Targeting Emerging RNA Viruses by Engineered Human Superantibody to Hepatitis C Virus RNA-Dependent RNA Polymerase

Kittirat Glab-ampai, Kanasap Kaewchim, Techit Thavorasak, Thanatsaran Saenlom, Watayagorn Thepsawat, Kodchakorn Mahasongkram, Kanyarat Thueng-In, Nitat Sookrung, Wanpen Chaicumpa and Monrat Chulanetra

RNA-dependent RNA polymerase (RdRp) is a unique and highly conserved enzyme across all members of the RNA virus superfamilies. Besides, humans do not have a homolog of this protein. Therefore, the RdRp is an attractive target for a broadly effective therapeutic agent against RNA viruses. In this study, a formerly generated cell-penetrating human single-chain antibody variable fragment (superantibody) to a conformational epitope of hepatitis C virus (HCV) RdRp, which inhibited the polymerase activity leading to the HCV replication inhibition and the host innate immunity restoration, was tested against emerging/reemerging RNA viruses. The superantibody could inhibit the replication of the other members of the Flaviviridae (DENV serotypes 1–4, ZIKV, and JEV), Picornaviridae (genus Enterovirus: EV71, CVA16), and Coronaviridae (genus Alphacoronavirus: PEDV, and genus Betacoronavirus: SARS-CoV-2 (Wuhan wild-type and the variants of concern), in a dose-dependent manner, as demonstrated by the reduction of intracellular viral RNAs and numbers of the released infectious particles. Computerized simulation indicated that the superantibody formed contact interfaces with many residues at the back of the thumb domain (thumb II site, T2) of DENV, ZIKV, and JEV, Picornaviridae (genus Enterovirus: EV71, CVA16), and Coronaviridae (genus Alphacoronavirus: PEDV, and genus Betacoronavirus: SARS-CoV-2). The superantibody binding may cause allosteric change in the spatial conformation of the enzyme and disrupt the catalytic activity, leading to replication inhibition. Although the speculated molecular mechanism of the superantibody needs experimental support, existing data indicate that the superantibody has high potential as a non-chemical broadly effective anti-positive sense-RNA virus agent.

Keywords: RNA viruses, RNA-dependent RNA polymerase, phage display, human single-chain antibody variable fragment, superantibody (cell penetrating antibody), computerized simulation, plaque-forming assay, focus-forming assay
INTRODUCTION

During the past two decades, several human and animal RNA viruses have emerged/reemerged to cause epidemics/epizootics or pandemics/panzootics that inflict a huge negative impact on the human and animal health as well as socioeconomics globally. Examples are influenza A viruses (IAV H5N1 and H1N1pdm2009) (Tang et al., 1998; Novel Influenza A/H1N1 Investigation Team, 2009); flaviviruses including dengue virus (DENV) (Kyle and Harris, 2008; European Centre for Disease Prevention and Control, 2020) and zika virus (ZIKV) (Noobrakhsh et al., 2019); ebola virus (EBOV) (World Health Organization Ebola Response Team, 2014); enteroviruses including EV71 and CVA16 (Schmidt et al., 1974); and coronaviruses (CoVs) including Alphacoronavirus (porcine epidemic diarrhea virus, PEDV), Betacoronavirus (severe acute respiratory syndrome virus, SARS-CoV; MERS-CoV; novel coronavirus 2019 or SARS-CoV-2), and Deltacoronavirus (porcine Deltacoronavirus, PDCoV) (Pensaert and de Bouck, 1978; Chan-Yeng et al., 2015; Hu et al., 2015; Jung et al., 2016; World Health Organization [WHO], 2019). Currently, the world population is facing the unprecedentedly scaled pandemic of coronavirus disease caused by the SARS-CoV-2, named COVID-19, that emerged in December 2019. The catastrophic COVID-19 pandemic caused by the SARS-CoV-2 mutated descendants (variants of concern, VOC) is still going on, although a large fraction of the world population has been vaccinated against the disease. As of March 10, 2022, more than 400 million people around the globe were infected by the SARS-CoV-2, and among them, more than 6 million were deceased. The world consternations frequently threatened by the emerging/re-emerging RNA viruses emphasize the need not only for effective vaccines but also for safe therapeutics to counteract the viruses, especially for those with severe morbidity.

RNA-dependent RNA polymerase (RdRp) is a highly conserved enzyme across all members of the RNA virus superfamilies (except Retroviridae), although the enzyme itself accounts for the rapid RNA virus mutations from the high rate of transcription errors. The RNA virus RdRps probably arose from a common ancestor (Payne, 2017). The enzyme is indispensable for the synthesis of the genomic RNA and the transcription process during the virus replication cycle (Payne, 2017). Positive-sense RNA viruses use their RNA genomes as mRNAs for protein synthesis, while the negative-sense RNA viruses use the genomic RNAs as templates of the RdRp-dependent transcriptional process in the generation of the plus-sense strand that functions as mRNAs. Some RNA viruses, including coronaviruses, use RdRp for subgenomic RNA synthesis. Although the RdRps of the RNA viruses are diverse in their amino acid sequences as well as the structural details (the RdRp molecule may be linked with other structures that perform other functions, such as methyltransferase, endonuclease, helicase, and nucleoside-triphosphatase), their catalytic modules are relatively conserved and composed of the palm, fingers, and thumb domains such that the overall architecture reminiscences encircled/cupped human right hand (Jia and Gong, 2019). The catalytic motif (active site) of the RdRp is surrounded by the palm, fingers, and thumb domains with seven catalytic motifs (motifs A–G) distributed within the palm (motifs A–E) and fingers (motifs F–G) (Poch et al., 1989; Gorbalenya et al., 2002; Brunnen, 2003; te Velthuis, 2014; Wu et al., 2015; Venkataraman et al., 2018; Jia and Gong, 2019). The viral RdRp lacks a human homolog. It is the essential and most conserved protein of RNA viruses (Jia and Gong, 2019). The RdRps of Flaviviridae, Hepatitis C virus (HCV), DENV, ZIKV, West Nile virus, share high percentages of identity with RdRp of the Coronaviridae members (e.g., SARS-CoV, MERS-CoV, and SARS-CoV-2) (Picarazzi et al., 2020); it is highly plausible that drugs or therapeutics that act on the RdRp of the former virus family may as well affect the RdRp of the latter, if not also other families. This speculation is well supported by the evidence that sofosbuvir (a small molecular inhibitor of HCV RdRp/NS5B protein in combination with daclatasvir/Daklinza) showed effectiveness in reducing the mortality rate of patients with severe COVID-19 (Abbass et al., 2021; Zein et al., 2021). In this study, therefore, we tested a previously generated cell-penetrating human single-chain antibody (superantibody) to HCV RdRp that has been shown to effectively interfere with the HCV replication and rescued the virally suppressed host innate immunity (Thueng-In et al., 2014), for replication inhibition of several other positive-sense RNA viruses. The ultimate purpose is to develop the broadly effective superantibody further toward a clinical use as a pan, direct-acting anti-positive-sense RNA virus agent.

