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Review

Engineering ACE2 decoy receptors to combat viral escapability

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Decoy receptor proteins that trick viruses to bind to them should be resistant to viral escape because viruses that require entry receptors cannot help but bind decoy receptors. Angiotensin-converting enzyme 2 (ACE2) is the major receptor for coronavirus cell entry. Recombinant soluble ACE2 was previously developed as a biologic against acute respiratory distress syndrome (ARDS) and verified to be safe in clinical studies. The emergence of COVID-19 reignited interest in soluble ACE2 as a potential broad-spectrum decoy receptor against coronaviruses. In this review, we summarize recent developments in preclinical studies using various high-affinity mutagenesis and Fc fusion approaches to achieve therapeutic efficacy of recombinant ACE2 decoy receptor against coronaviruses. We also highlight the relevance of stimulating effector immune cells through Fc-receptor engagement and the potential of using liquid aerosol delivery of ACE2 decoy receptors for defense against ACE2-utilizing coronaviruses.

Engineered ACE2 decoy receptors as an alternative strategy to overcome SARS-CoV-2 evolution

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was first identified in the Chinese city of Wuhan in late 2019. SARS-CoV-2 is a positive-stranded RNA virus that belongs to lineage B (sarbecovirus) of the betacoronavirus genus [1–3]. ACE2 (see Glossary) is the major receptor for SARS-CoV-2 cell entry. The virus attaches to host cells through its trimeric spike glycoprotein in which the S1 subunit binds to the receptor and the S2 subunit mediates membrane fusion [4]. Along with worldwide spread of the virus, viral RNA genome replication errors have continuously generated new mutants. Some of these have had advantages in viral transmissibility and have formed new waves of infections. To fight against COVID-19, intensive efforts have been made in the development of vaccines and drugs. The main drug targets are the site of initial interaction between the receptor binding domain (RBD) of the virus spike protein and host cell surface ACE2, and the main protease that cleaves virus nonstructural long polyproteins, as well as the cleaved product, RNA-dependent RNA polymerase (RdRp) [5–8]. Among drugs developed for SARS-CoV-2, monoclonal antibodies (mAbs) have gained a leading role as therapeutics against COVID-19. However, mAbs have an Achilles’ heel in that mutations in the spike gene can lead to the SARS-CoV-2 adaptation to neutralizing antibodies, termed escape mutation [9–11]. To prevent such viral evasion, some mAbs are administered in cocktail form and others are designed to target evolutionarily conserved regions [12,13]. With these efforts, mAbs have effectively prevented infection and clinical progression for most SARS-CoV-2 strains [14].

However, the striking evolution of omicron, which included numerous mutations in the spike, resulted in escape from most mAbs in clinical use, as well as from vaccinated sera [15–16].

Highlights

Recombinant angiotensin-converting enzyme 2 (ACE2) was developed as a biologic against acute respiratory distress syndrome and rapidly repositioned as a decoy receptor for SARS-CoV-2 after emergence of COVID-19.

High-affinity mutagenesis and fusion with IgG-Fc enables recombinant ACE2 to effectively neutralize SARS-CoV-2 in animal models of COVID-19.

ACE2 decoy receptors neutralize broadly SARS-CoV-2 variants as well as ACE2-utilizing sarbecoviruses.

Enhancement of Fc effector function has the potential to promote antiviral effects by the clearance of viral particles, cytotoxic elimination of virus-infected cells, and induction of antiviral T cell responses.

Direct drug delivery to infectious site by liquid aerosol inhalation potentially enhances therapeutic efficacy against COVID-19.
ACE2 decoy receptors are an alternative strategy to neutralize the virus in a manner that is robust against such escape [19,20]. The concept of decoy receptors has already been clinically applied to modulate autoimmune diseases and angiogenesis, with a focus on tumor necrosis factor receptor (TNFR), cytotoxic T lymphocyte associated antigen-4 (CTLA4) [21], or vascular endothelial growth factor receptor (VEGFR) [22,23]. In the case of viral infection, decoy receptors have a distinct advantage over mAbs in terms of resistance to escape mutations [24,25]. Viral mutations escaping from decoy receptors may appear, but in theory, they would have limited binding affinity toward the native receptors on host cells, leading to reduced or even absent infectivity [24]. In recent studies, virus culture treated with sparing concentrations of ACE2 decoy receptors has generated no escape mutants [24], and omicron and even other sarbecoviruses remain sensitive to ACE2 decoy receptors (Figure 1) [26,27].

