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ACE2-Fc fusion protein overcomes viral escape by potently neutralizing SARS-CoV-2 variants of concern

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ABSTRACT

COVID-19, an infectious disease caused by the SARS-CoV-2 virus, emerged globally in early 2020 and has remained a serious public health issue. To date, although several preventative vaccines have been approved by FDA and EMA, vaccinated individuals increasingly suffer from breakthrough infections. Therapeutic antibodies may provide an alternative strategy to neutralize viral infection and treat serious cases; however, the clinical data and our experiments show that some FDA-approved monoclonal antibodies lose function against COVID-19 variants such as Omicron. Therefore, in this study, we present a novel therapeutic agent, SI–F019, an ACE2-Fc fusion protein whose neutralization efficiency is not compromised, but actually strengthened, by the mutations of dominant variants including Omicron. Comprehensive biophysical analyses revealed the mechanism of increased inhibition to be enhanced interaction of SI–F019 with all the tested spike variants, in contrast to monoclonal antibodies which tended to show weaker binding to some variants. The results imply that SI–F019 may be a broadly useful agent for treatment of COVID-19.

1. Introduction

COVID-19 is an infectious disease caused by severe acute respiratory syndrome (SARS) coronavirus 2 (SARS-CoV-2). Complications of COVID-19 may include long-term lung damage, pneumonia, acute respiratory distress syndrome (ARDS), peripheral and olfactory nerve damage, multi-organ failure, septic shock, and death (Nalbandian et al., 2021). By March 11, 2020, the World Health Organization (WHO) declared the COVID-19 outbreak a pandemic (Cucinotta and Vanelli, 2020). As of February 02, 2022, more than 380 million cases had been reported across 188 countries and territories with more than 5.6 million deaths, of which more than 74.5 million cases and 880,580 deaths were reported by the United States (WHO COVID-19 Dashboard, 2020).

From a prevention standpoint, the CDC's vaccine effectiveness studies suggest that the available mRNA COVID-19 vaccines protect as well in real-world conditions as they have in clinical trial settings. The vaccines reduce the risk of COVID-19, especially severe illness, among people who are fully vaccinated. However, immunological studies have shown a steady decline of neutralizing antibody levels among vaccinated individuals in the long term (Naaber et al., 2021; Thomas et al., 2021).

Biologically, although the mechanism for SARS-CoV and -CoV-2 infection of humans has been studied in cell and animal models, many aspects of the disease are incompletely understood (Zhou et al., 2020; Jia et al., 2021). All the data suggest that binding to Angiotensin-converting enzyme 2 (ACE2) receptor is a critical initial step for entry of SARS-CoV family members, including SARS-CoV and CoV-2, to target cells. ACE2 is a zinc-containing metalloenzyme located

Abbreviations: ACE2, angiotensin-converting enzyme 2; ADE, antibody-dependent enhancement; ARDS, acute respiratory distress syndrome; CDC, Centers for Disease Control and Prevention; CRS, cytokine release syndrome; EMA, European Medicines Agency; Fc, crystallizable fragment; FcγR, Fc gamma receptor; FcRn, neonatal Fc receptor; FDA, Food and Drug Administration; IFN-γ, interferon γ; IL-6, interleukin 6; INN, international nonproprietary name; MERS, Middle East respiratory syndrome; MCP-1, monocyte chemoattractant protein 1; RBD, receptor-binding domain; SARS, severe acute respiratory syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; TNF-α, tumor necrosis factor α; WHO, World Health Organization.

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on the cell membrane of mainly alveolar cells of the lung, enterocytes of the small intestine, endothelial cells of the vasculature, smooth muscle cells of arteries, and other lineages of cells in the lungs, arteries, heart, kidney, intestines, and other tissues (Hoffmann et al., 2020). ACE2 regulates the renin angiotensin system by counter-balancing angiotensin-converting enzyme activity in the cardiovascular, renal and respiratory systems, indicating its important role in the control of blood pressure. Besides, ACE2 plays a protective role in the physiology of hypertension, cardiac function, heart function, and diabetes (Gheblawi et al., 2020).

SARS-CoV-2 has a tropism for ACE2-expressing epithelial cells of the respiratory tract. Patients with severe COVID-19 have symptoms of systemic hyperinflammation. Clinical laboratory findings of elevated IL-6, monocyte chemoattractant protein 1 (MCP-1), and tumor necrosis factor-α (TNF-α) indicative of cytokine release syndrome (CRS) suggest an underlying immunopathology (Lu et al., 2021; Liu et al., 2020). Cytokine release syndrome is a major adverse side effect that can limit the utility of treatment with biologics and is assessed using *in vitro* cytokine release assays.

