Interference of HCV replication by cell penetrable human monoclonal scFv specific to NS5B polymerase

Kanyarat Thueng-in,1,2 Jeeraphong Thanongsaksrikul,1,2 Surasak Jittavisutthikul,2,4 Watee Seesuay2 Monrat Chulanetra2 Yuwaporn Sakolvaree2 Potjanee Srimanote3 and Wanpen Chaicumpa2,3,*

1Department of Microbiology and Immunology; Faculty of Veterinary Medicine; Kasetsart University; Bangkok, Thailand; 2Laboratory for Research and Technology Development; Department of Parasitology; Faculty of Medicine Siriraj Hospital; Mahidol University; Bangkok, Thailand; 3Graduate Program in Biomedical Science; Faculty of Allied Health Sciences; Thammasat University; Pathumthani, Thailand; 4Graduate Program in Immunology; Department of Immunology; Faculty of Medicine Siriraj Hospital; Mahidol University; Bangkok, Thailand

Keywords: Hepatitis C, hepatitis C virus, NS5B polymerase, human single-chain variable antibody fragments (human scFv), phage display

Abbreviations: ABTS, 2, 2'-Azino-di(3-ethylbenzthiazoline-6-sulfonate); AE, adverse effects; BCIP/NBT, 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium liquid substrate system; bp, base pairs; CDR(s), complementarity determining region(s); cDNA, complementary deoxyribonucleic acid; DMEM, Dulbecco’s modified Eagle’s medium; DNA, deoxyribonucleic acid; DTT, dithiothreitol; E. coli, Escherichia coli; ELISA, enzyme-linked immunosorbent assay; Fc, fragment crystallizable of immunoglobulin; FR(s), immunoglobulin framework region(s); HCV, hepatitis C virus; HRP, horseradish peroxidase; scFv, single-chain variable antibody fragments; IPTG, isopropyl β-D-1-thiogalactopyranoside; LDH, lactic dehydrogenase; IFN-α, interferon-alpha; IgG, immunoglobulin G; kDa, kilo-Daltons; mM, millimolars; Ni-NTA, nickel-nitrilotriacetic acid; nM, nanomolars; nm, nanometers; NS, non-structural protein; NS5BΔ55, recombinant C-terminally 55 amino acid deleted NS5B; NTPs, nucleotide triphosphates; OD, optical density; PBS, 0.15 molar phosphate-buffered saline, pH 7.4; PCR, polymerase chain reaction; PDB, protein data bank; PEG, pegylated; pen/PEN, penetratin gene/protein; qPCR, quantitative real-time reverse transcription polymerase chain reaction; RBV, ribavirin; RdRp, RNA-dependent RNA polymerase; RFLP, restriction fragment length polymorphism; RT-PCR, reverse transcription polymerase chain reaction; scfv, gene sequence coding for scFv; SD, standard deviation; SDS, sodium dodecyl sulfate; SOC, standard-of-care; STAT-C, specifically targeted anti-viral therapy for hepatitis C; SVR, sustained virologic response; VH, variable heavy domain of conventional four-chain IgG; VL, variable light chain domain

A new class of hepatitis C virus (HCV)-targeted therapeutics that is safe, broadly effective and can cope with virus mutations is needed. The HCV’s NS5B is highly conserved and different from human protein, and thus it is an attractive target for anti-HCV therapeutics development. In this study, NS5B bound-phage clones selected from a human single chain variable antibody fragment (scFv) phage display library were used to transform appropriate E. coli bacteria. Two scFvs inhibiting HCV polymerase activity were selected. The scFvs were linked to a cell penetrating peptide to make cell penetrable scFvs. The transbodies reduced the HCV RNA and infectious virus particles released into the culture medium and inside hepatic cells transfected with a heterologous HCV replicon. They also rescued the innate immune response of the transfected cells. Phage mimotope search and homology modeling/molecular docking revealed the NS5B subdomains and residues bound by the scFvs. The scFv mimotopes matched residues of the NS5B, which are important for nucleolin binding during HCV replication, as well as residues that interconnect the fingers and thumb domains for forming a polymerase active groove. Both scFvs docked on several residues at the thumb armadillo-like fold that could be the polymerase interactive sites of other viral/host proteins for the formation of the replication complex and replication initiation. In conclusion, human transbodies that inhibited HCV RdRp activity and HCV replication and restored the host innate immune response were produced. They are potentially future interferon-free anti-HCV candidates, particularly in combination with other cognates that are specific to NS5B epitopes and other HCV enzymes.
**Introduction**

The hepatitis C virus (HCV) has infected over 170 million people worldwide, and new cases occur at a rate of 3–4 million each year. Although many of the newly infected subjects are asymptomatic, 60–80% develop chronic hepatitis, of which about 20% will progress to liver fibrosis and cirrhosis, with a 1–4% annual risk of developing fatal hepatocellular carcinoma among the latter. The standard-of-care (SOC) for HCV infected subjects consists of weekly injected pegylated interferon-α (PEG-IFN) in combination with oral daily ribavirin (RBV) for 24–48 wk. The aims are to enhance the host immunity (IFN-α) and interfere with the virus replication (RBV) in order to reduce the viral load and slow the end stage-liver disease progression. A significant proportion of the patients does not comply with this long-term and stringent treatment, not only because of the high cost burden, but also because of the adverse effects (AE) including IFN-α-related-flu-like symptoms, neuropsychiatric involvement, autoimmune development and ribavirin mediated-hematologic complications (leukopenia, thrombocytopenia and hemolytic anemia). In addition, genotype 1 HCV is relatively resistant to the SOC.