Figure 1. Comparison of engineered angiotensin-converting enzyme 2 (ACE2) decoy receptor and monoclonal antibody against neutralization of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). (A) The spike protein of SARS-CoV-2 binds to ACE2 on the surface of host cells through its trimeric spike glycoprotein and its interaction is essential for internalization of SARS-CoV-2 into host cells. The receptor binding domain (RBD) of spike protein is a direct binding site for ACE2 and a common target of neutralizing antibodies. (B) Evolutionary mutations in the spike gene can lead to the SARS-CoV-2 adaptation to neutralizing antibodies, termed escape mutation. In this case, existing monoclonal antibodies (mAbs) against a particular epitope of the original coronavirus spike RBD might inhibit infection by partially masking the viral spike RBD. Engineered high affinity ACE2 mutants decoy receptors, some fused with human Fc region of immunoglobulin, have been developed by directed evolution, deep mutational scanning, computer-assisted design, and a combination of these approaches to improve neutralization of viruses and escape mutants. (C) The effective recognition of monoclonal antibody against SARS-CoV-2 largely depends on the virus variants. Recently developed ACE2 decoy receptors have been shown to neutralize all variant of concerns including SARS-CoV-2 omicron variant in cultured cells, while existing mAbs therapy developed for Wuhan strain SARS-CoV-2, for example, imdevimab and casirivimab, show reduced affinity to SARS-CoV-2 omicron strain [26].
Historically, recombinant human soluble ACE2 (rhACE2) was developed as an anti-inflammatory drug for ARDS [28]. Its therapeutic potential as a decoy receptor was investigated in the early phase of the COVID-19 pandemic [19]. During the pandemic, several groups made efforts to engineer affinity-enhancing mutations or to fuse human Fc region of immunoglobulin with soluble ACE2 in order to improve its pharmacokinetics and virus neutralization efficacy. Emergence of the omicron variant reinforced the difficulty of drug development against mutational escape of viruses and increased interest in decoy receptors as potential universal effective drugs against current and future SARS-CoV-2 variants and coronavirus pandemics.

ACE2 as an enzymatic drug for ARDS and decoy receptor for COVID-19

ACE2, a homolog of ACE, is a zinc metallopeptidase and predominantly hydrolyzes angiotensin (Ang) II to Ang 1–7 [29]. Ang II is the major effector of the renin–angiotensin system (RAS) and plays a key role in maintaining fluid and salt balance. It also mediates proinflammatory actions including vasoconstriction, apoptosis, capillary leak, and fibroproliferation via the angiotensin type I receptor (AT1R), while Ang 1–7 induces anti-inflammatory responses through the Mas receptor pathway [28]. Since increased Ang II and imbalance between AT1R and Mas receptor signals are involved in the pathogenesis of ARDS, ACE2 which converts Ang II to Ang 1–7, is expected to resolve this imbalance and improve lung inflammatory injury [29,30]. Indeed, the development of rhACE2 was motivated by its therapeutic effect in animal models of ARDS [31]. In clinical studies, although rhACE2 was well-tolerated in both healthy human volunteers and ARDS patients, and treatment resulted in rapid conversion of Ang II to Ang 1–7, no beneficial changes in acute physiology or clinical outcomes was detected [28,32].

Following the emergence of SARS-CoV-2, rhACE2 was repositioned as a decoy receptor for COVID-19 therapy. Treatment of vessel and kidney organoids with rhACE2 in vitro effectively blocked SARS-CoV-2 infection [19], but Phase 2 trials failed to demonstrate significantly improved clinical outcomes in the treatment of hospitalized patients upon intravenous administration, even though there were small improvements in pulmonary gas exchange efficiency (ClinicalTrials.gov identifier NCT04335136). Because rhACE2 monomers have a fast clearance rate in human blood with a half-life of ~3 h, one likely reason for the lack of efficacy is inadequate drug concentration at the infection site [32,33]. Currently, Phase 1 trials to evaluate the safety and tolerability of rhACE2 inhalation, which is a more direct administration to site of infection, are ongoing (NCT05065645). In parallel, ACE2 mutagenesis to increase binding affinity and fusion to Fc for extended half-life, avidity effects, and antiviral immune responses, are expected to improve the therapeutic potential of rhACE2 as a decoy receptor against COVID-19.

