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Supporting Information

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Discovery of Hepatitis C Virus NS3 Helicase Inhibitors by a Multiplexed, High-Throughput Helicase Activity Assay Based on Graphene Oxide**

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Supporting Information

Methods

Preparation and characterization of graphene oxide (GO).

Graphene oxide nanosheets were prepared according to the previously reported Hummers method with sli ght modifications. Briefly, 0.5 g of natural graphite (FP 99.95% pure, Graphit Kropfmühl AG, Hauzenber g, Germany), 0.5 g of $NaNO_3$ (Junsei, Tokyo, Japan), and 23 mL of H_2SO_4 (Samchun, Seoul, Korea) were mixed in a beaker with vigorous stirring in an ice bath. 3 g of $KMnO_4$ (Sigma-Aldrich, MO, USA) was sl owly added to the mixture. Then, the mixed solution was transferred into 35 ^oC water bath with vigorous stirring. After an hour, 40 mL of distilled water was added to the mixed solution and stirred for 30 min at 90 ^oC. Another 100 mL of distilled water was added and followed by the drop-wise addition of 3 mL of 3 0% H_2O_2 (Junsei, Tokyo, Japan). During this addition, the color of the solution changed from dark brown to yellow. The mixture was purified by filtration with a Büchner funnel and washed with distilled water u ntil the filtrate was neutralized. The filtered cake was dried in a desiccator. The GO was exfoliated in disti lled water by sonication until there were no visible particles in the suspension. An atomic force microscop y image and a height profile of the prepared GO were taken with an XE-100 (Park System, Korea). The R aman spectra of graphite and the prepared GO were obtained with LabRAM HR UV/vis/NIR (Horiba Jobi n Yvon, France) using an Ar ion CW laser (514.5 nm) as the excitation source focused through a BXFM c onfocal microscope equipped with an objective (50X, numerical aperture=0.50). The FTIR spectrum of G O was obtained with an EQUINOX55 (Bruker, Germany) using the KBr pellet method.

Multiplexed graphene oxide based helicase assay (mGOHA).

To prepare the SARS CoV helicase substrate, a mixture of each strand of DNA was prepared by the additi on of 2 μL of 10 μM Cy3 labeled DNA strand (5′-Cy3-GGA ATT CTA ATG TAG TAT AGT AAT CCG C TC-3′, Genotech, Daejeon, Korea) to a 2-fold excess of complementary DNA strand (5′-TTT TTT TTT T TT TTT GAG CGG ATT ACT ATA CTA CAT TAG AAT TCC-3′, Genotech, Daejeon, Korea) in an annea ling buffer solution (50 mM Tris-HCl, pH 8.0) (Fisher, NJ, USA) and 50 mM NaCl (Junsei, Tokyo, Japa n)). Next, annealing was carried out using a heat block at 95 ^oC for 5 min, followed by slow cooling at roo m temperature over 30 min. The duplex fluorescence labeled DNA substrate for HCV NS3 helicase was p repared in the same manner with Cy5 labeled DNA (5′-Cy5-TGG CGA CGG CAG CGA GGC AGA GG A GCA GAG GGA GCA-3′, Genotech, Daejeon, Korea) and complementary strand (5′-GCC TCG CTG CCG TCG CCA-3′, Genotech, Daejeon, Korea). A solution of helicase substrate dsDNA was prepared by mixing 20 nM annealed SARS CoV helicase substrate and 20 nM HCV NS3 helicase substrate with 0.5 m M EDTA (pH 8.0) solution (BIO-RAD, CA, USA), 20 mM ATP (Sigma-Aldrich, MO, USA) in a 1x react ion buffer (50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol (Bio Basic Inc., ON, Canada) and 0.65

mM $MgCl_2$ (Junsei, Tokyo, Japan)). GO solution was prepared at 10 μg/mL in a 1x reaction buffer right before use from a 1 mg/ml GO solution in distilled water. 30 μL of the substrate solution and 15 μL of GO solution were mixed in 96 well plates. Then, 15 μL of a mixed solution of SARS CoV and HCV NS3 helicases (in 1x reaction buffer) were then added to the above mixture in the 96 well plates. The final concentrations of dsDNA substrate for each helicase, GO, SARS CoV helicase and HCV NS3 helicase were 10 nM, 2.5 μg/ml, 2 nM and 2 nM in a total volume of 60 μL with 1x reaction buffer. Next, the dsDNA unwinding activity of the helicase was measured over time by monitoring fluorescence intensities at Ex/Em= 550 nm / 570 nm (for Cy3, SARS CoV helicase) and at Ex/Em= 650 nm / 670 nm (for Cy5, HCV NS3 helicase) with a fluorometer, SynergyMx (BioTek, UK).

Measurement of the Z′-factor.

The substrate solution was prepared by mixing 20 nM SARS CoV helicase substrate, 20 nM HCV NS3 helicase substrate, 0.5 mM EDTA (pH 8.0), and 20 mM ATP in a 2x reaction buffer. After pre-incubation at room temperature for 10 min, 1.3 mM $MgCl_2$ was added to the mixture. For the positive control, 30 μL of substrate solution and 15 μL of GO solution (20 μg/mL GO in distilled water) were mixed in 96 well plates, followed by the transfer of 15 μL of 2 nM SARS and HCV helicases, respectively. For the negative control, 30 μL of substrate solution and 15 μL of GO solution (20 μg/mL GO in distilled water) were mixed in 96 well plates, followed by the transfer of 15 μL of 2 nM boiled inactive helicase for each. After the addition of the helicases and incubation for 30 min, the fluorescence intensities were measured (Ex/Em= 550 nm / 570 nm for Cy3 and Ex/Em= 650 nm / 670 nm for Cy5) (n=30). The Z′-factor was calculated using equation (1).

Screening of the small molecule compound library.

Basically, screening was performed by applying the same condition for mGOHA as described above except that 10,000 compounds from the DIVERSet chemical library (ChemBridge Chem., San Diego, CA, USA) were first mixed with the helicase solution at 1 mM before the addition of a mixture of GO and dsDNA substrates. After the addition of the mixture of helicases and library compounds to the mixture of the substrate and GO, the helicase activities for dsDNA unwinding were evaluated by measuring the fluorescence intensity at Ex/Em= 550 nm / 570 nm for Cy3 and at Ex/Em= 650 nm / 670 nm for Cy5 after 30 min of incubation at 25 ^{o}C using a fluorometer.

