Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Review

Engineering ACE2 decoy receptors to combat viral escapability

Takao Arimori,[1] Nariko Ikemura,[2] Toru Okamoto,[3,4] Junichi Takagi,[1,4,5] Daron M. Standley,[4,6] and Atsushi Hoshino [iD] [2,*]

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 [15–18].

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.

[1]Laboratory for Protein Synthesis and Expression, Institute for Protein Research, Osaka University, Osaka, Japan
[2]Department of Cardiovascular Medicine, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, Kyoto, Japan
[3]Institute for Advanced Co-Creation Studies, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan
[4]Center for Infectious Disease Education and Research (CiDER), Osaka University, Osaka, Japan
[5]Integrated Frontier Research for Medical Science Division, Institute for Open and Transdisciplinary Research Initiatives (OTRI), Osaka University, Osaka, Japan

Check for updates

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].

[6]Department of Genome Informatics, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

*Correspondence:
a-hoshi@koto.kpu-m.ac.jp (A. Hoshino).
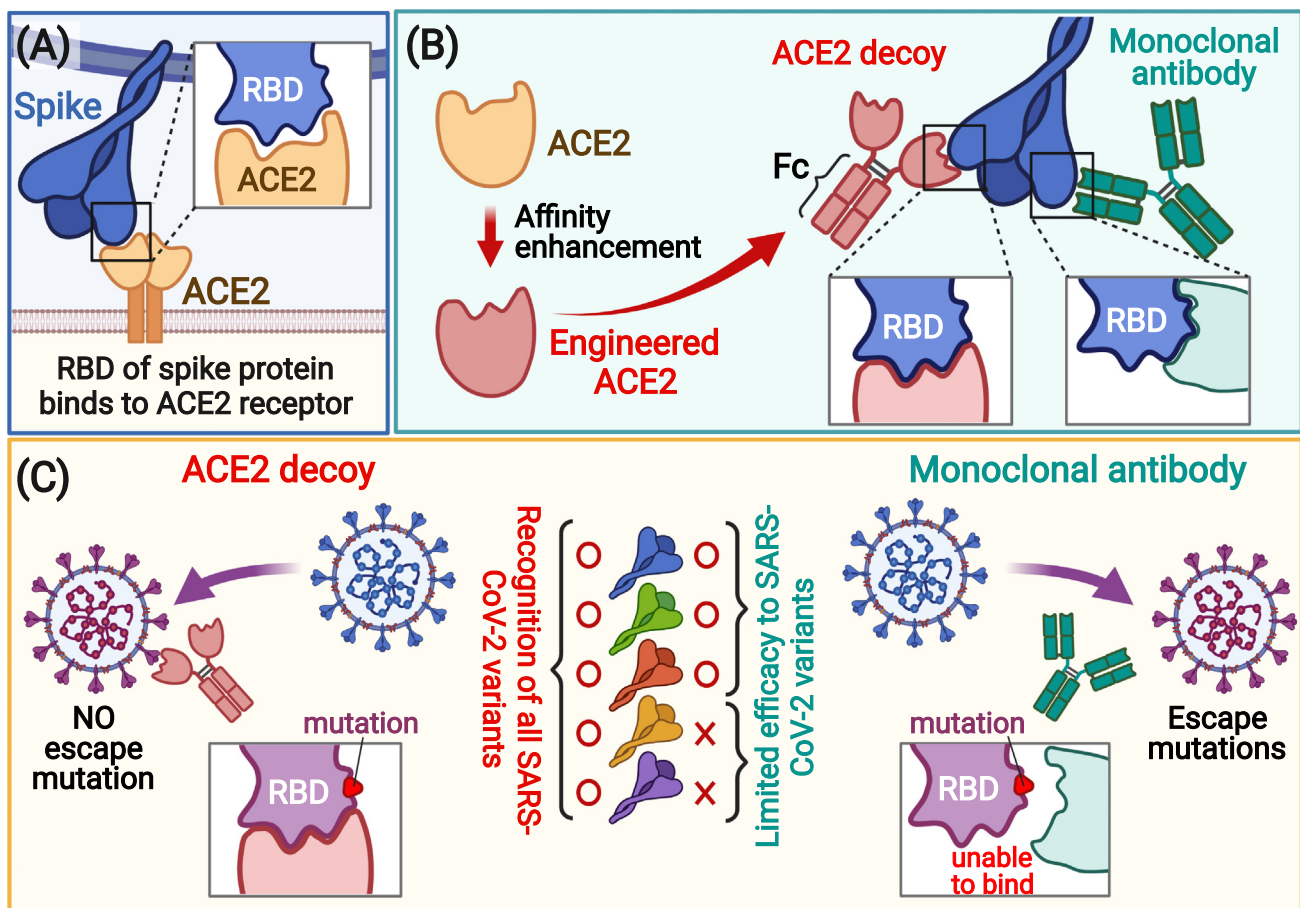
Figure 1. Comparison of engineered angiotensin-converting enzyme 2 (ACE2) decoy receptor and monoclonal antibody against neutralization of severe acute respiratory syndrome *coronavirus 2 (*SARS-CoV-2). (A) The spike protein of SARS-CoV-2 binds to ACE2 on the surface of host cells through its trimeric 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 main 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

