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Advances in developing ACE2 derivatives against SARS-CoV-2

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Extensive immune evasion of SARS-CoV-2 rendered therapeutic antibodies ineffective in the COVID-19 pandemic. Propagating SARS-CoV-2 variants are characterised by immune evasion capacity through key amino acid mutations, but can still bind human angiotensin-converting enzyme 2 (ACE2) through the spike protein and are, thus, sensitive to ACE2-mimicking decoys as inhibitors. In this Review, we examine advances in the development of ACE2 derivatives from the past 3 years, including the recombinant ACE2 proteins, ACE2-loaded extracellular vesicles, ACE2-mimicking antibodies, and peptide or mini-protein mimetics of ACE2. Several ACE2 derivatives are granted potent neutralisation efficacy against SARS-CoV-2 variants that rival or surpass endogenous antibodies by various auxiliary techniques such as chemical modification and practical recombinant design. The derivatives also represent enhanced production efficiency and improved bioavailability. In addition to these derivatives of ACE2, new effective therapeutics against SARS-CoV-2 variants are expected to be developed.

Introduction

The COVID-19 pandemic is a major challenge to human life. SARS-CoV-2, the causative agent, can be transmitted by aerosols and respiratory droplets—respiratory transmission is the dominant mode—granting the virus extensive disseminative capability.[1] Under current circumstances, COVID-19 still poses a real threat to human well being and an undeniable burden on health care.

Neutralisation of SARS-CoV-2 before its entry into human cells is a logical target choice for reducing mortality caused by viral penetration into the lungs.[2] Vaccine-induced immunity is well known to play a crucial role in preventing COVID-19 transmission. Unfortunately, many newly emerging variants can evade immunity;[3] therefore, new therapies that block viral cell entry are called for as a complementary resolution.

SARS-CoV-2 was shown to use angiotensin-converting enzyme 2 (ACE2) as its cell entry receptor.[4] In 2020, the binding pattern of SARS-CoV-2 spike protein and ACE2 at an atomic level was revealed by cryo-electron microscopy (figure 1).[5] The majority of α1 helix (mainly residues 21–43) and several residues scattered in α2, β3, and β4 of ACE2 are responsible for the one-to-one binding of ACE2 and SARS-CoV-2 spike protein monomer.[6] The binding of cell surface ACE2 is the leading stage of viral endocytosis;[7] therefore, blockage of the protein–protein interaction (PPI) of the receptor-binding domain (RBD) of ACE2 is an important method of clinical prevention of SARS-CoV-2.[8] This method of prevention is especially important given that the SARS-CoV-2 mutations that lead to immune evasion are also likely to reduce the viruses affinity to cell surface ACE2.

Using ACE2 derivatives as a viral antagonist is a practical strategy because SARS-CoV-2 tends to retain the ACE2-binding ability in evolution.[9] The clinical-grade human recombinant soluble ACE2 (hrsACE2) inhibited SARS-CoV-2 infections in Vero cells, engineered human blood vessels, and kidney organoids.[10] Other soluble ACE2 derivatives were developed to improve affinity and avidity towards spike protein as well as pharmacokinetics.[11] Hence, ACE2 derivatives earned their place in SARS-CoV-2 inhibitor development.

As many SARS-CoV-2 variants of concern (VOCs) spread aggressively, critical mutations were found responsible for immune evasion, bringing new challenges for COVID-19 treatment.[12] Researchers refocused on how RBD–ACE2 PPI changes upon mutation; revisiting the evolutionary trajectories of SARS-CoV-2 substantiated ACE2 binding as an evolvable feature.[13] It has been shown that circulating VOCs are characterised by enhanced ACE2 binding.[14] These discoveries clarify the potential of recombinant ACE2 and its mimetics as time-tested inhibitors.