MATERIALS AND METHODS

Cells, Viruses, and Virus Propagation

Human hepatocellular carcinoma cells (Huh7), human embryonic kidney (HEK) 293T cells, African green monkey kidney epithelial (Vero) cells, and Rhabdomyosarcoma (RD) cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, United States). Vero E6 cells were provided by Prasert Auewarakul, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10% fetal bovine serum (FBS) (HyClone; GE Healthcare Life Sciences, Marlborough, MA, United States), 100 units/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine (Gibco) (complete DMEM).

The viruses used in this study included HCV infectious particles, one isolate each of DENV serotypes 1-4; one isolate of ZIKV; one isolate of Japanese encephalitis virus (JEV); one isolate each of Wuhan wild-type, alpha (B.1.1.7), beta (B.1.351), delta (B.1.617.2), and omicron (B.1.1.529) of SARS-CoV-2; Enterovirus 71 (EV71, genotype A, BrCr strain, ATCCVR-1775TM); and Coxackievirus A16 (CVA16) and PEDV (P70 strain, GII field isolate from a deceased infected piglet in Thailand).

The HCV infectious particles were prepared as described previously (Thueng-In et al., 2014). Full-length cDNA of pJFH-1 (Wakita et al., 2005) was linearized by digesting the plasmid with XbaI endonuclease (Fermentas, Burlington, ON, Canada), and 1 µg was transcribed in vitro by using a Megascript T7 kit (Ambion, Carlsbad, CA, United States). The RNA transcript
incubated at 37°C for 5 days. The culture supernatant containing the HCV infectious particles was concentrated by using a centrifugal device (Pall, Port Washington, NY, United States). The virus titer was determined by focus-forming assay (FFA).

The enteroviruses (EV71 and CVA16) were propagated in RD cells grown in complete DMEM at 37°C in a 5% CO2 atmosphere until the CPE was clearly observed. The Kärber formula (World Health Organization [WHO], 2004) was used to calculate the virus CCID<sub>50</sub> (10<sup>-80</sup> cells/well) for each viral stock.

Porcine epidemic diarrhea virus (PEDV) was propagated in the permissive Vero cells as for the DENV, ZIKV, and JEV for 2 days. The amount of the PEDV in the harvested cell spent medium was determined by the plaque (syncytial)-forming assay (Thavarasak et al., 2022). The virus was kept at −80°C in small portions until use.

Preparation of Cell-Penetrating Human Superantibody to Hepatitis C Virus RNA-Dependent RNA Polymerase

HB2151 E. coli clone 34 that carried pCANTAB5E phagemid with inserted gene sequence coding for human single-chain antibody variable fragment (huscFv) specific to HCV RdRp (HuscFv34) was generated previously by using phage display technology (Thueng-In et al., 2014). Recombinant HCV NS5BΔ55 (RdRp) protein was used as an antigen in the phage bio-panning to select out the antigen-bound phage clones from the HuscFv phage display library (Kulkaw et al., 2009). One of the HB2151 E. coli clones (clone 34) infected with the antigen-bound phage produced HuscFv (HuscFv34) that inhibited the HCV RdRp activity in vitro (Thueng-In et al., 2014) and when the HuscFv34 was linked to a cell-penetrating peptide (CPP), i.e., penetratin (PEN), the PEN-HuscFv34 could enter the Huh7 cells (being superantibody). The superantibody not only inhibited HCV replication ex vivo but also rescued the host's innate immunity from the HCV suppression (Thueng-In et al., 2014).

In this study, the huscFv from the recombinant huscFv-phagemid of the HB2151 E. coli clone 34 was subcloned to recombinant pET23b+ plasmid backbone carrying a DNA insert coding for a protein transduction domain/cell-penetrating peptide, penetratin (PEN) (Poungpair et al., 2010), and the DNA construct was introduced to BL21(DE3) E. coli. Non-chromatographic purification of the E. coli inclusion body (IB) was used to isolate the PEN-HuscFv34 from the bacterial cells grown under IPTG induction conditions. Four grams of the bacterial pellet was resuspended with 20 mL of 1 × BugBuster® protein extraction reagent (Millipore, Merck KGaA, Darmstadt, Germany) dissolved in 50 mM tris(hydroxymethyl)aminomethane (Tris; Millipore, Merck KGaA), pH 8.0. After the bacterial pellet was completely resuspended, Lysonase™ bioprocessing reagent (Millipore, Merck KGaA) was added at 10 µL per gram of the wet bacterial pellet. After 20 min at room temperature (25 ± 2°C) on a slow setting shaking platform, the soluble fraction was removed from the preparation by centrifugation at 8000 × g for 30 min. The insoluble contents was washed with wash-100 reagent [50 mM phosphate buffer, pH 8.0, 500 mM sodium chloride (Kemaus, CherryBrook, NSW, Australia), 5 mM ethylenediaminetetraacetic acid (Kemaus), 8% (v/v) glycerol (Kemaus), and 1% (v/v) Triton X-100 (USB, Affymetrix, Thermo Fisher Scientific)] at 25°C and wash-114 reagent [50 mM Tris-HCl pH 8.0, 500 mM sodium chloride, 1% (v/v) Triton X-114 (Sigma Aldrich, St. Louis, MO, United States)] at 4°C. The preparation was spun down at 8000 × g for 30 min and the supernatant was removed. The inclusion body was washed with deionized distilled water and collected by centrifugation at 8000 × g for 30 min. The PEN-HuscFv34 was retrieved from the inclusion body by solubilization and refolding. The inclusion body solubilization was performed by dissolving the inclusion body in 50 mM N-cyclohexyl-3-aminopropansulfonic acid (CAPS) (Sigma Aldrich, St. Louis, MO, United States) buffer, pH 10.8 supplemented with 0.3% (w/v) sodium lauryl sarcosinate (Sigma Aldrich, St. Louis, MO, United States) and 1 mM dithiothreitol (DTT; USB, Affymetrix) at a protein concentration of 1 mg/mL. Solvation of the inclusion body was performed at room temperature for 15 min followed by keeping at 4°C for 16 h. The non-solubilized part was removed by centrifugation at 10,000 × g for 10 min. The preparation was immediately refolded by buffer exchange against 20 mM imidazole, pH 8.5 with and without 0.1 mM DTT. The refolded PEN-HuscFv34 was subsequently verified by SDS-PAGE and Coomassie Brilliant Blue G-250 (CBB) staining.