Glossary

Acute respiratory distress syndrome (ARDS): a life-threatening respiratory failure characterized by rapid onset of widespread inflammation in the lungs. Excessive fluid impairs gas exchange and induces symptoms including shortness of breath (dyspnea), rapid breathing (tachypnea), and bluish skin coloration (cyanosis).

Affinity: strength of interaction at a singular binding site.

Angiotensin-converting enzyme 2 (ACE2): a zinc metallopeptidase that catalyzes the hydrolysis of Ang II into Ang 1–7. This reaction counteracts the RAS and negatively regulates vasoconstriction and vascular permeability. ACE2 is expressed on the cell surface and some ACE2 is cleaved by ADAM17 to be released as a soluble form of ACE2. Both SARS-CoV-1 and SARS-CoV-2 use ACE2 as a cell-entry receptor.

Avidity: overall strength of protein–protein interaction with more than one biding site.

Clade: group of biological taxa or species that includes all the evolutionary descendants of a common ancestor.

Decoy receptors: soluble version of receptors that bind to viruses competitively with native receptors on host cells and inhibit viruses infection.

Deep mutational scanning (DMS): a method to comprehensively understand how amino acid substitutions at each position in a protein of interest affect its function. By combining protein mutagenesis, high-throughput functional analysis, and deep sequencing, the functional impact of a huge number of mutations can be quantitatively evaluated.

Disulfide bond: bridges formed between cysteine residues in proteins. This fundamental building block for the molecular architecture can play a key role in basic biological processes as well as stabilizing protein structure. The formation of a disulfide bond by two sulfur atoms of spatially proximal cysteines is processed by a two-electron oxidation from reduced sulfhydryl groups of cysteines (S-H) to the oxidized cystine (S-S) residue.

Fc: homodimeric domain consisting of the C-terminal half of the immunoglobulin heavy chain. Fc modulates antibodies efficacy by effector functions such as antibody-dependent cell-mediated cytotoxicity, antibody-dependent cellular

Box 1. Directed evolution

Directed evolution is an experimental method to improve protein function for desired purpose by mutagenesis and selection, imitating evolution in nature. Sol Spiegelman pioneered this technology in 1967 and Frances H. Arnold contributed to the development of artificial enzymes and received the Nobel Prize in Chemistry in 2018. Evolution in nature consists of repeated random mutations to the genome and selection in terms of adaptation to the surrounding environment. Directed evolution is, in other words, a method of evolving a protein *in vitro* with an accelerated time course. Here, we review the general process of directed evolution. First, a random mutation is introduced into the gene of the protein of interest to prepare a mutant library. One of the common methods for mutagenesis is error-prone PCR using low-fidelity DNA polymerase. DNA shuffling and StEP are other methods to obtain a chimeric gene through realignment of mutated DNA fragments by a PCR reaction [37]. The mutant library is then introduced into a host, such as yeast, *Escherichia coli*, phage, or mammalian cells, to express mutant proteins. Yeast has advantages in the library size, resistance to incorporation of multiple mutants due to the exclusive nature of different plasmids, and established systems for displaying the proteins on the cell surface. By contrast, screening with mammalian cells allows analysis of a wide variety of cellular phenotypes that include virus infection. More recently, a method for autonomously introducing random mutations in the host cell was developed. This method optimized base editor and protospacer adjacent motif (PAM)-free nuclease in CRISPR genome editing [93]. Here, after obtaining library cells, screening is performed under the desired selective pressure. In case of binding affinity analysis, it is useful to pool the library and collect the target cell population by fluorescence-activated cell sorting. The target phenotypic cells are collected and random mutations are reintroduced in the extracted DNA for the next round of mutagenesis and selection. The desired function becomes stronger upon repetition of this cycle.

phagocytosis, and complement-dependent cytotoxicity through the interaction with Fcγ receptors on immune cells and the complement protein C1q.

Receptor binding domain (RBD): key domain within a spike protein monomer that binds to the cell surface ACE2. Binding of the RBD to ACE2 induces a conformational change in the spike protein, leading to virus–cell membrane fusion followed by infection.

Spike glycoprotein: trimeric structure found on the outside of the virus particle and gives coronaviruses their crown-like appearance. It has two subunits, of which S1 binds to ACE2 and S2 facilitates membrane fusion to complete entry into host cells.