Current anti-HCV alternatives are small molecular drugs that interfere specifically with HCV protein activities (specifically targeted antiviral therapy for hepatitis C; STAT-C) or inhibitors/agonists of host factors that are involved in the HCV replication, e.g., cyclophilin and toll-like receptors. Anti-HCV drugs in the pipeline include first and second generation protease inhibitors, polymerase inhibitors (nucleoside and non-nucleoside) and inhibitors of NS5B and NS5A proteins. Two protease inhibitors (boceprevir and telaprevir) have recently been approved in many countries for clinical use in combination with SOC, especially for the genotype 1 infection. Although the triple therapy increased the percentage of the sustained virologic response (SVR) (non-detectable HCV RNA in the serum by PCR 24 wk after cessation of the therapy) compared with SOC, it causes additional AE, i.e., anemia, skin rash, pruritus and anorectal symptoms. The regimen is also contraindicated for patients with underlying conditions including decompensated liver/metabolic diseases, autoimmune conditions, chronic diseases of the kidney/heart/lung and pregnancy.

The protease inhibitors cannot be used as a sole therapeutic agent. Therefore there is a need for a novel anti-HCV remedy that is safe, tolerates virus mutations better and is broadly effective, such as a cocktail of the right HCV enzyme inhibitors.

Recently, humanized, cell penetrable single domain antibodies (VH/VH1) that bound specifically to epitopes located around the HCV polymerase catalytic groove and inhibited the RdRp activity leading to the suppression of the virus replication were reported. In this study, cell penetrable human single chain variable antibody fragment (scFv) molecules that bound to epitopes different from the previously reported NS5B specific-VH/VH1 were produced. A cocktail of human/humanized small antibodies to HCV NS5B polymerase that simultaneously interferes with different regions of the protein could be another future STAT-C candidate.

**Results**

Phage clones displaying NS5BΔ55-bound human scFvs and the scFv characteristics

C-terminally 55 amino acid deleted recombinant NS5B protein, i.e., NS5BΔ55, with inherent RdRp activity of the native HCV polymerase was used as antigen in the phage bio-panning for selecting the NS5B-bound phage clones from a previously established human naïve scFv phage display library. The antigen-bound phages were used to transfect the HB2151 E. coli and the bacteria were grown on a selective agar. Forty colonies were picked randomly from the plate and screened for the presence of the human scFv coding sequences (scfvs) by PCR using a phagemid specific primers, and 20 colonies (50%) were positive for the scfvs (~1,000 bp). The scfvs-positive E. coli clones were grown under IPTG induction, and 11 clones (27.5%) expressed soluble scFvs (25–30 kDa) as determined by western blotting using rabbit anti-E tag antibody as the scFv detection reagent. Binding of the scFvs to the NS5BΔ55 protein was tested by indirect ELISA using BSA as a control antigen and the scFvs that gave ELISA OD405 nm at least two times higher than to BSA were selected (Supporting Figure 1A). Antigenic specificity of the scFvs of clone nos. 14, 27, 28, 34, and 38 were verified by western blot analysis against the SDS-PAGE-separated the NS5BΔ55 (Supporting Figure 1B). The scfv sequences of these clones revealed three different DNA banding patterns (RFLP) (Supporting Figure 1C). However, after sequencing, the scfv sequences of clones nos. 27, 28 and 38 were truncated; thus, only the remaining clones (nos. 14 and 34) that showed complete scFv deduced amino acid sequences (three CDRs and four FRs of the VH and VL domains and a peptide linker between both domains) were studied further. The scFv14 contained V segments of IgHV5 and IgkV3 families while the V segments of scFv34 were IgHv1 and IgkV2 as analyzed by using http://www.vbase2.org.

Inhibition of HCV RNA-dependent RNA polymerase (RdRp) activity by NS5BΔ55 specific-human scFvs

At molar ratios of antibody:polymerase 2:1 and 4:1, the purified scFv14 showed 64.8 and 75.2% inhibition of the NS5BΔ55 polymerase activity, respectively, compared with the control scFv, which showed no inhibition (P < 0.05) when tested by the SLD3 RNA and biotinylated-CTP based-ELISA. The scFv34 inhibited the polymerase activity at molar ratios 2:1 and 4:1 by 83.8 and 82.2%, respectively, which was significantly different (P < 0.05) from the inhibition mediated by the scFv14 (Fig. 1).

Ability of cell penetrable peptide-linked scFvs to enter mammalian cells and their effects to the cells

The scfvs sequences of E. coli clone nos. 14 and 34 which their expressed scFvs inhibited the NS5BΔ55 RdRp activity in vitro were subcloned to the recombinant pET23b+ plasmid backbone carrying a DNA insert coding for a cell penetrating peptide, i.e., penetratin (PEN) (see Materials and Methods). The scfv was inserted at the SfiI and NotI sites downstream of the PEN coding sequence in order to produce cell penetrable scFvs (PEN-scFv fusion proteins or transbodies). After incubating the human
hepatic (Huh7) cells with 10 μg of the respective PEN-scFvs, it was found that the amounts of the PEN-scFvs of both clones recovered from the cells were more than 85% of the incubation amount (data not shown). The PEN-scFvs could readily enter the Huh7 cells (Fig. 2).

By the LDH assay, the Huh7 cells cultured in the medium containing 10 μg of PEN-scFvs of either clones for 24 h had no significant LDH release compared with the non-treated cell control (data not shown).

Inhibition of HCV replication by cell penetrable scFvs specific to HCV polymerase
The JFH-1 RNA transfected-Huh7 cells cultured in the medium containing 10 μg of PEN-scFv-14 and -34 and PEG-IFN+RBV had significantly less HCV RNA in culture supernatants (Fig. 3A) and inside the cells (Fig. 3B) than the transfected cells cultured in the medium alone (negative inhibition control) and the cells treated with control PEN-scFv (background inhibition control) ($P < 0.05$). The amounts of the HCV RNA inside the cells and culture fluids of the specific transbody-treated cells were not different from the cells treated with PEG-IFN+RBV ($P > 0.05$). Likewise, the numbers of the HCV

![Figure 1](image1.png)

**Figure 1.** Percent ELISA inhibition of RdRp activity of NS5BΔ55 after adding the scFvs of clone nos. 14 and 34 (bars 3 and 4) into the RdRp reaction mixture at the molar ratios of scFv:polymerase 2:1 and 4:1, respectively. Reaction mixture without antibody served as negative inhibition controls (bar 1). Reaction mixture mixed with heparin (bar 2) and control scFv (bars 5) served as positive and background inhibition controls, respectively.