Determination of the IC_{50}.

A substrate solution was prepared as described above. 30 μL of substrate solution and 15 μL of GO solution (20 μg/mL in distilled water) were mixed in 96 well plates, followed by mixing with a solution of 15 μL of 2 nM each helicase and 10~1000 μM of the selected 15 hit compounds from chemical library in 96 well plates. Fluorescence intensities were measured at Ex/Em= 550 nm / 570 nm (for Cy3) and at Ex/Em= 650 nm / 670 nm (for Cy5) after 30 min of incubation at 25 ^{o}C. The IC_{50} values were determined by four-

parameter logistic curve-fit method with Sigmaplot (Systat Software Inc., San Jose, CA, USA).

ATPase assay.

All the ATPase assays were carried out with the QuantiChromTM ATPase/GTPase Assay Kit (BioAssay Systems, Hayward, CA, USA). First, mixtures of helicases (2 nM each) and hit compounds (250 μM) were prepared and the substrate solution (20 nM of each DNA duplex) was added in the presence of ATP (10 mM). After 30 min incubation, 200 μL of the ATPase assay reagent was added to each reaction mixture and the absorbance was measured at 620 nm to determine the relative concentration of phosphate, the hydrolyzed product of ATP.

Cell culture.

The human hepatoma cell line Huh-7 carrying the HCV NS3 replicon RNA (kindly provided by Dr. Bartenschlager, University of Heidelberg, Germany) was maintained in Dulbecco's modified minimal essential medium (DMEM) (WelGENE Inc., Seoul, Korea) containing 4.5 g/L D-glucose supplemented with 10% fetal bovine serum (FBS) (WelGENE Inc., Seoul, Korea), 100 units/mL penicillin, 100 mg/mL streptomycin, and 500 μg/mL G418 (A. G. Scientific, Inc. USA) in 37 ^{o}C temperature and 5% CO_2 atmospheric condition unless otherwise stated.

MTT assay for cell viability test.

The Huh-7 cells containing the HCV replicon RNA were seeded in 96 well plates with 10,000 cells/well in G418 containing media with 1% DMSO to reach 70~80% confluency. After the cells were treated with 10~1000 μM of the hit compounds, the Huh-7 cells were washed with phosphate buffered saline (PBS, WelGENE, Korea) solution and incubated with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chemical Co., St. Louis, MO, USA) stock solution (5 mg/mL MTT dissolved in PBS) to analyze metabolically the active cells. MTT treated cells were incubated for 2~4 hrs until a purple color developed. Then, the media were discarded and 200 μL of DMSO was added to each well to dissolve the formazan salt. Finally, the absorbance was measured at 560 nm with a SynergyMx fluorometer.

Luciferase assay.

The HCV replicon Huh-7 cells were seeded in 24 well plates with 100,000 cells/well. The cells were grown for 24 hrs to reach 70~80% confluency. Cells were treated with 10~1000 μM of the hit compounds in serum-free media with 1% DMSO. Then, the cells were washed with PBS and lysed with 40 μL of 1X passive lysis buffer (Promega Corp., Madison, WI, USA) for 30 min at room temperature. Cell lysates were centrifuged at 10,000 rpm for 5 min. 20 μL of each supernatant of the cell lysates were transferred to 96 well plates, followed by the addition of 100 μL of luciferase assay reagent, luciferin (n=3). Relative light units were measured after a 10-sec delay using SynergyMx. The total protein concentration of the whole-cell extract lysates were quantified using the Bradford assay with BSA as a protein standard.

HCV NS3 serine protease assay.

Protease assays were carried out with the SensoLyte® 620 HCV protease assay kit (Anaspec, Fremont, CA, USA). The replicon Huh-7 cells were treated with the hit compounds (250 μM) for 24 hrs, washed and then lysed with 60 μL of 1x passive lysis buffer (Promega, WI, USA) for 30 min at room temperature after being rinsed with PBS. After centrifugation, 50 μL of the supernatant was transferred to 96 well black plates. Then, 50 μL of FRET peptide substrate solution (2 μM) was added to each well, followed by shaking the plate gently for 60 sec. Then, the reaction mixtures were incubated at room temperature for 60 min in the dark. The Fluorescence intensities were measured at Ex/Em=591 nm / 622 nm with a SynergyMx microplate reader.

NMR experiments

NMR spectra were acquired at using a Bruker AVANCE 900 MHz spectrometer (Korea Basic Science Institute). In order to check the interaction between dsDNA and antiSARS-HCV-Hel-1~5, 1D ^{1}H NMR titration experiment was performed at 283 K. The dsDNA was annealed in a buffer containing 10 mM Sodium phosphate (pH 6.0), 50 mM NaCl, 0.2 mM EDTA, 0.45 mM $MgCl_2$ by heating to 95°C and slowly cooling to the room temperature. The titration was performed to obtain 2:1 molar ratio of chemical: DNA. The spectra were analyzed to find any chemical shift change in the DNA spectrum after chemical binding.

ITC experiments

The interaction between antiSARS-HCV-Hel-1~5 and dsDNA were observed with a VP-ITC microcalorimeter (MicroCal. Piscataway, NJ) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10% glycerol, 0.2 mM EDTA, 0.45 mM $MgCl_2$. The DNA (10 μM) and the antiSARS-HCV-Hel1~5 (200 μM) were added to the reaction cell (1.4 mL) and the injector syringe (296 μL), respectively. 26 injections were done per experiment (1 μL for the first injection and 10 μL for the following 25 injections), and the stirring speed was at 350 rpm. The data was analyzed by the using the ORIGIN software provided by the manufacturer.