Valency: total number of binding sites involved in protein–protein interaction.

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 second version (v2) that contained four essential mutations for affinity enhancement. Among these, the 3N39v2 monomer (amino acids 18–615) harboring A25V/K31N/E35K/L79F substitutions exhibited the best K_D value (0.64 nM) for the SARS-CoV-2 spike RBD, which indicates a 100-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 CDY14HL that contains K31M/E35K/S47A/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 synergetic 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
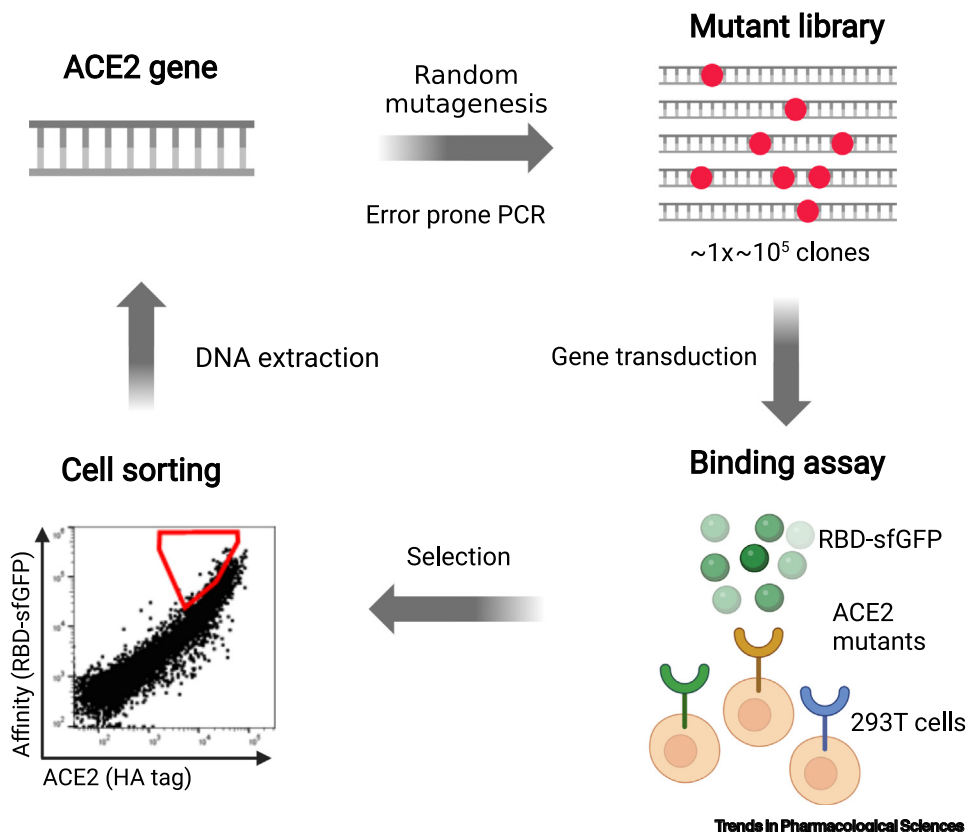
Figure 2. Directed evolution to enhance angiotensin-converting enzyme 2 (ACE2) affinity toward the spike receptor binding domain (RBD). An illustration depicting workflow for directed evolution of ACE2 as described in [24]. The protease domain (PD) of ACE2 is the interface to viral spike RBD. Random mutations are introduced to the N-terminal residues 18–102 of the PD by error-prone PCR at the rate of ~10 mutations per 1 kb (up to three mutations in residues 18–102). The PCR fragments are inserted into the backbone plasmid in the context of full-length ACE2 expression with an N-terminal HA tag. The resultant library are composed of ~10^5 ACE2 mutants and packaged into lentiviruses. The mutant library are transduced to 293T cells in less than 0.3 multiplicity of infection to yield no more than one mutant ACE2 per cell, followed by incubation with recombinant RBD of SARS-CoV-2 spike protein fused to superfolder GFP (sfGFP). Using fluorescence-activated cell sorting, the top 0.05% of cells showing higher binding affinity (sfGFP signal) relative to protein expression level (HA signal) are harvested from ~$5×10^7$ cells. To exclude mutants with impaired structural stability, only cells with preserved signal of surface ACE2 are gated. Genomic DNA are extracted from collected cells and used as the template for error-prone PCR to pursue the next round of mutagenesis and selection.

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].

