A previous report revealed the relationship between the position of guanine bases in the complementary strand of BODIPY FL-conjugated DNA and the quenching efficiency of BODIPY FL [18]. This finding indicates that the guanine bases at the complementary or at positions outside the 5′-end of the fluorescent probe quench the fluorescence. Thus, to optimize the fluorescence quenching efficiency of the dsDNA substrate, we examined the effect of the number of guanine bases at the 3′-end in the complementary strand at which fluorescence is quenched. The five strands complementary to the fluorescent strand with the 3′ overhang of guanine bases were prepared. The fluorescent strand was hybridized to excess of these complementary strands by heating and slow cooling, and the fluorescence intensity of the dye was measured (Table 1). Fluorescence quenching efficiency and signal-to-background ratio (S/B) were calculated. In the presence of complementary strands, the fluorescence intensities of the dye significantly decreased. The highest fluorescence quenching efficiency and S/B were observed when the complementary strand that consists of three guanine bases at the 3′-end was used. Fluorescence quenching efficiency and S/B slightly decreased when the complementary strands with four and five guanine bases at the 3′-end were used. In subsequent experiments, the dsDNA substrate containing three guanine bases at 3′-end was used.

Table 1
Effect of number of guanine bases at 3′-end in complementary strand that caused quenching of fluorescence.

Substrate	*F*	Fluorescence quenching efficiency (%)	S/B
ATC-5′ without complementary strand[a]	1.76 ± 0.01 (=F_0)	–	–
ATC-5′[a] TAG-3′[b]	0.339 ± 0.000	80.7 ± 0.0	5.19 ± 0.01
ATC-5′[a] TAGG-3′[b]	0.350 ± 0.004	80.1 ± 0.2	5.02 ± 0.05
ATC-5′[a] TAGGG-3′[b]	0.319 ± 0.001	81.8 ± 0.1	5.50 ± 0.03
ATC-5′[a] TAGGGG-3′[b]	0.381 ± 0.002	78.3 ± 0.1	4.62 ± 0.03
ATC-5′[a] TAGGGGG-3′[b]	0.389 ± 0.001	77.9 ± 0.1	4.52 ± 0.01

The fluorescent strand was modified with BODIPY FL at the 5′-end. Fluorescence quenching efficiency was calculated using the equation $(F_0 - F)/F_0 \times 100$. *F*, fluorescence intensity (arbitrary units); F_0, fluorescence intensity in the absence of complementary strand; S/B, signal-to-background ratio. Results represent the average ± SD (standard deviation) of triplicate samples.

[a] Fluorescent strand.

[b] Complementary strand.

Fluorescent helicase assay

The fluorescent helicase assay was then used for measuring the activity of NS3 helicase. When the recombinant NS3 protein was added, fluorescence was emitted from the assay mixture in the presence of ATP (Fig. 2). In the absence of ATP, no increase in fluorescence intensity was found. Moreover, no increase in fluorescence intensity was found in the presence of ATP without the NS3 protein. These results indicate that the observed fluorescence emission arose from dsDNA unwound by the NS3 helicase because ATP hydrolysis is required for dsDNA unwound by NS3 helicase [7–10]. The results also indicate that contaminating proteins such as nuclease that can disturb the assay were not present at detectable level in the NS3 protein.

Next, we evaluated the effect of NS3 protein concentration on the performance of fluorescent helicase assay. Serial dilutions of the NS3 protein were assayed, and the initial velocity of fluorescence emission (helicase reaction) in each test was measured. As a result, both fluorescence intensity and fluorescence emission rate increased with NS3 protein concentration (Fig. 2). This increase in initial fluorescence emission rate saturated in the presence of the NS3 protein at about 120 nM. These results further supported the finding that the assay can be used to quantitatively measure DNA unwinding activity due to NS3 helicase. Slight decreases in fluorescence emission rate were observed after 30 min, probably owing to the establishment of a steady-state equilibrium caused by ATP exhaustion and/or a slow degradation of both the NS3 protein and nucleotides [16]. To determine the optimum concentration of the NS3 protein for this assay, the Z' value [24], which is a statistical parameter (from 0 to 1) commonly used for the validation of reliability and reproducibility of assay conditions, was calculated. The assay that has a higher Z' is more reliable and reproducible. The calculated Z' values in triplicate determinations were 0.69, 0.45, 0.92, 0.91, and 0.86 for 40, 80, 120, 160, and 200 nM NS3, respectively. These values indicate that 120 nM was the optimum concentration of NS3 protein; therefore, we used 120 nM NS3 protein in subsequent experiments.