SARS-CoV-2 is one of seven known coronaviruses to infect humans, including SARS-CoV and MERS CoV viruses, that caused the outbreak of SARS in Asia in 2003 and in the Middle East in 2012 (Peiris et al., 2004; Zaki et al., 2012). The immune response to SARS-CoV-2 virus involves a combination of cell-mediated immunity and antibody production. Although more than 300 million people have recovered from COVID-19 (as of February 2022), it remains unknown if natural immunity to SARS-CoV-2 virus will be long-lasting in recovered individuals (WHO COVID-19 Dashboard, 2020). One of the concerns relates to the virus's continual accumulation of mutations, which may alter the spectrum of viral antigenicity and cause re-infection by mutant strains of the virus. These variant strains may harbor mutations that ultimately enhance viral recognition and infection into host cells, thereby increasing infectivity and/or pathogenicity. Early in the pandemic, there were few 'mutant' variant viruses because of the small number of people infected, and thereby fewer opportunities for escape mutants to emerge (Weisblum et al., 2020; Andreano et al., 2021; McCarthy et al., 2021). As time went on, SARS-CoV-2 started evolving to become more transmissible (Singh and Yi, 2021). Several SARS-CoV-2 variants are of particular importance due to their potential for increased transmissibility, increased virulence, or vaccine resistance. To classify SARS-CoV-2 variants, the ancestral type is type "A", and the derived type is type "B". The B-type mutated into further types including B.1, which is the ancestor of the major global variants of concern. WHO has named Alpha (B.1.1.7, December 2020) (Tang et al., 2021), Beta (B.1.351, January 2021) (Tegally et al., 2021), Gamma (P.1, January 2021) (Sabino et al., 2021), Kappa (B.1.617.1, 2020) (Ferreira et al., 2021), Delta (B.1.617.2, May 2021) (Mlcochova et al., 2021), Lambda (C.37, 2021) (Romero et al., 2021), Omicron (B.1.1.529, 2021) (Callaway, 2021), and other variants.

Both the Alpha variant and the Delta variant are notably more transmissible than the original virus identified in early 2020. The Delta variant is about 60% more contagious than the Alpha variant, and became the dominant strain during the spring of 2021 (Yang and Shaman, 2021). By late August 2021, the Delta variant accounted for 99% of U.S. cases and was found to double the risk of severe illness and hospitalization for those not yet vaccinated, and even vaccine protection by mRNA vaccines fell from 91% to 66% (Twohig et al., 2021; Fowlkes et al., 2021). Recent studies have demonstrated reduced vaccine efficacy of 53.1%, 42–76%, or 64.6%, with the decrease likely due to waning immunity combined with inferior protection against the highly infectious Delta strain (Nanduri et al., 2021; Puranik et al., 2021; Seppälä et al., 2021). In August 2021, the majority of cases and hospitalizations in Israel occurred in vaccinated individuals, highlighting the need for complementary modalities for treatment of COVID-19 (Bar-On et al., 2021; Wadman, 2021). At the time of manuscription preparation, the Omicron variant had become the dominant strain due to even higher transmissibility (Araf et al., 2022). Antibody neutralization was significantly lower for the Omicron variant compared to other variants after the original two-dose mRNA vaccine regimen, necessitating an additional booster dose (Garcia-Beltran et al., 2022). Despite the emergence of multiple new variants, fortunately, no reports have shown major changes in the specificity or mechanism of SARS-CoV-2 viral infection.

Another concern relates to the phenomenon of antibody-dependent enhancement (ADE) (Takada and Kawaoka, 2003; Wan et al., 2020). ADE occurs when the binding of suboptimal antibodies enhances viral entry into host cells. In coronaviruses, antibodies targeting the viral spike (S) glycoprotein promote ADE. In cases of SARS-CoV viruses, the antibodies that neutralized most variants were found to be able to enhance immune cell entry of the mutant virus, which, in turn, worsened the disease the vaccine was designed to protect against (Tetro, 2020; Lee et al., 2020). Although ADE is not thought to contribute to excess cytokine production in the context of SARS-CoV-2, IgG ligation of Fc receptors has been shown to contribute to modest ADE of infection (Maemura et al., 2021). Therefore, ADE can hamper vaccine development, as a vaccine may cause the production of suboptimal antibodies. In this context, any preventive strategy other than vaccines shall be considered as a viable alternative circumventing ADE, either before or after exposure to SARS-CoV-2 virus.

Based on these clinical observations and scientific reports, we developed an ACE2-Fc fusion protein, SI–F019, to serve as a therapeutic inhibitor of SARS-CoV-2. Despite several ACE2 or ACE2-like molecules being developed for COVID-19 treatment, early studies have not yet evaluated the neutralization efficacy toward different variants, or have utilized the wild-type Fc domain, which may cause undesirable ADE effects (Glasgow et al., 2020; Iwanaga et al., 2020; Zhang et al., 2021; Siriwattananon et al., 2021; Huang et al., 2021; Bernardi et al., 2020; Xiao et al., 2021). Through comprehensive characterization, we demonstrate that SI–F019 has strong binding and neutralization, and fortuitously, more potent neutralization of SARS-CoV-2 variants compared to the original strain. The data suggest that ACE2-Fc will be a promising therapeutic agent for COVID-19 treatment, which may retain efficacy against continuously developing variants.

