Cell Penetrable Humanized-VH/VH That Inhibit RNA Dependent RNA Polymerase (NS5B) of HCV

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Abstract

NS5B is pivotal RNA dependent RNA polymerase (RdRp) of HCV and NS5B function interfering halts the virus infective cycle. This work aimed to produce cell penetrable humanized single domain antibodies (SdAb; VH/VH) that interfere with the RdRp activity. Recombinant NS5BΔ55 of genotype 3a HCV with de novo RNA synthetic activity was produced and used in phage biopanning for selecting phage clones that displayed NS5BΔ55 bound VH/VH from a humanized-camel VH/VH display library. VH/VH from E. coli transfected with four selected phage clones inhibited RdRp activity when tested by ELISA inhibition using 3’ di-cytidylic 25 nucleotide directed in vitro RNA synthesis. Deduced amino acid sequences of two clones showed VHH H mark and were designated VHH6 and VHH24; other clones were conventional VH, designated VH9 and VH113. All VH/VH were linked molecularly to a cell penetrating peptide, penetratin. The cell penetrable VH9, VH113, VHH6 and VHH24 added to culture of Huh7 cells transfected with JHF-1 RNA of genotype 2a HCV reduced the amounts of RNA intracellularly and in culture medium implying that they inhibited the virus replication. VH/VH mimotopes matched with residues scattered on the polymerase fingers, palm and thumb which were likely juxtaposed to form conformational epitopes. Molecular docking revealed that the antibodies covered the RdRp catalytic groove. The transblasts further studies for in vivo role in inhibiting HCV replication.

Introduction

The NS5B protein has RNA-dependent RNA polymerase (RdRp) activity which is pivotal for de novo RNA synthesis of hepatitis C virus (HCV). The protein is an attractive target of developing anti-HCV agents [1]. Similar to other polymerases, the NS5B resembles human right hand structure consisting of finger, thumb, and palm domains [1]. The polymerase active site is located in the palm [1]. NS5B acquires two different crystal forms: active closed-form-I and inactive open-form-II [1]. The closed conformation mediated by anchoring of ?1 and ?2 subdomain loops of fingers to the thumb is believed to regulate entering of RNA template and ribonucleotide (rNTP) substrate into the catalytic cavity during RNA replication [2]. NS5B lacking a hydrophobic C-terminal 55 amino acid residues (NS5BΔ55) has higher polymerase activity than the full-length NS5B [3].

There is no vaccine against HCV infection. Combined pegylated-interferon (PEG-IFN) and ribavirin is used for intervening of the chronic hepatitis C progression to the end stage liver diseases including liver cirrhosis and hepatocellular carcinoma [4]. Rationales are to enhance the host immunity and inhibit the viral RNA synthesis. Weekly IFN injection and daily oral ribavirin are necessary throughout the 24–48 week treatment course in order to expect effectiveness [4]. Even with such intensive treatment, the success rate is only about 50% due to tolerance of some HCV genotypes (1 and 4) [5]. Many patients do not comply with this regimen, partly because of the severe adverse side effects. Moreover, the treatment cost is beyond affordability of many infected individuals of the developing part of the world where HCV infection is a real problem. As such, novel anti-HCV agent with improved treatment efficacy and safety and less expensive warrants development. Recently, telaprevir and boceprevir which are HCV protease inhibitors have been approved by US FDA [6] but these drugs are not yet widely available.

Recently, sera of camelids were found to contain not only the conventional four chain-immunoglobulin G (IgG) but also heavy chain antibody (HCAb) which each molecule consists of heavy (H) chain homodimers. The HCAb is soluble in serum in spite of the fact that the H chains do not have the linked light (L) chain partners. This is because the HCAb has mutated some hydrophobic amino acids at the former interface between the variable heavy chain domain (VH) and the variable light chain domain (VL) to be more hydrophilic; thus reducing aggregation [7–9]. This area is located on immunoglobulin framework-2 (FR2) of the antigen binding domain of HCAb, designated VH-H in order to
differentiate from the VH of the conventional four chain antibody. The V_{i}H FR2 area contains a tetrad amino acid hallmark, i.e., F/Y 12, E49, R/C50 and G/L52 which substitute for V_{i}H [7-9]. Besides, the third complementarity determining region (CDR3) of the V_{i}H is exceptionally long and can extend to cover the FR2 hydrophobic groove of the target enzyme and blocked specifically the catalytic enzymatic activity [10]. This functional mechanism of antibody has never been possible by large molecular sized conventional antibody. More recently, humanized-camel VH/V_{i}H that bound specifically to Cobra (Naja kaouthia) venom phospholipase A2 and inhibited the enzyme activity was reported [11]. It is now generally accepted that the V_{i}H is a potent enzyme inhibitor [12]. In this study, cell penetrable humanized VH/V_{i}H, synonym single domain antibodies (SdAb), that bound specifically to HCV NS5B and interfered with the native RdRp catalytic activity inside the HCV infected cells leading to inhibition of the HCV replication were produced. To our knowledge this is the first report on HCV polymerase neutralization by cell penetrable humanized-VH/ V_{i}H.

