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REVIEWS

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David versus goliath: ACE2-Fc receptor traps as potential SARS-CoV-2 inhibitors

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ABSTRACT

Anti-SARS-CoV-2 monoclonal antibodies and vaccines have shown improvement in lowering viral burden and hospitalization. However, emerging SARS-CoV-2 variants contain neutralizing antibody-escape mutations. Therefore, several reports have suggested the administration of recombinant angiotensin-converting enzyme 2 (rACE2) as a soluble receptor trap to block SARS-CoV-2 infection and limit viral escape potential. Several strategies have been implemented to enhance the efficacy of rACE2 as a therapeutic agent. Fc fusions have been used to improve pharmacokinetics and boost the affinity and avidity of ACE2 decoys for the virus spike protein. Furthermore, the intrinsic catalytic activity of ACE2 can be eliminated by introducing point mutations on the catalytic site of ACE2 to obtain an exclusive antiviral activity. This review summarizes different evolution platforms that have been used to enhance ACE2-Fc (i.e., immunoadhesins) as potential therapeutics for the current pandemic or future outbreaks of SARS-associated betacoronaviruses.

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Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has been identified as the seventh coronavirus that is known to cause human respiratory disease.[1] This novel virus, which belongs to the genus betacoronavirus, has a single-stranded positive-sense RNA genome. It is the causative agent of the coronavirus disease 19 (COVID-19) that emerged in China and swiftly spread to the rest of the world starting in late 2019.[2] According to the World Health Organization, COVID-19 has affected nearly every healthcare system in the world, and infection with the virus has led to millions of deaths.

The 30-kb SARS-CoV-2 genome encodes four main structural proteins: the spike (S) glycoprotein, membrane (M) protein, an envelope (E) protein and a nucleocapsid (N) protein.[3] The S protein on the surface of the virus is known to play the most important roles in viral attachment, fusion and entry. Therefore, S protein has been exhaustively studied as a key target for vaccine and therapeutic development.[4]

The entry of SARS-CoV-2 into the host cells is mediated by the binding of the S protein to the host cellular membrane-bound angiotensin-converting enzyme 2 (ACE2) receptors.[5–8] The S protein on the surface of the virus is composed of S1, which is responsible for binding, and S2, which is responsible for membrane fusion.[9] The S1 subunit contains an N-terminal domain (NTD) and a receptor-binding domain (RBD) at the C-terminal that contains the receptor-binding motif (RBM). However, the S2 subunit contains a fusion peptide (FP), heptad repeat 1 (HR1) and 2 (HR2) domains, a transmembrane (TM) and a cytoplasmic (CP) domain (Figure 1). Upon binding the RBD on the S1 to the peptidase domain (PD) of ACE2, the FP on the S2 site is inserted into the cell membrane to promote fusion with the viral membrane.[5,10] This process is primed by several host transmembrane protease serine proteases, including TMPRSS2 and TMPRSS4, to cleave S1 and S2 subunits.-[11–13]

Given the important role of RBD in initiating the invasion of SARS-CoV-2 into host cells, it is reasonable to define the RBD as the most promising target for the development of virus attachment inhibitors, neutralizing antibodies and vaccines. Therefore, several studies have suggested the administration of human recombinant ACE2 (rACE2) protein to block the RBD, and thereby prevent COVID-19.

Recombinant human ACE2

Human ACE2 is a monocarboxylic peptidase that is widely expressed in several organs, including the brain, heart, lung, gut, kidneys and testis,[14] and circulates in the plasma as a soluble form.[15,16] Physiologically, ACE2 converts angiotensin II into angiotensin 1–7, to counterbalance induction of the renin-angiotensin system (RAS) and thereby regulates hypertension, sodium-water retention and protects multiple organs, including the heart, kidneys and lung.[17] Therefore, rACE2 can be used to alleviate angiotensin II–induced diseases.

APN01 (alunacedase alfa, GSK-258688; Apeiron Biologics), a human soluble rACE2,[18–20] was evaluated in a Phase 2 clinical trial as a treatment for SARS-CoV-2 infection (NCT04335136).[21,22] Due to its catalytic activity, APN01 is able to regulate the RAS to minimize organ injuries and improve the symptoms in patients with severe COVID-19.[22,23]