2. Materials and methods

2.1. Cloning, expression and purification

The human ACE2 protein has at least three functional domains: a signal peptide (residues 1–17), zinc metallopeptidase domain (residues 18–615), and a TMPRSS2 protease cleavage site (residues 697–716) (Genbank number: NP_001358344.1). The experimental and simulation data all show the zinc metallopeptidase domain of ACE2 was the receptor for entry into lung epithelial cells. Therefore, this metallopeptidase domain was chosen for further study. Furthermore, each Fc fragment of IgG1 Fc region contains a cysteine at position 220 (according to EU numbering system), which may intrinsically form a disulfide bond with either kappa or lambda light chain. To reduce the risk of having a free cysteine that may destabilize and/or inactivate the Fc fusion protein, C220 may be substituted for serine (C220S) to avoid this issue. This clone was named SI–F019. The control molecule, SI-69R4, which carries a longer ACE2 domain, including TMPRSS2 protease cleavage site, was fused with wild type IgG1 Fc region. The materials we constructed are listed in Table 1.

The recombinant fusion genes encoding the fusion proteins were

Table 1
The cloning, expression, and purification of recombinant ACE2-Fc fusion proteins.

Recombinant fusion protein	Sample ID	Purified fusion protein
huACE2 (1–615) - IgG1 Fc null	SI–F019	huACE2 (18–615) - IgG1 Fc null
huACE2 (1–740) - IgG1 Fc (wt)	SI-69R4	huACE2 (18–740) - IgG1 Fc (wt)

cloned into either pTT5 vector and expressed in ExpiCHO cells (Thermo Fisher scientific) for transient expression or pCGS3.0 vector then transfected into CHOZN cell (Sigma-Aldrich) for stable cell line development. All the recombinant proteins were purified following standard protein expression protocols, via protein A affinity, CEX and CHT chromatography, sterilized using a 0.22 μm filter, and stored in a cryopreservation buffer at 4 °C. During the expression and purification, each recombinant fusion protein may undergo post-translational modification, including N-glycosylation and the cleavage of N-terminal signal peptide (17 amino acids).

Six control anti-SARS-CoV-2 antibodies, Bamlanivimab, Casirivimab, Etesevimab, Imdevimab, Cilgavimab, and Tixagevimab were expressed using amino acid sequences referenced from WHO International Nonproprietary Names (INN) database.

Putative 3D model of SI–F019 was built by PyMol (Schrödinger and DeLano), Chimera (Pettersen et al., 2004), and N-glycan was added by Glycosciences.DB (Böhm et al., 2018). The reference ACE2 structure is PDB number 1R42 (Towler et al., 2004), while Fc domain is from PDB number 1HZH (Saphire et al., 2001).

2.2. Protein quality validation by HPLC-SEC-MALS

To assess the actual molecular weight of SI–F019, analytical size exclusion chromatography (SEC) was used, in a combination with multi-angle light scattering (MALS), absorbance (UV), and/or refractive index (RI) concentration detector techniques. The method combines the chromatographic separation by molecular size and the determination of absolute molar mass by light scattering (LS) without the limitations of molecule weight standard calibration. The sample was analyzed by Waters ACQUITY Arc HPLC System (Waters Corp, Milford, MA) connected with Nano BioCore SEC-300 5 μm, 4.6mm × 300 mm (Nano-Chrom Technologies, Suzhou, China), sequential connection with miniDAWN TREOS multi-angle light scattering (MALS) detector and Optilab T-rEX differential refractometer. The running condition is 50 μg sample per injection in 125 mM sodium phosphate, 100 mM sodium chloride, pH 6.8 mobile phase with 0.3 ml/min flow rate. The standard dn/dc values for MALS evaluation is 0.185 ml/g for protein and is 0.145 ml/g for glycan. The chromatography data was collected by EMPOWER 3 software, and MALS analysis report is generated by ASTRA 7.1.4.8 (Wyatt Technology, Santa Barbara, CA).

2.3. TMPRSS2 protease hydrolysis assay

For the protease assay, each sample, SI–F019 and SI-69R4, treated with or without TMPRSS2 (weight ratio of TMPRESS2 to ACE2-Fc fusion variants is 1:100 (w/w)), were incubated at 37 °C for 16 h. Then deglycosylation by enzyme PNGase F treatment was employed before running SDS-PAGE to eliminate the smear background induced by high and heterogeneous glycan content. The protein samples were denatured and ran in reduced condition.

2.4. Octet binding assay

All protein binding experiments were conducted in Octet RED384 (Sartorius, Marl-borough, MA), with vendor-recommended buffers and sensors. The data were processed by ForeteBio Data Analysis (version 11.1.0.4).

Due to the different biological attributes of SI–F019, we evaluated the ACE2 and Fc region binding affinity and avidity, respectively. To put it simply, the definition of affinity term is to immobilize SI–F019 to AHC biosensor and probe the analyte: SARS-CoV RBD, SARS-CoV-2 RBD (and variants) or SARS-CoV-2 S1 protein, respectively; while the definition of avidity term is to immobilize biotinylated SARS-CoV RBD, SARS-CoV-2 RBD (and variants) or SARS-CoV-2 S1 protein to SA biosensor and probe the analyte, SI–F019. For the spike protein, we immobilized His tagged full length spike protein on the HIS1K biosensor and probed SI–F019 as analyte. For the Fcγ receptors (FcγRs) family binding study, we immobilized biotinylated Fcγ receptors (FcγRs) family to SA sensor, and flowed SI–F019 as analyte. All the recombinant SARS-related proteins were purchased from ACRO Bio-systems (Newark, DE) or Sino Biological Inc (Wayne, PA). Fcγ receptors (FcγRs) families were purchased from ACRO Biosystems. C1q protein was purchased from Abcam (Waltham, MA).