Materials and Methods

Recombinant NS5B.ΔSS Protein

Complementary DNA (cDNA) was synthesized from RNA extracted from the serum of patient infected with genotype 3a HCV (kindly provided by Professor Yong Poovorawan, Chulalongkorn Hospital, Bangkok) which is the predominant serotype. The cDNA was used as a template for amplification of NS5BΔSS cDNA by polymerase chain reaction (PCR). Oligonucleotide primers specific to nucleotide sequence coding for HCV NS5BΔSS protein were designed from the genotype 3a HCV nucleotide sequence of the database (GenBank NC_009824). The PCR amplicon was cloned into pET23b+ vector between EcoRI and XmaI sites and the recombinant plasmid was introduced into BL21 (DE3) E. coli. Transformed E. coli was grown and induced to over-express the recombinant protein by 0.2 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). The recombinant NS5BΔSS was purified from the bacterial lysate by using Ni-NTA beads (Invitrogen) and verified by gel-based liquid chromatography-tandem mass spectrometry [13].

RdRp Activity of the Recombinant NS5BΔSS

Enzyme linked immunoabsorbent assay (ELISA) was used for determining RdRp activity of the NS5BΔSS by detecting an incorporation of biotinylated-ctDNA (CTP) (Invitrogen) into a RNA template in the presence of the NS5BΔSS. The SLD3 RNA (5’GGGGCUUGCAUAGCAGUUGUGAGACC 3’) [14] was used as RNA template and the procedure described previously [15] was followed with modification. The SLD3 RNA (800 mU) was attached covalently to the surface of the Nucleolink module (Thermo Scientific Nunc, UK) via carbodiimide condensation [16]. Polymerase reaction mixture (80 μL) (300 mM of NS5BΔSS; 20 mM sodium glutamate, pH 8.2; 4 mM MgCl2; 12.5 mM DT; 0.5% (v/v) Triton X-100; 2 mM MnCl2; 40 units RNase inhibitor; 200 μM each ATP, UTP, GTP, and biotinylated-CTP) was added to the SLD3 RNA coated well and incubated at 37°C for 2 hours. Polymerase reaction mixture containing heparin (2 μM) which is polymerase quencher [17] was included in the assay as the RdRp inhibition control. Non-incorporated rNTPs were washed away before adding with streptavidin-horseradish peroxidase (HRP) conjugate (Southern Biotech, USA), followed by 2,2’-azino-di-(3-ethylbenzthiazoline-6-sulfonate (ABTS) substrate (KPL, USA). Wells containing buffer and normal BL21 (DE3) E. coli lysate were included as blank and negative control, respectively. Optical density at absorbance 405 nm (OD405 nm) of the content of each well was determined.

Humanized-camel VH/V_{i}H Phage Display Library

The library was constructed previously [10] using total RNA extracted from peripheral blood mononuclear cells of a naive male camel (Camelus dromedarius) and messenger RNA (mRNA) was reverse transcribed to cDNA. The gene fragments encoding variable domains of the camel VH/V_{i}H were PCR amplified using the cDNA as template and 14 forward and 3 reverse degenerate primers designed from all families of human immunoglobulin genes [18]. The human primer directed-camel vh/vhh cDNA amplicons were ligated into a pCANTAB5E phagemid vector and introduced into competent TG1 E. coli cells. The complete phage particles displaying humanized-camel VH/V_{i}H with integrated vh/vhh in the phage genomes were rescued from by co-infecting the vh/vhh-phagemid transformed E. coli with a helper phage.

Phage Biopanning and Preparation of the Humanized-VH/V_{i}H

A single round phage biopanning for selecting phage clones that displayed NS5BΔSS bound-VH/V_{i}H was performed as described previously [10] using 10 μg of purified NS5BΔSS as the panning antigen. Antigen bound phages were supplemented with log phase grown HB2151 E. coli. The phagemid transformed E. coli clones were selected on LB agar plate containing 100 μg/mL ampicillin and 2% glucose. E. coli clones carrying recombinant vh/vhh-phagemids were screened by PCR using phagemid specific RI and R2 primers [18]. Selected clones were grown individually under 0.5 mM IPTG induction and VH/V_{i}H proteins in bacterial lysates were partially purified by ion exchange (DEAE) column chromatography. Amount of VH/V_{i}H in each preparation was standardized.

Specific Binding of VH/V_{i}H to NS5BΔSS

Specific binding of VH/V_{i}H to NS5BΔSS was determined using indirect ELISA and Western blot analysis (WB) [10,18]. For ELISA, one μg of NS5BΔSS and antigen control, i.e., bovine serum albumin (BSA) was immobilized separately in wells of an ELISA plate. After blocking the empty sites on well surface with 5% BSA in PBS, standardized VH/V_{i}H contained in lysates of transformed E. coli were added to appropriate wells and kept at 37°C for 1 hour. Unbound VH/V_{i}H were removed; the bound VH/V_{i}H in each well was detected by adding rabbit anti-E tag antibody (Abcam, UK), goat anti-rabbit immunoglobulin-HRP conjugate (Southern Biotech), and ABTS substrate (KPL), respectively, with washing with phosphate buffered saline, pH 7.4 containing 0.5% Tween-20 (PBST) between steps. E. coli clones which their lysates gave OD405 nm to NS5BΔSS two times higher than to the BSA were selected.