In this Review, we discuss advances from the past 3 years in the design strategies and exemplification of ACE2-derived inhibitors, including clinical-grade recombinant ACE2, ACE2-loaded extracellular vesicles, ACE2-based neutralisation antibodies, peptides, and mini-protein mimetics of ACE2. Efficacy-enhancing modifications (figure 2) can further improve the binding affinity of ACE2 derivatives, suggesting their contribution as potential therapeutic methods. Furthermore, as ACE2 is a shared receptor protein among SARS-CoV-2 and some other coronaviruses,[4] the comprehensive illustration of ACE2 derivatives and exploration of future ACE2 mimetic drugs would most likely benefit broad-spectrum drug development against emerging coronaviruses.

Recombinant ACE2 protein

The protease TMPRSS2 cleaves human membrane-bound ACE2 (805 amino acid protein) into endogenous soluble ACE2 (sACE2) proteins and membrane anchors and could activate spike protein of SARS-CoV-2 for membrane fusion.[15] Since the cleaved sACE2 maintains the motifs mediating SARS-CoV-2 binding, it could theoretically inhibit viral binding to membrane-bound ACE2 by acting as a decoy to bind SARS-CoV-2 that is in blood circulation.[16] However, the concentration of circulating sACE2 is low.[17] More importantly, the physiological concentration (ie, ng/mL) of sACE2 has been reported to conversely

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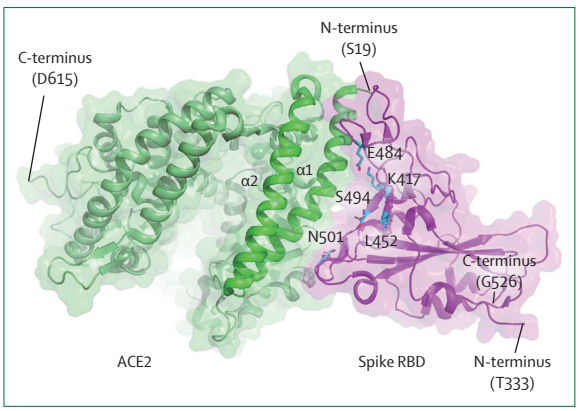

Figure 1: **Structure of the complex of ACE2 (green) and SARS-CoV-2 spike RBD (purple)**
Helices α1 and α2 within ACE2 are the major components involved in RBD binding. The main residues of the spike RBD that undergo mutation in variants of concern—ie, K417, L452, E484, S494, and N501—are coloured in blue. ACE2=angiotensin-converting enzyme 2. RBD=receptor-binding domain.

enhance SARS-CoV-2 infection in HK-2 cell lines from 10% to 30–50%.[18] In contrast, higher concentrations of sACE2 (ie, μg/mL) can reduce the chances of infection.[18] Therefore, supplementation with exogenous sACE2 has been considered a treatment for viruses that use ACE2 as a receptor, such as SARS-CoV and SARS-CoV-2, and it could also be considered for viruses that emerge in the future.[19]

Previous phase 1 and phase 2 clinical trials have shown the safety and tolerance of intravenously administered hrsACE2 (residues 1–740, also called APN01). The phase 1 trial was done in healthy individuals, and the phase 2 trial was done in people with acute respiratory distress syndrome.[20,21] In 2020, the clinical-grade hrsACE2 was reported to greatly inhibit the replication of wild-type strain SARS-CoV-2 (referred to as the reference strain for comparison with the VOCs) in cell culture, resulting in a 1000-to-5000-fold reduction in viral load.[10] Moreover, viral RNA detected by quantitative real-time PCR has characterised that hrsACE2 also inhibits SARS-CoV-2 infection in human capillaries and kidney organoids.[10] In addition, the positive results from biophysical affinity assays and live-virus neutralisation assays validated that hrsACE2 binds tightly to the spike protein and effectively blocks viral infection of variants such as alpha (B.1.1.7), beta (B.1.351), delta (B.1.617.2), and omicron (B.1.1.529),[22] paving the way for clinical use of hrsACE2 (table).