2.5. Pseudovirus assay

To test the ability of SI–F019 to prevent viral infection, viral infectivity was characterized using a luciferase reporter assay. SARS-CoV-2 S protein packaged pseudovirus (wild-type or variant strains, Sino Biological) containing a luciferase reporter gene was co-incubated with 293T cells overexpressing ACE2 (clone 3D4) and 1:3 serial titration (high dose started from final 30 μg/ml) of SI–F019. Expression of ACE2 on the transfected cells was confirmed by enzymatic and FACS assays. The pseudovirus may enter the ACE2-positive cells via Spike protein binding to ACE2, which leads to transgene expression, luciferase. Thus, luminescence is used as a readout of viral infectivity.

In particular, 10-fold stock solution of S protein pseudovirus was prepared in culture medium to a final virus load of 227–394 TCID50/well. SI–F019 in culture medium was serially diluted 3-fold with maximum concentration 150 μg/ml. 3D4 cells were harvested using dissociation buffer lacking trypsin. Pseudovirus (20 μl) and SI–F019 (30 μl) were combined in wells of a 96-well plate, mixed, and incubated for 1 h at room temperature. Then, 100 μl of harvested 3D4 cells were added to each well (20,000/well) and incubated for 18 h at 37 °C, 5% CO2. After incubation, supernatant was removed and 50 μl of luciferase substrate solution was added, mixed, and incubated for 1 min at room temperature. Luminescence was read using I3X plate reader, where the luminescence signal in RLU (relative luminescence units) is representative of S protein pseudovirus infectivity. Decrease in luminescence compared to the condition without SI–F019 treatment can be calculated to determine percent inhibition of infectivity. This data was then fit to a sigmoidal function in GraphPad Prism 8.0 and R version 4.12 with ggplot2 version 3.3.3 (Wickham, 2016) to extract IC50 values for SI–F019 inhibiting pseudovirus infectivity where the pseudovirus contained different variants of spike protein.

3. Results

The ACE2-Fc fusion protein, SI–F019, was developed to overcome issues of viral resistance while minimizing off-target effects. In our design, the ACE2 functional domain contains only binding and catalytic functions but not the undesired TMPRESS2 protease hydrolysis site, which may disrupt the intact protein structure. Furthermore, a silenced antibody Fc domain is fused to the functional domain of ACE2. The engineered IgG1 Fc contains the L234A and L235A mutations, disrupting interaction with Fcγ receptors (FcγRs) family, and K322A mutation, reducing interaction with complement component C1q, which may reduce the chances of undesirable ADE effects. Due to the intrinsic dimerization proclivity of Fc, the ACE2-Fc fusion protein will be present in homodimeric form to significantly increase avidity effects for binding to spike. Furthermore, the FcRn binding ability endows the agent with a long circulatory half-life while the Fc domain as a whole confers good biochemical and biophysical stability. Fig. 1a and b depict the construction and three-dimensional structure of SI–F019. After the GMP lot of SI-019 was purified, we performed detailed characterization and functional evaluation, as follows:

3.1. Protein quality validation by HPLC-SEC-MALS

HPLC-SEC-MALS analysis was performed to evaluate the size, composition, and quaternary structure of SI–F019. The result (Fig. 1c) shows that SI–F019 exhibited an average total molecular weight of

