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Design of a bifunctional pan-sarbecovirus entry inhibitor targeting the cell receptor and viral fusion protein

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ABSTRACT Development of highly effective antivirals that are robust to viral evolution is a practical strategy for combating the continuously evolved severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Inspired by viral multistep entry process, we here focus on developing a bispecific SARS-CoV-2 entry inhibitor, which acts on the cell receptor angiotensin converting enzyme 2 (ACE2) and viral S2 fusion protein. First, we identified a panel of diverse spike (S) receptor-binding domains (RBDs) and found that the RBD derived from Guangdong pangolin coronavirus (PCoV-GD) possessed the most potent antiviral potency. Next, we created a bispecific inhibitor termed RBD-IPB01 by genetically linking a peptide fusion inhibitor IPB01 to the C-terminal of PCoV-GD RBD, which exhibited greatly increased antiviral potency via cell membrane ACE2 anchoring. Promisingly, RBD-IPB01 had a uniformly bifunctional inhibition on divergent pseudo- and authentic SARS-CoV-2 variants, including multiple Omicron subvariants. RBD-IPB01 also showed consistently cross-inhibition of other sarbecoviruses, including SARS-CoV, PCoV-GD, and Guangxi pangolin coronavirus (PCoV-GX). RBD-IPB01 displayed low cytotoxicity, high trypsin resistance, and favorable metabolic stability. Combined, our studies have provided a tantalizing insight into the design of broad-spectrum and potent antiviral agent.

IMPORTANCE Ongoing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) evolution and spillover potential of a wide variety of sarbecovirus lineages indicate the importance of developing highly effective antivirals with broad capability. By directing host angiotensin converting enzyme 2 receptor and viral S2 fusion protein, we have created a dual-targeted virus entry inhibitor with high antiviral potency and breadth. The inhibitor receptor-binding domain (RBD)-IPB01 with the Guangdong pangolin coronavirus (PCoV-GD) spike RBD and a fusion inhibitor IPB01 displays bifunctional cross-inhibitions on pseudo- and authentic SARS-CoV-2 variants including Omicron, as well as on the sarbecoviruses SARS-CoV, PCoV-GD, and Guangxi pangolin coronavirus. RBD-IPB01 also efficiently inhibits diverse SARS-CoV-2 infection of human Calu-3 cells and blocks viral S-mediated cell-cell fusion with a dual function. Thus, the creation of such a bifunctional inhibitor with pan-sarbecovirus neutralizing capability has not only provided a potential weapon to combat future SARS-CoV-2 variants or yet-to-emerge zoonotic sarbecovirus, but also verified a viable strategy for the designing of antivirals against infection of other enveloped viruses.

KEYWORDS SARS-CoV-2, sarbecoviruses, receptor-binding domain (RBD), fusion inhibitor, bifunctional entry inhibitor

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to cause a global pandemic with more than 6.6 million deaths as of December 2022 (https://covid19.who.int/). In an effort to curb the pandemic, various prophylactic vaccines and therapeutics have been developed and authorized for emergency use (1, 2). However,

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the emergent SARS-CoV-2 variants of concern (VOCs), especially circulating Omicron lineages, have greatly impaired the purported efficacy of vaccinee's sera and neutralizing antibodies (NAbs) (3–7), thus being a major concern for the protective effects of vaccines and therapeutic NAbs. Thus, development of antivirals that are effective against a broad spectrum of sarbecoviruses, including SARS-CoV-2 and its variants, SARS-CoV, and SARS-related coronaviruses (SARSr-CoVs), is pivotal to prevent and control future outbreaks of emerging or re-emerging coronavirus diseases.

SARS-CoV-2 with a high dynamic mutation rate suggests that application of highly conserved target is the key to develop broad therapeutics. The cell entry of SARS-CoV-2 is mediated by viral spike (S) protein: the S1 subunit, containing a receptor-binding domain (RBD), is responsible for binding to the cell receptor angiotensin converting enzyme 2 (ACE2), while the S2 subunit, forming a six-helix bundle (6-HB) core structure via the interaction of heptad repeat 1 (HR1) and heptad repeat 2 (HR2), mediates fusion between viral and cellular membranes (8, 9). Consequently, RBD and 6-HB have been considered the important target sites for therapeutics development (10, 11). Disappointingly, multiple clinically RBD-targeted NAbs could not withstand RBD mutations (3, 12–15), and is likely to facilitate the emergence of accumulated escape mutations under strong selective pressure applied in the setting of therapeutics (3, 15–17), making these NAbs impracticable to release a broad-spectrum effect. Differently, ACE2 on the host cell surface can serve as an ideal target because initiation of virus infection is strictly ACE2-dependent. One of the strategies for ACE2 targeting uses a recombinant RBD protein that can block virus binding (18–20). Recently, our group and others demonstrated that 6-HB-targeted HR2-derived lipopeptide fusion inhibitors maintained the highly potent activities against a variety of SARS-CoV-2 variants, including Omicron subvariants, thus verifying their potential antiviral breadth for future emerging SARS-CoV-2 variants (21–23). Different from a native HR2 peptide, the lipopeptides can interact preferentially with the viral and cellular membranes, thus inhibiting virus-cell fusion with elevated local concentrations.

To combat against the persistently evolved SARS-CoV-2, here, we focus on developing a broad and potent bispecific entry inhibitor targeting cell ACE2 receptor and viral S2 fusion protein. The creation of dual-targeted entry inhibitors begins with the identification of the most effective RBD, named Guangdong pangolin coronavirus (PCoV-GD), from a panel of sarbecovirus RBD proteins. Four fusion proteins were then generated by linking the PCoV-GD RBD and a fusion inhibitor peptide IPB01 or IPB19 in different orders tethered via a GGGGS linker, and of them, a bispecific inhibitor RBD-IPB01 exhibited very potent and broad-spectrum inhibitory activities against SARS-CoV-2 and VOCs, as well as multiple other sarbecoviruses. RBD-IPB01 also exhibited low cytotoxicity, high trypsin resistance, favorable metabolic stability in human serum, and excellent thermostability. In conclusion, we developed a bifunctional entry inhibitor with a high resistance to SARS-CoV-2 escape and exceptional sarbecovirus breadth which highlights the potential clinical applications.