Then, to demonstrate the potential of this new assay for the quantitative determination of helicase activity, the NS3 protein was incubated with various concentrations of known helicase inhibitors, i.e., KCl, NaCl, and ATP-γ-S. As shown in Fig. 3, the increase in the concentrations of KCl, NaCl, and ATP-γ-S significantly decreased helicase activity. At 100 mM KCl and NaCl, approximately 80% of the original helicase activity was inhibited. These results are consistent with previous reports [6,8]. In addition, at 1 mM ATP-γ-S, approximately 50% of the original helicase activity was inhibited. This result is appropriate because ATP-γ-S is the competitive inhibitor of NS3 ATPase and 1 mM ATP was used in this experiment. Moreover, paclitaxel [25], which is another potential inhibitor of helicase activity, was also tested; however, no inhibitory effect was demonstrated for this candidate at a concentration of up to 50 μM (data not shown).

High-throughput screening for inhibition of NS3 helicase activity

With the aim of performing a high-throughput screening of potential NS3 helicase inhibitors, a helicase assay adapted to a 96- or 384-well plate format was established. By using this high-throughput assay, we screened for possible inhibitory substances from cell

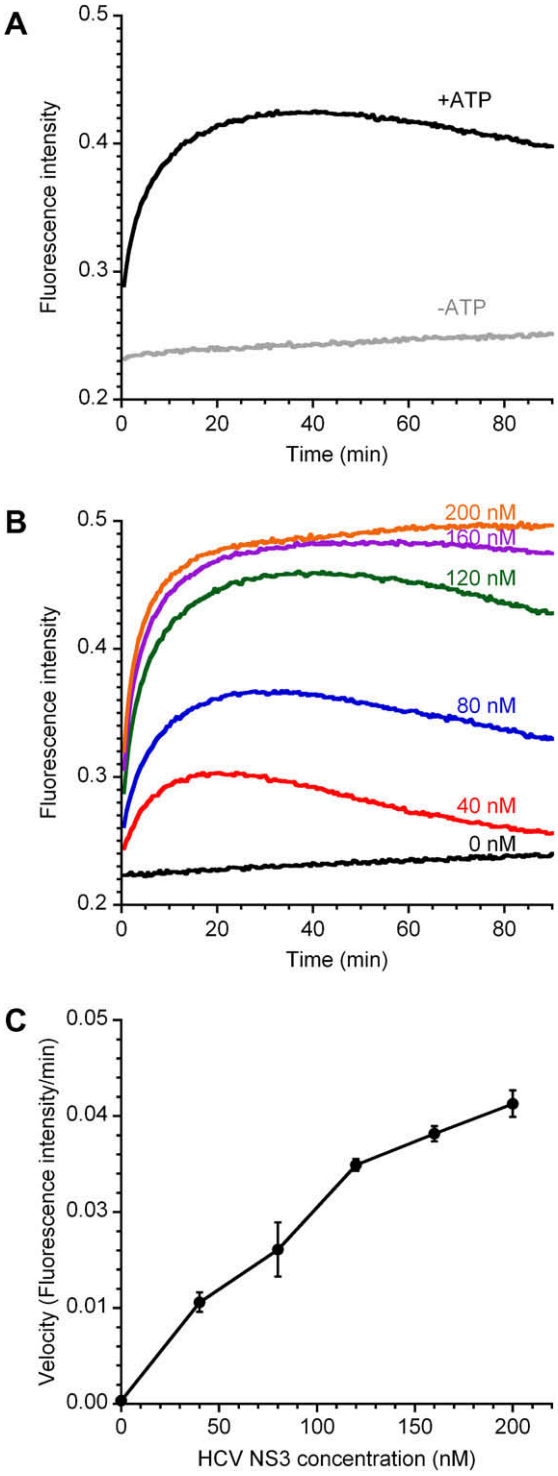

Fig. 2. Fluorescent helicase assay. (A) ATP dependence of NS3 helicase activity evaluated by fluorescent helicase assay. Black line, with 1 mM ATP; gray line, without ATP. The NS3 protein (120 nM) was incubated with the dsDNA substrate (10 nM) in the presence (black line) or absence (gray line) of 1 mM ATP. Effect of NS3 helicase concentration on kinetics (B) and rate (C) of fluorescence emission caused by DNA unwinding induced by helicase. Helicase reaction mixtures contained 1 mM ATP and the indicated concentrations of NS3. Experiments were performed in triplicates and data are presented as the mean initial velocity ± standard deviation (SD). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