Verification of the Cell-Penetrating Ability of the Penetratin-HuscFv34

Human hepatocellular carcinoma cells (1 × 10<sup>5</sup> cells) in complete DMEM were seeded onto a cover glass placed in a well of 24-well
cell culture plate and incubated at 37°C in a 5% CO₂ atmosphere overnight. The established cell monolayer was added with the PEN-HuscFv34 prepared from the E. coli inclusion body and kept at 37°C in a 5% CO₂ atmosphere for 24 h. The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeated with 0.1% Triton X-100 (USB, Affymetrix) in PBS. The cells were blocked with 5% BSA in PBS at room temperature for 20 min. After the excess BSA was removed by washing with PBS, rabbit anti-HuscFv34 was added to the cell monolayer and incubated for 1 h. Goat anti-rabbit Ig-AlexaFlour488 (1: 200; Thermo Fisher Scientific) was used as the secondary antibody, and DAPI was used to locate nuclei. After washing, the cells were mounted and observed under a confocal microscope (Nikon, Melville, NY, United States) for intracellular PEN-HuscFv34.

**Biocompatibility of the Penetratin-HuscFv34/Superantibody to Mammalian Cells**

Mammalian cells including A549, Huh7, Vero, and Vero E6 cells (4 × 10⁴) were seeded separately in a 96-well white plate (Corning, Thermo Fisher Scientific) and incubated at 37°C in the CO₂ incubator overnight. The fluids were discarded; the cells were replenished with a culture medium containing PEN-HuscFv34 (0.25, 0.5, 1.0, 1.5, and 2.0 µM) and kept at 37°C in the CO₂ incubator overnight. Cytotoxicity of the superantibody was determined by using Cytoxtox-Glo™ Cytotoxicity Assay (Promega, Madison, WI, United States). The assay buffer provided with the kit was added to each well (50 µL/well), and the plate was kept at room temperature for 15 min. Experimental dead cell luminescence was detected by using Multidetection Microplate Reader Synergy H1 (Biotek, Agilent Technology, Santa Clara, CA, United States). Lysate reagent of the test kit was then added to all wells (50 µL/well), and the plate was placed on an orbital shaker (100 rpm) for 15 min. Total dead cell luminescence was detected, also by the microplate reader. Viable cell luminescence (Test luminescence) was calculated: Test luminescence = Total dead cell luminescence – Experimental dead cell luminescence. Percent cell viability was calculated: (Test luminescence ÷ Normal cell luminescence) × 100.

**RNA Virus Replication Inhibition Mediated by Superantibody**

Ten micromolars of HCV-JFH1 RNA was transfected into Huh7 cells by electroporation. The transfected cells were immediately seeded to 12-well cell culture plate (2 × 10⁵ cells/well) and incubated at 37°C, 5% CO₂ for 6 h. After washing the cells, the complete DMEM containing various concentrations of superantibody (0.25, 0.5, 1.0, 1.5, and 2.0 µM) or medium alone was added. The treated cells were cultured at 37°C in a 5% CO₂ atmosphere for 5 days. The RNAs were extracted from the treated cells for viral RNA quantification by real-time PCR; the HCV infectious particles in the culture supernatants were enumerated by focus-forming assay (FFA).

Vero cells (3 × 10⁴ cells) were seeded to 12-well cell culture plates and incubated at 37°C in a 5% CO₂ incubator overnight. DENV (serotypes 1-4), ZIKV, and JEV at MOI 0.1 and PEDV at MOI 0.0005 were added individually to the Vero cells in different wells and incubated further for 1 h. After washing with plain DMEM, complete DMEM containing the PEN-HuscFv34/superantibody (0.25, 0.5, 1.0, 1.5, and 2.0 µM) or medium alone (negative control) were added to appropriate wells containing the infected cells and incubated for 24 h for the PEDV and 48 h for the other viruses. The culture supernatants and cells were collected, and RNA was extracted from each sample and subjected to real-time RT-PCR for viral RNA quantification. Infectious virus particles in the culture supernatant samples were enumerated by the plaque-forming assay (PFA).

The RD cells were transfected with 100 CCID₅₀/mL of EV71 strain BrCr or MOI 0.1 of CVA16. After 1 h incubation at 37°C in a 5% CO₂ incubator, cells were washed one time with plain DMEM and replaced with complete DMEM containing superantibody (0.25, 0.5, 1.0, 1.5, and 2.0 µM) or medium alone as a negative control. Cells were incubated further for 24 h. Then, RNA was extracted from the collection and the viral RNA was quantified by real-time RT-PCR. Infectious virus particles in the culture supernatants were enumerated by PFA.

For SARS-CoV-2 replication inhibition, 1.5 × 10⁵ cells of Vero E6 cells were seeded to wells of 24-well cell culture plate and incubated at 37°C, 5% CO₂ for 24 h. The plates were moved to the BSL-3 room to perform all the subsequent processes of the experiment. The seeded cells were infected with SARS-CoV-2 [Wuhan wild type, alpha (B.1.1.7), beta (B.1.351), delta (B.1.617.2), and omicron (B.1.1.529)] at 50 PFU/well. After 1 h incubation, the supernatants were removed and replenished with the superantibody (0.25, 0.5, 1.0, 1.5, and 2.0 µM) containing DMEM supplemented with the 2% FBS. The treated cells were incubated at 37°C, 5% CO₂ for 18 h. The RNAs were extracted from the cells for the real-time RT-PCR, and the culture supernatants were collected to detect the infectious particles by PFA for the Wuhan wild type and α, β, and δ variants and by FFA for the omicron variant (their plaques in the PFA were too tiny to be counted accurately).

**Real-Time RT-PCR**

The RNAs from the superantibody/medium-treated infected cells were extracted using TRizol® reagent (Invitrogen). The amounts of viral RNA were quantified by real-time RT-PCR using a 1-step brilliant III SYBR green RT-qPCR master mix (Agilent Technologies). The real-time RT-PCR primers for each virus and house-keeping gene control are listed in Supplementary Table 1. The copy numbers of viruses were calculated from the Cq value using a comparative method.

**Plaque-Forming Assay**

The Vero or Vero E6 cells were seeded into wells of 24-well culture plates (1.5 × 10⁵ cells per well) and kept in humidified 5% CO₂ incubator at 37°C overnight. The virus-containing samples were diluted 10-fold serially, and 250 µL aliquots were added to the wells containing the cell monolayer. Experiments involving SARS-CoV-2 were performed in BSL-3. The plates were incubated further for 1 h; the fluids were discarded; the infected cells were rinsed with sterile PBS before adding with 1.5% carboxymethyl cellulose (CMC) (Sigma Aldrich, St. Louis, MO, United States) in complete DMEM and the plates were incubated further for 3 days (SARS-CoV-2) or 7 days (DENV, ZIKV, and
JEV). After incubations, the infected cells were fixed with 10% formaldehyde at room temperature for 1 h (2 h for SARS-CoV-2). The cells were washed with distilled water five times to get rid of the CMC and stained with 1% crystal violet in 10% ethanol at room temperature for 15 min. After washing with distilled water, the plates were dried, and plaques were counted visually. The amount of the virus in the original sample was calculated: PFU/mL = plaque number/(infection volume × dilution factor).