MATERIALS AND METHODS

Cells and plasmids

HEK293T cells, human hepatocellular carcinoma cell line Huh-7, and African green monkey kidney-derived Vero E6 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Human colon carcinoma cell line Caco-2 and lung adenocarcinoma cell line Calu-3 were kindly provided by Wei Yang and Zhaohui Qian, respectively, at the Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical Colleges (Beijing, China). hACE2-overexpressing human embryonic kidney cell line 293T/ACE2 was produced and preserved in our laboratory. Cells were cultured in Dulbecco's minimal essential medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS), penicillin-streptomycin (100 U/mL), 2 mM l-glutamine, and 1 mM sodium pyruvate at 37°C and 5% CO_2. Plasmids encoding the S proteins

of SARS-CoV-2 WH-Hu-1 strain (wildtype, WT) and VOCs (Alpha, Beta, Gamma, Delta, Lambda, and the Omicron sublineages BA.1, BA.2, BA.2.12.1, BA.4/5, BF.7, and XBB.1.5) were kindly provided by Linqi Zhang at the Tsinghua University (Beijing, China). Plasmid encoding the S protein of Omicron sublineage BA.2.13 harboring a specific mutation (L452M) was constructed via PCR-based site-directed mutagenesis. The mammalian codon-optimized genes encoding RBD proteins from WT, Delta, BA.1, the SARS-CoV-2-like pangolin CoVs GX-P5L (QIA48632.1) and GD-MP789 (QIG55945.1), SARS-CoV GD03 (AY525636.1), the civet SARS-CoV SZ16 (AY304488), the bat SARS-like CoVs SHC014 (KC881005.1), and WIV1 (AGZ48828.1), the bat CoVs RaTG13 (MN996532.1), RmYN02 (EPIISL412977), RacCS203 (QQM18864.1), RsYN04 (QWN56242.1), RsYN06 (QWN56252.1), Rco319 (LC556375), and RShSTT200 (EPI_ISL_852605), and genes encoding the bispecific inhibitors (IPB01-RBD, RBD-IPB01, IPB19-RBD, and RBD-IPB19) were fused with a C-terminal His tag and synthesized by Tsingke Biotechnology Co (Tianjin, China). The designed genes were inserted into the eukaryotic cell expression vector pcDNA3.4 between the Xba I and Age I sites.

Expression and purification of recombinant RBDs and bifunctional inhibitors

To produce RBD or bispecific inhibitors, a corresponding plasmid was transfected into HEK293T cells. At 6 h posttransfection, the culture medium was replaced with fresh complete DMEM containing 1% FBS. After an additional 48-h culturing at 37°C in a 5% CO_2 incubator, inhibitors containing cell supernatants were collected by centrifugation at 10,000 rpm for 5 min and purified using nickel-chelated affinity column chromatography, according to the manufacturer's protocol (QIAGEN GmbH, Germany). Purified RBD and bispecific proteins buffers were exchanged with phosphate-buffered saline (PBS, pH 7.4) and concentrated using amicon ultra-15 10 k centrifugal filter device (Millipore, Billerica, MA, USA) and stored in PBS at −80°C. To check the purity and molecular weight of purified RBD and bispecific proteins, the protein samples were separated on a 10% SDS-polyacrylamide gel and the gel was analyzed with Coomassie staining.

Flow cytometry assay

To examine the binding activity of RBDs for cell surface hACE2, 5×10^5 of 293T/ACE2 cells were incubated with different concentrations (9, 3, or 1 µg) of RBDs of SARS-CoV-2 WT, PCOV-GD, Guangxi pangolin coronavirus (PCOV-GX), RaTG13, and RshSTT200 for 60 min at 4°C. Next, the cells were sequentially stained with a mouse anti-His tag antibody and P-phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody for 45 min at 4°C. The stained cells were then washed twice with flow cytometry (FACS) buffer (phosphate-buffered saline solution with 0.5% bovine serum albumin and 2 mM EDTA) and resuspended in 0.2 mL of FACS buffer. FACS analysis was conducted with a FACSCanto II instrument.

Inhibition of recombinant protein inhibitors on sarbecoviruses

The inhibitory activities of RBD and bispecific inhibitors on several sarbecoviruses were determined by a single-cycle infection assay as described previously (21). In brief, the corresponding pseudoviruses (PsV) of SARS-CoV-2, SARS-CoV, and PCoV were generated by cotransfecting of HEK 293T cells with a lentivirus backbone plasmid (pNL4-3.Luc.R-E-) and a viral S protein-expressing plasmid using a linear polyetherimide (PEI) transfection reagent. After 48-h culturing, the pseudovirus-containing supernatants were collected by centrifugation at 4,000 rpm for 10 min and stored in aliquots in a freezer at −80°C. To assess the inhibitory activities of RBD and bispecific inhibitors, 50 µL of inhibitors were 3-fold serially diluted with DMEM containing 10% FBS and mixed with 50 µL of pseudovirus. At 1 h postincubation, the inhibitor-virus mixture was then added to Huh-7 or Calu-3 cells (3×10^4 cells per well) for infection and incubated for an additional 48 h. The relative light units of cells were measured with a luciferase assay system and a luminescence counter (Promega, Madison, WI, USA). The percent inhibition of pseudovirus infection and 50% inhibitory concentration (IC_{50}) of an inhibitor were calculated using GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA).

Inhibition of recombinant protein inhibitors on S-mediated cell-cell fusion

The inhibitory activities of bispecific inhibitor along with two templates on viral S-mediated cell-cell fusion were determined by a dual-split protein (DSP)-based cell-cell fusion assay as described previously (24). In brief, HEK293T effector cells were seeded at 1.5×10^4 cells/well in 96-well culture plates; Huh-7 target cells were seeded at 3×10^6 cells/dish in 10 cm culture dish. After incubation overnight, effector cells were cotransfected with an S-expressing plasmid and a DSP_{1-7}-expressing plasmid, and target cells were transfected with a DSP_{8-11}-expressing plasmid and cultured at 37°C for 24 h. To evaluate the inhibitory activities of bispecific inhibitors, 50 µL of inhibitors were 3-fold serially diluted with DMEM containing 10% FBS and then added into effector cells. At 1 h postincubation, target cells were resuspended in prewarmed culture medium containing the EnduRen substrate (Promega). After 30 min of incubation, target cells (3×10^4 cells per well) were added to effector cells and spun down to accelerate cell-cell contact and then cocultured for 6 h. Luciferase activity was measured as described above.