For WB, NC strip blotted with SDS-PAGE separated-NS5BΔSS was blocked with 5% skim milk in Tris buffered saline (TBS) and kept at 25°C for 1 hour. After washing with TBS containing
ELISA Inhibition for Screening VH/VH \text{H} That Inhibited NSSB/V55 RdRp Activity

Test mixtures, i.e., partially purified VH/VH \text{H} mixed individually with NSSB/V55, and control mixtures, i.e., NSSB/V55 mixed with irrelevant VH \text{H} that specific to botulinum neurotoxin type A, VHH H17 [10] (background inhibition control) and NSSB/V55 mixed with antibody diluent (negative inhibition control or blank) were prepared. The mixtures were added separately to ELISA wells containing immobilized SLD3 RNA template. The polymerase reaction mixture prepared as above was added to each well and the ELISA procedure was similarly completed. OD 405 nm of the reaction mixture prepared as above was added to each well and OD 405 nm of the content of the test and control wells were measured against blank. Less OD 405 nm of the tests compared to the background and negative inhibition controls indicated that the VH/VH \text{H} could neutralize specifically the NSSB/V55 RdRp activity.

Production of Cell-penetrable VH/VH \text{H}

Gene sequence coding for the VH/VH \text{H} that inhibited the NSSB/V55 RdRp \text{in vitro} was subcloned to PEN-pET23b plasmid backbone [19] in order to produce cell-penetrable VH/VH \text{H}. The \text{vh}/\text{vhh} sequences were cloned into the recombinant plasmid backbone at downstream of the DNA sequence coding for a 16 amino acid cell-penetrating peptide, i.e., penetratin (PEN) via \text{Sfi} and \text{Nol} sites and introduced into BL21 (DE3) \text{E. coli}. PEN-VH/VH \text{H} fusion proteins were produced by transformed bacteria and purified by using Ni-NTA beads [19].

Inhibition of HCV Replication in Huh7 Cells by Cell-penetrable VH/VH \text{H}

The plasmid pJFH-1 containing full-length cDNA of the JFH-1 HCV of genotype 2a (GenBank AB047639) was kindly provided by Dr. Takaji Wakita, Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan and Professor Ralf Bartenschlager, Department of Molecular Virology, University of Heidelberg, Germany. To generate genomic HCV RNA, the pJFH-1 was linearized with \text{Xba} and transcribed \text{in vitro} using Megascript T7 kit (Ambion, USA). The transcribed RNA (10 \mu g) was put into Huh7 cells (4.0\times10^6 cells) by electroporation (single pulse at 0.27 kV, 100 milliseconds) using eukaryotic electroporation mode by Electroporator® (Eppendorf). The JFH-1 RNA transfected Huh7 cells were immediately transferred to 40 mL of complete (serum containing DMEM, 10 and 20 \mu g of purified PEN-VH/VH \text{H} preparations for 1 hour. Cell culture supernatants were collected. Cells in individual wells were washed with plain DMEM, added with fixed volume of PBS, homogenized by freezing and thawing repeatedly and the cell lysates were collected. Each culture supernatant/cell lysate (75 \mu L) was immobilized on ELISA well until dried. The amounts of the immobilized PEN-VH/VH \text{H} were quantified by indirect ELISA as described previously using mouse monoclonal anti-6x-histidine as the tracing antibody. OD 405 nm of the content of each wells were determined. Amount of VH/VH \text{H} in each preparation was determined from standard curve constructed from ELISA OD 405 nm of purified PEN-VH/VH \text{H} (ranged from 2.5 to 25 \mu g). Duplicate experiments were performed. The % cell internalization of the PEN-VH/VH \text{H} was calculated from the original 20 \mu g amount of antibody.

LDH Assay

Cytotoxicity of individual PEN-VH/VH \text{H} on naive Huh7 cells was determined by using CytoTox 96® non-radioactive cytotoxicity (LDH) assay (Promega, USA).

Restriction Fragment Length Polymorphism (RFLP) of \text{vh}/\text{vhh} Sequences

RFLP of \text{Mva}I digested DNA sequences coding for the individual VH/VH \text{H} were determined by 14% polyacrylamide gel electrophoresis followed by ethidium bromide staining [13].

Amino Acid Sequences, Immunoglobulin Frameworks (FRs) and Complementarity Determining Regions (CDRs) of the VH/VH \text{H}

The \text{vh}/\text{vhh} were sequenced and amino acids were deduced. All protein sequences were multiply aligned by ClustalW. Immunoglobulin frameworks (FRs) and complementarity determining regions (CDRs) of each VH/VH \text{H} were predicted by using the International Immunogenetics information system (IMGT) [21].
Mimotope Searching

Ph.D.-12™ phage display peptide library (New England Biolabs, USA) was used to determine VH/VHH bound phage mimotopes as described previously [10]. The mimotope peptide sequences were deduced from the phage DNA sequences by DNAMAN software version 4.15. The mimotopes were classified into groups by using Phylogeny ClustalW [22]. The sequences of the same mimotope group were multiply aligned with HCV NS5B sequence (Accession no. NP_751928) by Kalign [23].