ACE2 mutagenesis approaches to achieve increased affinity

In comparison to native ACE2, the higher affinity of ACE2 decoy receptors for SARS-CoV-2 spike protein is important for competitive neutralization of infectious virus at the host cell surface at standard biologic doses. The equilibrium dissociation constant ($K_d$) between ACE2 monomer and SARS-CoV-2 spike RBD ranges from ~10 to ~60 nM, depending on the virus variant [4,16,24,34,35]. By contrast, mAbs in clinical use for COVID-19 treatment have $K_d$ <10 nM [36]. Multifaceted approaches have been recently used to enhance binding affinity of ACE2 decoy receptors to the level of therapeutic antibodies, including directed evolution, deep mutational scanning (DMS) and computational design.

Directed evolution as an established protein engineering method

Directed evolution is a powerful method to design proteins with desirable functions by repeated random mutagenesis and selection (Box 1) [37]. Recent studies demonstrate the utility of this method to achieve high-affinity virus neutralizers. For example, Hoshino and coworkers...
conducted directed evolution of ACE2 in an assay using human cell line. They focused on the protease domain of ACE2 which is the interface for SARS-CoV-2 spike RBD binding and performed mutagenesis by error-prone PCR of the N-terminal residues 18–102 of the protease domain (Figure 2). Three cycles of such mutagenesis and selection experiments identified three major high-affinity ACE2 mutants, termed 3N39, 3J113, and 3J320. Each high-affinity ACE2 mutant contained six or seven mutations. Substitution with wild-type residues resulted in a n-fold increased affinity compared to wild-type ACE2 (Table 1) [24]. In another study, Wilson and coworkers performed directed evolution with error-prone PCR and staggered extension process (StEP) in yeast surface display and identified several high affinity ACE2 mutants. Among these, a dimer form with IgG4-Fc fusion version of CDY14H that contains K31M/E35K/L79F/L91P/N330Y; and H345L substitution, which confers enzymatic inactivation was shown to have 1000-fold improved affinity (31 pM) [38] (Table 1). This CDY14HL decoy was then applied as an adeno-associated virus (AAV)-mediated intranasal gene therapy in mice and nonhuman primates. The result showed significant prophylactic effect against SARS-CoV-2 challenge. Although directed evolution is a classical approach to protein engineering, these studies indicate that it is still one of the efficient methods to achieve the saturated binding affinity between virus glycoprotein and its receptor. (See Figure 3.)

DMS for comprehensive mutagenesis analysis

DMS is an approach to comprehensively evaluate the effect of all possible single amino acid substitutions on a protein property parameter such as binding affinity. Procko and coworkers developed a single mutant ACE2 library encompassing all 20 amino acid substitutions at 117 sites, that includes the interface with the SARS-CoV-2 spike RBD as well as those involved in ACE2 enzymatic activity. The results of this study revealed the advantage of N-glycosylation removal at N90 and amino acid substitutions at several other important sites that can increase affinity. Although the original paper mentioned no detailed methods for synergistic combination of affinity-enhancing single amino acid substitutions, several candidates made up of three or four mutations were further analyzed. Soluble ACE2 (sACE2)v2.4, containing mutations T27Y/L79T/N330Y and fused to the collectrin domain (amino acids 18–740), a homolog of collectrin (Tmem27) which induces ACE2 dimer formation [39], gained >10-fold higher virus-neutralizing
activity compared to wild-type ACE2 dimer, with ~40-fold enhanced monomeric affinity toward SARS-CoV-2 RBD (Table 1) [40]. Although DMS provides large-scale datasets of single amino acid substitutions to reveal intrinsic protein properties, the limitation of this approach is the lack of established method to know which mutations can be combined synergistically. One solution is the use of computational modeling to structurally predict the effective combination without compromising protein stability or binding free energy [41]. The other is the machine learning-guided approach. Datasets from DMS and empirical affinity test for multiple mutations are used to train machine learning algorithm, and the resultant machine learning model predicts the affinity value for all possible combination of mutations [42,43].