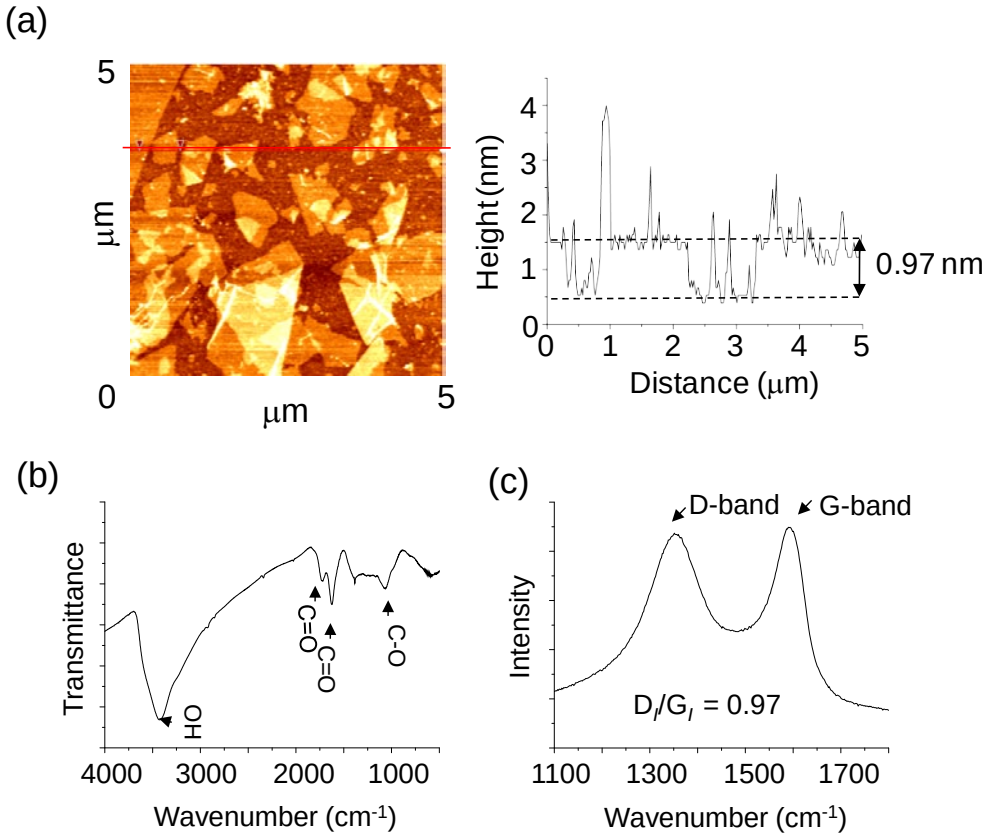


Figure S1. The prepared GO was characterized by using AFM, IR and Raman spectroscopy. Prepared GO sheets were 0.97 nm and 0.01~4 □ m dimension. Characteristic peaks corresponding to oxygen containing functional groups at 3,395, 1,716, 1,225 and 1079 cm^{-1} were observed in IR spectrum. Raman spectrum of GO showed characteristic D- and G-band at 1351 and 1589 cm^{-1} respectively.

(a)

SARS CoV Helicase Substrate (Cy3-DNA-SH)

5′-TTT TTT TTT TTT TTT GAG CGG ATT ACT ATA CTA CAT TAG AAT TCC-3′

3′-CTC GCC TAA TGA TAT GAT GTA ATC TTA AGG-5′-Cy3

HCV NS3 Helicase Substrate (Cy5-DNA-HH)

5′-GCC TCG CTG CCG TCG CCA-3′

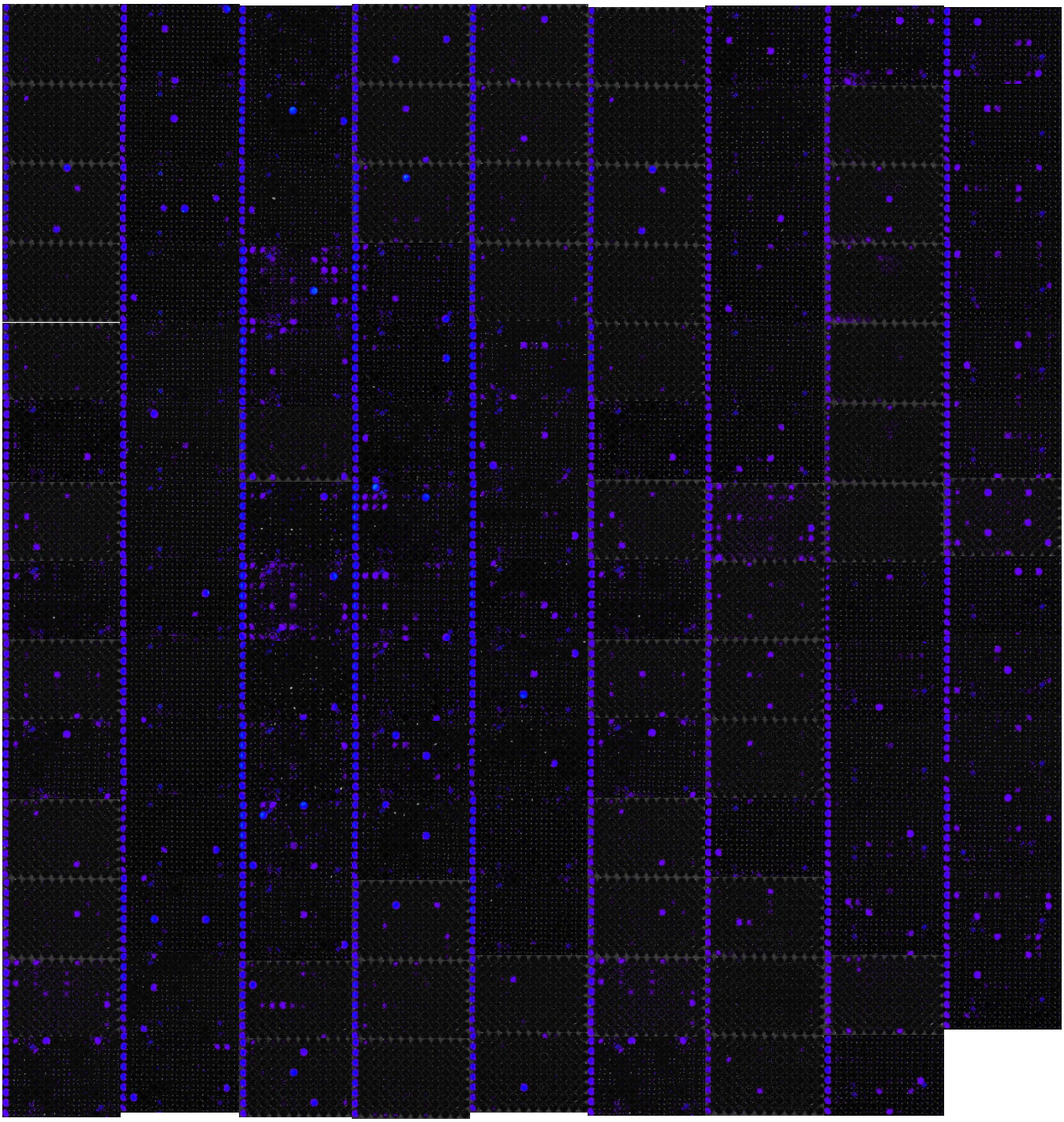
3′-ACG AGG GAG ACG AGG AGA CGG AGC GAC GGC AGC GGT-5′-Cy5