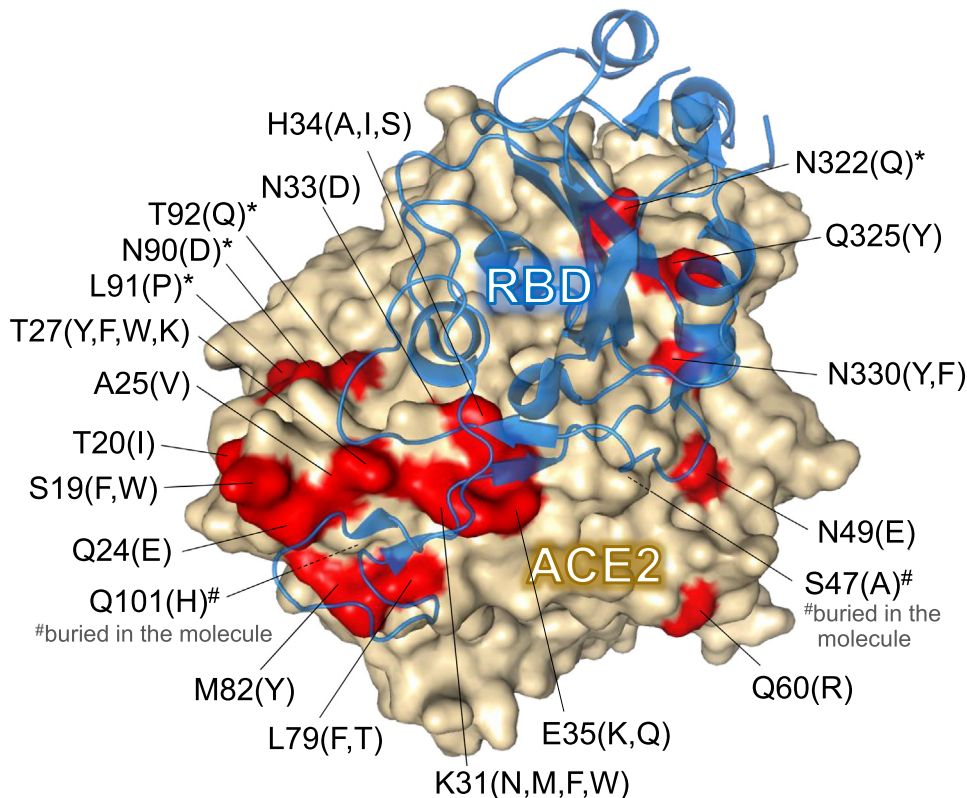### Computational design with growing sophistication
There has been progress in computer-aided drug design using structure-based modeling and molecular dynamics simulation in various drug modalities including small compounds, peptides,

Table 1. Summary of ACE2 mutants to neutralize SARS-CoV-2

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 17.6 (WT)	0.082 24.8 (WT)		Hamster, Wuhan, post-infection, 20mg/kg i.p.	[24]
3J113v2	K31M/E35K/Q60R/L79F (WT)			1.14	0.33			
3J320v2	T20I/H34A/T92Q/Q101H (WT)			3.98	0.068	Validated in *in vitro*		
3N39v4	A25V/K31N/E35K/T92Q (S138C/V343C)	740	WT	N.D.	0.036 0.45 (WT)	Validated in mice	Hamster and CAG-hACE2 mice, Omicron, post-infection, 20mg/kg i.p.	[26]
CDY14HL	K31M/E35K/S47A/L79F/ L91P/N330Y (H345L)	615	IgG4	0.031[a] 29[a] (WT)	0.037 11(WT)	Validated in *in vitro*	K18-hACE2 mice, rhesus/ cynomolgus macaques, AAV, pre-infection	[38]
Deep mutational scanning								
sACE2v2.4	T27Y/L79T/N330Y (WT)	740	WT	0.6[a] 22[a] (WT)	N.D.	Validated in *in vitro*	K18-hACE2 mice, Wuhan & Gamma, pre-/post-infection, 10 mg/kg i.v., Inhalation 3×	[27,40,58]
Computational design								
CVD293	K31F/H34I/E35Q (WT)	740	WT	0.23[a] 2.8[a] (WT)	0.036 0.7(WT)			[53]
CVD313	K31F/N33D/H34S/E35Q (H345L)			N.D.	0.028			
M81	L79F/M82Y/Q325Y (H374A/H378A)	740	GASDALIE[b]	2.74[a] 26.0[a] (WT)	N.D.		K18-hACE2 mice, Wuhan, pre-/post-infection, 12.5 mg/kg i.n., 6.25 mg/kg i.v.	[45]
FFWF	S19F/T27F/K31W/N330F (WT)	740	WT	1.8[a] 16[a] (WT)	N.D.			[50]
	S19W/N330Y (H374A/H378A/E402A)	615	WT	10.1 81.8 (WT)	0.09 15.5 (WT)			[47]
	S19W/T27W/N330Y (H374A/H378A/E402A)			19.3	0.53			
	Q24E/T27K/H34S/N49E/N90D (H374N/H378N)	615	WT	5.45[a] 11.6[a] (WT)	0.39 0.82 (WT)			[52]
	Deglycosylation (WT)	N.A	WT	7.6[a] 16.2[a] (WT)	N.D.			[49]
Multimer ACE2 and others								
	WT (H374N/H378N)	740	LALA-PG[b]	11.5[a] 18.3[a] (WT)	4.1 nM 5.1 nM (WT)		Hamster, Wuhan, post-infection, 5/50 mg/kg i.p.	[74]
MDR504	WT (H345A)	740	LALA[b]	N.D.	3.5nM[c] 7.5nM[c] (WT)		Ad5-hACE2-transduced/K18-ACE2 mice, Wuhan, pre-/post-infection, 15/30 mg/kg i.v.	[79]
	WT (H374N/H378N)	N.A	WT	8.23[a] 1.59[a] (WT)	4.17 1.02 (WT)		Ad5-hACE2-transduced mice, Wuhan, pre-/post-infection, 50mg/kg i.p.	[73]
	T27W-foldon (WT)	615	WT	0.06 76.8 (WT)	0.21 24.1 (WT)			[72]
	D30E-tetramer/hexamer (WT)	740	WT	N.D.	0.07/0.06 nM 0.68 nM (WT)			[69]