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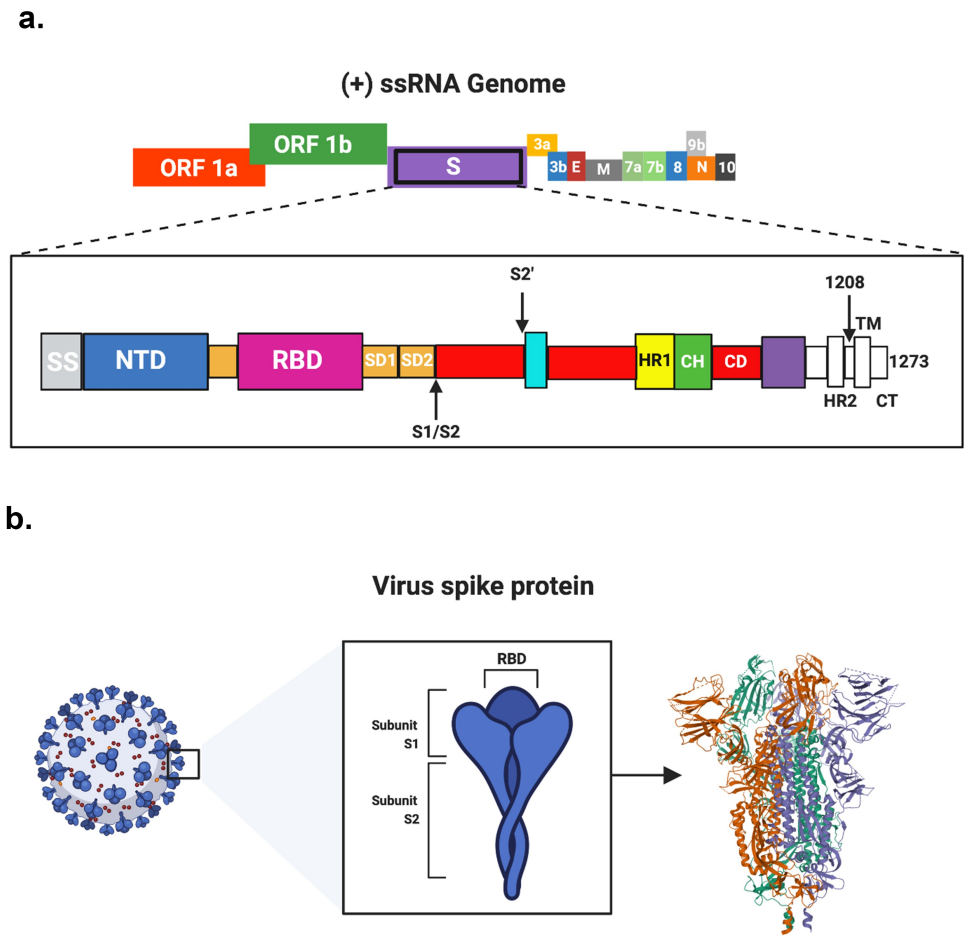

Figure 1. SARS-CoV-2 genome and the encoded S protein. A. Schematic representation of the single-stranded positive-sense RNA (+ ssRNA) genome of SARS-CoV-2 (27–32kb in length). Different domains are shown by different colors. ORF, open reading frame. The spike (s) protein consists of secretion signal (SS); N-terminal domain (NTD); receptor-binding domain (RBD); subdomain 1(SD1); subdomain 2 (SD2); protease cleavage site (S1/S2); heptad repeat 1 (HR1); central helix (CH); connector domain. (CD); heptad repeat (HR); transmembrane domain. (TM); and cytoplasmic tail (CT). B. The crystallographic primary structure of the S protein.