(b)

$$Z'\,factor = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{|\mu_{c+} - \mu_{c-}|}$$

Figure S2. (a) Cy3 and Cy5 fluorescence dye labeled SARS CoV and HCV NS3 helicase double stranded DNA sequences. (b) Z`-factor calculating equation from the measured calculated the mean (μ_{c+} and μ_{c-} for the positive and negative controls, respectively) and standard deviations (σ_{c+} and σ_{c-})

(a)



(b)

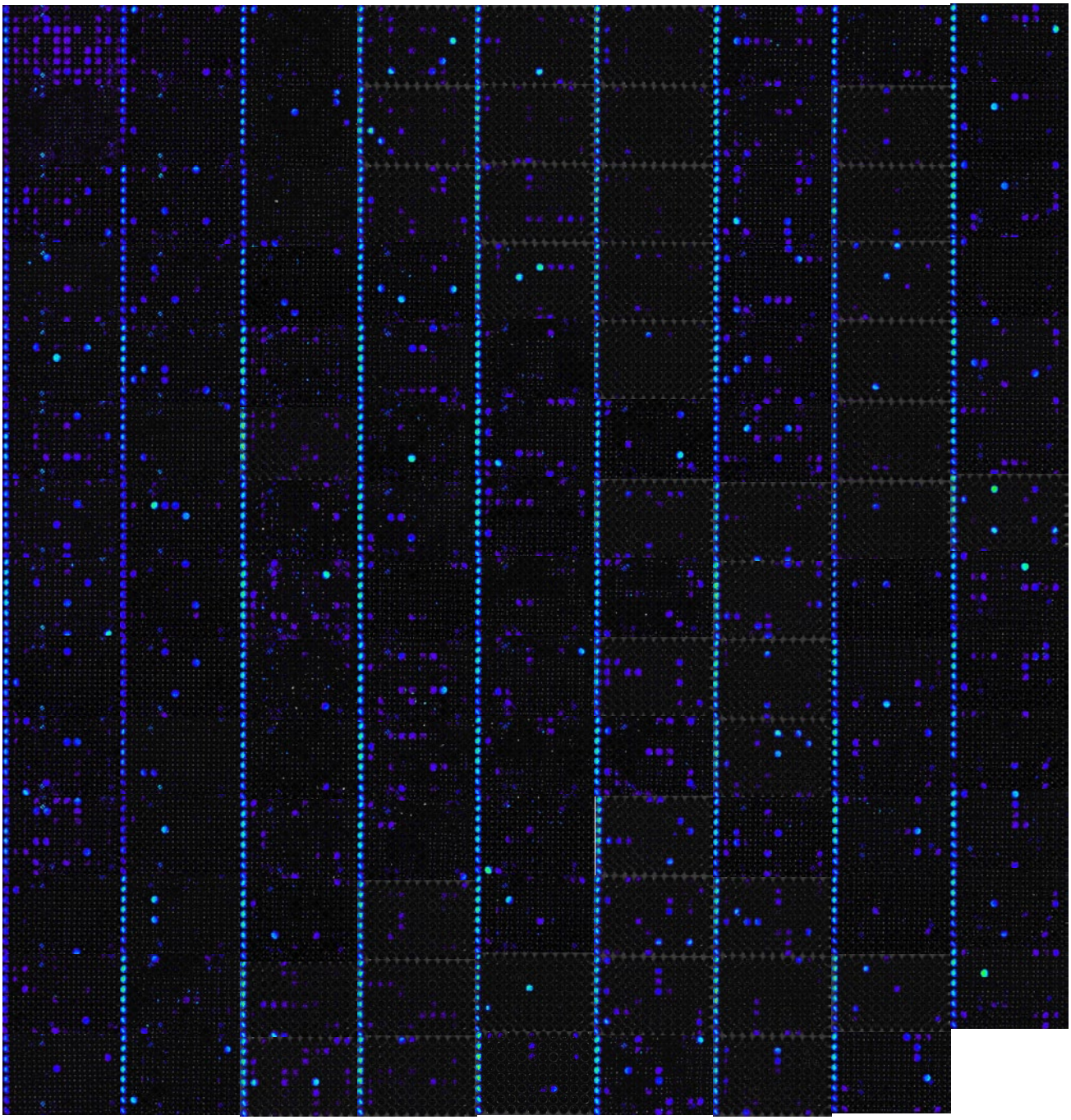


Figure S3. The present mGOHA-based high-throughput screening of 10,000 small molecules were performed in 96-well plates. The 125 plates were used and each helicase reaction mixture containing SARS CoV helicase, HCV NS3 helicase, DNA substrates specific for each helicase reaction mixture, GO and library small molecule was prepared in each well with negative controls and positive controls in first and last columns, respspectively. After 30 min of incubation at 25 ^{o}C, fluorescence images of whole plates corresponding to (**a**) Cy3 for Cy3-DNA-SH and (**b**) Cy5 for Cy5-DNA-HH were taken by using an in vivo fluorescence imaging system. In each image, bright spots represent maintenance of high fluorescence even after incubation with corresponding helicase, indicating presence of possible inhibitors of the helicase in the corresponding reaction mixture.