**Computational design with growing sophistication**

There has been progress in computer-aided drug design using structure-based modeling and molecular dynamics simulation in various drug modalities including small compounds, peptides,
| Name       | Mutations for affinity (mutations for enzyme) | Length | Fc | \(K_d\) (nM) | IC50 (μg/ml) | Efficacy for omicron | Animal model, virus strain, delivery time, route and dose | Refs |
|------------|----------------------------------------------|--------|----|---------------|-------------|---------------------|-------------------------------------------------------------|------|
| Directed evolution |                                           |        |    |               |             |                     |                                                             |      |
| 3N39v2     | A25V/K31N/E35K/L79F (WT)                     | 615    | WT | 0.64          | 0.082       | Hamster, Wuhan, post-infection, 20mg/kg i.p.                | [24] |
| 3J13v2     | K31M/E35K/Q60R/L79F (WT)                     | 1.14   |    | 0.33          |             |                                                                  |      |
| 3J32v2     | T20I/H34A/T92Q/Q101H (WT)                    | 3.98   |    | 0.068         |             | Validated in vitro                                           |      |
| 3N39v4     | A25V/K31N/E35K/T92Q                        | 740    | WT | N.D.          | 0.036       | Validated in mice                                            |      |
| CDY14HL    | K31M/E35K/S47A/L79F/L91P/N330Y (H345L)       | 615    | IgG4| 0.031*        | 0.037       | K18-hACE2 mice, rhesus/ cynomolgus macaques, AAV, pre-infection | [38] |
| Deep mutational scanning |                                             |        |    |               |             |                     |                                                             |      |
| sACE2v2.4  | T27Y/L79T/N330Y                              | 740    | WT | 0.6*          | 22*         | Validated in vitro                                           |      |
| Computational design |                                       |        |    |               |             |                     |                                                             |      |
| CVD293     | K31F/H34I/E35Q (WT)                          | 740    | WT | 0.23*         | 0.036       |                                                                  |      |
| CVD313     | K31F/N33D/H34S/E35Q (H345L)                  |        |    |               |             |                                                                  |      |
| M81        | L79F/M82Y/Q325Y (H374A/H378A)               | 740    | GASDALIE* | 2.74*       | 26.0*       | K18-hACE2 mice, Wuhan, pre-/post-infection, 12.5 mg/kg i.v., Inhalation 3x | [45] |
| FFWF       | S19F/T27F/K31W/N330F (WT)                    | 740    | WT | 1.8*          | 16*         |                                                                  |      |
|            | S19W/N330Y (H374A/H378A/E402A)               | 615    | WT | 10.1          | 0.09        |                                                                  |      |
|            | S19W/T27W/N330Y (H374A/H378A/E402A)          |        |    |               | 19.3        |                                                                  |      |
|            | Q24E/T27K/H34S/N40E/N80D (H374N/H378N)      | 615    | WT | 5.45*         | 0.39        |                                                                  |      |
| Deglycosylation (WT) |                                           | N.A    | WT | 7.6*          | 16.2*       |                                                                  |      |
| Multimer ACE2 and others |                                             |        |    |               |             |                     |                                                             |      |
|            | WT (H374N/H378N)                             | 740    | LALA-PQ* | 11.5*       | 18.3*       | Hamster, Wuhan, post-infection, 5/50 mg/kg i.p.              | [74] |
| MDR504     | WT (H345A)                                   | 740    | LALA*  | N.D.        | 3.5nM*       | Ad5-hACE2-transduced/K18-ACE2 mice, Wuhan, pre-/post-infection, 15/30 mg/kg i.v. | [73] |
|            | WT (H374N/H378N)                             | N.A    | WT | 8.23*         | 1.59*       | Ad5-hACE2-transduced mice, Wuhan, pre-/post-infection, 50mg/kg i.p. | [73] |
|            | T27W-foldon (WT)                             | 615    | WT | 0.06          | 76.8        |                                                                  | [72] |
|            | D30E-tetramer/hexamer (WT)                   | 740    | WT | N.D.          | 0.07/0.06   |                                                                  | [69] |

Notes to Table 1:

*aAvidity value of ACE2 dimer with collectrin domain and/or Fc.
*bGASDALIE: G236A/S239D/A330L/I332E, LALA: L234A/L235A, LALA-PG: L234A/L235A/P329G.
*cIC50 value against authentic virus.
Following the emergence of the COVID-19 pandemic, many protein structures related to COVID-19 have been rapidly deposited in some virtual databases, such as the Protein Data Bank, helping drug development efforts. Pazgier and coworkers took advantage of high-resolution ACE2-RBD structures (6m0j and 6vw1) to design and generate high-affinity ACE2. Noncovalent intermolecular forces, such as van der Waals force, electrostatic interactions, hydrogen bonding, and hydrophobic interactions, play essential roles in determining the protein-binding force. On the basis of these noncovalent interactions of amino acid side chains at the interface between SARS-CoV-2 RBD and ACE2, they discovered that ACE2 mutant, M81 with L79F/M82Y/Q325Y substitutions obtained an ~10-fold increased affinity for the spike RBD over wild-type ACE2 (Table 1). Also, Lu and coworkers performed structure-based computational virtual screening at the ACE2-RBD interface. Their algorithm identified S19W, T27W, and N330Y in ACE2 protein as key substitutions and their combinations achieved up to an eightfold increase in the affinity toward SARS-CoV-2 RBD over wild-type ACE2 (Table 1).