Several clinical trials are underway to evaluate the safety and effectiveness of hrsACE2 in patients with COVID-19. A double-blind, randomised, placebo-controlled phase 2 clinical trial registered with ClinicalTrials.gov (NCT04335136) assessed the safety, tolerability, and antiviral activity of hrsACE2 over 28 days in patients who are severely ill with COVID-19 and showed clinical benefits. During the study, fewer all-cause deaths or invasive mechanical ventilation were observed for patients treated with hrsACE2 than with placebo, but this difference was not statistically significant. In contrast, the group treated with hrsACE2 showed a statistically significant reduction in viral load on day 3 and day 5 and a significant improvement in mechanical ventilator-free days compared with the placebo group. Another encouraging therapeutic effect of intravenous hrsACE2 was documented in one 45-year-old female patient with severe COVID-19.[49] The patient was intravenously administered with hrsACE2 twice daily for 7 days, and had substantial decreases in viral load, angiotensin 2 concentration, and inflammatory cytokines concentration before clinical condition improved and later discharge from the hospital. Two mechanisms of hrsACE2 that benefit patients with COVID-19 are: (1) it neutralises SARS-CoV-2 by binding to spike protein; and (2) it downregulates the renin-angiotensin-aldosterone system to help prevent inflammation and organ injury.[50] Drug design and delivery methods are constantly evolving to improve the use and targeting of ACE2. To deliver the drug directly to the respiratory tract, a nebulised hrsACE2 formulation was designed, and in 2022 a double-blind, placebo-controlled phase 1 clinical trial (NCT05065645) for this formulation was done in healthy volunteers—the results of this study have not yet been released.[51] Moreover, the oral CTB-ACE2 chewing gum topical delivery approach was used to prolong contact with the oral viruses, reducing viral replication and transmission.[25] In this combination, ACE2 captures the virus in the oral cavity, and CTB prevents the virus from entering the host cells. Pseudovirus neutralisation assays have shown that CTB-ACE2 chewing gum can effectively prevent SARS-CoV-2 entry into Vero cells and Chinese hamster ovary cells more 95% of the time,[25] and as a result a phase 1 clinical trial (NCT05433181) is being set up to test this in people. An ACE2-like carboxypeptidase derived from the bacterium *Paenibacillus* species B38 (B38-CAP) was found to be protective against SARS-CoV-2-induced lung injury by reducing angiotensin 2 concentration without binding to SARS-CoV-2.[24] B38-CAP is undergoing two phase 1 clinical trials (NCT04375046 and NCT04382950) as a potential COVID-19 drug.

Beyond the original hrsACE2, modifications were applied to sACE2 to enhance its viral neutralisation performance. To this end, a high affinity (K_D=3·8 nM) and high expression mutant sACE2.v2.4 (1–615 amino acid residues, with mutations T27Y, L79T, and N330Y) was obtained using deep mutagenesis.[23] Extending sACE2.v2.4 to 732 amino acids can yield a stable dimer called $sACE2_2$.v2.4, of which the K_D value with RBD enhanced to 0·6 nM and the viral neutralisation ability against SARS-CoV-2 and SARS-CoV was enhanced.[23]

In summary, supplementing exogenous hrsACE2 can inhibit viral invasion and infection by neutralising SARS-CoV-2 in the body's circulation and can also ameliorate lung injury by reducing angiotensin 2 concentrations and decreasing inflammatory cytokine levels. As previously mentioned, hrsACE2 exerted a

clinically significant therapeutic effect in a patient with severe COVID-19,[49] and more clinical trials on hrsACE2 are underway. More importantly, hrsACE2, as a competitor of membrane-bound ACE2 (the intrinsic key host receptor for SARS-CoV-2), exhibits a promising therapeutic prospect regarding its non-destructive neutralisation of SARS-CoV-2, its variants, and other ACE2-targeting viruses, such as SARS-CoV.