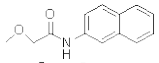
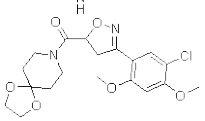
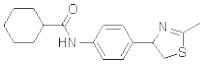
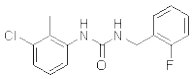
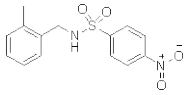
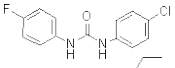
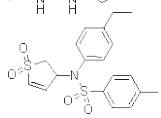
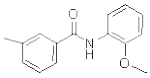
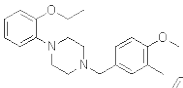
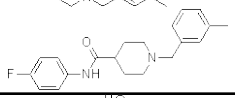
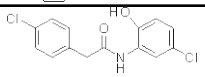
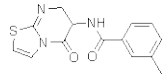
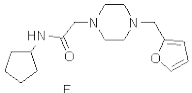
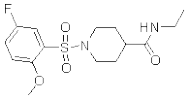
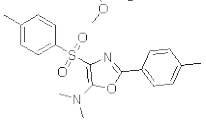
Target	Name	Structure	IC50, μM	
			SARS CoV Helicase	HCV NS3 Helicase
SARS CoV Helicase	antiSARS-Hel-1		49.3	-
	antiSARS-Hel-2		104.6	-
	antiSARS-Hel-3		102.3	-
	antiSARS-Hel-4		79.8	-
	antiSARS-Hel-5		84.6	-
HCV NS3 Helicase	antiHCV-Hel-1		-	51.6
	antiHCV-Hel-2		-	56.2
	antiHCV-Hel-3		-	57.4
	antiHCV-Hel-4		-	61.9
	antiHCV-Hel-5		-	92.0
SARS CoV & HCV NS3 Helicase	antiSARS-HCV-Hel-1		133.2	441.1
	antiSARS-HCV-Hel-2		537.5	152.9
	antiSARS-HCV-Hel-3		177.3	137.1
	antiSARS-HCV-Hel-4		103.8	103.5
	antiSARS-HCV-Hel-5		262.3	155.4

Figure S4. Selected Chemical structures and measured IC50 values of antiSARS-Helicase, antiHCV-Helicase and SARS CoV & HCV NS3-Helicase inhibitors from 10,000 of chemical compounds library.

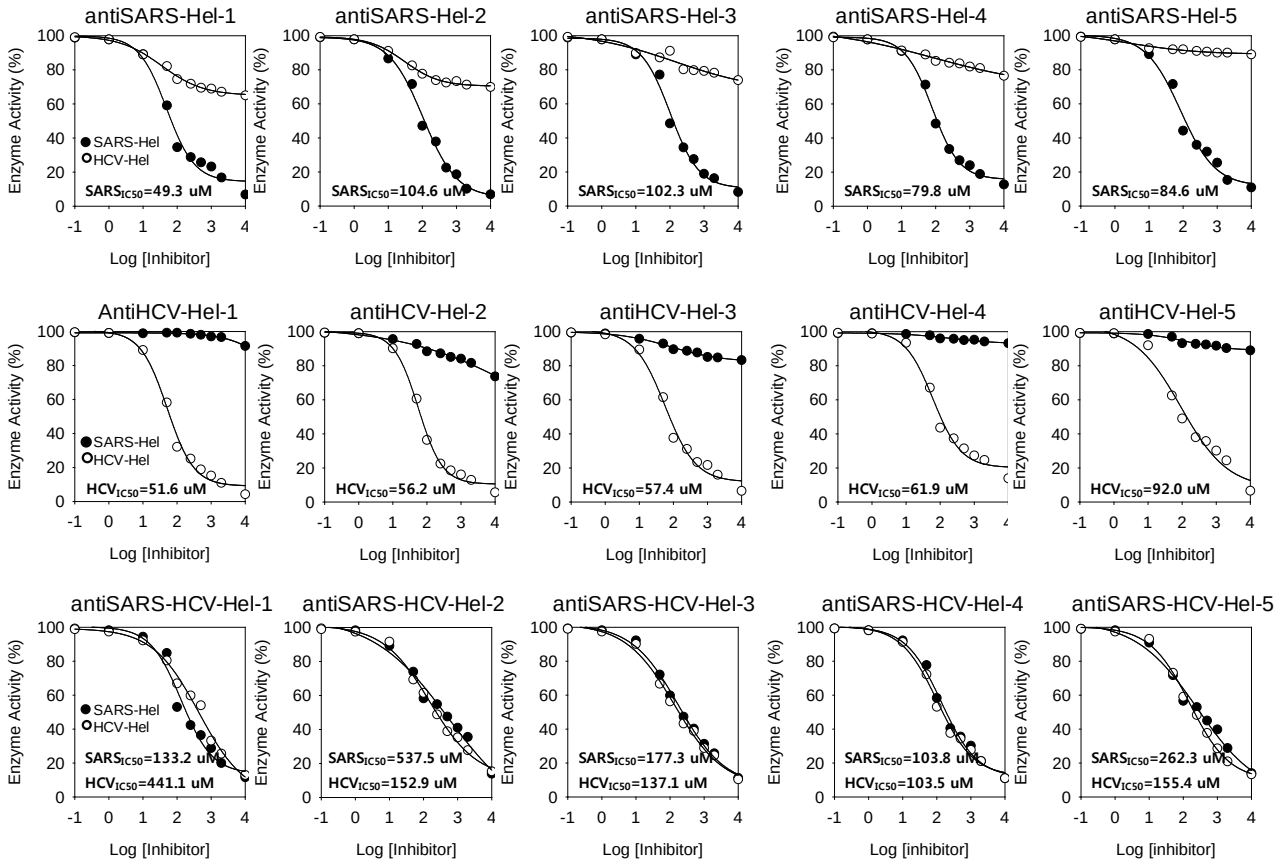


Figure S5. Dose-dependent inhibition of helicases by the discovered 15 compounds was measured and IC_{50} values were calculated. The antiSARS-Hels and antiHCV-Hels showed IC_{50} values of 49.3~104.6 μM and 51.6~92.0 μM for each helicase, respectively (first and second row). The antiSARS-HCV-Hel-1~5 had higher IC_{50} values than selective inhibitors, ranging from 103.8 to 537.5 μM for SARs CoV helicase and 103.5~441.1 μM for HCV NS3 helicase (third row).