![Figure 2](image2.png)

**Figure 2.** Intracellular localization of PEN-scFv34 revealed by laser sectional confocal microscopy. Huh7 cells treated with 10 μg of PEN-scFv34 were washed, fixed with cold methanol, permeated with 1% Triton X-100 before adding with mouse anti-His Tag and donkey anti-mouse immunoglobulin (DyLight® 488), respectively. DAPI was used to locate the cell nuclei. The stained cells were observed under 1 μm laser sectional confocal microscopy for localization of the PEN-scFvs at different cellular layers. Three upper panels show appearances of PEN-scFv34-treated cells at 2, 3 and 4 μm from the cell surface, respectively; the lowest panels show appearances of non-treated cells at 3 μm from the cell surface. Left blocks of all panels: cells stained with DAPI to locate the nuclei (blue); middle blocks of the upper three panels show intracellular localization of PEN-scFvs in the cells probed with mouse anti-His tag and stained with donkey anti-mouse immunoglobulin (DyLight® 488) (green fluorescence); middle block of the lowest panel, negatively stained control cells. Right blocks, the respectively merged left and middle blocks. The PEN-scFvs were scattered in cytoplasm of the cells at the three layers (the three upper right blocks).
infectious particles in the culture supernatants of the transfected cells treated with the PEN-scFv14, PEN-scFv34 and PEG-IFN-CRBV were significantly less than the transfected cells cultured in the medium or control PEN-scFv (P < 0.05) (Fig. 4). Appearances of the HCV foci inside the transfected Huh7 monolayer of all treatments are shown in Figure S2.

Response of the HCV transfected cells to treatment with PEN-scFvs

Expression levels of mRNAs of the innate immune response genes, i.e., TRAF and IRF3, after treating the JFH-1 RNA transfected Huh7 cells with PEN-scFv-14 and -34, control PEN-scFv, PEG-IFN+RBV and untreated cells are shown in Figure 5. There were significant increases of TRAF mRNA in the cells treated with PEN-scFv-14 and -34 and PEG-IFN+RBV (7.0, 5.0 and 4.5-folds, respectively) compared with the infected cells without any treatment (P < 0.05) (Fig. 5A). Likewise, the increases of IRF3 mRNA compared with the untreated infected cells were 7.0, 5.0 and 7.0 fold, respectively (Fig. 5B). There were no differences of TRAF and IRF3 mRNA expressions between the HCV transfected cells treated with control PEN-scFv and the untreated transfected cells. The infected cells treated with
PEN-scFv-14 and -34, irrelevant PEN-scFvs and PEG-IFN+RBV did not reveal significant increases of IFN-β mRNA compared with the untreated infected cells (data not shown). Results of agarose gel electrophoresis of TRAF and IRF3 qPCR amplicons of the infected cells treated with PEN-scFv-14 and -34, control PEN-scFv, PEG-IFN+RBV and medium alone and normal cells are shown in Figure 5C using the GAPDH gene for normalization.

Phage mimotopes and presumptive epitopes of the HCV polymerase specific-scFvs

The 12-mer phage mimotopic peptides that bound to the NS5B specific-scFvs were determined by using a Ph.D. - 12\textsuperscript{TM} phage display peptide library (see Materials and Methods). The phage peptides were classified into mimotope types. Only one mimotope type of the phage peptides deduced from the phages bound to scFv14 (designated M14). The M14 peptide, VEAAQTPILPYW, matched with amino residues 489–500 of the NS5B thumb domain (Fig. 6A). The mimotopes of scFv34 could be classified into three types: M34/1 (ALPSMGYHNSVY), M34/2 (NYPATNTHRTPY), and M34/3 (IPVKSWPIRPSS). The peptides of these mimotope types matched with amino residues 22–40 of a Δ1 loop interconnecting the fingers and thumb domains and amino residues 470–481 and 495–506 of the thumb domain, respectively (Fig. 6B).

Inhibition of the scFv binding to NS5BΔ55 by phage mimotopes and the NS5B peptides

The scFv-14 and -34 (5 μg in 50 μl PBS) were incubated separately with various amounts of the mimotopic phages (M14, M34/1, M34/2 and M34/3), M34-1, M34-2 and M34-3 mixture and synthetic peptides identical to the NS5B epitopes matched with the M14, M34/1, M34/2, and M34/3 phage
mimotopes, i.e., LRKLGCPLRAW (P14), PISPLNSLLRHHLTVY (P34–1), GLSAFTLSYSP (P34–2), and PPLRRAWHRARA (P34–3) and mixture of the peptides at 37°C for 1 h. Background and negative inhibition controls (maximum binding, 100%) consisted of the scFvs mixed with control phage/peptide and buffer only. Thereafter, all reaction mixtures were added appropriately to ELISA wells pre-coated with the N55BΔ55, Mouse monoclonal anti-E tag antibody, goat anti-mouse immunoglobulin-HRP conjugate and ABTS substrate were used for color development. The overall results of the competitive ELISA are shown in Figure 7. The bindings of the scFv-14 and -34 to the recombinant N55BΔ55 were blocked by the respective mimotopic phages and peptides. Mixtures of the M34 mimotopes and the peptide epitopes blocked the scFv34 binding to the N55BΔ55 slightly better than the individual mimotopes/peptides.