ACE2-loaded extracellular vesicles

Although sACE2 has shown excellent antiviral activity, it has the disadvantage of having a short duration of efficacy with an elimination half-life of approximately 10 h.[20] In a 28-day multicentre clinical phase 2 trial and a clinical case report, hrsACE2 was administered intravenously twice daily to maintain an effective drug concentration.[49] Therefore, to prolong the duration of ACE2, vesicles—a popular biotransfer tool—have been used for ACE2 protection by many researchers.[52]

Extracellular vesicles are nano-sized spherical cell secretions that encapsulate bioactive macromolecules and protect them from degradation by digestive enzymes, resulting in a long blood circulation half-life.[53] An extracellular vesicles-based treatment in animal models of lung injury suppressed inflammation and pulmonary fibrosis while promoting alveolar epithelium and microvascular angiogenesis, suggesting that extracellular vesicle-based treatments have considerable potential to combat COVID-19 since the lungs are the major organ of infection for SARS-CoV-2.[54] Herein, we focus on how the ACE2-loaded extracellular vesicles act as a so-called trap host against SARS-CoV-2 infection and transmission.

El-Shennawy and colleagues[26] found that in the plasma of patients with COVID-19, endogenous circulating extracellular vesicles harbouring ACE2 (evACE2) showed substantial upregulation, and the isolated evACE2 completely blocked cell death induced by SARS-CoV-2 infection. Moreover, Zhang and colleagues found that these extracellular vesicles containing ACE2 bind to spike proteins.[55] ACE2-containing and ACE2-TMPRSS2-containing extracellular vesicles also inhibited SARS-CoV-2 pseudovirus infectivity with an efficacy approximately 500–1500-times higher than hrsACE2.[27] Furthermore, evACE2 isolated from engineered cell lines was shown to be 135-times more effective than hrsACE2 in blocking the viral spike protein, as well as enhanced broad-spectrum neutralisation effect on SARS-CoV-2 reference strain, and alpha, beta, and delta variants; these results were verified by both pseudovirus and live-virus assays.[26] It was later shown that intranasal evACE2 can protect human ACE2 (hACE2) transgenic mice from SARS-CoV-2-induced lung injury and mortality.[26] Similarly, extracellular vesicles embedded with engineered ACE2 (EVs-ACE2) showed potent neutralisation ability against SARS-CoV-2 pseudovirus, both in vitro and in vivo. EVs-ACE2 administration reduced cellular luciferase activity by 96% in a pseudovirus neutralisation assay and blocked S-pseudovirus in nasal epithelial cells of mice 100-times more efficiently than non-treatment control groups.[28]

In addition, sACE2 and its mutants (ie, sACE2.v1 [H34A, T92Q, Q325P, and A386L] and sACE2.v2 [T27Y, L79T, N330Y, and A386L]), which have previously been shown to enhance binding to SARS-CoV-2 spike proteins,[23]