Notes to Table 1:
[a]Avidity value of ACE2 dimer with collectrin domain and/or Fc.
[b]GASDALIE: G236A/S239D/A330L/I332E, LALA: L234A/L235A, LALA-PG: L234A/L235A/P329G.
[c]IC$_{50}$ value against authentic virus.

Figure 3. Location of mutation sites for affinity enhancement in angiotensin-converting enzyme (ACE)2. ACE2 and receptor-binding domain (RBD) in the complex structure (PDB: 6m0j [20,34]) are shown as a light orange surface model and a blue translucent cartoon model, respectively. Mutation sites for affinity enhancement introduced in ACE2 are indicated in red. Reported amino acid substitutions of each residue are shown in parentheses. Asterisks indicate mutation sites for deletion of the N90 and N322 glycans.

and protein biologics [44]. 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 [34] and 6vw1 [35]) to design and generate a high-affinity ACE2 [45]. 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 [46]. 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) [45]. 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) [47].

Molecular dynamics simulation can provide plentiful kinetic information and calculate thermodynamic properties for the protein–drug interactions. [48]. 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

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 a 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.

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).

References

1. Zhou, P. *et al.* (2020) A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* 579, 270–273
2. Wu, F. *et al.* (2020) A new coronavirus associated with human respiratory disease in China. *Nature* 579, 265–269
3. Letko, M. *et al.* (2020) Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat. Microbiol.* 5, 562–569
4. Wrapp, D. *et al.* (2020) Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation. *Science* 367, 1260–1263
5. Banerjee, R. *et al.* (2021) Potential SARS-CoV-2 main protease inhibitors. *Drug Discov. Today* 26, 804–816
6. Qiao, J. *et al.* (2021) SARS-CoV-2 M(pro) inhibitors with antiviral activity in a transgenic mouse model. *Science* 371, 1374–1378
7. Drayman, N. *et al.* (2021) Masitinib is a broad coronavirus 3CL inhibitor that blocks replication of SARS-CoV-2. *Science* 373, 931–936
8. Robson, F. *et al.* (2020) Coronavirus RNA proofreading: molecular basis and therapeutic targeting. *Mol. Cell* 79, 710–727
9. Zhu, Q. *et al.* (2011) Analysis of respiratory syncytial virus preclinical and clinical variants resistant to neutralization by monoclonal antibodies palivizumab and/or motavizumab. *J. Infect. Dis.* 203, 674–682
10. Jensen, B. *et al.* (2021) Emergence of the E484K mutation in SARS-COV-2-infected immunocompromised patients treated with bamlanivimab in Germany. *Lancet Reg. Health Eur.* 8, 100164
11. Starr, T.N. *et al.* (2021) Complete map of SARS-CoV-2 RBD mutations that escape the monoclonal antibody LY-CoV555 and its cocktail with LY-CoV016. *Cell Rep. Med.* 2, 100255
12. Baum, A. *et al.* (2020) Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies. *Science* 369, 1014–1018
13. Rappazzo, C.G. *et al.* (2021) Broad and potent activity against SARS-like viruses by an engineered human monoclonal antibody. *Science* 371, 823–829
14. Kreuzberger, N. *et al.* (2021) SARS-CoV-2-neutralising monoclonal antibodies for treatment of COVID-19. *Cochrane Database Syst. Rev.* 9, CD013825
15. VanBlargan, L.A. *et al.* (2022) An infectious SARS-CoV-2 B.1.1.529 Omicron virus escapes neutralization by therapeutic monoclonal antibodies. *Nat. Med.* https://doi.org/10.1038/s41591-021-01678-y
16. Cameroni, E. *et al.* (2021) Broadly neutralizing antibodies overcome SARS-CoV-2 Omicron antigenic shift. *Nature* 602, 664–670
17. Hoffmann, M. *et al.* (2022) The Omicron variant is highly resistant against antibody-mediated neutralization: Implications for control of the COVID-19 pandemic. *Cell* 185, 447–456 e411
18. Dejnirattisai, W. *et al.* (2022) SARS-CoV-2 Omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. *Cell* 185, 467–484 e415
19. Monteil, V. *et al.* (2020) Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2. *Cell* 181, 905–913 e907
20. Lei, C. *et al.* (2020) Neutralization of SARS-CoV-2 spike pseudotyped virus by recombinant ACE2-Ig. *Nat. Commun.* 11, 2070
21. Smolen, J.S. *et al.* (2007) New therapies for treatment of rheumatoid arthritis. *Lancet* 370, 1861–1874