Molecular docking for determining the binding sites of the N55B specific-scFvs on the HCV polymerase

The models of the scFv-14 and -34 and HCV genotype 3a N55B were predicted by the I-TASSER and the most accurate data were extracted from the best scores for estimating the modeled quality. Table S1 shows the estimated accuracy of the modeled HCV genotype 3a NS3B and scFv-14 and -34. The molecular docking between the HCV NS5B of the genotypes 3a and 1b (PDB: 1C2P) with the scFvs performed by the ClusPro 2.0 server were predicted in four separate modes including, balance, electrostatic-favored, hydrophobic-favored and Van der Waals-electrostatic. The scFvs were found to dock on the same areas of the polymerases of both HCV genotypes. Therefore, the interactive complexes of the 1C2P with the scFvs with the lowest energy score averages were collected as representatives. The representative models were extracted from the best scores of the collected modes. The lowest energy for the complexes of C12P with the NS5B-scFv14 (-1,018.3 kcal/mol) and the NS5B-scFv34 (-1,273.3 kcal/mol) were chosen from cluster 3 of the electrostatic favored-mode and cluster 1 of the hydrophobic mode, respectively.

According to the docking outputs, the scFv14 was found to bind to four patched areas of the thumb domain of the 1C2P NS5B (Figs. 8A and 8B). These areas included: patch 1: α-helix U; patch 2: α-helix T; patch 3: α-helix S and patch 4: β15–16. The interactive residues between the NS5B and the scFv14 are detailed in Figure 8B and Table 1. For patch 1, K531 (R531 in other genotypes) and K533 located near the C-terminal of the α-helix U interacted with D106 and Y27 located in the VH-FR4 and VH-CDR1, respectively. For patch 2: residues of α-helix T, i.e., R498 interacted with S187 and G198 located in the VL-FR3 and R501 and R505 interacted with E102 of VH-CDR3 and T190 of VL-FR3 of the scFv, respectively. For patch 3, Y477 located near the N-terminal of α-helix S interacted with Y110 of VH-CDR3; N483 and S487 (G487 in other genotypes) of the α-helix S interacted with N164 and S163 of VL-CDR1, respectively, and R490 of the α-helix S interacted with S163 and D165 of VL-CDR1 and S201 of VL-FR3. For patch 4, A376 (D/N376 in other genotypes) located at β15–16 interacted with S31 of VH-CDR1 while R380 of the β16 interacted with Y52, D55 and D57 of the VH-CDR2.

The scFv34 bound also to four patches of the polymerase: three patches (1, 2 and 3) of the thumb domain and one patch of the finger domain (patch 4) (Fig. 8C and 8D). Figure 8D and Table 1 give details on the interactive residues of N55B of HCV genotype 3a with the scFv34 residues. For patch 1, K531 (R531 of other genotypes) located near the C-terminal of α-helix U interacted with Y111 of VH-FR4 of the scFv. For patch 2, three residues of α-helix T, i.e., R498 interacted with W36 of VH-CDR2 and R501 and R505 interacted with E108 and D110 of VH-FR4, respectively. For patch 3, S476 located near the N-terminal of α-helix S interacted with D106 of VH-CDR3 and P479 and N483 of α-helix S interacted with Y104 of VH-CDR3. For patch 4, N28 of α-helix A and R32 located near the C-terminal of the α-helix A (both residues are in the Δ1 of the fingers domain important for interconnecting with the thumb
domain for forming the active polymerase catalytic groove) interacted with G44 and Q43 of VH-FR2, respectively.

**Discussion**

The invention of hybridoma technology in 1975\(^{18}\) enabled the production of therapeutic mouse monoclonal antibodies, which led to the launch of the first therapeutic mouse monoclonal antibody in 1986.\(^{19}\) Nowadays, recombinant antibodies to any epitope can be rapidly produced in vitro (regardless of the tissue culture facility and immunogenicity and toxicity of the antigen) from *E. coli* bacteria transfected with the antigen-binding phage selected from an antibody phage display library.\(^{20-25}\) The engineered antibodies can also be made into intact four-chain molecules when the biological activities of the Fc fragment are needed for the therapeutic effect. Alternatively, smaller antibody fragments that are devoid of the Fc fragment or constant domains, e.g., scFv\(^{21,22}\) or single domain antibodies (nanobodies/VH/V\(_{H}\)H)\(^{13,23-25}\) can be used to increase the tissue penetrating ability and reduce tissue inflammation. The small antibodies have high solubility, reproducible refolding capacity and thermal stability, which ease the production process. Moreover, they can be made as cell penetrable molecules that can reach intracellular targets.\(^{26}\)

There is a need for a new remedy for the treatment of HCV infection that is safer than the existing regimens. The new therapy should also be broadly effective and able to cope better with the virus mutation. Many new anti-HCV pharmaceutical drugs for STAT-C are being developed and some have already become clinically available. The treatment solution that we are proposing consists of a combination of ready to use, engineered, cell penetrable small antibodies that are specific to multiple epitopes of HCV NS5B.\(^{27}\)
key HCV enzymes. These antibodies should not cause any adverse effects, e.g., serum sickness, that usually occur when animal derived immune globulins or murine monoclonal antibodies are used.19

NS5B polymerase is indispensable for the infectious cycle of HCV. The 3D structure of the HCV polymerase has the same right hand configuration as other polymerases with fingers, palm and thumb domains.15-17 Nevertheless, the catalytic site of the HCV polymerase is fully encircled by interaction of the fingers and thumb subdomains in order to create a tunnel for entering a single stranded RNA template.28,29 The protein is highly conserved and several residues in the functionally different domains/subdomains are potential targets for anti-HCV inhibitors.17,30 In this study, two scfV-phage transformed E. coli clones produced human scFvs, i.e., scFv-14 and -34, that not only inhibited the RdRp activity but also, in their cell penetrable format could inhibit replication of heterologous HCV (genotype 2) in the infected cells and rescue the cells’ innate immune response. The scFvs did not cause LDH leakage from the mammalian cells indicating that they were not cytotoxic and should be safe for human treatment.