Inhibition of bifunctional inhibitor on authentic SARS-CoV-2 infection

For measuring the inhibitory activity of a bispecific inhibitor against replicative SARS-CoV-2, focus reduction neutralization test (FRNT) was conducted in a certified biosafety level 3 laboratory as previously described with minor modification (25). In brief, serially 3-fold dilutions of RBD-IPB01 inhibitor were added to Vero E6 cells seeded in 96-well plates and incubated for 1 h at 37°C. Then, 200 foci-forming unit of a live virus (WT, Delta, Omicron BA.2 or BA.4) were added to the culture wells and incubated for 1 h at 37°C. After replacing the supernatants with medium containing 1.6% carboxymethylcellulose and 2% FBS, the plates were incubated at 37°C for an additional 24 h. The cells were then fixed with 4% paraformaldehyde solution, permeabilized with Perm/Wash buffer (BD Biosciences, Franklin Lakes, NJ, USA) containing 0.1% Triton X-100, followed by incubation with horse radish peroxidase (HRP)-conjugated anti-SARS-CoV-2 N protein antibody P301-F7. The reactions were developed using KPL TrueBlue peroxidase substrate (Seracare Life Sciences Inc., Cambridge, MA, USA). The number of virus foci was quantified using an EliSpot reader (Cellular Technology Ltd., Cleveland, Ohio, USA). The percent inhibition of virus infection and 50% inhibitory concentration of RBD-IPB01 were calculated using GraphPad Prism software (GraphPad Software).

Cytotoxicity of inhibitors

The cytotoxicity of inhibitors was measured by cell counting kit-8 (CCK-8) (Abbkine, Wuhan, China) as described previously (26). In brief, Huh-7, 293T/ACE2, Vero E6, Caco-2, and Calu-3 cells were seeded at a density of 25,000 cells/well in 96-well plates, and 50 µL of DMEM serially diluted protein or peptide inhibitors were added to the cells. After incubation at 37°C for 48 h, 20 µL of CCK-8 solution reagent was added into each well and incubated for an additional 4 h at 37°C. The absorbance was measured at 450 nm with a Multiscan MK3 microplate reader (Thermo Fisher Scientific, MA, USA), and cell viability (percentage) was calculated.

Stability of RBD-IPB01 inhibitor

To assess the stability of bifunctional inhibitor, the fusion protein (2 mg/mL) was prepared in PBS (pH 7.2) in the presence of 0.1 mg/mL trypsin or 20% human serum. Then, the samples were stored at 37°C for 0, 30, 60, 120, 240, and 360 min, respectively; whereas for long-term thermostability test, the samples without any treatment were stored at 37°C for 0, 3, 6, 15, or 24 days. Samples were collected at set time and centrifuged immediately at 10,000 rpm for 10 min, and their antiviral activities were determined on Huh-7 cells in single-cycle infection as described above.

RESULTS

Identification of recombinant RBD proteins as SARS-CoV-2 entry inhibitors

To identify an RBD protein that can block SARS-CoV-2 entry more efficiently, genes encoding a panel of spike RBD sequences from diverse SARS-CoV- and SARS-CoV-2-related sarbecoviruses were constructed with a C-terminal His tag in a pcDNA3.4 vector. All RBDs were transiently expressed in HEK293T cells and purified from culture supernatants with a Ni Sepharose 6 Fast Flow Column. Coomassie blue-stained SDS-PAGE revealed that all RBDs had correct size and high purity (Fig. 1A).

We then applied SARS-CoV-2 pseudovirus bearing a D614G mutation (PsV/D614G) to examine the antiviral activities of recombinant RBDs on Huh-7 cells by a single-cycle infection assay. As shown in Fig. 1B, while most RBDs showed no effective inhibition at a high concentration (left panel), seven RBDs possessed potent inhibitory activities with IC_{50} values in the 10.95–314.89 nM range (right panel). Specifically, three RBDs derived from SARS-CoV-2 WT, Delta, and Omicron exhibited comparable inhibitory potencies, with IC_{50} of 48.12, 30.19, and 39.58 nM, respectively; two RBDs of pangolin SARS-CoV-2-like viruses (PCoV-GD and PCoV-GX) inhibited the virus with IC_{50} of 10.95 and 25.68 nM, respectively, whereas two RBDs of bat SARS-CoV-like viruses (WIV1 and SHC014) had cross-inhibitory activities with IC_{50} of 314.89 and 70.94 nM, respectively. In order to explore the inhibitory mechanism, we examined the cell surface-binding activities of five representative RBD proteins by flow cytometry (Fig. 1C). As judged by the relative cell counts and fluorescence intensities, RBD-PCoV-GD showed the most robust binding,