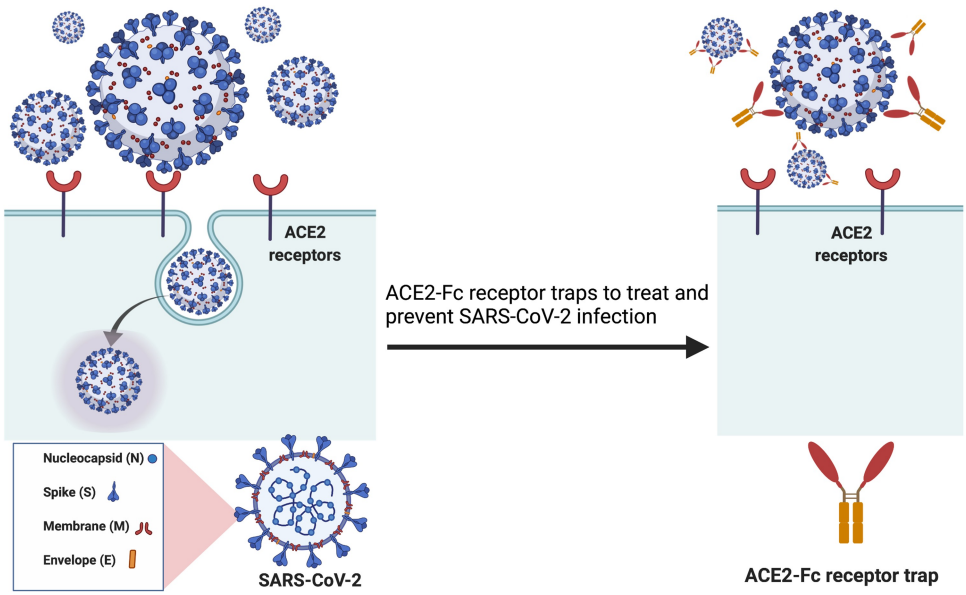

Figure 2. Soluble recombinant human ACE2-Fc protein as a decoy receptor to SARS-CoV-2. Recombinant human ACE2-Fc protein generated by fusing the C-terminus of the human ACE2 extracellular domain to a human IgG Fc region could work as a potential SARS-CoV-2 inhibitor.

The ACE2 cell receptor is a common entry gateway of several human coronaviruses, including SARS-CoV-1, HCoV-NL63 and SARS-CoV-2, wherein the viral spike proteins are used for receptor binding.[9,24,25] Therefore, rACE2 has been evaluated as a potential antiviral therapy in which the protein acts as a decoy to facilitate immune clearance of such viruses.[19,20,26,27] SARS-CoV-2 is an RNA virus that is expected to have a high mutation rate,[28] which might consequently enable the virus to acquire resistance against vaccines and antibodies.[29–34] Thus, using ACE2 as a decoy receptor for SARS-CoV-2 is particularly attractive as it aims to combat the interactions between SARS-CoV-2 S protein and cellular ACE2 receptor (Figure 2). Moreover, ACE2 has the ability to neutralize emerging SARS-CoV-2 variants of concern that harbor antibody-escape mutations, as has been observed for vaccine escape mutations.[33,35]

Several studies performed deep mutagenesis analysis to identify critical amino acid changes in ACE2 that could increase affinity for SARS-CoV-2 S protein. For example, the engineered decoy receptor ACE22.v2.4, showed high affinities to broadly diverse SARS-CoV-2 S proteins from humans and bats, despite the fact that the ACE2-binding surface region have high diversity. These results suggest that resistance to such engineered decoy receptors will most probably be rare and that they might be active against future emerging outbreaks of SARS-related betacoronaviruses.[36,37]

Other computational, site-directed mutagenesis and glycosidase treatment studies investigated S protein N-glycosylation involved in RBD-ACE2 interaction to engineer an ACE2 decoy receptor with enhanced S protein binding affinity and improved virus neutralization. Recent studies indicated that S N-glycans attached to N343, N165, N234, N90 and N322 positions of ACE2 play important role in determining spike binding, facilitating RBD opening and stabilizing RBD-ACE2 interaction.[38–41]

As with any therapeutic, there are strengths and limitations associated with the rACE2-based treatment. For example, supplementation of exogenous rACE2 might be a double-edged sword in COVID-19 patients with underline cardiovascular disorders. It has been reported that there are a close association between increased levels of circulating soluble ACE2 (sACE2) and cardiovascular diseases, which are known to be COVID-19 risk factors.[42,43] The elevated levels of sACE2 would initiate the formation of high numbers of circulating SARS-CoV2–sACE2 complex, which might be responsible for vascular occlusions, autoimmune inflammation, and organ ischemia. Therefore, further laboratory and clinical research are needed to assess the use of rACE2 to treat COVID-19 patients with cardiovascular diseases.

Additionally, pharmacological studies showed that rACE2 as a decoy receptor exhibits a short half-life in both human and mice and is limited by its fast clearance.[18] Moreover, there have been opinions that rACE2 may unintentionally upregulate endogenous ACE2 expression, alter the balance of ACE2 hormonal substrates and worsen COVID-19 recovery.[44,45] To overcome this, it has been suggested that fusion of enzymatically inactivated ACE2 to the Fc region of human immunoglobulin G offers superior pharmacokinetic and pharmacological benefits compared to rACE2-based therapy. Thus, this review is focused on different strategies used to engineer human rACE2 as Fc fusion proteins (i.e., immunoadhesins) to trap SARS-CoV-2.