Molecular dynamics simulation can provide plentiful kinetic information and calculate thermodynamic properties for the protein–drug interactions. Stadlmann and coworkers...
focused on glycan-mediated interactions between ACE2 and the spike protein [49]. In silico modeling and molecular dynamics simulations of fully glycosylated proteins have suggested that the spike–ACE2 complex formation is disrupted by glycans attached at N90 and N322 of ACE2. Also, the substitution of T92Q or N322Q to remove the respective glycans resulted in up to twofold increased affinity compared to wild-type ACE2. Full enzymatic deglycosylation further increased affinity for the spike RBD, as well as neutralization activity against SARS-CoV-2 (Table 1). Islam and coworkers also conducted computational mutagenesis and subsequent molecular dynamics for free-energy calculations on ACE2-SARS-CoV2/RBD interaction. The resultant ACE2 mutant, FFWF composed of S19F/T27F/K31W/N330F substitutions, achieved ninefold affinity enhancement over the wild-type ACE2 [50].

Multiple sequence alignment (MSA) is generally the alignment of three or more biological protein or DNA sequences, which infers the homology and the evolutionary relationships between the sequences [51]. Farzan and coworkers focused on the ACE2 protein sequences of horseshoe bats, because horseshoe bats are candidate reservoirs of SARS-CoV-2 and their spike RBD may have evolved to bind to horseshoe bat ACE2 [52]. Computational MSA of horseshoe-bat ACE2 revealed five consensus residues, Q24E/T27K/H34S/N49E/N90D. These residues were introduced in human ACE2, and they showed enhanced binding and neutralization efficacy (Table 1) [52].

Some other studies have combined multiple approaches to maximize the ACE2 binding toward SARS-CoV-2 RBD. Wells and coworkers combined computational modeling with sequential directed evolution based on yeast surface display [53]. They determined important amino acid side chains for binding of ACE2 to SARS-CoV-2 spike RBD by computational alanine scanning on the binding interface. Further free-energy calculations and computational saturation mutagenesis generated ACE2 mutant, CVD293 harboring K31F/H34I/E35Q substitutions. Directed evolution following computational design revealed another mutant, CVD313 with K31F/N33D/H34S/E35Q substitutions, which exhibited binding affinity and virus neutralization efficacy comparable to 3N39v2 [24] and sACE2v2.4 [40].

Currently, computational design generates only ACE2 mutants with weaker affinity to SARS-CoV-2 RBD compared with cell-based mutagenesis, and requires further fine adjustment from wet laboratory experiments [24,38,40,45,47,50,53]. However, the field of protein structure prediction and design is rapidly evolving, and new technologies based on deep learning that trains artificial neural network to approximate complicated unknown functions in a high-dimensional abstract space [54], are expected to play a major role in the near future.

**Fc fusion to manipulate pharmacokinetics, immunological activity, and avidity effect**

Fusion with the antibody Fc domain is a major modification to enhance drug concentration in target tissues and therapeutic effects. Many biologically active proteins or peptides have short serum half-lives due to their constant cellular uptake and subsequent lysosomal degradation, mainly in endothelial cells [55]. Human IgG antibody has a half-life of ~21 days. Its Fc domain allows it to bind to the neonatal Fc receptor (FcRn) in a pH-dependent manner. In pinocytosis-mediated IgG internalization and subsequent endosomal sorting, binding of Fc to FcRn at endosomal pH (5.5–6.0) prevents IgG from being directed to the endolysosome for degradation and enables its sorting to the plasma membrane. Upon fusion with the plasma membrane, IgG is released from FcRn at extracellular pH 7.4 and recycled back into circulation, thus prolonging its...
serum half-life [56,57]. For ACE2 decoys, IgG1-Fc fusion extended the half-life from ~3 to ~30 h, and enabled treatment of COVID-19 in animal models [24,32,58]. Also, IgG-Fc domain engages with Fcγ receptor (FcγR) family expressed on effector leukocytes and triggers the clearance of viral particles, cytotoxic elimination of virus-infected cells, and induction of antiviral T cell responses [59]. These Fc- and FcγR-mediated functions of antibody are termed Fc-effector function [60]. The importance of Fc-effector function is especially recognized in tumor immunotherapy [61] and some mAbs already approved for clinical use are engineered to enhance Fc-effector function for efficient elimination of cancer cells [62]. In case of virus diseases, FcγR interaction also has a risk of antibody-dependent enhancement (ADE) of virus infection to FcγR-positive immune cells, which has been, in fact, observed during secondary infection with dengue virus [63]. However, there are studies that indicate that there is no definite risk of ADE of SARS-CoV-2 infection and that Fc-effector function is important in the therapeutic and prophylactic administration of mAbs. Mouse studies have shown that mAbs containing mutated Fc lacking ability to bind to FcγR lose therapeutic efficacy and that mutations enhancing Fc-effector function further promote therapeutic effects of SARS-CoV-2 neutralizing mAbs [59,64,65]. Even in the prophylaxis of virus infection that mainly requires neutralizing activity [65,66], another study has reported that mAbs with mutations enhancing Fc-effector function improve prevention of COVID-19, similar to therapeutic administration [59].