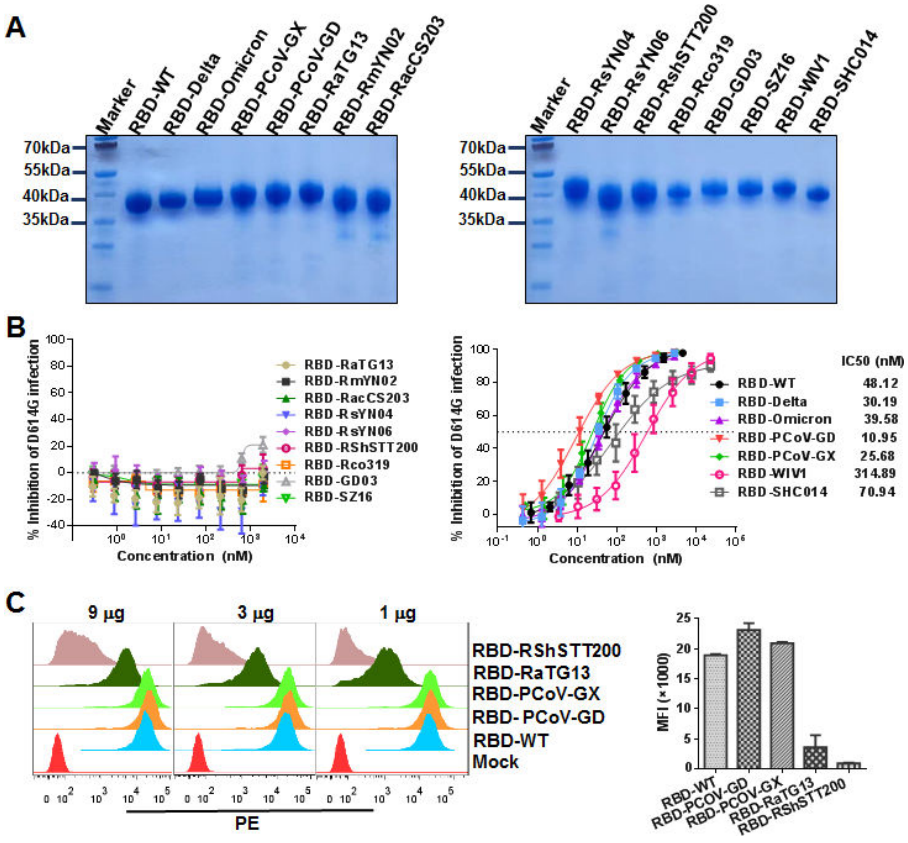

FIG 1 Expression and characterization of recombinant RBD proteins as SARS-CoV-2 entry inhibitors. (A) The purity and size of RBD proteins derived from SARS-CoV-2 WT, Delta, Omicron, PCoV-GX, PCoV-GD, RaTG13, RmYN02, and RacCS203 (left panel) and from RsYN04, RsYN06, RshSTT200, Rco319, GD03, SZ16, WIV01, and SHC014 (right panel) were analyzed by SDS-PAGE. (B) Inhibitory activities of RBD proteins on SARS-CoV-2 D614G pseudovirus were determined on Huh-7 cells by a single-cycle infection assay. Data are expressed as mean ± SD (n = 3). (C) The bindings of five representative RBD proteins to 293T/ACE2 cells were detected by FACS. MFI, mean fluorescent intensity.

whereas the RBD-RaTG13 and RBD-RShSTT200 bindings dramatically reduced, providing evidence for their antiviral efficacies. Therefore, the PCoV-GD RBD was identified with the most potent antiviral and cell receptor-binding activities, and thus, it was selected for designing bifunctional SARS-CoV-2 entry inhibitors.

Design and characterization of bifunctional inhibitors targeting host ACE2 and viral S2 protein

We previously reported that two HR2-derived peptides, IPB01 and IPB19, were effective inhibitors of SARS-CoV-2 and other human CoVs (24, 27). To construct an efficient bispecific SARS-CoV-2 entry inhibitor, the sequence encoding IPB01 or IPB19 was genetically connected to the N- or C-terminus of PCoV-GD RBD via a flexible $(GGGGS)_4$ linker, thus generating four fusion proteins designated RBD-IPB01, IPB01-RBD, RBD-IPB19, and IPB19-RBD (Fig. 2A). Similarly, the recombinant proteins were expressed and purified from HEK293T cells and analyzed by SDS-PAGE (Fig. 2B), which demonstrated their molecular integrity. In the single-cycle infection assay (Fig. 2C), the fusion proteins could potently inhibit the D614G pseudovirus infection, with RBD-IPB01 showing the highest potency. Comparing the IC_{50} values, RBD-IPB01 was about 15-fold more potent than the parental RBD inhibitor and at least 7,000-fold more potent than the fusion inhibitor peptide, which indicated its effectiveness as a bispecific inhibitor. Moreover, we determined whether the length of a flexible linker between the peptide sequence and RBD molecule could further improve the antiviral activity of RBD-IPB01. To this end, the linkers with one or seven repeats of GGGGS were applied to generate two new fusion

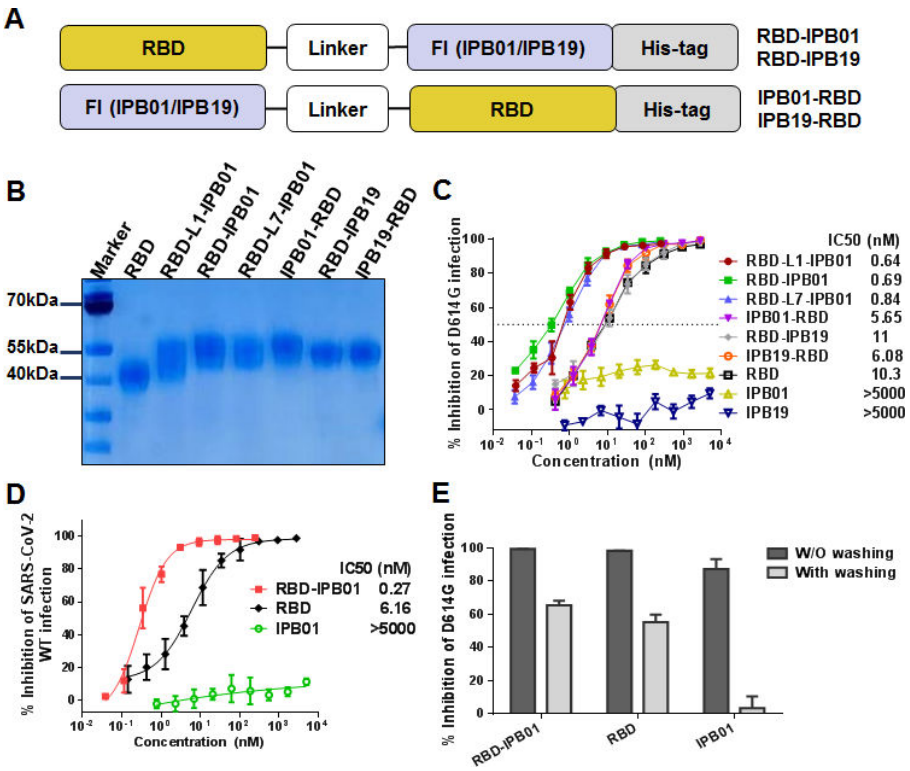

FIG 2 Design and characterization of bifunctional SARS-CoV-2 inhibitors targeting ACE2 receptor and S2 fusion protein. (A) Schematic diagram of design strategy of bispecific entry inhibitors. RBD-IPB01 and RBD-IPB19 represent two inhibitors by coupling IPB01 or IPB19 to the C-terminus of RBD. IPB01-RBD and IPB19-RBD represent two inhibitors by coupling IPB01 or IPB19 to the N-terminus of RBD. A His tag was incorporated into the C-terminus of the fusion proteins for easy purification and detection. (B) The analysis of purity and size of bispecific recombinant proteins by SDS-PAGE. (C) Inhibition of fusion proteins and parental inhibitors on SARS-CoV-2 D614G pseudovirus. (D) Inhibition of RBD-IPB01 fusion protein and parental inhibitors on SARS-CoV-2 WT pseudovirus. (E) Inhibitory activities of cell membrane-anchored inhibitors against SARS-CoV-2 D614G pseudovirus were measured on Huh-7 cells. W/O, without. Data are expressed as mean ± SD (n = 3).

proteins, termed RBD-L1-IPB01 and RBD-L7-IPB01. Assessment of their antiviral activities against the D614G pseudovirus suggested that linker length had no apparent effect on the efficacy of RBD-IPB01 (Fig. 2C). Consistently, RBD-IPB01 also showed a dramatically increased potency on ancestral SARS-CoV-2 WH-Hu-1 strain (WT) relative to RBD and IPB01 inhibitors (Fig. 2D).