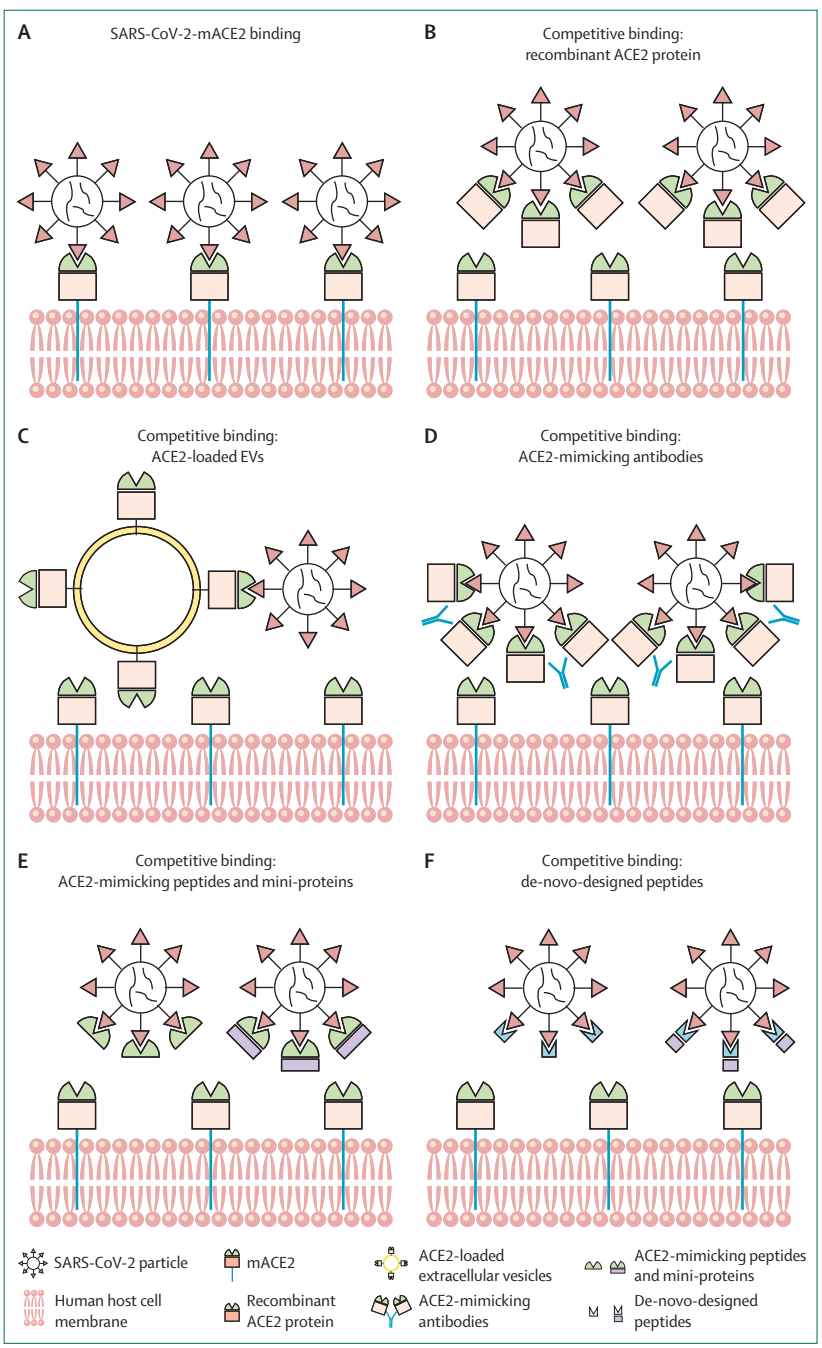

Figure 2: **Schematics of SARS-CoV-2 binding patterns with native ACE2 and its derivatives**
ACE2=angiotensin-converting enzyme 2. mACE2=membrane-bound ACE2.