ACE2-Fc immunoadhesin as a decoy receptor

ACE2-Fc immunoadhesins, as antibody-like molecules, offer substantial advantages over other traditional antiviral treatments. The effector functions of the Fc domain allow the recruitment of several phagocytic immune cells, including dendritic cells, macrophages and natural killer cells, and facilitate the activation of the host antiviral immune response against the virus.[46,47] Additionally, fusing ACE2 with Fc would improve the recombinant protein half-life, binding affinity, long-acting time, serum stability, antiviral specificity, neutralization efficacy and transport into the lung.[48–51] For example, neonatal Fc receptors (FcRn) are widely expressed by a variety of cell types, including endothelium and pulmonary epithelial cells, and thus these cells are capable of transporting IgG and Fc fusion molecules into the respiratory tract through the mucosal barriers.[52–54]

Immunoadhesin-based drugs have been widely used in modern biopharmaceuticals. Around 13 immunoadhesins have been approved in the United States or Europe and are currently marketed as treatments for different disease conditions, including systemic lupus erythematosus, multiple sclerosis and rheumatoid arthritis, and many more are in different phases of clinical trials.[55–58] However, no antiviral immunoadhesins have been approved, though at least one has entered clinical trials for human immunodeficiency virus (HIV) treatment.[59] While HIV is not kinetically and clinically similar to the SARS-CoV-2, previous research on HIV suggests that decoy receptor could be a potential therapeutic strategy against SARS-CoV-2.

Recent research showed that making a cocktail therapeutic by mixing neutralizing antibodies that do not bind to the RBD with RBD-targeting antibodies and engineered ACE2-Fc would generate synergistic inhibitory effect against viral infections.[60] Furthermore, it has been found that a ACE2-Fc fusion protein induced a broad neutralization capacity against many SARS-CoV-2 variants, including D614G, B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.1 (Kappa) and B.1.617.2 (Delta).[61] Thus, engineering immunoadhesins as receptor traps and stockpiling them would be critical for a fast, preemptive approach against emerging and reemerging betacoronaviruses to help control future outbreaks.

Inactivating ACE2 intrinsic catalytic activity

ACE2 at optimum concentration can lower virus infectivity in vitro, in a similar pattern as neutralizing antibodies.[21,62–64] Moreover, it has an intrinsic catalytic activity that regulates cardiovascular functions and fluid balance.[65] However, the role of ACE2 receptors for viral entry is not directly linked with their enzymatic and nonenzymatic actions. Thus, it is critical to introduce individual point mutations on the catalytic site of ACE2 to inactivate the unnecessary carboxypeptidase activity, and to achieve an exclusive antiviral effect.

The ACE2 metallopeptidase activity requires a divalent cation such as Zn^{2+}, wherein the zinc ion binding site is buried in the catalytic cleft within the proximal lobe, which is close to the viral binding site on ACE2. Three proximal lobe residues, His374, His378 and Glu402, which control the Zn^{+2} binding with their side chains, are the apparent candidates to be

mutagenized to generate enzymatically inactive ACE2 mutants. For example, Lei et al. constructed an ACE2 immunoadhesin by fusing the extracellular domain of a modified version of the human rACE2 (1–740) to the Fc region of the human immunoglobulin IgG1.[63] The modified ACE2-Fc variant includes two specific mutations for histidine residues 374 and 378 to asparagine (H374N and H378N) to reduce the intrinsic ACE2 catalytic activity. The fusion protein has shown high binding affinity toward the RBDs of SARS-CoV-1 (170 nM) and SARS-CoV-2 (11 nM), which was determined with BIAcore-binding assay. The ACE2-Fc receptor trap was also able to neutralize and inhibit S protein-mediated fusion of both SARS-CoV-1 and SARS-CoV-2, despite the H374N and H378N mutation, suggesting that fusing recombinant sACE2 to the Fc region of the human IgG1 can remarkably improve the neutralization potency and pharmacokinetics of the receptor traps.[63,66]

Although mutations at the Zn^{+2} ion binding sites H374 and H378 can produce enzymatically inactive mutants, mutations within zinc-binding pocket may cause protein instability because the divalent cation is essential for metallopeptidase protein structure and stability.[67–69] Therefore, mutations within the ACE2 substrate-binding pocket are less likely to cause construct instability or affect the binding affinity. A study performed by Liu et al. investigated individual point mutations using alanine within the catalytic site of ACE2 to inactivate the unnecessary carboxypeptidase activity and to achieve stable and effective version of the receptor trap.[62] They were able to engineer enzymatically inactive ACE2-Fc immunoadhesin variants that can block and neutralize the virus without the potential adverse side effects from its catalytic activities. Six other residues located in the proximal and distal lobe (Glu145, Arg273, His345, Pro346, Asp368 and His505) were also mutagenized to produce enzymatically inactive ACE2 mutants because their side chains formed direct interactions with hormonal substrates.[62] Among all enzymatically inactive ACE2-Fc variants, the Arg273Ala mutant, which is a substrate-binding residue, showed complete inhibition of the peptidase activity and is expected to maintain protein stability and binding affinity to the viral RBD. There are, however, compelling arguments that ACE2 catalytic activity can help alleviate COVID-19 symptoms through blocking receptor-binding sites on the S protein to neutralize SARS-CoV-2 infection or promoting angiotensin II breakdown and angiotensin-1-7 production.[22]