To certify whether the dual-functional antiviral activity of RBD-IPB01 was a result of cell-surface-tethered IPB01 through RBD-ACE2 interaction, Huh-7 cells were pretreated with all inhibitors at a saturated concentration in a 96-well plate at 37°C for 1 h, followed by thorough washing with PBS solution to remove unbound inhibitors. After being challenged with a D614G virus, the antiviral activity of cell membrane-bound inhibitors was assessed. As shown in Fig. 2E, after the inhibitor-incubated cells were thoroughly washed, RBD-IPB01 and RBD displayed a largely sustained antiviral efficacy, whereas IPB01 had a dramatically reduced inhibitory activity, as anticipated. Taking these results together, it was validated that RBD-IPB01 exerted its bifunctional antiviral activity through cell membrane ACE2 receptor anchoring.

RBD-IPB01 is a highly potent inhibitor of divergent SARS-CoV-2 variants

We next sought to examine the inhibitory activities of RBD-IPB01 along with RBD protein and IPB01 peptide against diverse SARS-CoV-2 variants. A panel of pseudoviruses corresponding to SARS-CoV-2 variants, including Alpha, Beta, Gamma, Delta, Lambda, and Omicron sublineages BA.1, BA.2, BA.2.12.1, BA.2.13, BA.4/5, BF.7, and XBB.1.5, was prepared and used in the single-cycle infection assay. As shown in Fig. 3A; Table 1, RBD-IPB01 exhibited the significantly improved activity against all tested SARS-CoV-2 variants compared to its parental inhibitors. Specifically, IPB01 peptide could not effectively inhibit all viruses at a tested concentration. In sharp contrast, RBD-IPB01 and RBD potently inhibited 12 mutant viruses with mean IC_{50}s of 0.46 and 8.95 nM, respectively. In comparison, RBD-IPB01 was about 19-fold more potent than RBD in inhibiting a variety of viral variants. As lung cells are the physiologically primary target of SARS-CoV-2 infection (28, 29), we also determined the antiviral efficacy of RBD-IPB01 on Calu-3 cells. As shown, RBD-IPB01 effectively inhibited five representative pseudoviruses, including D614G, Alpha, Delta, BA.2, and BA.4/5, with IC_{50}s in the range of 0.33 to 0.67 nM (Fig. 3B).

Moreover, we were interested to characterize the inhibitory activity of RBD-IPB01 by a DSP-based cell fusion assay. As shown in Table 2, RBD-IPB01, RBD, and IPB01 were effective against diverse viral S-mediated cell-cell fusion, with mean IC_{50}s of 0.63, 9.28, and 29.77 nM, respectively. In comparison, RBD-IPB01 also showed superior efficiency with about 15-fold or 47-fold increased inhibitory activity than RBD and IPB01, respectively, further verifying its dramatic improved potency and breadth over the parental inhibitors.

RBD-IPB01 is a highly effective inhibitor of authentic SARS-CoV-2 infection

Having demonstrated that RBD-IPB01 was consistently effective in inhibiting diverse SARS-CoV-2 S-pseudotyped viruses, we were intrigued to know whether RBD-IPB01 could effectively inhibit live SARS-CoV-2 infection. To this avail, an FRNT was conducted, in which four representative live strains, including WT, Delta, BA.2, and BA.4, were applied to infect Vero E6 target cells in the presence of RBD-IPB01 inhibitor. In line with the results above, RBD-IPB01 could potently block the infection of four live SARS-CoV-2 at subnamolar concentrations, with IC_{50}s of 0.24, 0.33, 0.29, and 0.26 nM, respectively (Fig. 3C), indicating that it was a uniformly potent inhibitor of authentic SARS-CoV-2 infection.

RBD-IPB01 possesses broad-spectrum inhibitory activities against SARS-CoV and pangolin CoVs

Given the high pandemic potential of zoonotic and epidemic sarbecoviruses, we next went on to characterize whether RBD-IPB01 was also effective in blocking a broad