ELISA Inhibition Assay for Validation of the Phage Mimotopes

Phage clones displaying the representative mimotopes of mimotope groups were propagated in ER2738 E. coli and the titers of the amplified phages were determined according to manufacturer’s instruction (New England Biolabs, USA). Phage mimotope preparations (50 µL) at various amounts (10^5, 10^7 and 10^8 plaque forming unit; pfu) were mixed individually with fixed amount of 50 µL VH/VHH (5 µg) and incubated at 37°C for 1 hour. The VH/VHH mixed with M13KO7 phage served as background binding control. NS5BΔ55 coated wells added with the VH/VHH served as 100% binding (maximum binding). After washing, rabbit anti-E tag antibody, goat anti-rabbit immunoglobulin-HRP conjugate and ABTS substrate were added respectively. OD405 nm of the content of each wells were determined. The % ELISA inhibition was calculated:

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% \text{ELISA inhibition} = \left[ \frac{\text{OD}_{405 \text{ nm of maximum binding}} - \text{OD}_{405 \text{ nm of test}}}{\text{OD}_{405 \text{ nm maximum control}}} \right] \times 100.
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Homology Modeling and Molecular Docking

Deduced amino acid sequences of NS5B and VH/VHH were subjected to basic local alignment search (BLAST). The sequences with maximum identities were used as templates for homology modeling. The constructed models were validated by using PROCHECK [24]. Three dimensional structure of the protein complex was predicted by protein docking technique. ZDOCK and RDOCK modules embedded on Discovery Studio program were used for docking. NS5B and VH/VHH were set as input receptor and input ligand, respectively. Each ZDOCK docking result was subjected to structure refinement by using RDOCK module. After RDOCK calculation, the dock pose with lowest RDOCK energy was analyzed for the binding interaction.

Statistical Analysis

Means and standard deviations of three independent experiments were used for comparison between tests and controls. P values <0.05 of unpaired t-test was considered significant difference.

Results

Recombinant NS5BΔ55

NS5BΔ55 (~60 kDa) of HCV genotype 3a was successfully produced and purified from the lysate of a selected transformed BL21 (DE3) E. coli carrying the recombinant NS5BA55-pET23b⁺ plasmids. Deduced amino acid sequence of the NS5BΔ55 showed 98% identity to the sequence of HCV genotype 3 NS5B protein (Accession no. YP_001491557.1) and approximately 80% identity to the NS5B protein sequences of various other HCV genotypes including 1a, 1b, 6b, 6c and 6m (Figure S1). The protein was verified by LC-MS/MS as the HCV NS5B (data not shown).

SLD3 RNA and biotinylated-CTP based-ELISA showed that the recombinant NS5BA55 acquired RdRp activity (Figure 1). The colorimetric values of the newly synthesized biotinylated RNA increased steadily when the amounts of NS5BΔ55 were increased from 50 to 600 nM. The RdRp activity of the NS5BΔ55 was quenched by the presence of heparin (2 µM) which was known to be the polymerase trapping reagent.
Phage Clones that Displayed NS5B.155-bound VH/VH\(_9\) and the VH/VH\(_{11}\) Characterization

From 40 selected HB2151 E. coli colonies grown on the selective agar, 29 clones were positive by PCR for \(\alpha/\gamma/\delta\) sequences (~600 bp) and 26 clones could express VH/VH\(_9\) (13-25 kDa) as determined by WB. They were designated clones no. 1-26. VH/VH\(_{11}\) in the lysates of 10 of the 26 clones bound to NS5B.J55 by indirect ELISA (Figure 2A) as well as by WB (Figure 2B). The \(\alpha/\gamma/\delta\) sequences of the 10 clones revealed 10 different DNA banding patterns (RFLP) (Figure 3A). Multiple alignments showed that all clones had different amino acid sequences especially at the CDR domains (Figure 3B). The deduced amino acid sequences of two clones had the characteristic amino acid tetrad of VH\(_9\); they were designated clones VH\(_9\)H6 and VH\(_9\)H24, while the other clones had conventional VH feature; thus, designated clones VH1, VH3, VH8, VH9, VH13, VH18, VH20, and VH25 [7,10]. Sequences of these 10 humanized-camel VH/VH\(_9\) showed high homology with human VH sequences (Table 1).

Humanized-VH/VH\(_9\) Mediated Neutralization of NS5B.J55 RdRp Activity

Partially purified VH/VH\(_9\) of the 10 HB2151 transformed E. coli clones were screened for NS5B.J55 RdRp activity by ELISA inhibition. At 2-4 \(\mu\)g, VH\(_9\)H6, VH\(_9\)H13 and VH\(_9\)H24 inhibited the RdRp activity of 300 nM NS5B.J55 by 10-69% (data not shown). The \(\alpha/\gamma/\delta\) sequences of these clones were subcloned into PEN-pET23b\(^*\) backbone. The PEN-VH/VH\(_9\) expressed from the IPTG induced transformed BL21 (DE3) E. coli carrying the respective plasmids were purified and tested for their ability to inhibit the NS5B.J55 RdRp activity by the SL2D RNA and biotinylated-CTP-based ELISA. At a molar ratio 3:1 of antibody to NS5B, the PEN-VH/VH\(_9\) showed high inhibition activity on the HCV replication was not clearly validated by using PROCHECK, and it has no residue in the medium alone.