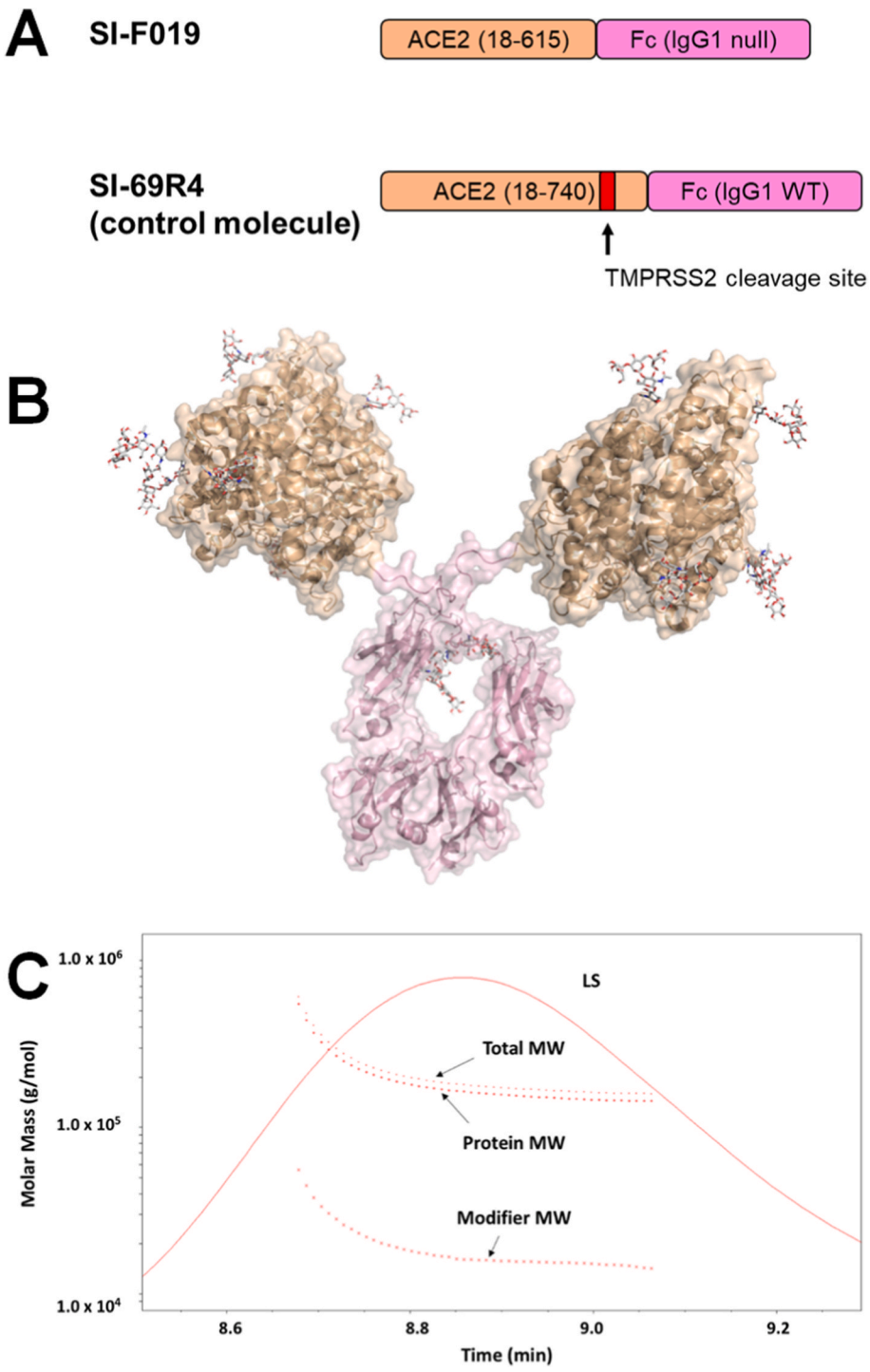

Fig. 1. shows (1a) design of recombinant fusion proteins comprising ACE2 functional domain and engineered Fc fragment (SI–F019 and SI-69R4), (1b) the modeled structure of a SI–F019 homodimer, and (1c) the SEC-MALS chromatogram of SI–F019 fusion protein complex.

209.6 kDa (main peak), of which the molecule weights of the SI–F019 dimer and its modifiers (i.e. glycans) were measured at 189.3 kDa and 20.3 kDa, respectively. In the theoretical calculation of its amino acids, the molecule weight of the SI–F019 monomer is 95.1 kDa. Thus, the HPLC-SEC-MALS data confirms that purified SI–F019 fusion protein complex is a highly glycosylated homodimeric protein.

3.2. TMPRSS2 protease hydrolysis assay

SI–F019 and control protein SI-69R4 were treated with TMPRSS2, then assessed by SDS-PAGE to determine their susceptibility to protease cleavage (Fig. S1a). SI–F019, which lacks the proteolysis site, is intact even when treated with TMPRSS2 protease (lane 1 and 2). However, the control protein SI-69R4, which contains the native TMPRSS2 recognition sequence, is clearly hydrolyzed in the presence of TMPRSS2 (lane 4), and kept intact without protease addition (lane 3). That SI-69R4 was digested into multiple fragments, further confirms that the TMPRESS2 cleaving site exists in the original ACE2 full length protein, but not in our design for SI–F019. From this experiment, we can confirm that SI–F019 is resistant to TMPRSS2 proteolysis, while the control molecule, SI-

69R4, is efficiently cleaved.

3.3. Octet binding to Fc receptors

To further understand the therapeutic potential of SI–F019 for neutralizing SARS-CoV-2, we used Bio-Layer Interferometry (BLI), Octet, to measure the *in vitro* binding affinity and avidity.

The Fc region interacts with multiple Fcγ receptors (FcγR) and complement protein C1q and mediates immune effector functions, which are important for many therapeutic applications, e.g., elimination of targeted cells via antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) or complement-dependent cytotoxicity (CDC). Generally, immunomodulatory antibodies are of IgG isotypes that have low, or no, binding to the FcγRs that trigger cell-mediated cytotoxic effector functions. These immunologically silent isotypes are used to mitigate the risk of depleting the effector cells upon which such antibodies depend for their mechanism of action, and to minimize FcR-mediated distribution. Herein, we adapted another strategy to eliminate the binding by mutation. The L234A, L235A and K322A mutations in IgG1 Fc region can abrogate binding to Fcγ receptors (FcγRs) and complement protein C1q, and thus abolish immune effector functions to reduce undesired FcγR activation and Fc-mediated toxicity.