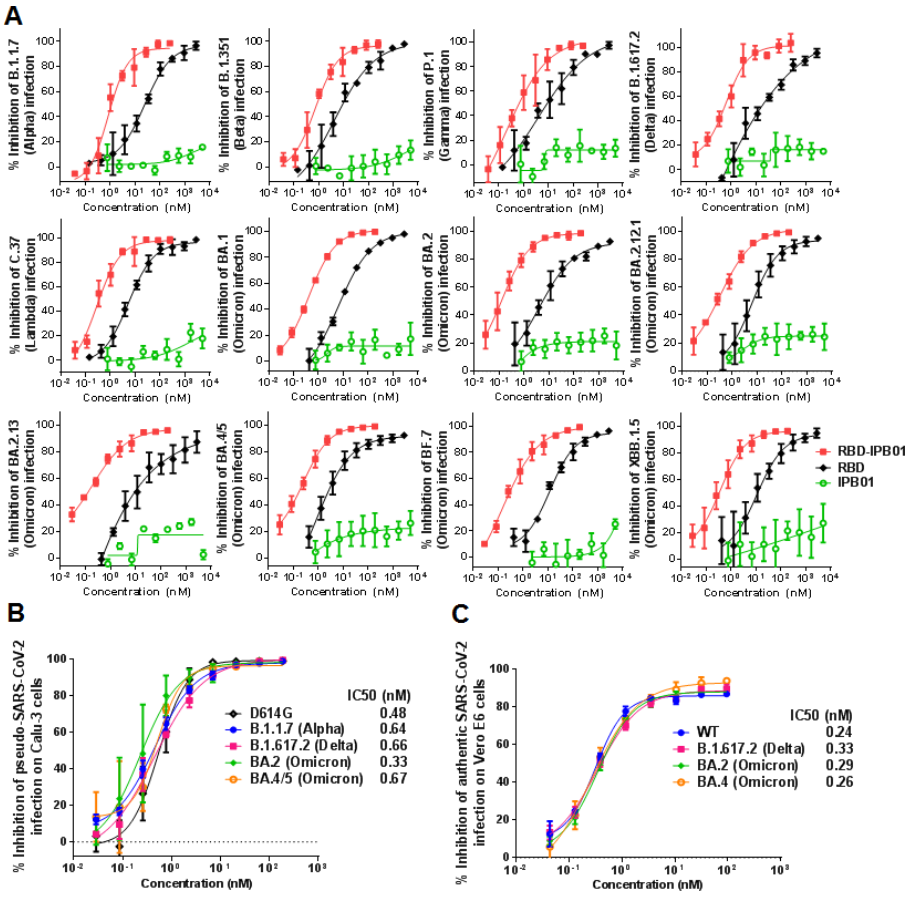

FIG 3 Inhibitory activities of RBD-IPB01 against divergent SARS-CoV-2 variants. (A) The inhibitory activities of RBD-IPB01 against a multitude of SARS-CoV-2 variants including Alpha, Beta, Gamma, Delta, Lambda, BA.1, BA.2, BA.2.12.1, BA.2.13, and BA.4/5 were determined on Huh-7 cells by a pseudovirus-based single-cycle infection assay. RBD protein and IPB01 peptide were used as control inhibitors. (B) Inhibitory activities of RBD-IPB01 against SARS-CoV-2 WT, Alpha, Delta, BA.2, and BA.4/5 pseudoviruses were measured on Calu-3 cells by the single-cycle infection assay. (C) Inhibitory activities of RBD-IPB01 against live SARS-CoV-2 WT, Delta, BA.2, and BA.4 strains were measured on Vero E6 cells by a focus reduction neutralization test assay. The experiments were repeated at least two times, and data are expressed as the means ± SD.

spectrum of sarbecoviruses. The pseudoviruses harboring the S proteins of SARS-CoV, PCoV-GD, and PCoV-GX were prepared and single-cycle infection assay was similarly performed on Huh-7 cells. Compared to the parental inhibitors RBD and IPB01, RBD-IPB01 displayed dramatically improved potencies against the infections of tested three viruses with mean IC_{50} values in the 0.16 to 0.72 nM range (Fig. 4A through C). Comparing the IC_{50} values, RBD-IPB01 was 3- to 9- fold more potent than RBD inhibitor and at least 6,900-fold more potent than IPB01 inhibitor. Combined, these results also confirmed that RBD-IPB01 was a highly effective bifunctional entry inhibitor of pan-sarbecoviruses.

RBD-IPB01 exhibits low cytotoxicity and high stability

We continued to characterize RBD-IPB01 for its cytotoxicity and biophysical stability. To validate the potent antiviral activity of RBD-IPB01, its potential side effects on several target cell lines (Huh-7, 293T/ACE2, Vero E6, Caco-2, and Calu-3) were assessed using CCK8 assay. As shown in Fig. 5A through D, similar to RBD and IPB01 controls, RBD-IPB01 did not show appreciable cytotoxicity at high concentrations, thus having a very high selectivity index (>6,800). The thermostability of RBD-IPB01 was detected by assessing its antiviral activities during 24 days of incubation at 37°C. As shown in Fig. 5E, the inhibitory

TABLE 1 Inhibitory activity of bifunctional inhibitor against divergent SARS-CoV-2 variants

Pseudovirus	Mean IC_{50} ± SD (nM)		
	RBD-IPB01	RBD	IPB01
B.1.1.7 (Alpha)	0.77 ± 0.06	26.01 ± 2.5	>5,000
B.1.351 (Beta)	0.71 ± 0.14	8.88 ± 0.21	>5,000
B.1.1.28 (Gamma)	0.52 ± 0.23	11.94 ± 8.52	>5,000
B.1.617.2 (Delta)	0.65 ± 0.38	10.09 ± 1.88	>5,000
C.37 (Lambda)	0.44 ± 0.25	7.22 ± 2.83	>5,000
BA.1 (Omicron)	0.48 ± 0.08	7.38 ± 1.18	>5,000
BA.2 (Omicron)	0.18 ± 0.05	5.47 ± 0.58	>5,000
BA.2.12.1 (Omicron)	0.53 ± 0.08	6.94 ± 0.97	>5,000
BA.2.13 (Omicron)	0.40 ± 0.12	8.82 ± 0.71	>5,000
BA.4/BA.5 (Omicron)	0.30 ± 0.08	3.01 ± 0.93	>5,000
BF.7 (Omicron)	0.42 ± 0.15	13.64 ± 0.55	>5,000
XBB.1.5 (Omicron)	0.53 ± 0.15	13.03 ± 2.17	>5,000
No. of viruses	12	12	12
Median IC_{50} (nM)	0.50	8.85	>5,000
GM[b] IC_{50} (nM)	0.46	8.95	>5,000
CC_{50} (nM)[a]	>3137.75	>11273.96	>50761.42
Selectivity index (SI)	>6,800	>1,200	NA[c]

[a]CC_{50}, the half-cytotoxic concentration of inhibitors on Huh-7, 293T/ACE2, Caco-2, and Calu-3 cells tested.
[b] GM indicates geometrical mean.
[c] NA indicates not determined.

activity of RBD-IPB01 against SARS-CoV-2 pseudovirus remained unchanged over the time. The metabolic stability of RBD-IPB01 in human serum was further assessed. After incubation at 37°C for 6 h, its inhibitory activity (IC_{50}) on SARS-CoV-2 infection was not altered (Fig. 5F and H), indicating that RBD-IPB01 had favorable metabolic stability. Moreover, RBD-IPB01 was treated with trypsin at 37°C, followed by collection of samples at different post-treatment time points for detection of their inhibitory activity against SARS-CoV-2 infection. Comparing the IC_{50} values, the inhibitory activity of RBD-IPB01 gradually decreased by 1.7- to 5-fold with prolonged incubation time (Fig. 5G and H). These results indicated that RBD-IPB01 is a highly potent entry inhibitor of diverse sarbecoviruses with low toxic effect and satisfactory stability.