Cell Entering Efficiencies of the PEN-VH/VH\(_9\)

After treating the Huh7 cells with 20 \(\mu\)g of PEN-VH9, PEN-VH13, PEN-VH\(_{11}\)H6 and PEN-VH\(_{11}\)H24, 16.4, 17.0, 16.6 and 17.2 \(\mu\)g of the antibodies were recovered from the cell lysates, calculated to be 82%, 85%, 83% and 86%, respectively The PEN-VH/VH\(_9\) could not be detected in the culture supernatants.

Correlation of the intracellular amounts of the SaAbs with their inhibitory activity on the HCV replication was not clearly demonstrated (Figure 4) although the PEN-VH9 which had the lowest cellular entering capacity had the lowest HCV inhibitory activity.

The PEN-VH/VH\(_9\) at the amounts up to 10 \(\mu\)M did not cause any detectable LDH leakage from the Huh7 cells after 24 hour incubation indicating their innocuousness (Figure S3).

VH/VH\(_9\) Bound Phage Mimotopes and Tentative Epitopes of the VH/VH\(_9\) on NS5B

The 12 mer peptides deduced from genomes of the phages that bound to VH/VH\(_9\) (phage mimotopes) could be classified into several homology groups; individual mimotope peptides are shown in Table S1. Sequences of each mimotope group were aligned with NS5B sequence of the database to locate the tentative VH/VH\(_9\) peptide epitopes on the HCV NS5B (Figure 5A). VH9 mimotopes matched with amino residues: 206–219 and 341–352 of palm and 304–395, 306–397, 413–424 and 493–504 of thumb; VH13 mimotopes matched with the residues: 20–31 of a loop interconnected fingers and thumb, 261–272 of the α-helix that links fingers and palm, 290–301 of palm and 434–435 of thumb; VH\(_{11}\)H6 mimotopes matched with residues: 226–237, 251–260 and 261–272 in the α-helix that links fingers and palm, and 413–424 and 530–530 of thumb; and VH\(_{11}\)H24 mimotopes matched with residues: 93–104 and 95–106 of fingers and 380–401 and 491–502 of thumb. The results indicate that the VH/VH\(_9\) bound to conformational epitopes of the NS5B.

Inhibition of the VH/VH\(_9\) Binding to the NS5B.J55 by Phage Mimotopes

Representative results of the ELISA inhibition for determining the ability of phage mimotopes in inhibiting the VH/VH\(_9\) binding to the NS5B.J55 are shown in Figure S2. Binding of the VH\(_9\)H6 to the NS5B.J55 was inhibited by the VH\(_9\)H6-phage mimotope groups 1 (M6-7: ALWPPNLHAWVVP), 2 (M6-5: -FWSPN-HLMMNNL), 3 (M6-1: -TLHLSHTWSSL), 4 (M6-12: HYPTAQPHIKQVQ) and 5 (M6-16: -YSAHNNYIDSGR) implying that the mimotopes carried the native HCV NS5B polymerase which validated the mimotope search results.

Interface Binding of VH/VH\(_9\) with the NS5B

The amino acid sequence of NS5B genotype 3a has 73% identity with hepatitis C virus NS5B RNA polymerase [PDB code 2HAI]. The Ramachandran plot of structure of NS5B was validated by using PROCHECK, and it has no residue in disallowed region (complete match). The Ramachandran plots of
Figure 2. Results of experiments for selection and characterization of VH/VH expressed E. coli clones. (A) Indirect ELISA results for detecting the binding of VH/VH in lysates of 26 vh/vh-phagemid transformed HB2151 E. coli clones to NS5BΔ55. Lysates of 10 clones (no. 1, 3, 6, 8, 9, 13, 18, 20, 24, and 25) gave OD_{405nm} to the immobilized NS5BΔ55 two times higher than to BSA control (asterisks). HB, negative VH/VH control which lysate of normal HB2151 E. coli. (B) Western blot result for confirming the binding of the VH/VH of the 10 ELISA positive clones to SDS-PAGE separated NS5BΔ55; VH/VH of all 10 clones bound to NS5BΔ55 (arrow). Lanes 1–10, VH/VH of clones no. 1, 3, 6, 8, 9, 13, 18, 20, 24, and 25, respectively. P, positive control which was SDS-PAGE separated NS5BΔ55 probed with anti-6x histidine tag. N, negative control which was SDS-PAGE separated NS5BΔ55 probed with lysate of normal HB2151 E. coli and detected by anti-E tag.

doi:10.1371/journal.pone.0049254.g002
A

B

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1

FR1

CDR1

FR2

CDR2

3  EVQLVESGGGLVQPGGSLRLSCAAS GFTFSH--YW MNWVRQAPKQGKLEWAVN IKQDGEYK 58
8  QVQLVESGGGLVQPGGSLRLSCAAS GFTFNS--YA MNWVRQAPKQGKLEWAVSA INSQGGST 58
25 EVQLVESGGGLVQPGGSLRLSCAAS GFTFNT--YY MNWVRQAPKQGKLEWAVST ISSDGKNT 58
9  QVQLVESGGALVQPGGSLRLSCAAS GTIGK--YY MNWVRQAPKQGKLEWAVSG IKKDGTRT 58
6  QVQLVESGGQVAGGSLRLSCMAS GTTFVG--PA MGYKHAPTECKGLSS ISGSGT--T 57
24 EVQLVESGGDSVQAGGSLRLCSRS GTFRG--YS WGWFRQAPKQGKEVRAGA ILSQGSGT 58
13 EVQLVESGGAEVKPKESLRISCKAS GYTFSS--YW IANVRQMPKGKLEWMGIG YIPGDFDT 58
18 QVQLVESGGEGEUKKELQSLKISCKTS GYXFAV--YW IANVRQMPKGKLEWMGIG YIPGGRST 58
1  QMLVQWGAGLKLPPSETLSTLTCAV GSFSFG--YY WSIRQPQPKQLLEWIEG INHSGS--T 57
20 EVQLVESGPGLVQLPQLTTLTCVW GSITSSYYG WSVIQPPQKQGLEWGMG IAAYSST--T 59