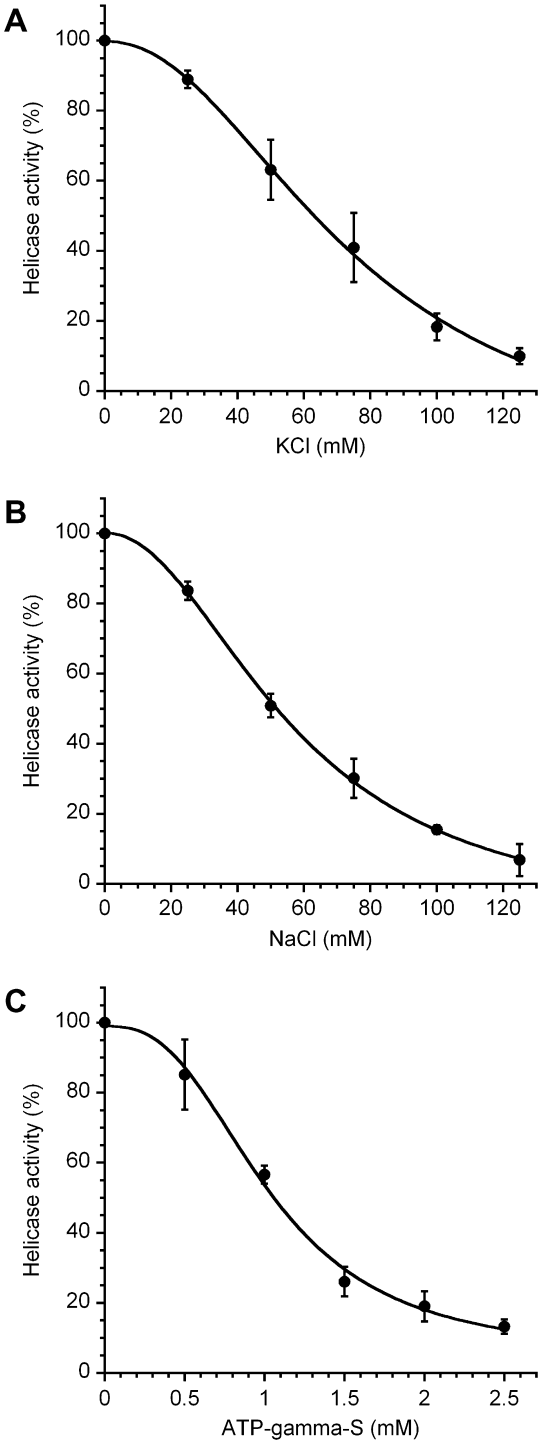

Fig. 3. Inhibition of helicase activity by (A) KCl, (B) NaCl, and (C) ATP-γ-S. Each inhibitor titration was carried out in triplicate and the data are presented as mean ± SD. The NS3 protein (120 nM) was incubated with various concentrations of known inhibitors as indicated. Helicase activity was calculated from control reactions without inhibitors.

extracts of microorganisms (*Streptomyces* cell extracts). The results of an initial screening of 19 randomly selected samples are shown in Fig. 4. The blank control, which contains only the medium components for culturing the microorganisms, slightly decreased the fluorescent emission signal intensity (20% decrease in emission rate). Four out of the 19 samples showed less than 15% of the original fluorescent emission rate (samples no. 14, 15, 16, and 19), possibly indicating the presence of potential inhibitory substances for helicase activity in these samples. We therefore concluded that