The RD cells were seeded on a 24-well culture plate (1.5 × 10^5 cells per well) and incubated at 37°C in a 5% CO_2 incubator overnight. After discarding the supernatant, the 10-fold serially diluted supernatants of the superantibody-mediated virus replication inhibition experiments were added into appropriate RD cell-containing wells, and the plates were incubated further for 1 h. The fluids were removed and 1.5% CMC in complete DMEM was added to each well and incubated further for 72 h. The cells were fixed with formalin and stained with crystal violet dye as described earlier. Plaque number were counted by eyes, and the number of viruses in the original sample was calculated.

For PEDV, after incubating with 10-fold diluted samples, the extracellular fluids were discarded; the cells were rinsed with sterile PBS, added with 1.5% CMC (Sigma Aldrich, St. Louis, MO, United States) in DMEM containing N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) trypsin, and incubated at 37°C in a 5% CO_2 atmosphere for 2 days. The cells were fixed with formalin and stained with crystal violet dye as described earlier. The CPE (syncytial formation) was enumerated under a microscope × 5 magnification), and the number of viruses in the original preparation was calculated.

**Focus-Forming Assay**

Vero E6 cells (4 × 10^4 cells/well) were seeded to wells of a 96-well cell culture plate and incubated at 37°C, 5% CO_2 for 24 h. The cell-seeded plates were moved to the BSL-3 room. The samples containing viruses (SARS-CoV-2 omicron variant) were 10-fold serially diluted. The fluids were removed from the cell-containing wells and replaced with 50 μL of the diluted virus samples. After 1 h incubation, the fluids were removed, replaced with 1.5% CMC in complete DMEM, and incubated further at 37°C, 5% CO_2 for 3 days. The CMC was removed from wells; the cells were fixed with 10% formaldehyde at room temperature for 2 h, washed with PBS three times, and permeated with 0.1% Triton X-100 in PBS at room temperature for 20 min. After washing twice with PBS, the cells were blocked with 5% BSA in PBS and stained with mouse anti-SARS-CoV-2 nucleoprotein antibody (1:5000) at room temperature for 1 h followed by incubating with goat anti-mouse IgG-HRP conjugate (SouthernBiotech, Birmingham, AL, United States). After the 1-h incubation, the cells were washed with PBS and the foci were developed by adding TMB sure blue substrate (SeraCare Life Sciences, Milford, MA, United States). The focal numbers were counted under an inverted light microscope, and the FFU/mL was calculated as mentioned earlier.

**Computerized Simulation to Determine Presumptive Interaction Between the Viral RNA-Dependent RNA Polymerase and the Human Single-Chain Antibody Variable Fragment**

Amino acid sequences of the HuscFv34 and three-dimensional (3D) structures of RdRp of DENV serotypes 1 and 4 and of PEDV were submitted for protein modeling using AlphaFold2 (Jumper et al., 2021) available in ColabFold’s online notebook (Mirdita et al., 2022). Modeled 3D structure of the HuscFv34 was docked against existing crystal structures of RdRp of different viruses, [HCV (PDB ID: 1QUV), DENV serotype 2 (PDB ID: 6IZY), DENV serotype 3 (PDB ID: 2J7U), ZIKV (PDB ID: 6LD1), JEV (PDB ID: 4MTP), EV71 (PDB ID: 3N6L), CVA16 (PDB ID: 5Y6Z), and SARS-CoV-2 (PDB ID: 6M71)], and the predicted 3D structures of RdRp of DENV serotypes 1 and 4 and PEDV, via HADDOCK server version 2.4 (van Zundert et al., 2016). The parameters from the HADDOCK (HADDOCK scores, van der Waals energy, electrostatic energy, desolvation energy, restraint violation energy, buried surface area, and Z-Score) were collected. The intermolecular docking that showed the best HADDOCK score was selected. PyMol software (The PyMOL Molecular Graphics System, Version 2.5.2, Schrodinger, LLC, NY, United States) was used for building the molecular interactive protein structure models. The docked structures were further submitted to the PRODIGY server to predict the binding energy ΔG (kcal per mol) and K_d (M) at 25°C (Honorato et al., 2021).

**Statistical Analysis**

GraphPad Prism version 9 software (GraphPad Software, San Diego, CA, United States) was used for the calculation of the half-maximal effective dose (EC_{50}) of the superantibody. Mean values and standard deviations (SD) of each treatment group from three independent experiments were compared using a one-way ANOVA. P-values of 0.05 or lower were considered statistically different: p > 0.05 (ns, not significant); p ≤ 0.05 (**), p ≤ 0.01 (***), p ≤ 0.001 (****), and p ≤ 0.0001 (*****).

1[www.graphpad.com](http://www.graphpad.com)
RESULTS

Penetratin-Linked HuscFv34 to Hepatitis C Virus RNA-Dependent RNA Polymerase

Penetratin-linked human single-chain antibody variable fragments (PEN-HuscFv34) was produced from transformed BL21(DE3) E. coli grown under an IPTG-induced condition. The yield of the bacterial inclusion body obtained from the E. coli homogenate was 7.029 g/L of the bacterial culture. After the solubilization and refolding, the total protein obtained from each mg of the inclusion body was 680 µg. The CBB-stained SDS-PAGE-separated purified preparation revealed a single protein band with a relative mass of 30 kDa (Figure 1A), the correct molecular mass for PEN-HuscFv, in which each molecule consists of a VH domain linked to the VL domain via the (Gly₄Ser)₃ linker plus 16 amino acids (RQIKIWFQNRRMKWKK) of penetratin (Pourangpair et al., 2010). As shown in Figure 1B, the PEN-HuscFv34 (green) could enter the mammalian cells (being cell-penetrable antibody/superantibody). The superantibody did not cause cytotoxicity to the mammalian cells that were tested, as determined by using the CytoTox-Glo™ Cytotoxicity Assay, based on the Practical Guide to ISO 109903-5 (Wallin, 1998; Figure 1C). The superantibody-treated cells appeared unchanged in their morphology under the light microscope (data not shown).

Inhibition of RNA Virus Replication by Superantibody

The ability of the superantibody specific to HCV RdRp (PEN-HuscFv34) in inhibiting replication of the homologous virus and
Inhibition of the plus-sense RNA virus replication by the superantibody shown as percent recovered viral RNAs inside the superantibody-treated infected cells, when compared with the infected cells in the medium alone. (A) Viruses of the family Flaviviridae (HCV, DENV1–4, ZIKV, and JEV); (B) Enteroviruses of the family Picornaviridae (EV71 and CVA16); (C) Members of the family Coronaviridae (genus Betacoronavirus: SARS-CoV-2 Wuhan wild-type and variants of concerns: α, β, δ, and omicron; and genus Alphacoronavirus: PEDV); (D) Half-maximal effective dose (EC$_{50}$) of the superantibody against the tested viruses. Other plus-sense RNA viruses in the family Flaviviridae (DENV1–4, ZIKV, and JEV), Picornaviridae (EV71 and CVA16), and Coronaviridae (PEDV and SARS-CoV-2) was determined. Details of the fold reduction of virus RNA from infected cells treated with the medium containing different concentrations of superantibody when compared with infected cells treated with the medium alone are shown in Supplementary Figures 1–3, and details of the reduction of released infectious viral particles (PFU/mL or FFU/mL) from the virus-infected cells treated with the medium-containing different concentrations of superantibody to RdRp when compared with infected cells treated with the medium alone are shown in Supplementary Figures 4–6.