Substitution of critical amino acids in Fc to modulate binding to specific receptors have been applied in clinical mAbs against COVID-19 considering the risk of ADE of virus infection, even though mouse studies and SARS-CoV-2 vaccine efficacy have disproved it. Etesevimab and cilgavimab (AZD1061) have LALA (L234A/L235A) and TM (L234F/L235E/P331S) mutations, respectively, and both mutations inhibit the binding of Fc to FcγR [67]. Also, to extend the half-life of COVID-19 mAbs, sotrovimab (VIR-7831) and tixagevimab (AZD8895) contain FcRn-oriented LS (M428L/N434S) and YTE (M252Y/S254T/T256) mutations, respectively, [57,67]. One open question in this area is whether enhancement of Fc-effector function or FcRn orientation is more suitable for COVID-19 therapy and prophylaxis. Another question is whether these Fc mutations have synergistic effects on antiviral efficacy. VIR-7832 is a derivative of sotrovimab (VIR-7831) and carries FcRn-oriented LS (M428L/N434S) mutation, as well as GAALIE (G236A/A330L/I332E) mutation to enhance Fc-effector function [68]. VIR-7832 is currently being evaluated in a Phase 2 study, directly comparing it with its parent, sotrovimab (VIR-7831), to assess the potential synergistic benefit of Fc-effector function and FcRn-related half-life extension in humans (NCT04746183).

Fusing IgG-Fc domain to other proteins also enhances avidity, that is the overall binding strength of protein interaction, due to bivalent target binding domain. Further increasing the valency of target binding domain by making quadrivalent and hexavalent sACE2-Fc proved beneficial in achieving more efficient neutralization [69,70]. Trivalent sACE2 is also effective when spatially aligned to the spike trimer [71,72]. These multivalent approaches are promising but these benefits need to be weighed against disadvantages such as low production yield.

**Dual aspects of ACE2 enzymatic activity on drugs for COVID-19**

ACE2 enzymatic activity has both the potential of lung protection and risk of adverse effects. Considering the therapeutic potential of rhACE2 for ARDS via conversion of proinflammatory Ang II to Ang 1-7, the enzymatic activity of the ACE2 decoy is expected to ameliorate lung inflammatory injury due to SARS-CoV-2 infection. Although there is extensive evidence to support the efficacy of rhACE2 in animal models of ARDS [30,31], Phase 2 trials have found no improvement in clinical outcomes of ventilated ARDS patients. It is possible that the dosage of rhACE2 may
have been insufficient to show beneficial effects [30]. If ACE2 decoy were to go to the clinic, the estimated dose of ACE2 decoy for COVID-19 would be ~tenfold higher than that used in ARDS clinical trials [24,28]. This dose may be sufficient to show protective effects against lung inflammation [27]; however, enzymatic activity could also lead to hemodynamic instability and unexpected adverse effects due to overconversion of Ang II to Ang 1–7. One approach to resolve the excessive enzymatic activity is the elimination of ACE2 metallopeptidase activity. Disruption of ACE2 zinc-binding motif, responsible and critical for its metallopeptidase activity inactivates it. Following this concept, H374N/H378N substitution was used in some studies; however, ACE2 without zinc-binding motif becomes structurally unstable [20,53,73,74]. Another strategy is introducing a mutation in ACE2 to block the binding with its substrate, Ang II. Wells and coworkers successfully designed a ACE2 decoy with H345L mutation. This mutation preserved ACE2 decoy thermostability and clearly blocked Ang II binding [53]. The introduction of a disulfide bond with S128C/V343C substitutions in ACE2 can also obstruct ACE2 substrate binding by closing the binding pocket [24]. The resultant ACE2 closed conformation not only completely eliminates enzymatic activity, but also greatly improves thermostability, which is a desirable attribute for biotherapeutics.