FR3

CDR3

3  Y FEDSVKGLFTISRDNAXNKSLYLMQMSLRAEDTAVYCYC ARVPEQVITVTPQGPLPYYGFQMDV 118
8  Y YTDSSSVKGRFTISRDNAXNKSTNYLQLNLKTEDTAMYCYC AKVGBIWIIH--YOMDY 118
25 Y YTDSSVDRFTISRDNAXNMLYLMQMSLRFDTALACYC S--SDSWSWSSS------105
9  Y YADSVKGRFTISRDNAXNLLYLMQMSLNAEDTALACYC ATSYYNYEYLSN------ALDL 116
6  Y YADSVKGRFTISRDNAXNLLYLMQMSLNSKDTQMGYCC AADVSEGGRWLC------DAFGA 120
24 Y YAGSVKGRFTISRDNAXNLQMSLQPSLTPEDTALYYC AAAGPSQTPQFPYLR--TSWNY 116
13 K YSPSERQLQVRQISRDSNLASTDQLWSSLKASSDTLVYYCC ARSPGT------TYWYHFD 111
18 K YSPSERQGKVFSVDTINTNYLQWSSLKASDTLVYYCC ARQ-------------ELVNY 104
1 N YNPVSLSRVLSIATVLQKQFSLKNLSSVSAADTAVYYC AREDRDLYYVFSAIRQGNFDP 117
20 Y YSPSLKSVTSTSRDTSSKNSQFSLQLSGVTPEATVYYCC ARSNG------YNYVAYG-MMN 114

FR4

3  WGGQTTVTVSS 129
8  WQRTGTVTVSS 124
25 -GPTMVTVSS 115
9  WGGQTTVTVSS 123
6  WGGQTVTVSS 123
24 WQRTMVTVSS 127
13 WQRTTSSVSS 122
18 WQRTMVTVSS 115
1 WQRTTSSVSS 128
20 WQRTTSSVSS 125

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Figure 3. Different RFLP patterns of DNA sequences coding for the VH/VHH of clones no. 1, 3, 6, 8, 9, 13, 18, 20, 24, and 25, respectively. (A). Multiple alignment of amino acid sequences for determining immunoglobulin frameworks (FRs) and complementarity determining regions (CDRs) of the 10 VH/VHH by using the International Immunogenetics Information System sever (B). Clones no. 6 and no. 24 have the tetrad amino acid hallmark of VHH in FR2 (bold letters) and were designated VHH6 and VHH24; the rests were conventional VH, designated VH1, VH3, VH8, VH9, VH13, VH18, VH20, and VH25. Asterisk indicates identical amino acids; colon indicates conserved amino acid substitution; and dot indicates a semiconserved amino acid substitution.

doi:10.1371/journal.pone.0049254.t001

Table 1. Percent amino acid homology of the VH/VHH sequences with the closest human V region frameworks.

| VH/VHH clone number | Closest human V region | Percent amino acid homology with human FRs |
|---------------------|------------------------|------------------------------------------|
|                      | FR1       | FR2        | FR3        |
| VH1                 | 92.00     | 100.00     | 97.37      |
| VH3                 | 100.00    | 94.12      | 92.11      |
| VHH6                | 86.21     | 41.18      | 78.95      |
| VHH8                | 96.00     | 100.00     | 84.21      |
| VHH                | 92.00     | 88.24      | 86.84      |
| VH13                | 88.00     | 94.10      | 84.21      |
| VH18                | 80.00     | 88.24      | 71.05      |
| VH20                | 88.00     | 88.24      | 78.95      |
| VHH24               | 80.00     | 59.24      | 81.58      |
| VHH25               | 96.00     | 82.35      | 84.21      |

*Indicates allele polymorphism.

doi:10.1371/journal.pone.0049254.t001
clones had conventional VH feature. Multiple alignments revealed that all 10 VH/VHH sequences were diverse especially in the CDRs implying that they might bind to different epitopes of the NS5B and conferred different inhibitory efficacy on RdRp activity. When antibodies from all clones were screened at the same weight basis for their ability to inhibit the NS5B RdRp activity by the SLD3 based-ELISA inhibition, only four clones, VH9, VH13, VHH6 and VHH24 could inhibit the polymerase while the rests were refractory. Thus these four clones were tested further for inhibition of native HCV RdRp in the hepatic cells transfected with genomic replicon of heterologous HCV, i.e., JFH1 RNA of genotype 2a, that are readily available and widely used in the HCV biology research and anti-HCV drug development [29–31].