Computerized Simulation to Determine Presumptive Interaction Between the Viral RNA-Dependent RNA Polymerase and the HuscFv34

In this study, the in-silico interactions of the homology modeled HuscFv34 3D structure with RdRp of the HCV and other plus-sense RNA viruses were determined by using the available crystal structures of HCV, DENV serotypes 2 and 3, ZIKV, JEV,
Reduction of the infectious viral particles released from the infected cells after treatment with various concentrations of the superantibody when compared with infected cells in the medium alone. (A) Viruses of the family Flaviviridae; (B) Enteroviruses of the family Picornaviridae; (C) Viruses of the family Coronaviridae.

**TABLE 1** | EC$_{50}$ (nM) of penetratin (PEN)-HuscFv34 in replication inhibition of the tested viruses.

| Flaviviridae | | | | | | | |
|---|---|---|---|---|---|---|---|
| Virus name | HCV | DENV1 | DENV2 | DENV3 | DENV4 | ZIKV | JEV |
| EC$_{50}$ | 65.6 | 232 | 553.6 | 336.3 | 282.5 | 473.9 | 464.4 |

| Picornaviridae | | | | | | |
|---|---|---|---|---|---|---|
| Virus name | EV71 | CVA16 |
| EC$_{50}$ | 322.4 | 369.6 |

| Coronaviridae | | | | | |
|---|---|---|---|---|
| Betacoronavirus | | | | |
| SARS-CoV-2 | Wuhan | α (B.1.1.7) | β (B.1.351) | δ (B.1.617.1) | omicron (B.1.1.529) |
| Variant | | | | | |
| EC$_{50}$ | 356.4 | 413.4 | 355.7 | 597.7 | 831.6 | 186.3 |

EV71, CVA16, and SARS-CoV-2 and modeled 3D structures of DENV serotypes 1 and 4 and PEDV, for which the crystal structures were not yet available. The data for computerized prediction of HuscFv34 and RdRp models and their interaction are summarized in Supplementary Table 2. The computerized models of interaction between the HuscFv34 and the RdRp of the studied viruses are shown in Figure 4. The details on the residues and domains of the RdRp of the viruses that formed contact interface with residues in the CDRs of the HuscFv34 are given in Table 2.
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FIGURE 4 | Computerized models of interaction between HuscFv34 and viral RdRp. (A–G) Viruses of the family Flaviviridae (HCV, DENV1–4, ZIKV, and JEV); (H,I) viruses of the family Picornaviridae (EV71 and CVA16); and (J,K) viruses of the family Coronaviridae (SARS-CoV-2 and PEDV). The RdRp are shown as cartoons: fingers domains (deep blue), palm domains (orange), and thumb domains (pink). The cartoon colored in red represents the contact interface between the HuscFv34 (green cartoon structure) and the target RdRp. Gray cartoons in DENV, ZIKV, and JEV are N-terminal S-adenosyl methionine methyltransferases (MTases). Gray cartoons in SARS-CoV-2 are the beta-hairpin that sandwiches with the palm domain, the Nidovirus-specific extension domain (NIRAN) domain, and the interface subdomain of the viral nsp12.

DISCUSSION

RNA-dependent RNA polymerase (RdRp) is an inscribed protein of RNA viruses that is indispensable for the virus replication cycle. The protein is a principal component of the replicase/transcriptase complex that generates new genomic RNA and virus proteins which assemble to form virus progeny for further spread. RdRp is structurally conserved among the RNA viruses with no human homolog. Therefore, it is a potential target for a pan-anti-RNA virus agent. Currently, several small chemical inhibitors (both nucleoside and non-nucleoside inhibitors) that target the RdRp have been developed and tested for the treatment of the RNA virus infections; some of them have been approved and launched for clinical use while the others are at various phases of clinical trials or were discontinued (Tian et al., 2021). Examples of the nucleoside inhibitors that target RdRp are sofosbuvir (Sovaldi/PSI-7977/GS-7977) for the treatment of hepatitis B and C, favipiravir (T-705/Avigan/Favipiravir, Favi-lavir) for the treatment of influenza (repurposed for COVID-19 treatment), ribavirin (ICN-1229/Tribavirin) for the treatment of influenza, hepatitis C, and respiratory syncytial virus (RSV) infection (repurposed for the treatment of SARS in 2003 and COVID-19), and remdesivir for COVID-19 and other infections. More recently, a few non-nucleoside inhibitors of RdRp have been launched for the treatment of hepatitis C including dasabuvir (Exviera/Viekira Pak/Viekira XR/ABT-333) and lomibuvir (VX-222/VCH-222) (Tian et al., 2021). Limitations of the chemical inhibitors besides their off-target
and adverse side effects such as teratogenicity, hemolytic anemia, gastro-intestinal disturbance, and others that preclude patients' compliance are their susceptibility to virus mutation; thus, often they must be used in combined medication among themselves, with other drugs or an interferon, for the treatment of viruses of the drug-resistant phenotypes, such as genotype I HCV.

Antibodies have been used for the treatment of human diseases including infectious, non-infectious, and toxin/venom-mediated maladies. For safety issues, the therapeutic antibodies or antibodies for passive immunization should have negligible immunogenicity in the recipients, implying that the fully human isotype is the safest antibody format. Although the penetratin (PEN) that has been linked to the HuscFv34 is derived from the third helix of *Drosophila* Antennapedia homeodomain protein (Derossi et al., 1994), it has been shown that dendritic cells (DCs) pulsed with this peptide could not activate autologous T cells, implying that the peptide is not immunogenic (Brooks et al., 2015). Currently, the PEN has been used in several vaccine studies to deliver tumor-associated antigens into antigen-presenting cells (APCs), and as a non-viral gene delivery vehicle in DNA vaccines, as well as carrying therapeutic substances into cellular compartments (reviewed by Brooks et al., 2010; Yang et al., 2019). However, in preclinical and clinical trials, the immunogenicity and biocompatibility of the PEN-HuscFvs must be investigated.