To assess the mechanisms of the scFv-mediated inhibitions of the RdRp activity and the HCV replication, the regions and residues of the NS5B molecule bound by the scFvs were determined by means of 12 mer-peptide phage mimotope search21,22 and computerized homology modeling and molecular docking.31-38 The NS5B of HCV genotype 3a’s linear sequence (accession number NP751928) was used in the alignment with the scFv mimotope sequences for tentative epitope identification and the 3D structure of PDB 1C2P was used in the molecular docking with the scFv 3D structures. Although the principles of the two methods are different (the phage mimotope search was based on the linear sequences of both the mimotopes and the NS5B while the computerized molecular docking encompassed 3D structures and conformational epitopes), the results were, more or less, conformed.

Interaction with the NS5B stretch at amino acids 500WRHRARS506 with host cell nucleolin (especially at the

![Figure 8. Illustrations of the bindings between HCV NS5B polymerase and the NS5B specific-scFvs. (A) Human scFv14 bound to the polymerase thumb domain. (B) Four patch areas of the NS5B polymerase that were bound by the scFv14 (patch 1: α-helix U; patch 2: α-helix T; patch 3: α-helix S and patch 4: β15–16) and the interactive residues. (C) Human scFv34 docked on the NS5B polymerase. (D) Four patch areas of the NS5B polymerase that were bound by the scFv34 (patch 1: α-helix U; patch 2: α-helix T; patch 3: α-helix S and patch 4: Δ1 interconnecting loop between fingers and thumb domains of the polymerase) and the interactive residues.](image-url)
W500 and the three arginines) is important for HCV replication. It is plausible that the binding of the PEN-scFvs to these critical residues interfered with the NS5B-nucleolin binding and thus impaired the HCV replication in the Huh7 cells transfected with the HCV replicon. It is also known that the interaction between the finger’s extension loop (D1) from residues I11-S46 and the back of the HCV polymerase thumb domain makes the molecule more rigid and active (closed, form 1). Mutation of Leu30 to polar Ser or Arg of HCV genotype 1 polymerase resulted in a non-functional enzyme implying that local perturbation of the D1 loop may disturb the enzymatic activity. The scFv34 interacted with residues in this region (indicated by both mimotope search and molecular docking) and thus it possibly interfered with the polymerase activity by disturbing the configuration of the enzymatic groove.

Previous studies have demonstrated that two non-nucleoside inhibitors, i.e., thioephene-2-carboxylic acids A and B, inhibited the polymerase of HCV genotypes 1b and 2a. The binding pocket of the inhibitors was located in the thumb domain formed by amino acids I419, R422, M423, L474, H475, T476, Y477, L482, L497, R498, K501 and W528. The phage mimotope M14 of the scFv14 matched with the NS5B sequence extended from residues 489–500 of NS3B, which encompassed L497 and R498. Also demonstrated by molecular docking, the scFv14 interacted with the epitope containing Y477 near the N-terminal of α-helix S and R498 and R501 of α-helix T. The scFv34 docked on S476, R498 and R501 of the NS5B and the M34/3 encompassed residues 470–481 and 495–506. Therefore, both scFvs, which likely interacted with several residues in the binding pocket of the thiophene-2-carboxylic acids A and B, might inhibit the HCV genotype 3a polymerase activity by the same mechanism as the two inhibitors. Moreover, the back surface of the thumb domain has the armadillo-like fold which could be the site of polymerase interaction with other viral/host cellular proteins during the formation of the replication complex and replication initiation. It is plausible that the scFvs might interfere with the polymerase interaction with the cognate protein(s) or the host factor and, as a consequence, inhibited the HCV replication.

HCV evades the host’s intracellular immune responses by using multiple strategies mediated by several viral proteins including NS3/4A protease, NS5A, E2. To our knowledge, the direct role of NS5B in subverting the type-1 interferon pathway has not been demonstrated. This study showed that treating HCV replicon transfected Huh7 cells for five days with cell penetrating scFv-14 and -34 specific to NS5B resulted in the restoration of TRAF and IRF-3 mRNA expressions indicating that the NS5B may be involved (indirectly) in the innate immune evasion of the HCV. The five day treatment might be too early for IFN-α expression which is downstream in the signaling pathway and thus an increase in IFN-β mRNA was not seen.

In conclusion, cell penetrable human single chain antibodies (transbodies) that inhibited HCV RdRp activity and HCV replication and restored the innate immune response of the host were

### Table 1. Residues and motives of HCV NS5B bound by residues and domains of human ScFv-14 and -34

| HCV NS5B          | HuScFv14       |
|-------------------|---------------|
| Amino acid        | Motif         | Amino acid(s) | Domain |
| K531              | Near to C-terminal of α-helix U | D106 | VH-FR4 |
| K533              | Near to C-terminal of α-helix U | Y27 | VH-CDR1 |
| R498              | α-helix T     | S187 and G198 | VL-FR3 |
| R501              | α-helix T     | E102 | VH-CDR3 |
| R505              | α-helix T     | T190 | VL-RF3 |
| Y477              | Near N-terminal of α-helix S | Y103 | VH-CDR3 |
| N483              | α-helix S     | N164 | VL-CRD1 |
| S487              | α-helix S     | S163 | VL-CRD1 |
| R490              | α-helix S     | S163 and D165 | VL-CDR1VL-FR3 |
|                   |               | S201 |      |
|                   |               | S31  | VH-CDR1 |
|                   |               | Y52, D55 and D57 | VH-CDR2 |
| A376              | β15-16        |      |      |
| R380              | β16           |      |      |

| HCV NS5B          | HuScFv34       |
|-------------------|---------------|
| Amino acid        | Domain         |
| K531              | Near to C-terminal of α-helix U | Y111 | VH-FR4 |
| R498              | α-helix T     | W36  | VH-CDR2 |
| R501              | α-helix T     | E108 | VH-FR4 |
| R505              | α-helix T     | D110 | VH-FR4 |
| S476             | Near to N-terminal of α-helix S | D106 | VH-CDR3 |
| P479             | α-helix S     | Y104 | VH-CRD3 |
| N483              | α-helix S     | Y104 | VH-CRD3 |
| N28               | α-helix A in Δ1 of finger domain | G44  | VH-FR2 |
| R32               | Near C-terminal of α-helix A | Q43 and G44 | VH-FR2 |

*, S476 of genotype 1b C1P2 was S479 of genotype 3a (accession no. NP751928). **, P479 of genotype 1b C1P2 was P481 of genotype 3a. Human scFv34 used only VH domain in binding to the target protein.
produced. Although the molecular mechanisms underlying the scFv-mediated interference with the HCV infectious cycle needs elucidation, the transbodies have a high potential for further development into an interferon-free STAT-C, particularly in combination with other counterparts specific to other polymerase epitopes and HCV enzymes.