TABLE 2 Inhibitory activity of bifunctional inhibitor against divergent SARS-CoV-2 variants S-mediated cell-cell fusion

Spike	Mean IC_{50} ± SD (nM)		
	RBD-IPB01	RBD	IPB01
WT	0.44 ± 0.10	5.74 ± 1.60	57.44 ± 16.57
D614G	0.51 ± 0.06	7.08 ± 4.05	36.34 ± 8.40
B.1.1.7 (Alpha)	0.34 ± 0.12	4.62 ± 0.49	48.15 ± 8.99
B.1.351 (Beta)	0.38 ± 0.03	7.72 ± 1.34	33.19 ± 10.61
B.1.1.28 (Gamma)	0.86 ± 0.20	10.21 ± 8.14	42.40 ± 23.18
B.1.617.2 (Delta)	0.90 ± 0.29	10.82 ± 4.64	38.23 ± 10.51
C.37 (Lambda)	0.95 ± 0.04	23.96 ± 5.42	32.26 ± 5.94
BA.1 (Omicron)	0.76 ± 0.15	11.85 ± 3.68	28.42 ± 9.82
BA.2 (Omicron)	0.39 ± 0.08	5.84 ± 0.30	15.11 ± 4.51
BA.2.12.1 (Omicron)	0.88 ± 0.15	12.91 ± 2.43	22.97 ± 7.78
BA.2.13 (Omicron)	0.97±0.11	8.25 ± 2.19	17.97 ± 6.48
BA.4/BA.5 (Omicron)	0.77±0.22	14.40 ± 1.52	17.23 ± 2.82
No. of spike	12	12	12
Median IC_{50} (nM)	0.77	9.23	32.73
GM IC_{50} (nM)	0.63	9.28	29.77

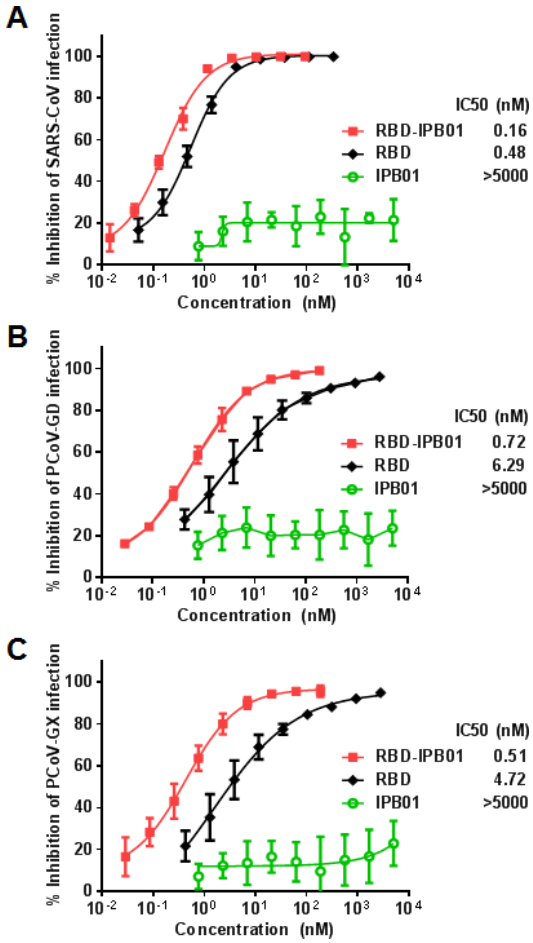

FIG 4 Inhibitory activities of RBD-IPB01 and parental inhibitors on SARS-CoV and pangolin coronaviruses. The inhibitory activities of RBD-IPB01 against SARS-CoV (A), PCoV-GD (B), and PCoV-GX (C) were measured on Huh-7 cells by a single-cycle infection assay. Data are expressed as mean ± SD (n = 3).

DISCUSSION

Inspired by the multistep nature of the SARS-CoV-2 entry process and multiple viable molecular targets involved in this pathway, here, we have applied our expertise to rationally design a potent and broad bispecific pan-sarbecovirus entry inhibitor that can inhibit two crucial stages of SARS-CoV-2 infection: the binding of viral spike to its receptor ACE2 and the 6-HB structure formation of S2 fusion protein. From a panel of RBD proteins, RBD of PCoV-GD spike was demonstrated as the most active inhibitor against SARS-CoV-2 infection. Then, four tandem molecules with $(GGGGS)_4$ linker were obtained by genetically linking this RBD with a fusion inhibitor (IPB01 or IPB19) in different orders. Of note, RBD-IPB01 displayed the dramatically improved anti-SARS-CoV-2 potency over RBD or IPB01 alone. We also found that the linker length between RBD and IPB01 did not affect the antiviral activity, implying the structure and activity relationship (SAR) of such a design approach. Importantly, RBD-IPB01 could dual-functionally inhibit various variants of concern, including Alpha, Beta, Gamma, Delta, and several Omicron sublineages. RBD-IPB01 also showed cross-inhibitory potency in blocking the infections of other sarbecovirus, including SARS-CoV and SARSr-CoVs from pangolins. Moreover, RBD-IPB01 was characterized with very low cytotoxicity in multiple target cells, high stability under 37°C and in human serum, as well as considerable resistance to trypsin digestion. Collectively, by acting on distinct targets within the same entry cascade and with consistent cross-inhibitory potency, RBD-IPB01 provides an