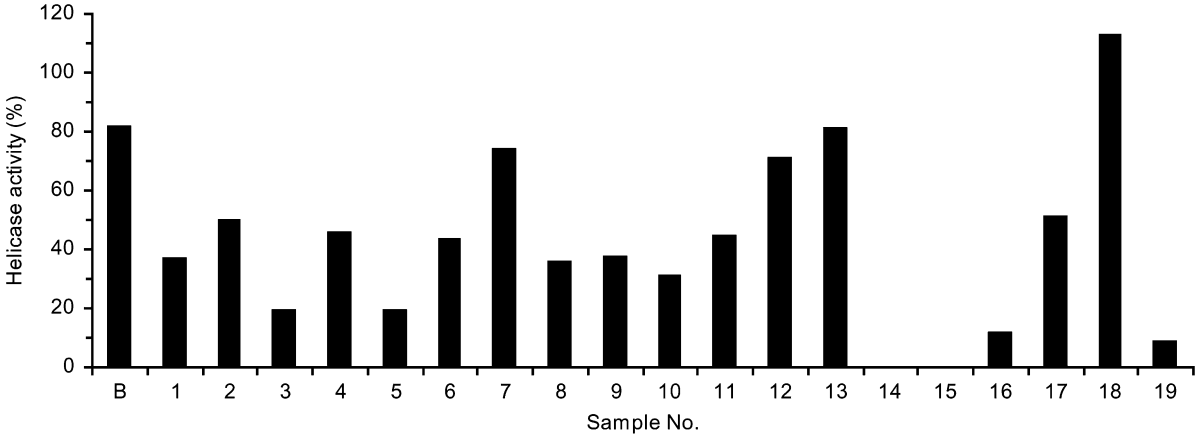

Fig. 4. High-throughput screening for inhibition of NS3 helicase activity by cell extracts from *Streptomyces* spp. The reaction was performed in 20 μL in individual wells of 384-well plates containing 120 nM NS3 protein and 1 μL of cell extracts. Fluorescence emission rate (helicase activity) was calculated relative to the control value examined without inhibitors. Sample B is the blank control that includes only the medium components for cultures.

the new assay is useful for high-throughput screening for NS3 helicase inhibitors.

Advantages of new assay

In this study, we have developed a high-throughput screening assay of HCV NS3 helicase inhibitors using the fluorescence-quenching phenomenon occurring between a fluorescent dye and guanine bases. This assay has several major advantages as follows (i) this assay is rapid, simple, safe, and cost-effective. The assay does not require complex procedures as in the case of other methods and does not require radioisotopes. This assay requires only one fluorescent dye, and so it is more cost-effective than FRET-based assays [16]. (ii) This assay enables the measurement of the real-time kinetics of helicase activity quantitatively. (iii) This assay has the potential for multiplex analysis. Although we used only the fluorescent dye BODIPY FL (excitation/emission = 505/513 nm) in this study, we can also use other dyes such as Pacific Blue (410/455), BODIPY R6G (517/543), and TAMRA (555/580), which have fluorescence spectra different from that of BODIPY FL and are strongly quenched by guanine bases at a particular position [18]. Moreover, if the autofluorescence of certain compounds affects the tests, this problem may be partially solved using the above-mentioned fluorescent dyes and appropriate sets of excitation/emission filters selected on the basis of their autofluorescence spectra.

In conclusion, our results demonstrate that this new assay is suitable for the quantitative assay of HCV NS3 helicase activity and is useful for high-throughput screening for its inhibitors. We believe that the present assay will accelerate the finding of possible anti-HCV drugs targeting HCV NS3 helicase.