Engineering ACE2-Fc immunoadhesin through guided evolution platforms

The main focus of engineering ACE2 as receptor traps is to enhance their binding affinity, avidity, and specificity, as well as pharmacokinetic and pharmacological efficacy.[36,47,63,70] Wild-type ACE2 is not an optimal anti-SARS-CoV-2 biotherapeutic due to its modest affinity toward the virus RBD.[71–73] Therefore, it is essential to engineer ACE2 to have a binding affinity (K_D) in the low- to sub-nanomolar range, which is comparable to SARS-CoV-2 S protein-specific monoclonal antibodies.[74–78]

The crystal structure of the SARS-CoV-2/human ACE2 complex revealed that the interacting segments of ACE2 include the residues S19 to A614,[79] which indicates the amino acid side mutations that are crucial for enhancing the ACE2–RBD interaction. As a result, different directed evolution approaches were performed to enhance ACE2 properties as receptor traps such as using artificial intelligent (AI), mutagenesis and display technologies. For instance, Glasgow et al. described a novel approach to generate numerous affinity matured and enzymatically inactive recombinant ACE2 immunoadhesins that act as receptor traps to block and prevent SARS-CoV-2 entry into host cells.[66] In their two-phase stepwise approach, AI was first used to engineer ACE2 variants by introducing specific amino acid changes that have resulted in several-folds tighter binding to the RBD compared to the wild-type ACE2. Secondly, they used the re-engineered receptor traps as templates for affinity maturation using yeast surface display, which enabled the isolation of variants with binding affinities that have several folds higher to the RBD compared to the original templates. A human ACE2 that comprises residues 18 to 614 extracellular domain [ACE2(614)] was used as a template for the computational affinity optimization strategy.[66] A computationally guided selection followed by affinity maturation using yeast surface display were used to generate variant 313 [K31F, N33D, H34S, E35Q ACE2(614)], which was one of the best clones in terms of binding affinity and neutralization potency. The computational design methods based on AI bypass the bias that can arise from using experimental affinity maturation platforms alone, and remarkably accelerate the overall process. Yeast was used as a eukaryotic display system due to its effective surface display and its ability to produce glycosylated proteins.[80]

The ACE2 ectodomain (18 to 740) contains an enzymatic domain (18–615) and a collectrin-like domain (CLD). Interestingly, Glasgow et al. also observed a significant enhancement for the ACE2-Fc decoy receptors affinity, avidity, and stability by including the natural ACE2 CLD [ACE2(740)-Fc]. This is consistent with another study that showed that ACE2(740)-Fc is more effective in blocking viral infection compared to ACE2(614)-Fc.[81] Also, to avoid the off-target effects that accompanied ACE2 catalytic enzyme activity without affecting the binding affinity toward RBD and ACE2(740)-Fc scaffold, they inactivated the peptidase activity by including an H345L mutation, which is important for substrate binding.-[82] Noteworthy, replacing the leucine with alanine for His345 residue did not inhibit the ACE2-Fc catalytic activity toward angiotensin II, but, on the contrary, it enhanced the construct activity toward the substrate compared to the wild-type.[83,84] Further investigations are required for better understanding of ACE2 mechanism and substrate specificity.

The computationally designed, affinity maturation, and enzymatic inactive variant 313 [K31F, N33D, H34S, E35Q, H345L ACE2(740)-Fc] was able to bind the RBD of SARS-CoV-2 a hundred times higher than wild-type ACE2-Fc and neutralize pseudoviruses and authentic SARS-CoV-2 virus with half-maximal inhibitory concentrations (IC_{50}) of less than 100 ng/mL.

Higuchi et al. demonstrated another strategy to enhance the binding affinity of ACE2 to the SARS-CoV-2 S RBD. They performed a mammalian cell (HEK-293 T)-based guided evolution using surface display of mutagenized library in association with fluorescence-activated cell sorting (FACS).[85]

Screening system based on mammalian cells were used instead of yeast display to isolate ACE2 variants with proper posttranslational modifications patterns and favorable biophysical and biological attributes.[86,87]

The viral S protein interface is found in the protease domain, which is located in the top-middle segment of the ACE2 ectodomain. Thus, Higuchi et al. mutagenized the protease domain using error-prone PCR. They generated small plasmid library (~10^5 mutants), which were transformed into competent cells and packaged into lentivirus before being expressed in human HEK-293 T cells. The library was then screened for 3 cycles and only the highest binding cells to SARS-CoV-2 RBD-GFP were collected by FACS. Mutant 3N39v2 (A25V, K31N, E35K, L79F) showed binding affinity at sub-nanomolar levels to SARS-CoV-2 RBD (K_D ~0.64 nM) due to its slow dissociation rate. This is because 3N39v2 saturate all three RBDs on the S protein trimer complex, while wild-type ACE2 binds to S protein mainly as 1:1.[85,88] 3N39v2 has also demonstrated potent neutralization of SARS-CoV-1 and SARS-CoV-2 pseudoviruses and authentic SARS-CoV-2 virus. In a COVID-19 hamster model, the 3N39v2 fusion protein has showed efficacy in mitigating lung abnormalities, viral RNA copies and cytokine expression, which are associated with COVID-19 severity.