22. Kaiser, P.K. (2013) Emerging therapies for neovascular age-related macular degeneration: drugs in the pipeline. *Ophthalmology* 120, S11–S15

23. Gaya, A. and Tse, V. (2012) A preclinical and clinical review of aflibercept for the management of cancer. *Cancer Treat. Rev.* 38, 484–493

24. Higuchi, Y. *et al.* (2021) Engineered ACE2 receptor therapy overcomes mutational escape of SARS-CoV-2. *Nat. Commun.* 12, 3802

25. Chan, K.K. *et al.* (2021) An engineered deco receptor for SARS-CoV-2 broadly binds protein S sequence variants. *Sci. Adv.* 7 Published online December 2021. https://doi.org/10.1126/sciadv.abf1738

26. Ikemura, N. *et al.* (2022) An engineered ACE2 decoy neutralizes the SARS-CoV-2 Omicron variant and confers protection against infection *in vivo*. *Sci. Transl. Med.* 14, eabn7737

27. Zhang, L. *et al.* (2022) An engineered ACE2 decoy receptor can be administered by inhalation and potently targets the BA.1 and BA.2 omicron variants of SARS-CoV-2. *bioRxiv* Published online March 28, 2022. https://doi.org/10.1101/2022.03.28.486075

28. Khan, A. *et al.* (2017) A pilot clinical trial of recombinant human angiotensin-converting enzyme 2 in acute respiratory distress syndrome. *Crit. Care* 21, 234

29. Vickers, C. *et al.* (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.* 277, 14838–14843

30. Zhang, H. and Baker, A. (2017) Recombinant human ACE2: acing out angiotensin II in ARDS therapy. *Crit. Care* 21, 305

31. Kuba, K. *et al.* (2021) Angiotensin-converting enzyme 2 (ACE2) in the pathogenesis of ARDS in COVID-19. *Front. Immunol.* 12, 732690

32. Haschke, M. *et al.* (2013) Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. *Clin. Pharmacokinet.* 52, 783–792

33. Huentelman, M.J. *et al.* (2004) Structure-based discovery of a novel angiotensin-converting enzyme 2 inhibitor. *Hypertension* 44, 903–906

34. Lan, J. *et al.* (2020) Structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor. *Nature* 581, 215–220

35. Shang, J. *et al.* (2020) Structural basis of receptor recognition by SARS-CoV-2. *Nature* 581, 221–224

36. Cao, Y. *et al.* (2021) Omicron escapes the majority of existing SARS-CoV-2 neutralizing antibodies. *Nature* 602, 657–663

37. Packer, M.S. and Liu, D.R. (2015) Methods for the directed evolution of proteins. *Nat. Rev. Genet.* 16, 379–394

38. Sims, J.J. *et al.* (2021) Intranasal gene therapy to prevent infection by SARS-CoV-2 variants. *PLoS Pathog.* 17, e1009544

39. Zhang, H. *et al.* (2001) Collectrin, a collecting duct-specific transmembrane glycoprotein, is a novel homolog of ACE2 and is developmentally regulated in embryonic kidneys. *J. Biol. Chem.* 276, 17132–17139

40. Chan, K.K. *et al.* (2020) Engineering human ACE2 to optimize binding to the spike protein of SARS coronavirus 2. *Science* 369, 1261–1265

41. Jing, W. and Procko, E. (2021) ACE2-based decoy receptors for SARS coronavirus 2. *Proteins* 89, 1065–1078

42. Chu, H.Y. and Wong, A.S.L. (2021) Facilitating machine learning-guided protein engineering with smart library design and massively parallel assays. *Adv. Genet.* 2, 2100038

43. Saito, Y. *et al.* (2021) Machine-learning-guided library design cycle for directed evolution of enzymes: the effects of training data composition on sequence space exploration. *ACS Catal.* 11, 14615–14624