Because the antibody must reach the intracellular NS5B in order to inhibit the de novo RdRp activity, the DNA sequences coding for all four VH/VHH clones were linked molecularly to a DNA sequence coding for a 16 amino acid cell penetrating peptide (penetratin, PEN) in a plasmid backbone constructed previously in our laboratory [19]. The cell penetrable VH/VHH (transbodies) specific to NS5B, i.e., PEN-VH9, PEN-VH13, PEN-VHH6 and PEN-VHH24, were successfully produced as bacterial inclusions by the transformed E. coli. Fortunately, after refolding all of the transbodies still retained the NS5B RdRp inhibitory activity similar to their original molecules. When added to the cell culture medium of Huh7 cells transfected with the JFH1 RNA, the cell penetrable VH/VHH suppressed replication of the HCV RNA replicon, albeit in different degrees, as shown by reduction of both intracellular and released viral RNA copies. Quantification of the HCV phenotypes inside the respective VH/VHH exposed transfected cells and in the culture fluids conformed to the results on the viral RNA detection. Overall data indicate the cross-genotypic inhibitory activity of the transbodies. Unfortunately genomic replicons of other HCV genotypes are not available for testing the cross-genotype inhibition. Nevertheless, the high amino acid sequence homology was observed among NS5B proteins of different HCV genotypes deposited in the Genbank database. Moreover, all HCV genotypes share identical RdRp D55–58 and GDD motifs of the catalytic groove [32]. Thus, it is optimistically envisaged that the cell penetrable VH/VHH produced in this study should also cross-neutralize the RdRp activity of other heterologous HCV genotypes.

Figure 4. Percent ELISA inhibition of RdRp activity of NS5BΔ55 mediated by VH9, VH13, VHH6, and VHH24 (bars 2–5, respectively). ELISA mixture containing NS5BΔ55 alone (bar 1) served as negative inhibition control. Bar 6, reaction mixture containing PEN-VH17 specific to botulinum neurotoxin type A [10] was included as background inhibition control. OD_{405 nm} of bars 1–6 were 0.151±0.053, 0.165±0.108, 0.175±0.076 0.174±0.078 and 0.508±0.061, respectively, while the OD of the ELISA without the SdAb was 0.514±0.111. *, different significantly from the negative inhibition control.
doi:10.1371/journal.pone.0049254.g004

Because the antibody must reach the intracellular NS5B in order to inhibit the de novo RdRp activity, the DNA sequences coding for all four VH/VHH clones were linked molecularly to a DNA sequence coding for a 16 amino acid cell penetrating peptide (penetratin, PEN) in a plasmid backbone constructed previously in our laboratory [19]. The cell penetrable VH/VHH (transbodies) specific to NS5B, i.e., PEN-VH9, PEN-VH13, PEN-VHH6 and PEN-VHH24, were successfully produced as bacterial inclusions by the respective transformed E. coli. Fortunately, after refolding all of the transbodies still retained the NS5B RdRp inhibitory activity similar to their original molecules. When added to the cell culture medium of Huh7 cells transfected with the JFH1 RNA, the cell penetrable VH/VHH suppressed replication of the HCV RNA replicon, albeit in different degrees, as shown by reduction of both intracellular and released viral RNA copies. Quantification of the HCV phenotypes inside the respective VH/VHH exposed transfected cells and in the culture fluids conformed to the results on the viral RNA detection. Overall data indicate the cross-genotypic inhibitory activity of the transbodies. Unfortunately genomic replicons of other HCV genotypes are not available for testing the cross-genotype inhibition. Nevertheless, the high amino acid sequence homology was observed among NS5B proteins of different HCV genotypes deposited in the Genbank database. Moreover, all HCV genotypes share identical RdRp D55–58 and GDD motifs of the catalytic groove [32]. Thus, it is optimistically envisaged that the cell penetrable VH/VHH produced in this study should also cross-neutralize the RdRp activity of other heterologous HCV genotypes.

Phage peptides bound to the NS5B specific-VH/VHH (mimotopes) were searched for predicting the locations of the NS5B protein interacted by the antibodies which would enlighten the molecular RdRp inhibitory mechanism of the antibodies. Multiple alignments of the mimotopes indicated that the VH/VHH bound to discontinuous (conformational) epitopes on the NS5B molecule which the antibody contact residues scattered on the polymerase, either on thumb and palm (VH9) or on palm, thumb and finger domains) [33]. The findings by indirect ELISA inhibition that representative phage clones displaying the mimotope groups could inhibit the VH/VHH binding to the NS5BΔ55 indicate the amino acids on the HCV polymerase analogous to the mimotope
peptides are the presumptive VH/VH epitopes. The multiple contact points of the VH/VH to the target NS5B would render them high tolerability to the HCV mutations [27]. It is noteworthy that the mimotopes of the clone VH9 which conferred the least HCV replication inhibition among the four antibody clones are located on the thumb and palm only, while mimotopes of the other clones interacted also with the interconnecting loop between fingers and thumb (VH13), α-helix that links fingers and palm (VH6) or fingers (VH24a). It is known that the RdRp HCV is active under the closed configuration (forming a tunnel for template and ribonucleotides accommodation) of the polymerase protein formed by anchoring of the ?1 and ?2 loops of the fingers and the thumb [2]. Thus it is plausible that the observed higher inhibitory activity of the VH13, VH6 and VH24a than the VH9 on the HCV replication was due to their interference with the RdRp tunnel formation which was likely to be more readily than the VH9.