Engineering ACE2-Fc immunoadhesin using mutations based on host-ortholog receptors

The binding domain of the viral cellular receptor may be used to render immunoadhesins. However, zoonotic viruses can bind to their animal-derived ortholog cellular receptors with higher affinities than human cell-surface receptors resulting from natural evolution.[89] Therefore, it was proposed that immunoadhesins built with mutations based on host-ortholog receptors can provide stronger antiviral therapeutics.

SARS-CoV-2 has a genome that is close to bat-derived SARS-like coronaviruses, which make them a probable origin of SARS-CoV-2.[90] Since ACE2 orthologs from different species may serve as SARS-CoV-2 entry receptors, these species may have served as intermediate reservoir hosts before virus spreading to humans.[90] For SARS-CoV-2, the human-ACE2 receptor has been shown to be a suboptimal receptor.[36] As a result, it was proposed that human-ACE2 can be re-designed to have a higher binding affinity for SARS-CoV-2 in order to establish an efficient immunotherapy that can effectively block and prevent virus infection.

According to Cohen-Dvashi et al., sequence alignment of many ACE2 orthologues derived from mammals revealed many non-conserved residues of the RBD-recognition site on ACE2 receptor,[91] suggesting that there are several potential sequence mutations in ACE2 that can be implemented to enhance the binding affinity to SARS-CoV-2. Cohen-Dvashi et al., therefore, subjected tens of orthologous ACE2 genes with at least 80% similarity to human-ACE2 to Rosetta atomistic modeling calculations to find beneficial ACE2 mutations that could maximize construct stability and binding affinity to the virus RBD. They generated potent, enzymatic inactivated, affinity-matured human ACE2-Fc immunoadhesin variant with eight incorporated mutations (T27L, D30E, Q42R, E75R, L79Y, N330F, T92R and E375L). For example, T92R mutation enhanced the affinity to SARS-CoV-2 RBD because of the elimination of N-linked glycan site, which imposes steric constraints for the binding to the RBD, while the arginine can form polar interactions with proximate glutamine. The modified ACE2-Fc variant demonstrated superior IC_{50} and K_D values compared to the unmodified ACE2-Fc.

In another report, Mou et al. tested HEK-293 T cells expressing several ACE2 orthologs for their ability to bind to the recombinant SARS-CoV-2 RBD.[92] The study showed that human, pangolin, and horseshoe-bat ACE2 orthologs bind to SARS2-CoV-2 RBD more efficiently compared to other organisms. Bats have been suggested to be the species of origin of SARS-CoV-2 since the virus genome has more than 95% similarity with other bat coronaviruses,[25] while pangolin have been suggested to be the intermediate host of SARS-CoV-2 rather than a long-term reservoir.[93] Mou et al. also suggested that SARS-CoV-2 S protein is not completely adapted to human ACE2,[92] and, therefore, mutations derived from pangolin and horseshoe-bat ACE2 orthologs were implemented to augment binding affinity and neutralization potency of human ACE2-Fc to SARS-CoV-2. Their report showed that the five bat-derived mutations (Q24E, T27K, H34S, N49E and N90D) enhanced ACE2-Fc binding affinity toward SARS-CoV-2 RBD (5.454 nM) compared to the wild-type ACE2-Fc (11.64 nM). Furthermore, the capacity of the modified ACE2-Fc variant to neutralize SARS-CoV-2 pseudo- and live viruses was significantly more potent than wild-type ACE2-Fc.