**Broad-spectrum efficacy against SARS-CoV-2 variants and sarbecoviruses**

As with most neutralizing antibodies, the main therapeutic target for ACE2 decoys is blocking the interaction of virus with native ACE2. In contrast to therapeutic mAbs, the concern of viral escape is expected to be low for decoy receptors. Virus mutants escaping from decoy receptors would have limited binding affinity toward the native ACE2 receptors, which results in impaired infectivity. This idea is supported by a recent study where ACE2 decoy receptors showed no signs of emerging escape mutants when treated at sparing concentration during long-term SARS-CoV-2 culture [24]. Although many existing mAbs showed less neutralization efficacy against omicron, ACE2 decoy versions fused with IgG1-Fc (3N39v4, 3J320v2, and sACE2v2.4) exhibited similar neutralization activity against the original Wuhan strain and multiple variants, including omicron in cultured cells [26]. Comprehensive mapping by DMS of ACE2 and the spike RBD is a useful tool not only to improve binding affinity, but also to identify escape mutations from immunized sera and neutralizing agents [25,40,76–78]. DMS of SARS-CoV-2 spike RBD to evaluate escape from the 3N39v4 decoy revealed that L455Y and N487Q mutations in the RBD partially reduced the neutralization efficacy of both 3N39v4 and wild-type ACE2 decoys. However, the 3N39v4 decoy preserved functional neutralization against viruses harboring these mutations in the RBD, indicating that no single-residue mutation in RBD achieved complete escape from engineered ACE2 decoy receptors, unlike in the case of mAbs [26].

In theory, ACE2 decoys would neutralize other sarbecoviruses that use ACE2 as an entry receptor. In fact, 3N39v4 decoy shows therapeutic efficacy against both SARS-CoV-1 and SARS-CoV-2 clades, including pangolin CoV (GD-1 and GX-P5L) and bat CoV (RaTG13, RsSHC014, and WIV1) [26]. sACE2v2.4 decoy also exhibit a better $K_D$ value for the RBD of some sarbecoviruses than wild-type ACE2 decoy does [25]. These results indicate the potential efficacy of engineered ACE2 receptor decoys against future zoonotic coronavirus diseases [13].

**Antiviral effects of ACE2 decoy receptors in animal models**

Pharmacokinetics and tissue absorption are important factors affecting in vivo therapeutic efficacy. The half-life of rhACE2 (i.e., non-Fc fusion) is reported to be ~3 h in humans [32,33]. However, fusion with wild-type IgG1-Fc and LALA-mutated Fc extended the half-life to 30–145 h in mice [24,74,79]. Lung distribution was also sufficient to achieve effective neutralization [24,79]. Wild-type ACE2 decoys composed of wild-type or LALA-mutated Fc protect lungs from the original Wuhan strain in both prophylactic pre-infection and therapeutic post-infection regimens in hamsters [74], as well as K18-hACE2 transgenic or Ad5-hACE2 transduced mice (Table 1) [79].
The 3N39v4 decoy also exhibits therapeutic efficacy in hamsters infected with Wuhan [24], as well as omicron [26] strains. sACE2v2.4 decoy was evaluated in epithelium-specific human ACE2 expressing (K18-hACE2 transgenic) mice that exhibited severe lung edema and death upon SARS-CoV-2 infection [80]. Prophylactic and therapeutic administration of sACE2v2.4 decoy improved pneumonia and survival from Wuhan and gamma strain infections (Table 1) [58]. sACE2v2.4 and 3N39v4 decoys exhibited antiviral effects at a dose of 10–20 mg/kg in rodent models of COVID-19, similar to therapeutic antibodies [81–83]. Considering the successful clinical outcome of mAbs [84–86], ACE2 decoy receptors are also expected to have sufficient efficacy to treat COVID-19.

Improved pharmacokinetics of ACE2 decoys has the potential to ameliorate lung inflammation due to effective conversion of Ang II to Ang 1–7 by sustained ACE2 enzymatic activity. A recent study demonstrated additional therapeutic benefits of the ACE2 bioactive form in the lethal COVID-19 model of K18-hACE2 transgenic mice [27]. However, the superiority of bioactive form should be comprehensively evaluated considering parameters that include structural thermostability and risks of unexpected adverse effects.