44. Temml, V. and Kutil, Z. (2021) Structure-based molecular modeling in SAR analysis and lead optimization. *Comput. Struct. Biotechnol. J.* 19, 1431–1444

45. Chen, Y. *et al.* (2021) Engineered ACE2-Fc counters murine lethal SARS-CoV-2 infection through direct neutralization and Fc-effector activities. *bioRxiv* Published online November 24, 2021. https://doi.org/10.1101/2021.11.24.469776

46. Gong, X. *et al.* (2014) Direct measurements of particle-surface interactions in aqueous solutions with total internal reflection microscopy. *Chem. Commun. (Camb.)* 50, 6556–6570

47. Ye, F. *et al.* (2021) S19W, T27W, and N330Y mutations in ACE2 enhance SARS-CoV-2 S-RBD binding toward both wild-type

48. Decherchi, S. and Cavalli, A. (2020) Thermodynamics and kinetics of drug-target binding by molecular simulation. *Chem. Rev.* 120, 12788–12833

49. Capraz, T. *et al.* (2021) Structure-guided glyco-engineering of ACE2 for improved potency as soluble SARS-CoV-2 decoy receptor. *eLife* 10, e73641

50. Havranek, B. *et al.* (2021) Computationally designed ACE2 decoy receptor binds SARS-CoV-2 spike (S) protein with tight nanomolar affinity. *J. Chem. Inf. Model.* 61, 4656–4669

51. Kapli, P. *et al.* (2020) Phylogenetic tree building in the genomic age. *Nat. Rev. Genet.* 21, 428–444

52. Mou, H. *et al.* (2021) Mutations derived from horseshoe bat ACE2 orthologs enhance ACE2-Fc neutralization of SARS-CoV-2. *PLoS Pathog.* 17, e1009501

53. Glasgow, A. *et al.* (2020) Engineered ACE2 receptor traps potently neutralize SARS-CoV-2. *Proc. Natl. Acad. Sci. U. S. A.* 117, 28046–28055

54. Ding, W. *et al.* (2022) Protein design via deep learning. *Brief. Bioinform.* 23, bbac102

55. Kontermann, R.E. (2011) Strategies for extended serum half-life of protein therapeutics. *Curr. Opin. Biotechnol.* 22, 868–876

56. Huang, C. (2009) Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. *Curr. Opin. Biotechnol.* 20, 692–699

57. Lee, C.H. *et al.* (2019) An engineered human Fc domain that behaves like a pH-toggle switch for ultra-long circulation persistence. *Nat. Commun.* 10, 5031

58. Zhang, L. *et al.* (2022) Engineered ACE2 decoy mitigates lung injury and death induced by SARS-CoV-2 variants. *Nat. Chem. Biol.* 18, 342–351

59. Yamin, R. *et al.* (2021) Fc-engineered antibody therapeutics with improved anti-SARS-CoV-2 efficacy. *Nature* 599, 465–470

60. van Erp, E.A. *et al.* (2019) Fc-mediated antibody effector functions during respiratory syncytial virus infection and disease. *Front. Immunol.* 10, 548

61. DiLillo, D.J. and Ravetch, J.V. (2015) Differential Fc-receptor engagement drives an anti-tumor vaccinal effect. *Cell* 161, 1035–1045

62. van der Horst, H.J. *et al.* (2020) Fc-engineered antibodies with enhanced Fc-effector function for the treatment of B-cell malignancies. *Cancers (Basel)* 12, 3041

63. Narayan, R. and Tripathi, S. (2020) Intrinsic ADE: the dark side of antibody dependent enhancement during dengue infection. *Front. Cell. Infect. Microbiol.* 10, 580096

64. Bournazos, S. *et al.* (2020) Fc-optimized antibodies elicit CD8 immunity to viral respiratory infection. *Nature* 588, 485–490

65. Winkler, E.S. *et al.* (2021) Human neutralizing antibodies against SARS-CoV-2 require intact Fc effector functions for optimal therapeutic protection. *Cell* 184, 1804–1820 e1816

66. Ju, B. *et al.* (2020) Human neutralizing antibodies elicited by SARS-CoV-2 infection. *Nature* 584, 115–119

67. Corti, D. *et al.* (2021) Tackling COVID-19 with neutralizing monoclonal antibodies. *Cell* 184, 4593–4595

68. Cathcart, A.L. *et al.* (2022) The dual function monoclonal antibodies VIR-7831 and VIR-7832 demonstrate potent *in vitro* and *in vivo* activity against SARS-CoV-2. *bioRxiv* https://doi.org/10.1101/2021.03.09.434607