It is well documented that SARS-CoV-2 uses the ACE2 receptor as a gateway to infect host cells.[94] However, it is not confirmed until now if domestic animals have a role in SARS-CoV-2 transmission.[95–97] Nonetheless, since wild animals such as bats have been shown to play an essential part in the transmission of notorious coronaviruses,[81] it is vital to study the virus's distributions among different hosts. Thus, the capacity of SARS-CoV-2 to bind to ACE2 animal orthologs was performed to assist identifying potential animal hosts. Accordingly, Le et al. evaluated 16 ACE2 orthologs to determine if they support SARS-CoV-2 entry by using recombinant RBD-IgG, pseudovirures and live virus.[81] The purified SARS-CoV2 RBD-IgG was able to bind to human ACE2 and ACE2 orthologs from a variety of domestic mammals, including camels, horses, cats, and rabbits, suggesting that these animals can be infected and might act as intermediate hosts for SARS-CoV2 viruses. Moreover, they tested the neutralization efficiency of different forms of ACE2-IgG against SARS-CoV-2 pseudovirures and live virus.[81] ACE2(615)-IgG and ACE2 (740)-IgG variants were designed to inactivate ACE2s' intrinsic enzymatic activity and enhance the immunoadhesins affinity toward the SARS-CoV-2 RBD. These mutations were based on adding hydrophobic residues (Y83W, H34Y and M82K) or enhancing the salt-bridge interactions (D30E). The ACE2 (740)-IgG variants were substantially more potent than ACE2 (615)-IgG variants. Additionally, compared to all other ACE2-IgG variants, ACE2(740)-D30E-IgG mutant had the best neutralizing activity against SARS-CoV-2. Furthermore, they engineered a more potent ACE2(740)-DE30-IgG version that has four sACE2 domains in a single molecule instead of two. This quadruple ACE2(740)-DE30-IgG showed more than several-

Table 1. Examples of modified ACE2-Fc as possible anti-COVID-19 agents.

Candidate	Modification strategy	ACE2 mutations	Binding affinity (K_D)[a]	Pseudovirus neutralization[b]	Authentic virus neutralization[c]	*In vivo* study	Ref.
ACE2(740)-IgG1-Fc (CVD313)	Computational-guided selection followed by yeast display	K31F, N33D, H34S, E35Q, H345L	Decreased off-rate hindered accurate estimation	↑ 25-fold	Efficiently neutralized SARS-CoV-2[b]	NA	66
ACE2(740)-IgG1-Fc_{LALA} (MDR504)	ACE2 fused to Fc-IgG1 with L234A/L235A mutations	H345A	NA	↓ 1.2-fold	↑ 2-fold	K18-hACE2 transgenic mice: ↓ Lung damage ↓ Viral load	64
ACE2(740)-IgG1-Fc	Alanine scanning at the substrate-binding residues for peptidase activity inhibition	R273A	NA	↓ 1.46-fold	NA	NA	62
ACE2(615)-IgG1-Fc (Coronacept)	Computational-guided selection based on orthologous ACE2 genes	T27L, D30E, Q42R, E75R, L79Y, N330F, T92R E375L	↑ 300-fold	↑ 17-fold	NA	NA	91
ACE2(740)-IgG1 (ACE2-Ig-v3)	Adopting IgG-like tetravalent configuration	D30	NA	↑ 19-fold	↑ 5-fold	NA	81
ACE2(615)-IgG-Fc	Mutations derived from different horseshoe-bat ACE2 orthologs	Q24E, T27K, H34S, N49E, N90D	↑ 2-fold	↑ 5-fold	↑ 2.3-fold	NA	92
ACE2(615)-IgG1-Fc (3N39v2)	Mammalian cell-based guided evolution in association with fluorescence-activated cell sorting	A25V, K31N, E35K, L79F	↑ 27-fold	↑ 300-fold	↑ 100-fold	Hamster: ↓ Lung damage ↓ Viral load	85
ACE2(732)-IgG4-Fc_{S228P}	ACE2 variants fused to Fc-IgG4 with S228P mutation	± H374N, H378N	No change	↓ 1.46-fold	↓ 1.5 to 2-fold[c]	NA	103
ACE2(740)-$Fc_{LALA\text{-}PG}$	ACE2 fused to Fc-IgG1 with L234A/L235A/P329G mutations	H374N, H378N	↓ 2-fold	Efficiently neutralized different SARS-CoV-2 variants	Efficiently neutralized SARS-CoV-2[b]	Hamster: ↓ Lung damage ↓ Viral load	109

[a]Binding affinities and neutralization potencies of modified ACE2-Fc immunoadhesins listed in the table were reported as fold change relative to the wild-type (WT) ACE2-Fc.
Ref., reference(s).
NA., data not available
[b]Neutralization potency of WT ACE2-Fc was not reported.
[c]Neutralization potency of the fusion proteins against several SARS-CoV-2 variants of concern

fold improvement in IC_{50} compared to the original dimer version in pseudovirus neutralization assay. Additionally, ACE2(740)-DE30-IgG with antibody-like configuration showed more potent neutralization of live virus infection at 0.16 µg/ml compared to bivalent ACE2(740)-DE30-IgG, which makes it a powerful entry inhibitor against SARS-CoV-2 virus.