Antibody uses several residues in multiple CDRs in synergistic binding to the target, causing difficulty for the pathogens to create an antibody-escape target mutant that retains the inherent functional activity, particularly the proteins that require high conservation. The main concern in using therapeutic antibodies in the treatment of the virus infection is the antibody-dependent enhancement (ADE) (Kulkarni, 2020) that often aggravates the morbidity. Conventional antibodies elicit ADE by different mechanisms. For *Flavivirus* infection, the Fc fragments of the virus-antibody complexes bind to the Fc-receptors and enhance the virus entry to myeloid cells, leading to increment of the virus replication and viral load (extrinsic ADE) (Khandia et al., 2018). The intracellular virus may inhibit type 1 interferon response and activates the production of interleukin-10 that causes a type 2 (Th2) immune response bias, which heightens virus production and release (intrinsic ADE); the intrinsic ADE enhanced more DENV replication than the extrinsic ADE (Narayan and Tripathi, 2020). For other viruses, including respiratory viruses such as RSV, influenza virus, and coronavirus, the bi-/multi-valent antibodies may form large immune complexes that activate complement, causing the release/formation of anaphylatoxins, chemotaxis, and membrane attack complexes (MAC) that recruit immune and inflammatory cells to the infected areas and exacerbate the tissue inflammation, cytokine storm, cellular apoptosis, and multi-organ damage, i.e., the so-called immune enhancement ADE (Sánchez-Zuno et al., 2021). The antibody may promote virus entry to host cells by other mechanisms besides the Fc-mediated; for SARS-CoV-2, non-neutralizing antibodies to an epitope in the N-terminal domain (NTD) of the S1 subunit of the spike protein promote an upstanding/open form of the RBD by cross-linking two adjacent spike trimers, which then

| Residue | Region | Interaction bond |
|---------|--------|-----------------|
| A25     | Finger | V167 VL-CDR1   | Alkyl |
| N28     | Finger | Q164 VL-CDR1   | Hydrogen |
| N28     | Finger | G165 VL-CDR1   | Hydrogen |
| S29     | Finger | H168 VL-CDR1   | Hydrogen |
| S29     | Finger | H169 VL-CDR1   | Hydrogen |
| R32     | Finger | Q235 VL-CDR3   | Hydrogen |
| R32     | Finger | S137 VH-CDR3   | Hydrogen |
| R32     | Finger | F237 VL-CDR3   | Hydrogen |
| R32     | Finger | N138 VH-CDR3   | Hydrogen |
| S431    | Thumb  | H169 VL-CDR1   | Hydrogen |
| R490    | Thumb  | Q62 VH-CDR2    | Hydrogen |
| R498    | Thumb  | N57 VH-CDR2    | Hydrogen |
| R498    | Thumb  | T58 VH-CDR2    | Hydrogen |
| V498    | Thumb  | F236 VL-CDR2   | Pi-Alkyl |
| H502    | Thumb  | D33 VH-CDR1    | Hydrogen |
| H502    | Thumb  | W50 VH-CDR2    | Pi-Pi |
| R503    | Thumb  | D103 VH-CDR3   | Hydrogen |
| R503    | Thumb  | H169 VL-CDR1   | Pi-Alkyl |
| R503    | Thumb  | T234 VL-CDR3   | Hydrogen |
| K531    | Thumb  | N54 VH-CDR2    | Hydrogen |

| Residue | Region | Interaction bond |
|---------|--------|-----------------|
| H800*   | Thumb  | G165 VL-CDR1   | Hydrogen |
| T805*   | Thumb  | F236 VL-CDR3   | Pi-Sigma |
| E806*   | Thumb  | Q164 VL-CDR1   | Hydrogen |
| D807*   | Thumb  | H169 VL-CDR1   | Hydrogen |
| D807*   | Thumb  | H168 VL-CDR1   | Hydrogen |
| L809*   | Thumb  | H169 VL-CDR1   | Hydrogen |
| S810    | Thumb  | V167 VL-CDR1   | Hydrogen |
| S810    | Thumb  | H169 VL-CDR1   | Hydrogen |
| S810    | Thumb  | H168 VL-CDR1   | Hydrogen |
| R814    | Thumb  | G165 VL-CDR1   | Hydrogen |
| V829    | Thumb  | H169 VL-CDR1   | Pi-Anion |
| S830    | Thumb  | H169 VL-CDR1   | Pi-Anion |
| S892    | Thumb  | N54 VH-CDR2    | Electrostatic |
| D890    | Thumb  | S55 VH-CDR2    | Electrostatic |
| L898    | Thumb  | D103 VH-CDR3   | Hydrogen |
| W899    | Thumb  | D103 VH-CDR3   | Hydrogen |

| Residue | Region | Interaction bond |
|---------|--------|-----------------|
| K719    | Thumb  | Q235 VL-CDR3   | Hydrogen |
| R770    | Thumb  | H169 VL-CDR1   | Hydrogen |
| E834    | Thumb  | H168 VL-CDR1   | Salt bridge,Electrostatic |
| E834    | Thumb  | H169 VL-CDR1   | Hydrogen |
| Y838    | Thumb  | R238 VL-CDR3   | Electrostatic |
| R856    | Thumb  | H169 VL-CDR1   | Electrostatic |
| R856    | Thumb  | Y102 VH-CDR3   | Pi-Alkyl |
| A860    | Thumb  | Y102 VH-CDR3   | Hydrogen |
| K861    | Thumb  | G105 VH-CDR3   | Hydrogen |
| K861    | Thumb  | D106 VH-CDR3   | Salt bridge,Electrostatic |
| N868    | Thumb  | N54 VH-CDR2    | Hydrogen |
| D881    | Thumb  | N54 VH-CDR2    | Hydrogen |
| D881    | Thumb  | S55 VH-CDR2    | Hydrogen |
| D881    | Thumb  | N57 VH-CDR2    | Hydrogen |

(Continued)
| DENV3 RdRp | HuscFv34 | Interactive bond |
|------------|----------|------------------|
| residue    | region   |                  |
| T806       | Thumb    | H169 VL-CDR1    | Hydrogen         |
| E807       | Thumb    | H169 VL-CDR1    | Hydrogen         |
| D808       | Thumb    | H169 VL-CDR1    | Hydrogen, Electrostatic |
| T809       | Thumb    | H169 VL-CDR1    | Hydrogen         |
| W833       | Thumb    | H169 VL-CDR1    | Hydrogen, Electrostatic |
| E834       | Thumb    | H169 VL-CDR1    | Hydrogen, Electrostatic |
| E834       | Thumb    | S31 VH-CDR1     | Hydrogen         |
| E834       | Thumb    | H169 VL-CDR1    | Electrostatic, Hydrogen |
| A860       | Thumb    | S55 VH-CDR2     | Hydrogen         |
| Q681       | Thumb    | R72 VH-CDR2     | Hydrogen         |
| L684       | Thumb    | N57 VH-CDR2     | Hydrogen         |
| E878       | Thumb    | Q164 VL-CDR1    | Hydrogen         |
| E878       | Thumb    | Q235 VL-CDR3    | Hydrogen         |
| L880       | Thumb    | H168 VL-CDR1    | Hydrogen         |
| D881       | Thumb    | F236 VL-CDR3    | Pi-Anion, Pi-Sigma |
| Y882       | Thumb    | H169 VL-CDR1    | Hydrogen         |
| M883       | Thumb    | N52 VH-CDR2     | Hydrogen         |