69. Li, Y. *et al.* (2020) SARS-CoV-2 and three related coronaviruses utilize multiple ACE2 orthologs and are potently blocked by an improved ACE2-Ig. *J. Virol.* 94. https://doi.org/10.1128/JVI.01283-20

70. Miller, A. *et al.* (2021) A super-potent tetramerized ACE2 protein displays enhanced neutralization of SARS-CoV-2 virus infection. *Sci. Rep.* 11, 10617

71. Guo, L. *et al.* (2021) Engineered trimeric ACE2 binds viral spike protein and locks it in "Three-up" conformation to potently inhibit SARS-CoV-2 infection. *Cell Res.* 31, 98–100

72. Xiao, T. *et al.* (2021) A trimeric human angiotensin-converting enzyme 2 as an anti-SARS-CoV-2 agent. *Nat. Struct. Mol. Biol.* 28, 202–209

73. Zhang, Z. *et al.* (2021) Potent prophylactic and therapeutic efficacy of recombinant human ACE2-Fc against SARS-CoV-2 infection *in vivo*. *Cell Discov.* 7, 65

and antibody-resistant viruses and its molecular basis. *Signal. Transduct. Target Ther.* 6, 343

74. Ferrari, M. *et al.* (2021) Characterization of a novel ACE2-based therapeutic with enhanced rather than reduced activity against SARS-CoV-2 variants. *J. Virol.* 95, e0068521

76. Starr, T.N. *et al.* (2020) Deep mutational scanning of SARS-CoV-2 receptor binding domain reveals constraints on folding and ACE2 binding. *Cell* 182, 1295–1310 e1220

77. Starr, T.N. *et al.* (2021) Prospective mapping of viral mutations that escape antibodies used to treat COVID-19. *Science* 371, 850–854

78. Greaney, A.J. *et al.* (2021) Antibodies elicited by mRNA-1273 vaccination bind more broadly to the receptor binding domain than do those from SARS-CoV-2 infection. *Sci. Transl. Med.* 13, eabi9915

79. Iwanaga, N. *et al.* (2022) ACE2-IgG1 fusions with improved *in vitro* and *in vivo* activity against SARS-CoV-2. *iScience* 25, 103670

80. Oladunni, F.S. *et al.* (2020) Lethality of SARS-CoV-2 infection in K18 human angiotensin-converting enzyme 2 transgenic mice. *Nat. Commun.* 11, 6122

81. Baum, A. *et al.* (2020) REGN-COV2 antibodies prevent and treat SARS-CoV-2 infection in rhesus macaques and hamsters. *Science* 370, 1110–1115

82. Kreye, J. *et al.* (2020) A therapeutic non-self-reactive SARS-CoV-2 antibody protects from lung pathology in a COVID-19 hamster model. *Cell* 183, 1058–1069 e1019

83. Su, W. *et al.* (2021) Neutralizing monoclonal antibodies that target the spike receptor binding domain confer Fc receptor-independent protection against SARS-CoV-2 infection in Syrian hamsters. *mBio* 12, e0239521

84. Weinreich, D.M. *et al.* (2021) REGEN-COV antibody combination and outcomes in outpatients with Covid-19. *N. Engl. J. Med.* 385, e81

85. Gottlieb, R.L. *et al.* (2021) Effect of bamlanivimab as monotherapy or in combination with etesevimab on viral load in patients with mild to moderate COVID-19: a randomized clinical trial. *JAMA* 325, 632–644

86. Boggiano, C. *et al.* (2022) Update on and future directions for use of anti-SARS-CoV-2 antibodies: National Institutes of Health summit on treatment and prevention of COVID-19. *Ann. Intern. Med.* 175, 119–126

87. Parray, H.A. *et al.* (2021) Inhalation monoclonal antibody therapy: a new way to treat and manage respiratory infections. *Appl. Microbiol. Biotechnol.* 105, 6315–6332

88. Piepenbrink, M.S. *et al.* (2021) Therapeutic activity of an inhaled potent SARS-CoV-2 neutralizing human monoclonal antibody in hamsters. *Cell Rep. Med.* 2, 100218

89. Kleinstreuer, C. *et al.* (2008) Targeted drug-aerosol delivery in the human respiratory system. *Annu. Rev. Biomed. Eng.* 10, 195–220

90. van der Straten, K. *et al.* (2022) Optimization of anti-SARS-CoV-2 neutralizing antibody therapies: roadmap to improve clinical effectiveness and implementation. *Front. Med. Technol.* 4, 867982

91. Syed, Y.Y. (2021) Regdanvimab: first approval. *Drugs* 81, 2133–2137

92. Paul, S. *et al.* (2019) Major histocompatibility complex binding, eluted ligands, and immunogenicity: benchmark testing and predictions. *Front. Immunol.* 10, 3151

93. Richter, M.F. *et al.* (2020) Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nat. Biotechnol.* 38, 883–891