For the binding assay to a panel of FcγRs, representative FcγR family members, including FcγRI/CD64, FcγRIIa/CD32a, FcγRIIb/CD32b, and FcγRIIIa/CD16a, as well as C1q, have no observed response, as expected based on Fc engineering to abolish these interactions (See Table 2). For the FcRn binding study, the data shows a KD value of 37.6 nM for SI–F019, which is comparable to reports of conventional monoclonal antibodies (Neuber et al., 2014). In support of the Octet binding data for Fc γ receptors, a cytokine release assay (Fig. S4) was conducted. Results demonstrated that SI–F019 (containing null Fc mutations) had significantly less release of inflammatory cytokines, including IFN-γ, TNF-α, IL-6, and MCP-1, compared to SI-69R4 (containing wild-type IgG1 Fc). SI–F019 did not induce any of the tested cytokines when exposed to WBC in plate-bound format at 2000 nM concentration with levels similar to buffer control in all conditions. In contrast, positive control mAb TGN1412 (αCD28 superagonist)(Attarwala, 2010) strongly induced most of the cytokines, which is in an agreement with previously published results. Some intermediate production of IFN-γ and TNF-α were detectable when SI-69R4 was used to stimulate the WBC indicating the increased safety of the Fc null portion of SI–F019.

3.4. Octet binding to SARS-CoV family spike protein domains

For the Spike-related protein binding study, we compared the SI–F019 binding affinity and avidity toward different strain subunits, namely the receptor-binding domain (RBD), intact spike (S1) protein, and spike trimer. In Table 3, SI–F019 is shown to bind to SARS-CoV RBD, -CoV-2 RBD and -CoV-2 S1 domain with similar KD values, in the ~20 nM range, characterized by high association rate. For the avidity study, not only each subunit, but also the full-length extracellular domain (ECD) domain of CoV-2 spike protein, all show extremely high binding. The KD values are in the sub-nanomolar range. Based on this binding data, SI–F019 may be a potential neutralizing agent for COVID-19 treatment.

Table 2
The effect of Fc null mutations on SI-F019 binding to Fc receptors.

Fc Receptor	KD (nM)	kon (1/ms)	kdis (1/s)
FcγRI/CD64	Not detectable		
FcγRIIα/CD32α	Not detectable		
FcγRIIβ/CD32β	Not detectable		
FcγRIIIα/CD16α	Not detectable		
C1q	Not detectable		
FcRn	37.6	4.51E+05	3.52E-0.2

Table 3
The affinity and avidity of SI–F019 binding to viral proteins.

	Affinity			Avidity		
	KD (nM)	kon (1/Ms)	kdis (1/s)	KD (nM)	kon (1/Ms)	kdis (1/s)
CoV-2 S1	14.7	3.37E+05	4.93E-03	0.29	1.49E+05	4.72E-05
CoV-2 RBD	21.8	4.26E+05	9.26E-03	0.11	8.63E+05	9.82E-05
CoV RBD	14.0	4.26E+05	5.97E-03	0.33	5.18E+05	1.71E-04
CoV-2 Spike Trimer	NA	NA	NA	0.18	4.20E+04	1.12E-05

Due to the emergent COVID-19 variants, which are more contagious than the original strain, we executed side-by-side binding studies using the mutant RBD domains, to evaluate the potential for resistance (See Tables 4 and 5). As comparators, we also included six FDA-approved monoclonal antibodies (see Tables S1 and S2). After initial experiments were completed, the Omicron variant emerged, and affinity experiments were repeated using Omicron RBD (Fig. S6). In Table S1, Bamlanivimab, the key component of Eli Lilly's anti-COVID-19 cocktail regimen, totally loses binding to Beta, Kappa, Gamma, Lambda, and Omicron strains. Casirivimab, the key component of Regeneron's anti-COVID-19 cocktail regimen, as well as Etesevimab, the other key component of Eli Lilly's anti-COVID-19 cocktail, lose binding to Beta and Omicron strains. Imdevimab completely loses binding to the Omicron variant. However, remarkably, SI–F019 shows comparable, or even stronger binding toward all variants. This result suggests that SARS-CoV-2 cannot evolve to escape the ACE2-Fc inhibitor without also losing its ability to bind the natural ACE2 entry receptor. Monoclonal antibodies binding to spike, on the other hand, can clearly be evaded by viral escape variants.

For the avidity study, all the COVID-19 variants bind to SI–F019 more tightly, as observed in the affinity study. Again, some antibodies lose binding avidity or response to mutant virus; for example, Bamlanivimab and Etesevimab no longer bind to the Beta variant, as listed in Table S2. Through this BLI-based protein binding study, we accumulate solid evidence that SI–F019 has robust affinity and avidity for the SARS-CoV and CoV-2 spike proteins and their subunits, and in some cases even

Table 4
Binding kinetics (affinity) of SI–F019, indicating that SI–F019 binds with increased affinity to variant forms of RBD relative to the wild-type RBD, driven largely by slower dissociation rate. *Omicron was tested separately after the initial experiment. The original WT pseudovirus was used as a bridging control in the later assay.

WHO Designation	RBD Mutation	KD (M)	kon (1/Ms)	kdis (1/s)
Original	WT	2.18E-08	4.26E+05	9.26E-03
Original*	WT	8.09E-09	5.12E+05	4.15E-03
Alpha	N501Y	4.54E-09	3.96E+05	1.80E-03
Delta	L452R, T478K	7.26E-09	6.11E+05	4.44E-03
Kappa	L452R, E484Q	9.07E-09	5.12E+05	4.65E-03
Gamma	K417T, E484K, N501Y	4.03E-09	5.30E+05	2.13E-03
Beta	K417N, E484K, N501Y	8.49E-09	4.24E+05	3.61E-03
Lambda	L452Q, F490S	1.35E-08	4.59E+05	6.21E-03
Omicron*	(Many)	4.08E-09	1.04E+06	4.24E-03

Table 5
Biolayer interferometry was used to quantify binding kinetics (avidity) of SI–F019 to different variants of S protein RBD.