Therapeutic mAbs are typically administered by intravenous or intramuscular injection, but such systemic delivery is inefficient in achieving optimal drug concentration at local sites of infection. Liquid aerosol inhalation is a promising noninvasive strategy to enhance efficacy against respiratory infections like COVID-19. The major challenge of inhalation is the short half-life, 16–24 h for inhaled mAbs in humans [87]. Even though the clearance rate is fast, as shown in a study by Kobie and coworkers using a hamster model of COVID-19, the inhalation delivery route achieved ~100-fold more efficient delivery to the lungs than intravenous administration did. The beneficial therapeutic effect was observed in a single inhaled dose of only 0.6 mg/kg [88]. Inhalation of engineered ACE2 decoy receptors exhibited similar efficacy with an estimated dose of ~0.5 mg/kg in mice [27]. Since the aerosol drug delivery rate to lungs is ~50% in humans [88,89], inhalation is expected to have dose-sparing benefits. Moreover, it is convenient for self-administration on an outpatient basis or at home; preventing hospitalization to conserve critical hospital resources, especially during waves of infection. Several Phase 1 studies have already demonstrated the safety and tolerability of mAbs as inhaled aerosols [90,91]. Based on these considerations, it is reasonable to argue that engineered ACE2 decoy receptor should also be developed as an inhaled formulation to treat COVID-19.

Concluding remarks and future perspectives

Advances in innovative technologies for therapeutic antibody development in the past 20 years accelerated the development and use of mAbs against SARS-CoV-2 in clinical settings within the first year of the COVID-19 pandemic. Cocktails of mAbs successfully neutralized earlier SARS-CoV-2 variants; however, the novel omicron variant exhibits extensive evasion of vaccines and therapeutic mAbs, even in cocktail form, due to the unprecedented evolution in the spike. ACE2 decoy receptors are expected to be free from complete virus escape, which is supported by evidence from results of DMS of the spike RBD and SARS-CoV-2 mutagenesis with sparing ACE2 decoy doses. Studies discussed in this review have shown that engineered ACE2 decoy receptors neutralize omicron and other sarbecoviruses that use ACE2 as an entry receptor, indicating broad efficacy against future variants and zoonotic coronavirus diseases.

Although the safety of rhACE2 for ARDS has been verified in Phase 1 human clinical trial, engineered ACE2 decoy receptors against COVID-19 would require higher doses than were used in clinical trials for ARDS, raising concerns about adverse effects. These concerns need to be addressed to gain clinical use approval (see Outstanding questions). One of them is overconversion of Ang II to Ang 1–7 due to excessive enzymatic activity of higher dose of

**Outstanding questions**

Is the elimination of ACE2 enzymatic activity required for preventing hemodynamic dysregulation and other unexpected effects?

What is the best approach to eliminate ACE2 enzymatic activity in terms of efficiency, antigenicity, and structural stability?

Which Fc modulation is more suitable for COVID-19 therapy and prophylaxis, Fc-effector enhancement or FcRn orientation?

Whether Fc-effector enhancement and FcRn orientation have synergistic effects on antiviral efficacy?

Can high affinity decoy receptors be applied for other virus diseases once virus receptors are identified?
ACE2 decoy receptors, which could induce hemodynamic dysregulation or unexpected adverse effects. One approach to avoid this concern is the elimination of enzymatic activity of ACE2 decoy receptors. Among the proposed options to modulate ACE2 enzymatic activity, Ang-II-binding pocket closure by disulfide bond formation benefits biologic use because it not only blocks the enzymatic activity but also significantly enhances thermostability [24].

For concerns about drug toxicity, immunogenicity and off-target binding to endogenous proteins are the main issues that must be investigated. Mutations in ACE2 protein have the risk of stimulating host immunity toward the drug and crossreactive responses to host tissues. Fortunately, immunogenicity can be predicted by T cell activation experiments and computational analysis [92], and high-risk mutations can be excluded from engineered ACE2 decoy receptors in advance. Also, the controversies surrounding the use of the modified Fc domain, expected dose, and the optimal route of drug administration has been partially resolved by studies that have shown the advantage of enhancing Fc-effector function and aerosol inhalation in COVID-19 disease models. Comprehensive screening for engineered ACE2 decoy receptors binding to endogenous proteins, especially membrane proteins, will help to assess the risk of off-target effects. ACE2 decoy receptors are promising modality against current and future coronavirus diseases. It is crucial that studies continue to accumulate clinical data in various conditions to optimize the antiviral effects of engineered ACE2 decoy receptors and translation to the clinic.

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Declaration of interests

A.H., J.T., and T.O. are the inventors on a patent filed by Kyoto Prefectural University of Medicine and Osaka University (ACE2 mutant protein, PCT/JP2021/031372).

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