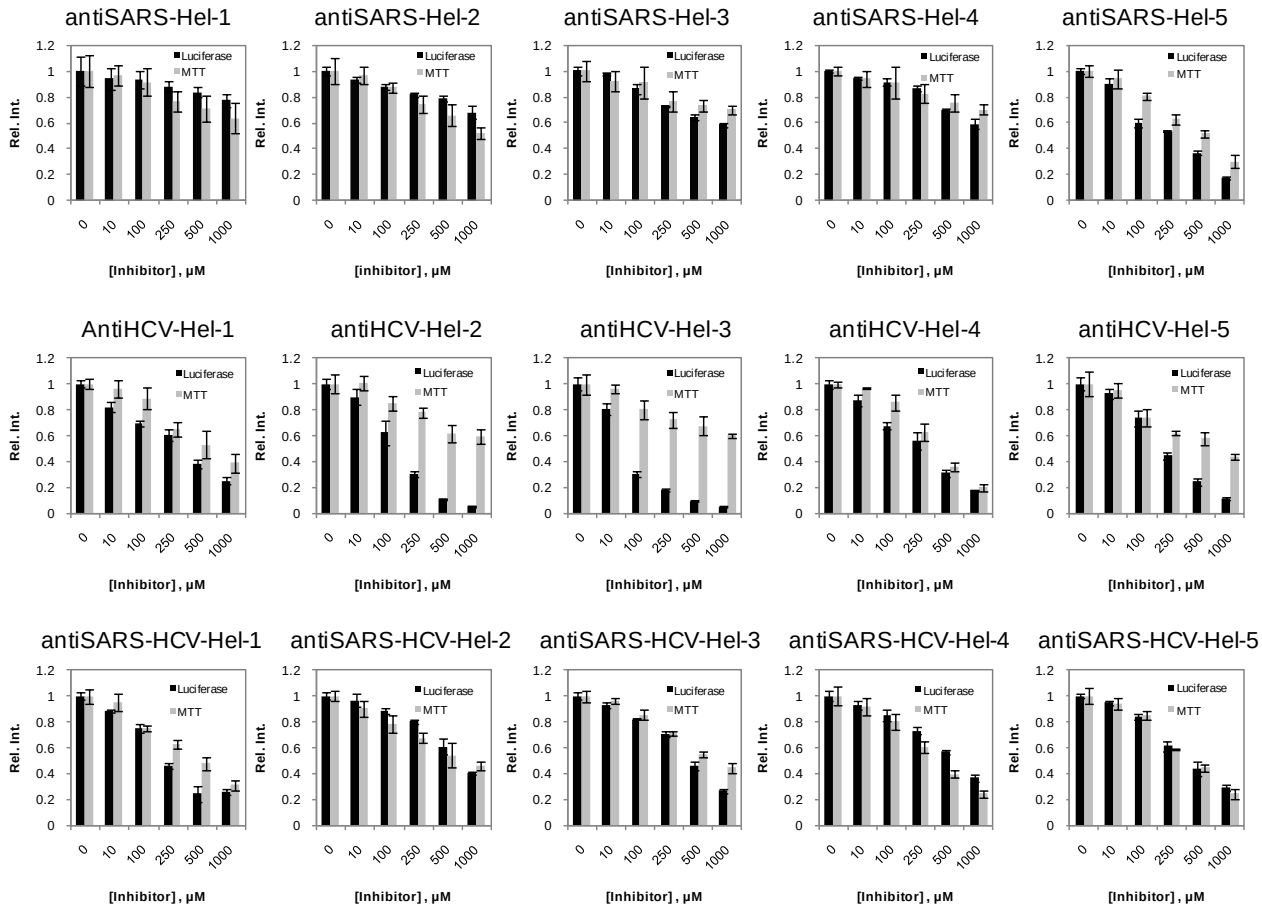


Figure S6. Cytotoxicity of the selected 15 compounds was measured by using MTT assay. Luciferase signal which represents expression level of HCV RNA in the replicon Huh-7 liver cells was also measured after treatment of each compound to the Huh-7 cells. The antiSARS-Hel compounds induced little reduction in HCV RNA replication (1~4) or showed high cytotocixity (5) (first row). Among antiHCV-Hels, #2 and #3 showed dramatic reduction of luciferase signal down to 10%, while keeping cell viability more than 60% at 1 mM (second row). The antiSARS-HCV-Hels were too cytotoxic to be further investigated for HCV drug development (third row).