| DENV4 RdRp | HuscFv34 | Interactive bond |
|------------|----------|------------------|
| residue    | region   |                  |
| K812       | Thumb    | D106 VH-CDR3    | Hydrogen, Electrostatic |
| K812       | Thumb    | E108 VH-CDR3    | Hydrogen, Electrostatic |
| P830       | Thumb    | T28 VH-CDR1     | Hydrogen         |
| H832       | Thumb    | H32 VH-CDR1     | Hydrogen         |
| H832       | Thumb    | G26 Electrostatic | Hydrogen         |
| H832       | Thumb    | T28 VH-CDR1     | Hydrogen, Electrostatic |
| E835       | Thumb    | Y104 VH-CDR3    | Hydrogen         |
| D836       | Thumb    | T30 VH-CDR1     | Hydrogen         |
| R872       | Thumb    | N170 VL-CDR1    | Hydrogen         |
| Y880       | Thumb    | N172 VL-CDR1    | Hydrogen         |
| D882       | Thumb    | N172 VL-CDR1    | Hydrogen         |
| P885       | Thumb    | Y102 VH-CDR2    | Pi-Alkyl         |
| R888       | Thumb    | N52 VH-CDR2     | Hydrogen         |
| E895       | Thumb    | H168 VL-CDR1    | Hydrogen         |
| E895       | Thumb    | Q235 VL-CDR3    | Hydrogen         |
| E895       | Thumb    | F236 VL-CDR3    | Hydrogen         |
| Y890       | Thumb    | Y102 VH-CDR3    | Hydrogen         |
| A892       | Thumb    | Y102 VH-CDR3    | Pi-Alkyl         |

| ZIKV RdRp | HuscFv34 | Interactive bond |
|-----------|----------|------------------|
| residue    | region   |                  |
| K721       | Thumb    | H169 VL-CDR1    | Hydrogen         |
| L776       | Thumb    | G171 VL-CDR1    | Hydrogen         |
| K943       | Thumb    | N170 VL-CDR1    | Pi-Cation        |
| K943       | Thumb    | Q192 VL-CDR2    | Hydrogen         |
| G854       | Thumb    | Y174 VL-CDR1    | Hydrogen         |
| A962       | Thumb    | Y102 VH-CDR3    | Hydrogen         |
| A962, E863 | Thumb    | Y102 VH-CDR3    | Hydrogen         |
| E863       | Thumb    | F236 VL-CDR3    | Hydrogen         |
| E863       | Thumb    | V167 VL-CDR1    | Pi-Alkyl         |
| E863       | Thumb    | S137 VH-CDR3    | Hydrogen         |
| I865       | Thumb    | D103 VH-CDR3    | Amide-Pi Stacked |
| K866       | Thumb    | D103 VH-CDR3    | Hydrogen, Alkyl  |
| K866       | Thumb    | Y104 VH-CDR3    | Hydrogen, Pi-Alkyl |
| K866       | Thumb    | G105 VH-CDR3    | Hydrogen         |
| K866       | Thumb    | Y107 VH-CDR3    | Hydrogen         |
| K866       | Thumb    | D33 VH-CDR1     | Hydrogen         |
| D884       | Thumb    | S55 VH-CDR2     | Salt bridge, Electrostatic |
| D884       | Thumb    | N57 VH-CDR2     | Electrostatic    |

| PEDV RdRp | HuscFv34 | Interactive bond |
|-----------|----------|------------------|
| residue    | region   |                  |
| K412       | Fingers  | H169 VL-CDR1    | Hydrogen         |
| E413       | Fingers  | H169 VL-CDR1    | Electrostatic, Hydrogen |
| E413       | Fingers  | Y102 VH-CDR3    | Hydrogen         |
enhances the virus entry (Liu et al., 2021). For the influenza virus, the non-neutralizing antibody promotes the virus entry by increasing hemagglutinin stem flexibility and virus fusion to the cell membrane (Winarski et al., 2019). The antibodies may by increasing hemagglutinin stem flexibility and virus fusion to the virus, the non-neutralizing antibody promotes the virus entry enhances the virus entry (Liu et al., 2021). For the influenza virus, the non-neutralizing antibody promotes the virus entry by increasing hemagglutinin stem flexibility and virus fusion to the cell membrane (Winarski et al., 2019). The antibodies may by increasing hemagglutinin stem flexibility and virus fusion to

**TABLE 2** (Continued)

| PEDV RdRp Residue | HuscFv34 Region | Interactive bond |
|-------------------|----------------|------------------|
| E420 Fingers H169 | VL-CDR1        | Electrostatic, Hydrogen |
| E420 Fingers Q235 | VL-CDR3        | Hydrogen          |
| H885 Thumb D103  | VH-CDR3        | Salt bridge, Electrostatic, Hydrogen |
| K888 Thumb D103  | VH-CDR3        | Salt bridge, Electrostatic |
| K988 Thumb D33   | VH-CDR1        | Electrostatic     |
| K888 Thumb W50   | Pi-Alkyl       |
| Q886 Thumb Y104  | VH-CDR3        | Hydrogen          |
| N919 Thumb Y104  | VH-CDR3        | Hydrogen          |
| A992 Thumb Y104  | VH-CDR3        | Hydrogen          |
| E996 Thumb D106  | VH-CDR3        | Hydrogen          |
| E996 Thumb H32   | VH-CDR1        | Electrostatic     |

| SARS-CoV-2 RdRp Residue | HuscFv34 Region | Interactive bond |
|-------------------------|----------------|------------------|
| F415 Fingers N54        | VH-CDR2        | Hydrogen         |
| N416 Fingers S55        | VH-CDR2        | Hydrogen         |
| D418 Fingers S55        | VH-CDR2        | Hydrogen         |
| D418 Fingers Y104       | VH-CDR3        | Hydrogen         |
| K426 Fingers Y102       | VH-CDR3        | Hydrogen         |
| K499 Thumb S55          | VH-CDR2        | Hydrogen         |
| Q686 Thumb H169         | VL-CDR1        | Hydrogen         |
| R889 Thumb H169         | VL-CDR1        | Hydrogen, Pi-Alkyl |
| K990 Thumb D103         | VH-CDR3        | Salt bridge, Electrostatic |
| K990 Thumb H169         | VL-CDR1        | Pi-Alkyl         |
| D990 Thumb H168         | VL-CDR1        | Salt bridge, Electrostatic |
| E994 Thumb N57          | VH-CDR2        | Hydrogen         |
| E994 Thumb F236         | VL-CDR3        | Pi-Sigma         |

*Priming loop of the thumb domain.