Limitation of the ACE2-Fc immunoadhesin

One potential limitation of the ACE2-Fc immunoadhesin strategy is that it could inadvertently activate the immune system. ACE2 is secreted in all human tissues, including the heart, kidney, and gastrointestinal tissues. Therefore, the elevation of circulating extracellular ACE2 domain for a prolonged time via Fc domain extended half-life could have unknown long-term clinical outcomes.

Another potential concern is that the presence of Fc might increase complement and cytokine responses, which might exacerbate the inflammation, worsen the patient's condition, leading to infection advancement.[98–100] Although the constant heavy-chain regions of the IgG isoforms have similar amino acid sequences, they exert different levels of effector functions. IgG1- and IgG3-Fc are known for strong complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP) and antibody-dependent cell-mediated cytotoxicity (ADCC), whereas, IgG4-Fc has lower Fc-mediated antibody effector functions.[101,102] Therefore, Svilenov et al. engineered IgG4-Fc-based ACE2(732)-Fc fusion constructs to avoid Fc-mediated cytotoxic side effects.[103] Nonetheless, the importance and the potential roles of Fc-associated effector functions in virus elimination should be further studied, as they could contribute to ACE2-Fc inhibitory effects against SARS-CoV-2.

Moreover, naturally occurring IgG4 antibodies are less stable than IgG1 versions, which makes them less valuable as biopharmaceutical products.[104–107] Therefore, Svilenov et al. incorporated the immunoglobulin Fc region of an IgG4 isotype with an S228P sequence mutation in the hinge region to minimize Fab arm exchange and increase the stability of the ACE2-IgG4-Fc fusion protein.[108] The ACE2(732)-IgG4-Fc_{S228P} construct was successfully expressed and purified with high binding affinity (K_D ~4 nM) to immobilized SARS-CoV-2 RBD and potent antiviral activity at nano- to picomolar levels.

Ferrari et al. demonstrated a catalytically inactive ACE2 (740)-H374N:H378N-Fc decoy receptor with abrogated FcγR interaction to reduce the risk of antibody-dependent enhancement.[109] They engineered an Fc L234A/L235A/P329G mutant in the CH2 domain (ACE2(740)-H374N:H378N-$Fc_{LALA\text{-}PG}$), which showed complete abrogation of human FcγR engagement while preserving the FcRn interaction to provide extended half-life. The generated construct displayed a potent neutralization activity in vitro against four SARS-CoV-2 variants, including D614G, B.1.1.7, B.1.351 and P.1. Additionally, administration of ACE2(740)-H374N:H378N-$Fc_{LALA\text{-}PG}$ in a SARS-CoV-2 hamster model showed significant reduction in viral RNA copy as well as lung damage.

Recombinant ACE2-Fc is expected to have an acceptable safety profile based on APN01 clinical trials, wherein no serious undesirable reactions were reported.[22,110] However, similar to monoclonal antibodies, patients receiving ACE2-Fc immunoadhesins might produce anti-drug antibodies, which would negatively affect the pharmacokinetic, safety and efficacy of the therapeutic molecule.[111,112] While acquiring immunological cross-reactivity against endogenous ACE2 after receiving recombinant ACE2-Fc would have a long-term devastating effect on patients, clinicians can use well-evaluated methods to delineate and reduce any undesirable immunological reactions of therapeutic biologics during the course of ACE2-Fc treatment.[113]

Conclusion

ACE2-Fc immunoadhesins offer considerable advantages over other therapeutics that aim to neutralize SARS-related betacoronaviruses. Here, we discussed different optimization strategies that helped generate several modified ACE2-Fc versions as promising anti-SARS-CoV-2 candidates (summarized in Table 1). Beyond coronaviruses, ACE2-Fc-based therapeutics could be used as a quick therapeutic option for future viral outbreaks that use the ACE2 receptor for entry without risk of virus mutational escape. Moreover, engineered ACE2-Fc as an antibody-like biomolecule would have the potential to be used as a prophylactic agent for those who are at high risk of COVID-19, such as healthcare workforces especially in the absence of an effective vaccine against new variants.

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Author contributions

Conceptualization – MA; Original draft Preparation – MA, AZ, SA; Review and editing – MA, AZ, SA, AH; Figures and visualizations – MA. All authors have read and agreed to the published version of the manuscript.

Abbreviations

ACE2, Angiotensin-converting enzyme 2; K_D, Binding affinity; CLD, Collectrin-like domain; IC_{50}, Half-maximal inhibitory concentrations, RBD, Receptor-binding domain; rACE2, Recombinant ACE2, SARS-CoV-2, Severe acute respiratory syndrome coronavirus 2; S, Spike protein.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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