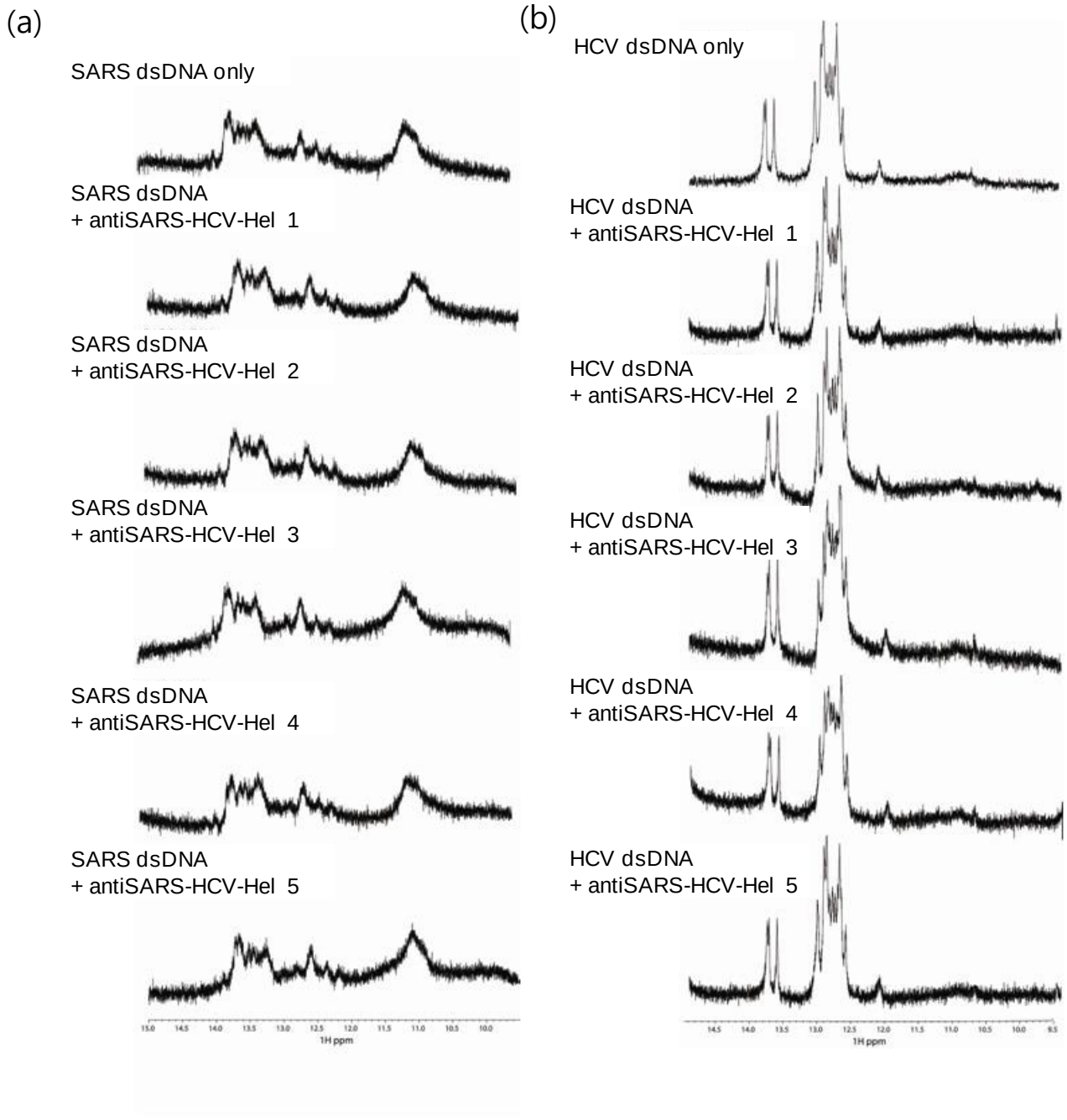


Figure S7. Imino proton NMR spectra of free dsDNA and of dsDNA in the presence of two equivalents of antiSARS-HCV-Hel 1~5. 1D 1H-NMR titration of (a) SARS dsDNA and (b) HCV dsDNA with antiSARS-HCV-Hel 1~5 were performed. The sodium phosphate buffer was used instead of Tris buffer which used in vitro helicase assay because high pH and Tris concentration hampered observation of proton peaks in the imino proton region (9-15 ppm). The imino proton peaks are expected to undergo changes such as peak shift or broadening upon chemical binding if there is interaction between the DNA and chemicals. However, upon addition of antiSARS-HCV-Hel 1~5, the spectra showed no changes, indicating that antiSARS-HCV-Hel 1~5 do not bind to the dsDNAs.