The HCV RdRp epitope bound by the HuscFv34 was identified previously (by phage mimotope search using 12-mer peptide phage display library and competitive peptide ELISA) as a conformational epitope that is composed of residues in the finger's tip of the finger domain and helix O of the thumb domain of the HCV RdRp (NS5B protein), which were juxtaposed upon the protein folding to form the roof of the active enzymatic groove (closed catalytic tunnel) (Thuong-In et al., 2014). There were three phage mimotopic peptides (mimotopes 1–3; M1-M3) derived from the mimotope search that matched with the stretched sequence of the HCV RdRp, including M1: ALPFMGYHNSVY matched with 22PISPLNSLRLHHNLVY40 of the Δ1 loop of finger domain; M2: NYPATNHTRTYP matched with residues 470GLSALTLHYFT481; and M3: IPVKSWPJRSPSS matched with residues 495PPRLAWHRARRA506 of the thumb domain (based on the identical, conserved, and semiconserved amino acid residues upon the pairwise alignment) (Thuong-In et al., 2014). In this study, the computerized simulation of the HuscFv34-HCV RdRp interaction was performed to verify the results of the previous finding. We did not model the interaction of the superantibody (PEN-HuscFv) with the target RdRps because the penetran (PEN) was linked to the HuscFv by a flexible linker and another end of the PEN was free. Besides, the PEN itself is not structured. Therefore, it should be inappropriate to fix the PEN in the rigid model for modeling and intermolecular docking as in reality the PEN would move freely while the HuscFv would be the principal part involved in target binding. By the in-silico analysis, the HuscFv34 interacted with residues of the HCV RdRp fingers domain, i.e., A25, N28, S29, and R32, located at the finger’s Δ1 extension loop [residues 111-146] that usually packs against the thumb domain to form active closing (form 1) of the HCV RdRp channel (Bressanelli et al., 1999). Binding of the HuscFv34 at the finger's Δ1 extension loop could disturb the conformation and rigidity of the enzymatic groove (Biswal et al., 2005). Besides the finger tip, the HuscFv34 also formed a contact interface with many residues at the back of the thumb domain. Previous evidence has shown that interaction of the HCV NS5B (RdRp) with a host component, nucleolin, is indispensable for HCV replication (Shimakami et al., 2006). Residue W500 and three arginines (R498, R501, and R503) at the armadillo-like arm repeats of the thumb domain (Bressanelli et al., 1999) are important for the nucleolin binding and the HCV replication (Kusakawa et al., 2007). The HuscFv34 interaction with several residues in this region of the thumb domain (shown in Figure 4A and Table 2) may interfere with the RdRp-host nucleolin interaction, hence HCV replication inhibition.

For dengue viruses, the RdRp is located at the C-terminal residues 270 to 900 of the bifunctional NS5 protein that contains 900 amino acids (the N-terminal residues of the NS5 form the enzyme S-adenosyl methionine transferase) (Yap et al., 2007). The thumb domain (residues 706-900) of the RdRp contains a motif (motif E/primer grip) that lies between the palm domain and α-helices of the thumb domain (Yap et al., 2007). There is a loop that spans amino acids 782 to 809 of the thumb
domain, called a priming loop. The priming loop together with another loop of the finger domain form the roof of the tunnel that regulates RNA entry and exit from the RdRp active site (Yap et al., 2007). Several residues of the priming loop protrude into the RdRp active groove and stabilize the NTPs on the RNA template at the initial stage of the de novo RNA synthesis; these residues also pad alongside the RNA template during the process of the RNA synthesis (Yap et al., 2007; Gong and Peersen, 2010). The thumb domain is also involved in the motility of the newly synthesized RNA. Various interactive bonds (hydrogen, salt bridge, stacking interaction) between amino acid residues of the priming loop, including Thr794 and Ser796, Glu807 and Arg815, and Arg749 and Trp787, contribute to maintaining the orientation of the RdRp protein (Yap et al., 2007). From the in-silico prediction, the HuscFv34 interacted with several residues in the priming loops of DENV1 and DENV3 of the thumb domain (asterisks in Table 2) that may interfere with their functional activity and/or cause a structural change of the protein, leading to impairment of the RdRp activity, hence the DENV replication inhibition.

From the in-silico analysis, the HuscFv34 is also predicted to form interaction with many residues at the back surface of the thumb domains of DENV1, DENV3, PEDV, and SARS-CoV-2 and interacted solely with the C-terminal helices of thumb domains of DENV2, DENV4, ZIKV, JEV, EV71, and CVA16, which could be the site of the polymerase interaction with other viral/host cellular proteins during the formation of the replication/transcriptase complex and replication initiation (Bressanelli et al., 1999). Several non-nucleoside chemical inhibitors have been shown to bind to allosteric sites on the outer surface of the thumb subdomain (Thumb II or T2) and cause changes in the spatial conformation of the enzyme, rendering it inactive and reducing the viral load (Le Pogam et al., 2006; De Clercq, 2013; Li et al., 2016; Lim et al., 2016; Tian et al., 2021).

The EC50 of the superantibody (PEN-HuscFv34) was found in the nanomolar range for all of the tested RNA viruses, ranging from 65.6 nM for the homologous HCV to 831.6 for SARS-CoV-2 omicron variant, which was comparable to the chemical nucleoside and non-nucleoside inhibitors: favipiravir EC50 for SARS-CoV-2 was 61.88 µM (Wang et al., 2020); cytosine analog (NHC, EIDD-1931) EC50 values for SARS-CoV-2 and MERS-CoV were 0.3 and 0.56 µM, respectively (Sheahan et al., 2020); EC50 values of remdesivir (GS-5734) in inhibiting SARS-CoV and MERS-CoV in human airway epithelial cells (HAE) were 0.069 and 0.07/0.074 µM, respectively (Sheahan et al., 2017; Agostini et al., 2018), and SARS-CoV-2 in Vero E6 cells were 0.77 µM (Wang et al., 2020) and 23.15 µM (Choy et al., 2020); EC50 value of ribavirin in inhibiting SARS-CoV-2 in Vero E6 cells was 109.5 µM (Wang et al., 2020; Frediansyah et al., 2021).

**CONCLUSION**

The cell-penetrating human single-chain antibody variable fragments (superantibody) specific to NS5B (RdRp) of HCV that were found previously to inhibit the HCV replication and that rescued the HCV suppressed host innate (anti-viral) immunity were tested against other RNA viruses of the families Flaviviridae (DENV1-4, ZIKV, JEV), Picornaviridae (EV71 and CVA16), and Coronaviridae (genus Alphacoronavirus: PEDV and genus Betacoronavirus: SARS-CoV-2 including Wuhan wild type and variants of concerns including alpha, beta, delta, and omicron). The superantibody inhibited replication of all RNA viruses that were tested in a dose-dependent manner. In-silico analysis indicated that the superantibody interacted mainly with the armadillo-like arm repeats at the back of the RdRp thumb domain, which may cause allosterical changes in the spatial conformation of the RdRp, rendering the enzyme inactive, hence virus replication inhibition. Although the molecular mechanisms of the superantibody against the viruses await experimental elucidation, data of this study persuade testing the superantibody further toward clinical application as a pan-direct acting anti-RNA virus agent.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

WC, MC, and KG-A contributed to the conceptualization, funding acquisition, resources, and project administration. MC, NS, and WC contributed to the methodology, data curation, formal analysis, supervision, visualization, and writing and editing the manuscript. KG-A, TT, TS, WT, KM, and MC contributed to the investigation, methodology, and visualization. KK and MC did the computerization. All authors have read and agreed to the published version of the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.926929/full#supplementary-material

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