**Materials and Methods**

**Production and characterization of human scFvs that bound to NS5BΔ55**

Purified recombinant C-terminally 55 amino acid deleted NS5B, *i.e.*, NS5BΔ55, of HCV genotype 3a with the RdRp activity was prepared. Phage clones that displayed the NS5BΔ55 bound-scFvs were selected from a human scFv phage display library constructed previously. The phage library was added to an ELISA well pre-coated with 10 μg of purified NS5BΔ55. The antigen-unbound phages were removed by washing and a log phase-grown HB2151 *E. coli* culture was added to the well containing the antigen-bound phages. The phage infected *E. coli* were grown on a selective agar and the resulting colonies on the overnight plate were screened for the presence of the human scFv coding sequence (*scfvs*) by PCR using a phagemid specific primers. The HB2151 *E. coli* clones carrying the *scfvs* were grown under 0.2 mM IPTG induction and the soluble scFvs were purified from the *E. coli* lysates using Ni-NTA affinity resin. Binding of the scFvs by individual *E. coli* clones to the NS5BΔ55 protein was determined by indirect ELISA and western blot analysis using BSA as control antigen.

The restriction fragment length polymorphism (RFLP) of the *scfvs* coding for the NS5BΔ55 specific-scFvs was determined by digesting the *scfv* PCR amplicons with *MvaI* restriction endonuclease. The digested DNA fragments were subjected to a 14% PAGE and ethidium bromide staining. The *scfvs* were also sequenced and their deduced amino acids were analyzed and multiply aligned by ClustalW to determine the sequence diversity. The International Immunogenetics Information system (IMGT) was used to predict the complementarity determining regions (CDRs) and the immunoglobulin framework regions (FRs) of the scFvs.

**In vitro neutralization of HCV RdRp activity by the human scFvs**

An ELISA inhibition was performed to determine the inhibitory activity of the NS5BΔ55 specific-scFvs on the RdRp activity. The NS5BΔ55 and SLD3 RNA (5′ GGCCUUGCAU AGCAAGUCUG AGACC 3′) were used as a source of active polymerase and as RNA template, respectively. A polymerase reaction mixture (80 μl containing 300 nM of NS5BΔ55, 20 mM sodium glutamate pH 8.2, 4 mM MgCl₂, 12.5 mM DTT, 0.5% (v/v) Triton X-100, 2 mM MnCl₂, 40 units RNase inhibitor, and 200 mM each of ATP, UTP, GTP, and biotinylated-CTP) was added to the SLD3 RNA coated well and incubated at 37°C for 2 h. In the presence of active RdRp, newly synthesized RNA containing biotinylated-CTP was detectable by adding streptavidin-HRP conjugate (Southern Biotech, USA) and ABTS substrate (KPL, USA), respectively. The OD₄₀₅nm of the content of each well was determined. In the ELISA inhibition, purified NS5BΔ55 specific-scFvs from *E. coli* clones (tests), control scFv (non-NS5BΔ55 binding; background inhibition control), 2 mM polymerase quencher, *i.e.*, heparin (RdRp positive inhibition control), buffer (blank) and normal BL21 (DE3) *E. coli* lysate (negative antibody control) were mixed individually with 300 nM of NS5BΔ55 (molar ratios of scFv:NS5BΔ55 were 2:1 and 4:1) before adding each to the polymerase reaction mixture. In the presence of the active polymerase inhibitor, the OD₄₀₅nm of the newly synthesized RNA containing biotinylated-CTP was reduced compared with the non-inhibition controls.

**Production of cell penetrable human scFvs specific to HCV NS5B**

A recombinant pET23d+ plasmid backbone with an insert of a gene sequence coding for a 16 amino acid cell penetrating peptide, penetratin (PEN), was constructed previously. The *scfv* sequences of individual *E. coli* clones that inhibited the NS5BΔ55 RdRp activity in vitro were subcloned to the recombinant pen-pET23d+ plasmid backbone at the *SfiI* and *NotI* sites downstream of the PEN coding sequence in order to produce cell penetrable human scFvs. The recombinant vectors were put into BL21 (DE3) *E. coli*. The PEN-scFv fusion proteins produced by the selected transformed bacteria were purified by Ni-NTA resin.

**Cell internalization of the PEN-scFvs**

Huh7 monolayer were incubated with 10 μg of individual PEN-scFv preparations for 1 h. Cell culture supernatants were collected; the cells were washed with plain DMEM, added with a fixed volume of PBS, homogenized and the lysates were collected after centrifugation. The amounts of PEN-scFv in the cell lysates were quantified by indirect ELISA as described previously. The intracellular localization of the PEN-HuScFvs was revealed by laser sectional confocal microscopy. Huh7 cells were grown on glass coverslips in tissue culture wells. After incubating with 10 μg of individual PEN-scFv at 37°C in a 5% CO₂ incubator for 1 h, the cells were washed with PBS, fixed with cold methanol for 20 min, washed again and permeated with 1% Triton X-100 for 30 min before blocking with 3% BSA. After washing, mouse anti-6×His tag (1:1,000) and donkey anti-mouse immunoglobulin (DyLight® 488) (1:1000) were added, respectively, to the cells with washing between the steps. DAPI (Invitrogen) was used to locate the cell nuclei. The stained cells were observed with 1 μm laser sectional confocal microscopy to localize the PEN-scFvs at different cellular layers.