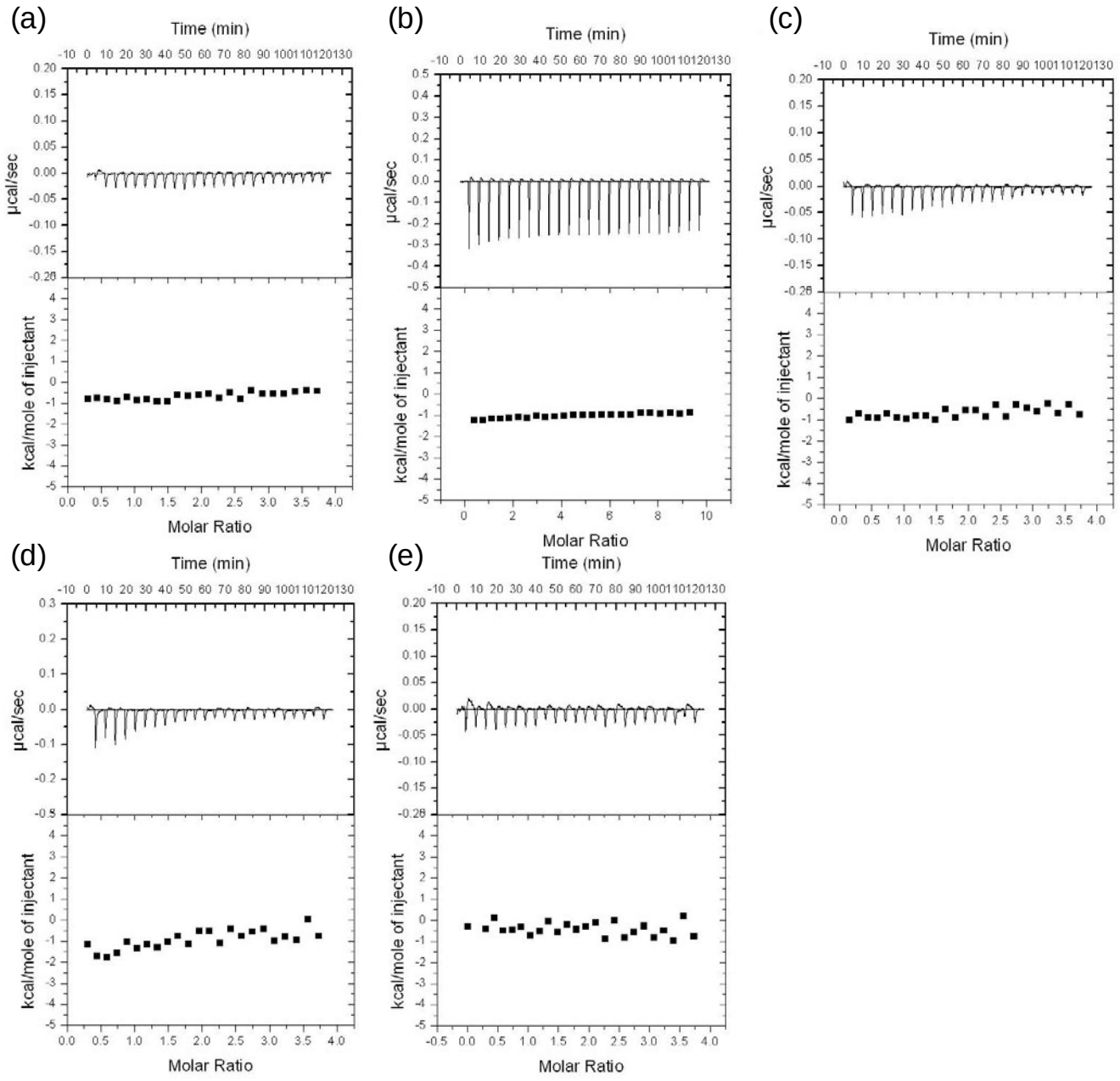


Figure S8. The interactions between SARS Hel substrate dsDNA and (a) anti-SARS-HCV-Hel-1, (b) anti-SARS-HCV-Hel-2, (c) anti-SARS-HCV-Hel-3, (d) anti-SARS-HCV-Hel-4, (e) anti-SARS-HCV-Hel-5 were measured by isothermal titration calorimetry (ITC). Raw data from the ITC experiment and the plotted molar heat versus molar ratio are shown in the upper and lower panel, respectively. The ITC profiles with the integrated heat data as presented in the lower panels showed linear line rather than saturation curve, indicating that binding of antiSARS-HCV-Hel-1~5 to SARS dsDNA is too weak to measure using ITC.

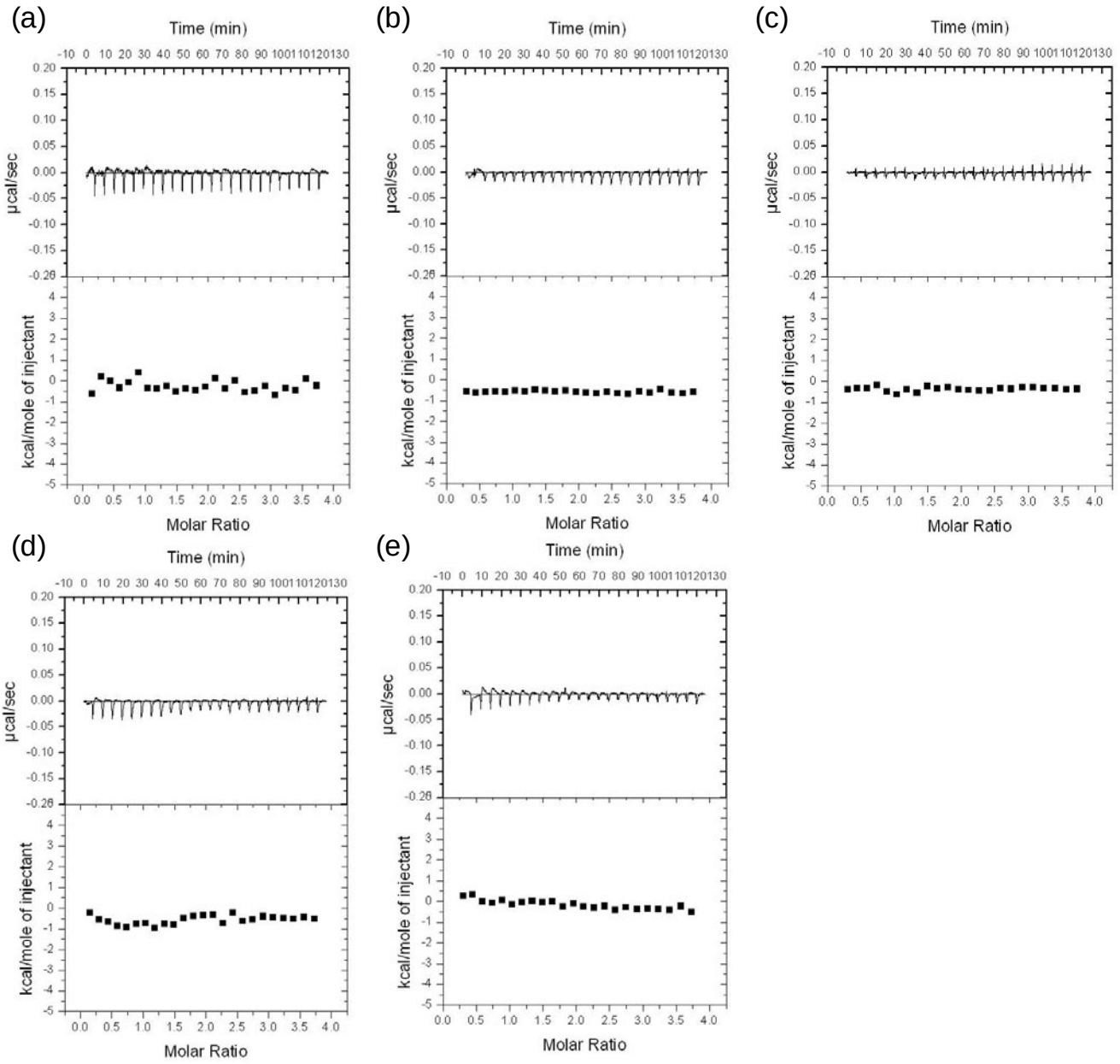


Figure S9. The interactions between HCV Hel substrate dsDNA and (a) anti-SARS-HCV-Hel-1, (b) anti-SARS-HCV-Hel-2, (c) anti-SARS-HCV-Hel-3, (d) anti-SARS-HCV-Hel-4, (e) anti-SARS-HCV-Hel-5 were measured by isothermal titration calorimetry (ITC). Raw data from the ITC experiment and the plotted molar heat versus molar ratio are shown in the upper and lower panel, respectively. The ITC profiles with the integrated heat data as presented in the lower panels showed linear line rather than saturation curve, indicating that binding of antiSARS-HCV-Hel-1~5 to HCV dsDNA is too weak to measure using ITC.