WHO Designation	RBD Mutation	KD (M)	kon (1/Ms)	kdis (1/s)
Original	WT	1.14E-10	8.63E+05	9.82E-05
Alpha	N501Y	1.90E-11	1.02E+06	1.94E-05
Delta	L452R, T478K	1.41E-11	8.58E+05	1.21E-05
Kappa	L452R, E484Q	<1.0E-12	1.01E+06	<1.0E-07
Gamma	K417T, E484K, N501Y	6.23E-11	1.00E+06	6.26E-05
Beta	K417N, E484K, N501Y	6.35E-11	8.93E+05	5.66E-05
Lambda	L452Q, F490S	8.92E-11	9.13E+05	8.15E-05

higher affinity for the contagious variants.

3.5. Pseudovirus neutralization assay

Pseudovirus neutralization is a standard assay to detect the presence and magnitude of functional antibodies or binders that can prevent infectivity of a virus, e.g. SARS-CoV-2 (Kalkeri et al., 2021). Therefore, to cross validate the binding data, we further examined SI–F019 potency by the pseudovirus neutralization assay. Viral inhibition data are plotted in Fig. 2A and IC50 values are tabulated in Table 6. Since the Omicron variant emerged after completion of initial experiments, the assay was repeated using Omicron pseudovirus along with the wild-type pseudovirus as a bridging control. The results show that SI–F019 is capable of inhibiting the infectivity of all variants with an increased potency from 2- to 15-fold when compared to its potency against the wild-type strain. The linear association between IC50 and KD values of either affinity or avidity (Fig. 2b and c) is indicative of competitive inhibition by SI–F019. Furthermore, the IC50 values are correlated with the date of emergence of variants of concern (Fig. S5), especially Alpha, Beta, Gamma, Delta, and Omicron variants (O'Toole et al., 2021). The *in vitro* data demonstrate that ACE2 protein, in this case SI–F019 fusion protein, is capable of not only tightly binding to but also inhibiting variants of SARS-CoV-2 virus where evolutionary enhancement of the RBD-ACE2 interaction necessarily increases the potency of ACE2-Fc neutralization.

4. Conclusions

Our design shows multiple favorable properties with regard to COVID-19 treatment. First, the functional ACE2 domain and engineered IgG1 Fc are derived from humans, reducing the probability of an anti-drug antibody (ADA) response. From the bio-layer interferometry (BLI) binding result, we also demonstrated that SI–F019 exhibits no binding to major Fc gamma receptors, mitigating the risk of ADE or immune cell-derived side effects. Third, the binding results demonstrate that SI–F019 has nanomolar (nM) affinity for the original SARS-CoV-2 strain, and extremely high avidity in the sub-nanomolar range. For all the variants, the affinity and avidity are unexpectedly higher than for the original strain. This phenomenon was not observed for the FDA-approved monoclonal antibodies, which lost binding to some variants, and implies that SI–F019 will be a promising treatment for these contagious variants, and even for continuously developing strains.

Human recombinant soluble ACE2 (hrsACE2) has been shown to be safely tolerated in healthy patients, and a case study demonstrated amelioration of severe COVID-19, highlighting the utility of the ACE2 moiety as a potential therapeutic inhibitor of SARS-CoV-2 (Zoufaly et al., 2020). While preparing this manuscript, other articles reported results for similar ACE2-Fc fusion proteins to be used as COVID-19 therapeutics. In one case, a wild-type IgG1 Fc was used, while in another the IgG4 subtype was employed (Zhang et al., 2021; Svilenov et al., 2021). However, even the more immunologically silent IgG4