**Cytotoxic assay**

The PEN-scFvs were tested for toxicity to Huh7 cells using a CytoTox96 assay (Promega, USA). The cells were maintained in DMEM (Gibco, Stockholm, Sweden) supplemented with 10% heat inactivated fetal calf serum (HyClone, USA), penicillin (50 units/ml) and streptomycin (50 μg/ml). The grown cells were added to the wells of a 96-well tissue culture
Quantitative RT-real time PCR

The PEN-scFvs were tested for their ability to inhibit HCV replication in Huh7 cells transfected with a JFH-1 RNA of HCV genotype 2a isolate (GenBank AB047639) as described previously.13 The pJFH-1 replicon was kindly provided by Dr. Takaji Wakita of the Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan and Prof. Dr. Ralf Bartenschlager, Department of Molecular Virology, University of Heidelberg, Germany. The full-length pJFH-1 cDNA was linearized by XbaI (Fermentas, Ontario, Canada) digestion and subjected to in vitro transcription using a Megascript T7 kit (Ambion, NY, USA). The RNA transcript (10 µg) was electroporated into Huh7 cells (4.0 × 10⁶ cells); the transfected cells were immediately transferred to 40 ml of serum supplemented-DMEM and seeded into wells of a 12-well culture plate (Corning), 2.0 × 10⁵ cells/well. After 24 h, the monolayer was washed with PBS and mixed with complete DMEM containing 20 µg of purified PEN-scFvs of individual E. coli clones or control PEN-scFv (background antibody control). Cells added with 100 units of PEG-IFN+50 nM RBV and medium alone served as positive and negative inhibition controls, respectively. Five days post-transfection, the total RNA was extracted from the cells using Trizol® reagent (Invitrogen) and then quantified with a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific). Primers specific to TRAF, IRF3 and IFN-β coding sequences, i.e., TRAF: forward 5'-AAGTGCCACC TGTTGCTGT-3' and reverse 5'-AACGATGCTC TCTTGA-CACG-3'; IRF3: forward 5'-CTTGGAGACG CCGGCTAC-3' and reverse 5'-CGGAAATTC CCTTCCAGGT-3' and IFN-β: 5'-GTCTCATCC AGCCAGTCT-3' and reverse 5'-TGCGAAATGA ATGGGAGGCT-3', were designed from the GenBank (accession nos. NM_145725.2, Z56281 and NM_002176, respectively) using Primer3 software.21 The PCR reaction mixture (12.5 µl) consisted of 1 × Brilliant II SYBR® Green QRT-PCR Master Mix (Agilent), RT/RNase block enzyme, 200 nM of each primer and 300 ng of RNA solution. The qPCR was performed using the Mx3000P QPCR System machine (Agilent) at 42°C for 1 h, 55°C for 30 min, initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. To analyze the dissociation curve, a thermal profile consisting of 95°C for 1 min, then ramped down to 55°C (0.5°C/s) for 45 s and ramped up to 95°C was used.

Foci assay

The foci assay was used to determine the numbers of HCV particles in the cell culture supernatants and the HCV plaques inside the transfected cells. The JFH-1 RNA transfected cells that had been treated with the PEN-scFvs, irrelevant PEN-scFv, PEG-IFN+RBV and medium alone for 5 d and their spent culture fluids were collected for the foci assay. After collecting the culture fluid, the infected cells in each well were rinsed with sterile PBS, fixed with absolute methanol and blocked with 3% BSA before incubating with mouse immune serum to HCV C protein (prepared by injecting recombinant C protein of HCV genotype 3a mixed with alum intramuscularly into BALB/c mice; two booster doses were given to the primed mice at two week intervals and the mice were bled at day 7 post-last booster for immune serum collection) at 37°C for 2 h in order to locate the infected cells. After rinsing, the cells were added with goat anti-mouse immunoglobulin-AP conjugate and BCP/NBT substrate, respectively, with washing between the steps. The foci were observed and counted by using inverted fluorescence microscope, NIS-Element D version 4.10.0.8310 W/camera (Ti-S Intensilight R1 NIS-D, Nikon, Japan) at 10 × original magnification. For enumeration of the HCV infectious particles in the culture supernatants, culture fluids from individual wells were diluted 1:10 with fresh culture medium before adding a diluted aliquot to the Huh7 cell monolayer. After 3 h incubation, the fluid in each well was discarded; the cells were rinsed thoroughly with PBS and cultured for 3 d before the virus foci were determined as above.

Response of HCV transfected cells to treatment with polymerase specific-PEN-scFvs

Expression levels of mRNA of innate immune response genes including TRAF, IRF3 and IFN-β in JFH-1 RNA transfected Huh7 cells after incubating with purified PEN-scFvs were determined in comparison to the controls. The transfected cells were cultured in complete DMEM in a 12-well culture plate (2.0 × 10⁵ cells/well) for 24 h before incubating with 10 µg of PEN-scFvs. The controls consisted of transfected cells treated with control PEN-scFv (background control), 100 units of PEG-IFN+50 nM RBV (positive control) and medium alone (infection control). Five days post-transfection, the total RNA was extracted from the cells using Trizol® reagent (Invitrogen) and then quantified with a Nano-Drop ND-1000 Spectrophotometer (Thermo Scientific). Primers specific to TRAF, IRF3 and IFN-β coding sequences, i.e., TRAF: forward 5'-AAGTGCCACC TGTTGCTGT-3' and reverse 5'-AACGATGCTC TCTTGA-CACG-3'; IRF3: forward 5'-CTTGGAGACG CCGGCTAC-3' and reverse 5'-CGGAAATTC CCTTCCAGGT-3' and IFN-β: 5'-GTCTCATCC AGCCAGTCT-3' and reverse 5'-TGCGAAATGA ATGGGAGGCT-3', were designed from the GenBank (accession nos. NM_145725.2, Z56281 and NM_002176, respectively) using Primer3 software.21 The PCR reaction mixture (12.5 µl) consisted of 1 × Brilliant II SYBR® Green QRT-PCR Master Mix (Agilent), RT/RNase block enzyme, 200 nM of each primer and 300 ng of RNA solution. The qPCR was performed using the Mx3000P QPCR System machine (Agilent) at 42°C for 1 h, 55°C for 30 min, initial denaturation at 95°C for 10 min and 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. To analyze the dissociation curve, a thermal profile consisting of 95°C for 1 min, then ramped down to 55°C (0.5°C/s) for 45 s and ramped up to 95°C was used.