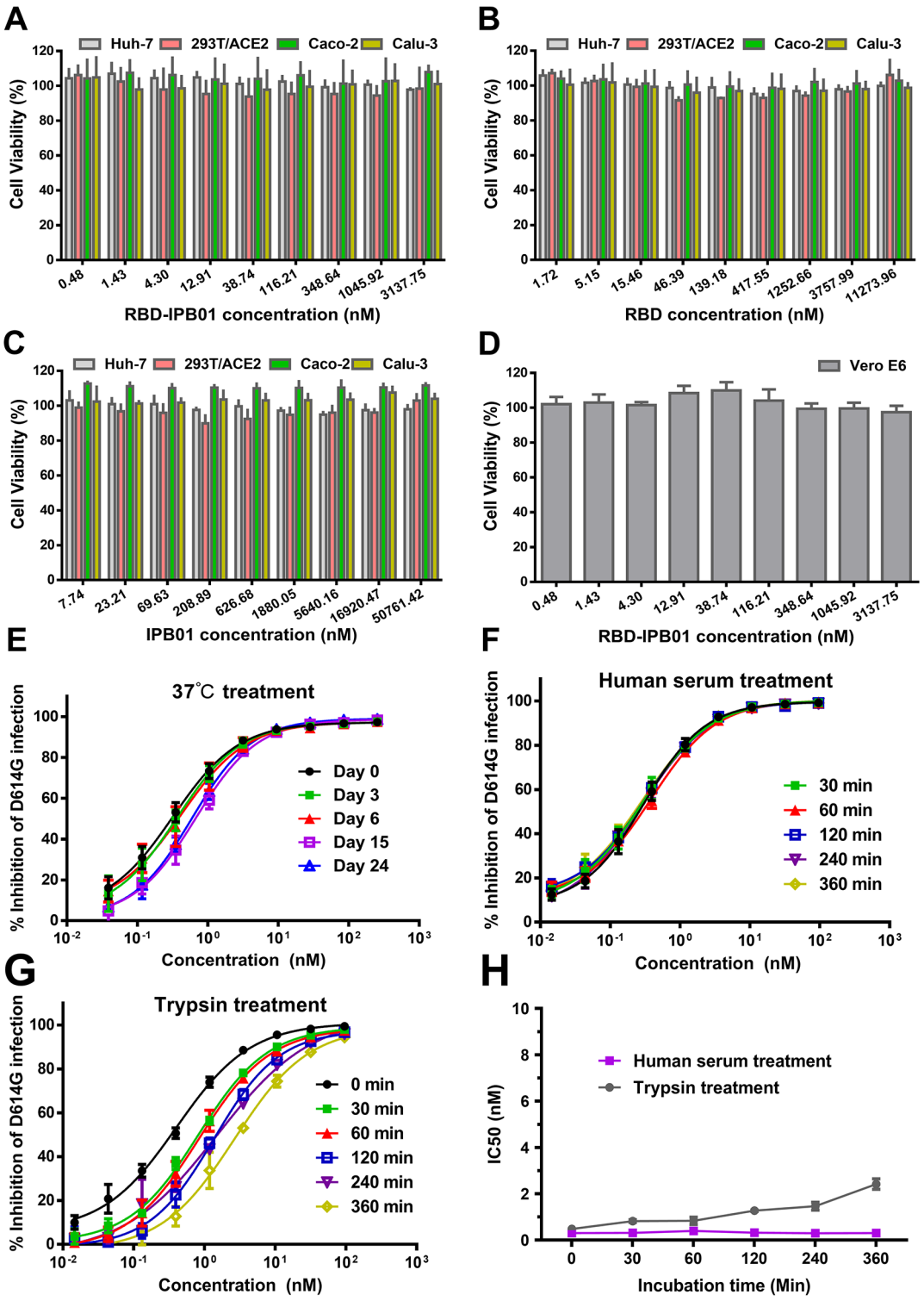

FIG 5 Cytotoxicity and biophysical properties of RBD-IPB01 inhibitor. (A) The cytotoxic effects of RBD-IPB01 (A), RBD protein (B), and IPB01 peptide (C) on Huh-7, 293T/ACE2, Caco-2, and Calu-3 cells were analyzed by CCK8 assay. (D) The cytotoxicity of RBD-IPB01 on Vero E6 cells was determined at different concentrations by CCK8 assay. The inhibitory activities of RBD-IPB01 after being stored at 37°C for 24 days (E), treated with human serum (F) or trypsin digestion (G), were determined by a pseudovirus-based single-cycle assay. (H) The antiviral curves of RBD-IPB01 treated with human serum and trypsin. Data are expressed as mean ± SD ($n = 3$).

effective and reliable armament for combating a multitude of sarbecoviruses, including circulating SARS-CoV-2 variants.

After SARS-CoV-2 was identified as the pathogen of COVID-19, the scientific community has been committed to the development of multifarious specific drugs and vaccines (30–32). The viral S protein has an important role in membrane fusion and viral infection, thus being a primary target for the development of diverse entry inhibitors, including peptide- and antibody-based drugs. In consideration of better bioavailability, antibody therapy seems to be the most promising therapeutic strategy, and there are many efforts toward developing effective neutralizing antibodies (33). However, the continuous evolution and mutation of SARS-CoV-2 have led to the emergence of new viral variants that exhibit remarkable resistance to many currently available NAbs (12, 34). Especially, several groups recently demonstrated that circulating Omicron viruses almost escaped from all reported broadly NAbs (7, 35–38). In this study, the antiviral potency of RBD-IPB01 and RBD was not affected by mutations on all VOCs, and even by other sarbecoviruses, indicating that RBD- and IPB01-based inhibitors are a promising strategy for combating SARS-CoV-2. The recent findings on an *in vitro* evolved RBD protein (RBD-62) (19), two ACE2-targeting antibodies (3E8 and h11B11) (39, 40), and HR2-based lipopeptides (21–23, 41), which showed effective inhibition of divergent SARS-CoV-2 VOCs, also corroborated the rationality of RBD-IPB01 strategy. As reported by Chaouat et al., fusing RBD with Fc generated a fusion protein RBD-Ig that was highly effective in inhibiting SARS-CoV-2 infection *in vivo* (42). Taken together, an RBD and a fusion peptide as two pharmacophores with reliable genetic barriers, when incorporating into one molecule to wield bifunctional action, is a tantalizing strategy. Chaouat and colleagues also demonstrated that RBD-Ig did not significantly alter the expression level of cell surface ACE2 receptor (42). In this study, when investigating the effect of RBD-IPB01 on the cell surface ACE2, we did not find an effective commercial anti-ACE2 antibody available without interfering with the binding of RBD-IPB01 to ACE2 (data not shown). Despite showing no appreciable cytotoxicity on different target cells, whether RBD-IPB01 affected ACE2 receptor expression on the cell surface should be determined to rule out the safety concern in our future study, because ACE2 is physiologically important to counterbalance induction of the renin-angiotensin system that regulates hypertension, sodium-water retention, and protect multiple organs (43). In this regard, Gao et al. recently demonstrated that the ACE2 receptor expression on the surface of ACE2-expressing cells was significantly affected by infection of live virus, rather than by the exogenous addition of viral S protein (44), which further reinforces the usability of RBD-IPB01 as a specific antiviral.

The outbreaks of SARS-CoV and SARS-CoV-2 give us a lesson that antivirals with broad activity across SARS-related sarbecoviruses would be useful to combat potential future spillovers. Very recently, the discovery of ACE2-utilizing infection by Middle East respiratory syndrome (MERS)-CoV-related viruses also suggests that broad-spectrum antivirals are highly desired (45). Considering the broad cross-neutralization and high potency of bifunctional molecule RBD-IPB01 described here, in combination with the concern over the antiviral efficacy of multiple currently available therapeutic NAbs against future emergent variants, we believe that RBD-IPB01 is a promising bifunctional pan-sarbecovirus entry inhibitor that could be stockpiled as part of a pandemic readiness toolbox. In addition, this strategy could be extended to the design and development of highly effective therapies for combating other enveloped viruses, such as HIV-1, Ebola virus, Marburg virus, Zika virus, influenza virus, hepatitis B virus, and respiratory syncytial virus.

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DATA AVAILABILITY

All data are fully available without restriction.

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