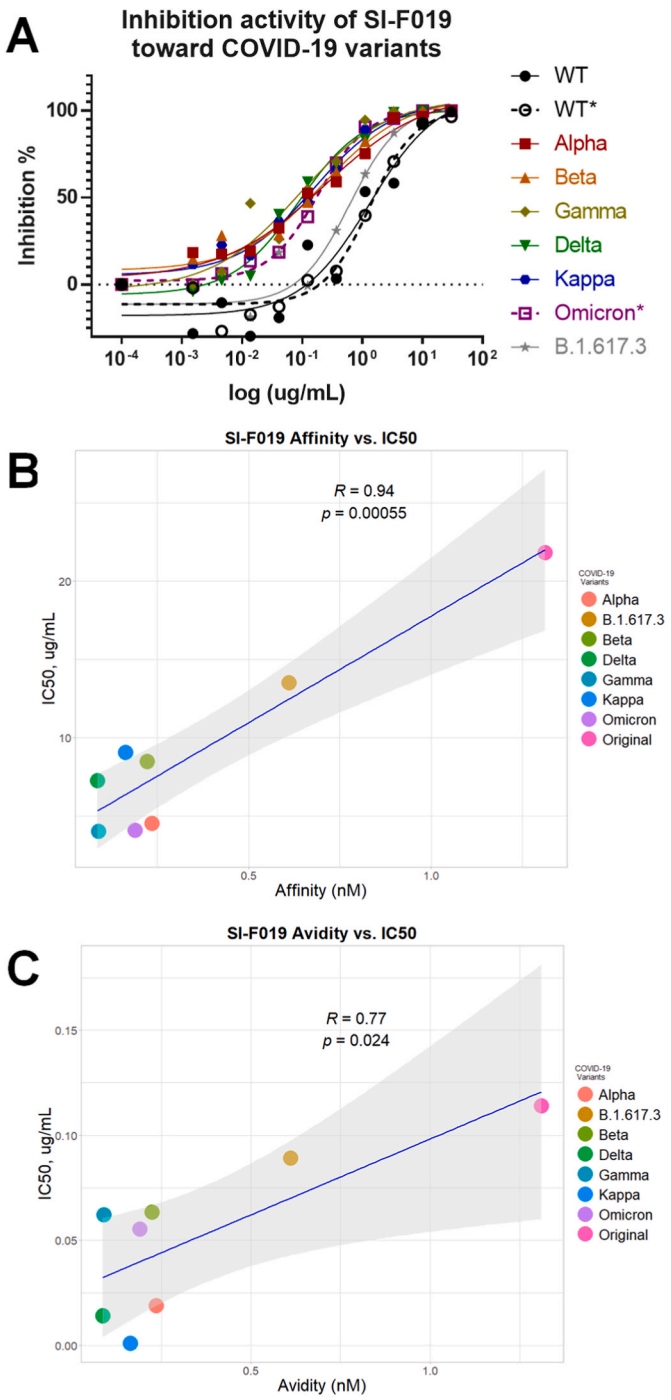

Fig. 2. demonstrates the potency of SI–F019 in protecting ACE2-expressing 293T cells from viral infection using variants of S protein packaged pseudovirus (NICPBP) in a luciferase reporter assay (2a), and the linear correlation between IC50 and the binding affinity (2b) or avidity (2c) indicative of competitive inhibition by SI–F019. *Omicron was tested separately after the initial experiment. The original WT pseudovirus was used as a bridging control in the later assay.

version had 40–50 nM affinity for FcγRI, demonstrating the potential for immune cell activation, albeit at a much lower propensity than for more active subtypes like wild-type IgG1. In contrast, our ACE2-Fc fusion utilized mutations to abolish FcγR binding and demonstrated undetectable cytokine release *in vitro*. Thus, the version with silent Fc domain may be safer or more tolerable due to low immune cell engagement.

Interestingly, the pseudovirus neutralization data is highly

Table 6
IC50 values for inhibition of viral infectivity in luciferase reporter assay using S protein packaged pseudovirus (NICPBP) to infect 293T cells expressing ACE2. Notably, SI–F019 inhibition of pseudovirus containing variant forms of S protein is more potent than inhibition of pseudovirus containing wild-type S protein based on lower IC50 values. *Omicron was tested separately after the initial experiment. The original WT pseudovirus was used as a bridging control in the later assay.

WHO Designation	SARS-CoV-2 Pseudovirus	IC_{50} (μg/ml)
Original	WT Wuhan-Hu-1	1.311
Original*	WT Wuhan-Hu-1	1.320
Alpha	B.1.1.7	0.235
Delta	B.1.617.2	0.087
Kappa	B.1.617.1	0.163
Gamma	P.1	0.089
Beta	501Y.V2	0.223
Omicron*	B.1.1.529	0.184
n/a	B.1.617.3	0.611

associated with the Octet binding affinity and avidity, with R square values of 0.94 and 0.77, respectively. To our knowledge, this is first time *in vitro* protein-protein binding data has been so closely reflected in in vitro protein-pseudovirus interaction data. In fact, the variants with later dates of emergence tended to have stronger affinity for ACE2, a trend also observed by other groups and suggesting evolution of the interaction over time. This finding may allow for generation of predictable models for therapeutic regimen based on virus variant sequence, which is not provided by monoclonal antibody-based treatment. Compared to treatment with FDA-approved monoclonal antibodies, which requires complex cocktails which may lose binding activity, e.g. Bamlanivimab for Beta, Gamma, Lambda, and Omicron strains, SI–F019 shows good neutralization efficacy for the original strain, and even better neutralization for variants. As there are limitations with using a pseudovirus assay in place of authentic live virus, future experiments could be used to further investigate the activity of SI–F019 and validate the pseudovirus data.

Collectively, these results suggest that SI–F019 will be a promising treatment for COVID-19 which may provide an additional modality to address the ongoing immune escape issue of evolving variants. Future studies will seek to demonstrate whether SI–F019 retains strong binding to the growing list of novel variants of concern. In the meantime, a phase I clinical trial evaluating safety of SI–F019 in healthy patients has been conducted (ClinicalTrials.gov Identifier: NCT04851444) and is being analyzed in preparation for future clinical studies.

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Declaration of competing interest

T.T., J.K., M.G., A.W., D.C., H.Z., A.M., Y.Z., D.G. are employees of SystImmune, Inc, while S.Z., Y.Z. and M.D. are employees of Sichuan Baili-Bio (Chengdu) Pharmaceutical Co., Ltd. Two patent applications have been filed by SystImmune, Inc. for content disclosed herein. SI–F019 is in ongoing clinical trials (NCT04851444).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2022.105271.

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