Determination of phage mimotopes that bound to N55B specific-human scFvs and validation of the mimotopes

Phage mimotopic peptides that bound to the N55B specific-human scFvs were determined by a Ph.D. - 12™ phage display peptide library (New England Biolab, USA) as described
previously. Wells of a 96-well ELISA plate were coated separately with scFv-14 and -34 (1 μg in 100 μl) at 4°C overnight. After washing with Tris buffered saline, pH 7.5 (TBS) containing 0.1% Tween-20 (TBST), each well was blocked with 200 μl of 0.5% BSA in TBS for 1 h and washed. The phage display 12-mer peptide library (diluted 1:100) that had been subtracted with lysate of original BL21 (DE3) E. coli was added to the wells coated with the scFvs and the plate was kept at 25°C for 1 h. Unbound phages were removed and the wells were washed with the TBST. The scFv-bound phages were eluted with 0.2 M glycine-HCl solution and the pH was brought up by adding a few drops of 2 M Tris base solution. The phages from each well were inoculated into 20 ml of log phase grown ER2738 E. coli and incubated at 37°C for 4.5 h. The bacterial cells were removed by centrifugation at 12,000 × g; the supernatants containing amplified phage particles were precipitated by adding PEG/NaCl and kept at 4°C overnight. Individual precipitates were re-suspended in 100 μl of TBST and used for the next panning round. Three rounds of the panning were performed. The eluted phages from the third round were used to infect the ER2738 E. coli in top agarose overlay on the LB agar plates containing IPTG and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and incubated at 37°C overnight. Twenty blue plaques on each plate were picked randomly, inoculated individually into 1 ml of 1:100 diluted log phase grown ER2738 E. coli culture and incubated at 37°C with shaking for 4.5 h. DNA of each phage clone was extracted from the culture supernatant by using the phenol/chloroform method and sequenced. Peptide displayed by each phage clone (phage mimotope) was deduced from the DNA sequence by using DNAMAN software version 4.15. Thereafter, the deduced peptides were classified into mimotope types using Phylogeny ClustalW. The sequences of the mimotope types were multiply aligned with HCV genotype 3a NS5B linear sequence (accession number NP751928) by Kalign in order to locate the NS5B regions analogous to the phage’s mimotopic peptides, i.e., presumptive scFv binding sites on the NS5B (presumptive epitopes).

A competitive ELISA as described previously was used for validating the phage mimotopes and the NS5B epitopes bound by the scFvs. The mimotopic phages (M14, M34/1, M34/2 and M34/3) were propagated in ER2738 E. coli and their titers were determined. The peptides identical to the NS5B epitopes matched with the respective phage mimotopes were synthesized (GenScript, USA). Peptides LRLKLGCPPLRAW (P14) matched with the M14, and PISPLSNLLRHHNLYV (p34-1), GLSAFTLHSVSP (P34–2) and PLLRAWRHRARA (P34–3) matched with the M34/1, M34/2 and M34/3, respectively. Peptide MTTLLLTLVVVTIV was used as control. Various amounts of the phages/peptides or their respective mixtures (50 μl) were incubated with fixed amount of the respective scFv (5 μg in 50 μl) at 37°C for 1 h. Background control and negative inhibition (maximum binding, 100%) consisted of the scFvs mixed with control phages/peptide and buffer, respectively. After incubating, the individual mixtures were added to the NS5B pre-coated wells, all wells were washed and mouse monoclonal anti-E tag antibody, goat anti-mouse immunoglobulin-HRP conjugate and ABTS substrate were added, respectively, with washing between the steps. The OD405nm of the content of each well was determined against the negative inhibition control. The % ELISA inhibition was calculated by: % ELISA inhibition = (OD405nm of maximum binding–OD405nm of test) / (OD405nm of maximum binding) × 100.

Homology modeling and molecular docking of NS5B and scFvs

Homology models of the three dimensional structures of genotype 3a NS5B and of the scFvs were predicted by the I-TASSER (http://zhanglab.ccmb.med.umich.edu/I-TASSER/). The crystal structure of the HCV genotype 1b NS5B RdRp, i.e., PDB 1C2P, was gained from the RCSB protein data bank (http://www.rcsb.org). The molecular interactions and the docking simulations were performed by the ClusPro 2.0 server. The amino acid sequence of HCV genotype 3a of the NZL1 isolate (UniProt ID Q81258) was used for indicating NS5B residues that interacted with the scFvs. The amino acids in the fingers, palm and thumb subdomains of the polymerase were numbered according to Lesburg et al. and Bressanelli et al.16

Statistical analysis

Means and standard deviations (SD) of three independent experiments were used for comparing tests and controls. A P value ≤ 0.05 of the unpaired t-test was considered significantly different.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Professor Yong Poovorawan for supplying serum samples of patients infected with HCV genotype 3a. Thanks are also due to Dr. Takaji Wakita of the Department of Microbiology, Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan and Prof. Dr. Ralf Bartenschlager, Department of Molecular Virology, University of Heidelberg, Germany, for providing the pJFH1 and Dr. Markus Roselieb for reading the manuscript.

Funding

This study was funded by the Thailand Research Fund (DPG5380001), the NSTDA Chair Professor grant and the National Research University (NRU) project of the Office of Commission on Higher Education (CHE), Ministry of Education, Thailand.

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.