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References

[1] S.L. Tan, A. Pause, Y.U. Shi, N. Sonenberg, Hepatitis C therapeutics: current status and emerging strategies, Nat. Rev. Drug Discov. 1 (2002) 867–881.
[2] X. Forns, J. Bukh, R.H. Purcell, The challenge of developing a vaccine against hepatitis C virus, J. Hepatol. 37 (2002) 684–695.
[3] D.E. Baker, Pegylated interferon plus ribavirin for the treatment of chronic hepatitis C, Rev. Gastroenterol. Disord. 3 (2003) 93–109.
[4] M.W. Fried, Side effects of therapy of hepatitis C and their management, Hepatology 36 (2002) 237–244.
[5] Q.L. Choo, G. Kuo, A.J. Weiner, L.R. Overby, D.W. Bradley, M. Houghton, Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral-hepatitis genome, Science 244 (1989) 359–362.
[6] P. Gallinari, D. Brennan, C. Nardi, M. Brunetti, L. Tomei, C. Steinkuhler, R. De Francesco, Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus, J. Virol. 72 (1998) 6758–6769.
[7] D.W. Kim, Y. Gwack, J.H. Han, J. Choe, C-terminal domain of the hepatitis-C virus NS3 protein contains an RNA helicase activity, Biochem. Biophys. Res. Commun. 215 (1995) 160–166.
[8] C.L. Tai, W.K. Chi, D.S. Chen, L.H. Hwang, The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3), J. Virol. 70 (1996) 8477–8484.
[9] Y. Gwack, D.W. Kim, J.H. Han, J. Choe, Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein, Biochem. Biophys. Res. Commun. 225 (1996) 654–659.
[10] Y. Gwack, D.W. Kim, J.H. Han, J. Choe, DNA helicase activity of the hepatitis C virus nonstructural protein 3, Eur. J. Biochem. 250 (1997) 47–54.
[11] P.S. Pang, E. Jankowsky, P.J. Planet, A.M. Pyle, The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding, EMBO J. 21 (2002) 1168–1176.
[12] A.D. Wardell, W. Errington, G. Ciaramella, J. Merson, M.J. McGarvey, Characterization and mutational analysis of the helicase and NTPase activities of hepatitis C virus full-length NS3 protein, J. Gen. Virol. 80 (1999) 701–709.
[13] A.A. Kolykhalov, K. Mihalik, S.M. Feinstone, C.M. Rice, Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo, J. Virol. 74 (2000) 2046–2051.
[14] B.H. Gu, C.B. Liu, J. Lin-Goerke, D.R. Maley, L.L. Gutshall, C.A. Feltenberger, A.M. Del Vecchio, The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhea virus NS3 protein are essential for viral replication, J. Virol. 74 (2000) 1794–1800.
[15] C.C. Hsu, L.H. Hwang, Y.W. Huang, W.K. Chi, Y.D. Chu, D.S. Chen, An ELISA for RNA helicase activity: application as an assay of the NS3 helicase of hepatitis C virus, Biochem. Biophys. Res. Commun. 253 (1998) 594–599.
[16] A.M. Boguszewska-Chachulska, M. Krawczyk, A. Stankiewicz, A. Gozdek, A.L. Haenni, L. Strokovskaya, Direct fluorometric measurement of hepatitis C virus helicase activity, FEBS Lett. 567 (2004) 253–258.
[17] M. Torimura, S. Kurata, K. Yamada, T. Yokomaku, Y. Kamagata, T. Kanagawa, R. Kurane, Fluorescence-quenching phenomenon by photoinduced electron transfer between a fluorescent dye and a nucleotide base, Anal. Sci. 17 (2001) 155–160.
[18] S. Kurata, T. Kanagawa, K. Yamada, M. Torimura, T. Yokomaku, Y. Kamagata, R. Kurane, Fluorescent quenching-based quantitative detection of specific DNA/RNA using a BODIPY FL-labeled probe or primer, Nucleic Acids Res. 29 (2001) e34.
[19] H. Tani, T. Kanagawa, S. Kurata, T. Teramura, K. Nakamura, S. Tsuneda, N. Noda, Quantitative method for specific nucleic acid sequences using competitive polymerase chain reaction with an alternately binding probe, Anal. Chem. 79 (2007) 974–979.
[20] H. Tani, T. Teramura, K. Adachi, S. Tsuneda, S. Kurata, K. Nakamura, T. Kanagawa, N. Noda, Technique for quantitative detection of specific DNA sequences using alternately binding quenching probe competitive assay combined with loop-mediated isothermal amplification, Anal. Chem. 79 (2007) 5608–5613.
[21] S. Okuniki, K. Nakamura, M. Kawaharasaki, H. Tanaka, H. Uchiyama, N. Noda, Quantification of *Rhodocyclus*-related and actinobacterial polyphosphate-

accumulating organisms in an enhanced biological phosphorus removal process using quenching probe PCR, Microbes Environ. 22 (2007) 106–115.
[22] F. Nishikawa, K. Funaji, K. Fukuda, S. Nishikawa, In vitro selection of RNA aptamer against the HCV NS3 helicase domain, Oligonucleotides 14 (2004) 114–129.
[23] H. Tran, M. Schilling, C. Wirbelauer, D. Hess, Y. Nagamine, Facilitation of mRNA deadenylation and decay by the exosome-bound, DExH protein RHAU, Mol. Cell 13 (2004) 101–111.
[24] J.H. Zhang, T.D.Y. Chung, K.R. Oldenburg, A simple statistical parameter for use in evaluation and validation of high throughput screening assays, J. Biomol. Screen. 4 (1999) 67–73.
[25] P. Borowski, R. Kuhel, O. Mueller, L.H. Hwang, J. Schulze Zur Wiesch, H. Schmitz, Biochemical properties of a minimal functional domain with ATP-binding activity of the NTPase/helicase of hepatitis C virus, Eur. J. Biochem. 266 (1999) 715–723.