The results of homology modeling and molecular docking confirmed that the VH/VH mediated interface binding to the NS5B molecule by occupying multiple areas around the RdRp template channel [34] and also covered the RdRp active site. The findings presumptively indicate that the NS5B specific VH/VH suppressed the polymerase activity by preventing accessibility of the template/substrate to the enzyme catalytic cavity which consequently incapacitated the HCV RNA replication.

A unique feature that distinguishes the active HCV RdRp from the other polymerases is the closed hand conformation of the former as opposed to the openhand structure of the latter. Alteration of the closed configuration of HCV RdRp impaired the RNA synthetic activity [35]. Legacy from evolutional studies have documented the un-relatedness of eukaryotic RdRp, viral RdRp and DNA-dependent RNA polymerase (DdRp) [36]. The β’ subunit of the DdRp and eukaryotic RdRp contain a signature motif DbDGD (b is a bulk residue) which contributes to the polymerase catalytic activity via divalent cation coordination, whereas the core catalytic groove of HCV RdRp contains Dx4–5D and GDD motif in the palm domain [37]. No other similarity was detected between DdRp and RdRp [38]. Moreover, the biochemical activity of the HCV RdRp is also different enough from the host DNA polymerases. The HCV NS5B does not express in normal human cells. Thus, the HCV NS5B specific cell penetrable VH/VH that interfere HCV RdRp function produced in this study should not inhibit the host cellular enzymes and should be harmless. Preliminary result of LDH assay performed on Huh7 cells indicated that the cell penetrable VH/VH at the amount as high as 10 μM were not toxic to the cells implying the antibody innocuousness.

Figure 5. Results of qPCR for determining the log_{10} of amounts of intracellular HCV RNA (A) and released HCV RNA in culture fluids (B) of the pJFH-1 RNA transfected Huh7 cells cultured in the medium (1) which was the negative inhibition control, medium containing 10 and 20 μg of cell-penetrable PEN-VH9, PEN-VH13, PEN-VH6 and PEN-VH24 (2–5, respectively), medium containing 20 μg of irrelevant PEN-VH17 which served as the background inhibition control (6) and medium containing ribavirin + PEG-IFN which was positive inhibition control (7). Penetratin added to the cell culture medium did not cause any inhibition of the HCV replication (data not shown). (C) Amounts of HCV core antigen (ng/mL) in cell culture supernatant of pJFH-1 transfected Huh7 cells cultured in the medium (1); medium containing 20 μg of PEN-VH9, PEN-VH13, PEN-VH6, and PEN-VH24 (2–5, respectively); medium containing 20 μg of irrelevant PEN-VH17 (6); and medium containing ribavirin + PEG-IFN (7) quantified by using QuickTiter HCV core antigen ELISA kit. (D) Numbers of HCV foci in transfected Huh7 cells (1), transfected Huh7 cells exposed to 20 μg of PEN-VH9, PEN-VH13, PEN-VH6, PEN-VH24a and irrelevant PEN-VH17 (2–6, respectively) and ribavirin + PEG-IFN (7). Means ± SD of the HCV foci in 100 microscopic fields (magnification 200×) were 1.55×10^3±41, 1.14×10^3±18, 1.36×10^3±29, 1.21×10^3±29, 1.60×10^3±35 and 1.00×10^3±17, respectively. *, different significantly from (1); #, different significantly from (7).

doi:10.1371/journal.pone.0049254.g005
Figure 6. Tentative locations of amino acid residues on NS5B primary sequence matched with the respective VH9, VH13, VHH6 and VHH24 phage mimotopes, i.e., tentative epitopes of the antibodies. Fingers, palm, thumb, loop interconnecting fingers and thumb, α-helix linking pairs of the fingers, and β-loop insertion are colored in blue, red, green, cyan, black, and dark green, respectively. B. Hypothetical models showing binding sites of the ribbons of VH9 (orange), VH13 (magenta), VHH6 (yellow), and VHH24 (grayish blue) on molecular surface of NS5B RNA duplexes. Finger, palm and thumb of the NS5B are colored in blue, red, and green, respectively.

doi:10.1371/journal.pone.0049254.g006

Supporting Information

Figure S1  Multiple alignments of the cloned NS5B amino acid sequence with the NS5B sequences of various HCV genotypes/subtypes of the database. The homology of the cloned sequence with the heterologous genotype/subtype sequences was approximately 80%.

(TIF)

Figure S2  Percent ELISA inhibition of the VHH6 binding to the NS5B. M55 binding to the NS5B demonstrated by ELISA well containing immobilized NS5B. NS5B binding sites of the phages. (ZIF)

Table S1  Phage mimotope groups, respective 12-mer peptides displayed on the phages that bound to VH9, VH13, VHH6 and VHH24 and mimotope-matched peptides (tentative epitopes of the antibodies) on NS5B primary protein sequence.

(DOC)

Author Contributions

Conceived and designed the experiments: WC KT JT. Performed the experiments: KT JT KB. Analyzed the data: WC KT JT KB. Contributed reagents/materials/analysis tools: SM. Wrote the paper: WC KT. Experiments: KT JT KB. Manuscript preparation and graphics: SM. Constructed the pen-pet23b+ plasmid backbone: OP. Supervised KB on computer modeling: KC.

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