	Sequence	Pseudovirus neutralisation assay	Live virus neutralisation assay	Animal models	Clinical trial	Study
Recombinant ACE2 protein						
hrsACE2 (APN01)	1–740 amino acids	..	Wild type, alpha, beta, gamma, delta, and omicron	Mice	Phase 2	Monteil et al (2020)[10] and Monteil et al (2022)[22]
sACE2 or $sACE2_2$ (dimer)	1–615 amino acids or 1–732 amino acids	..	Wild type	..	..	Chan et al (2020)[23]
B38-CAP	..	..	Negative	Hamsters and mice	Phase 1	Yamaguchi et al (2021)[24]
CTB-ACE2	Full-length	Wild type	Wild type	..	Phase 2	Daniell et al (2022)[25]
ACE2-loaded extracellular vesicles						
evACE2	Full-length	Wild type, alpha, beta, and delta	Wild type, alpha, beta, and delta	Mice	..	El-Shennawy et al (2022)[26]
ACE2-EVs and ACE2-TMPRSS2-EVs	Full-length	Wild type	..	..	..	Cocozza et al (2020)[27]
EVs-ACE2	Full-length	Wild type	..	Mice	..	Wu et al (2022)[28]
sACE2-sEVs	sACE2 and its mutants	Wild type, D614G, beta, and delta	Wild type and delta	Mice	..	Kim et al (2022)[29]
PM-ACE2-EVs	..	Wild type	Wild type	Mice	..	Xie et al (2021)[30]
ACE2-mimicking antibodies						
P2C-1F11	19–615 amino acids	Wild type, alpha, beta, and gamma	..	Mice	..	Wang et al (2021)[31] and Ge et al (2021)[32]
S2K146	..	Wild type, alpha, beta, gamma, delta, epsilon, and omicron	Wild type, alpha, beta, delta, and kappa	Beta variant in hamsters	..	Park et al (2022)[33] and Cameroni et al (2022)[34]
Peptide or mini-protein mimetics of ACE2						
AHB1 series or LCB1 series	23–46 amino acids and its mutants or de-novo design	..	Wild type	..	..	Cao et al (2020)[35]
P series	19–45 amino acids and its mutants	..	Wild type	..	..	Karoyan et al (2021)[36]
Spike S1-interacting domain of ACE2 receptor	37–42 amino acids	Wild type	..	Mice	..	Paidi et al (2021)[37]
SI series	24–45 amino acids and its mutants	..	Coronavirus GX_P2V	..	..	Pei et al (2022)[38]
Pep series	24–42 amino acids	Wild type	..	..	..	Zhu and Zhou (2022)[39]
D-peptide inhibitors	21–45 amino acids	..	Wild type, alpha, and beta	..	..	Valiente et al (2021)[40]
Chimeric X series	21–42 and 64–88 amino acids	..	Wild type	..	..	Bibilashvili et al (2021)[41]
NYBSP series	20–49 amino acids	Wild type	Wild type	..	..	Curreli et al (2020)[42]
TRI series	..	Wild type, D614G, alpha, beta, gamma, and delta	WA1/2020, alpha, beta, gamma, delta, and omicron	Alpha, beta, gamma, and delta in mice	..	Hunt et al (2022)[43]
Kansetin	..	Wild type	Wild type, alpha, beta, delta, and omicron	Mice	..	Lv et al (2022)[44] and Zhang et al (2022)[45]
De-novo-designed peptides						
SIH series	LCB1 derivatives	Wild type	..	Hamsters	..	Khatri et al (2022)[46]
LCB1 derivatives	..	..	Wild type	WA1/2020 and alpha variants in mice	..	Case et al (2021)[47]
CTC-445 series	..	Wild type	Wild type	Hamsters	..	Linsky et al (2020)[48]

SARS-CoV-2 variant Pango lineages are alpha (B.1.1.7), beta (B.1.351), gamma (P.1), delta (B.1.617.2), epsilon (B.1.429), kappa (B.1.617.1), and omicron (B.1.1.529). evACE2=extracellular vesicles harbouring ACE2. EVs-ACE2=extracellular vesicles embedded with engineered ACE2. ACE2=angiotensin-converting enzyme 2. hrsACE2=human recombinant soluble ACE2. sACE2=soluble ACE2. *Only experimentally validated inhibitors are listed.

Table: **ACE2-based inhibitors with suppressive effects on SARS-CoV-2 wild type and its variants***

were conjugated to a truncated form of CD9 with transmembrane region 4 deleted (CD9ΔTM4) on the extracellular vesicles surface to generate engineered extracellular vesicles.[29] In cellular experiments, the engineered sACE2.v1-extracellular vesicles and sACE2.v2-extracellular vesicles showed an increased affinity for spike proteins and greater neutralisation of pseudoviruses and authentic viruses compared with sACE2(WT)-extracellular vesicles. Furthermore, the engineered sACE2.v1-extracellular vesicles also conferred protective efficacy against SARS-CoV-2 infection in K18-hACE2 mice.[29] Meanwhile, post-translational modifications of ACE2 also influence the effect of extracellular vesicles. Palmitoylation at Cys141 and Cys498 residues of ACE2 promotes ACE2-targeting cell membranes and secretion to extracellular vesicles.[30] Compared with EVs-ACE2, engineered EVs-ACE2 that is fused with S-palmitoylation-dependent plasma membrane targeting sequence has greater neutralisation potency against both pseudo-typed SARS-CoV-2 and authentic viruses in vitro and in hACE2 transgenic mice.[30]

Extracellular vesicles are becoming one of the most desirable candidates for developing drug delivery vehicles and targeted drug delivery due to their intrinsic advantages, such as excellent biocompatibility, low immunogenicity, size at the nanometre level, and long blood circulation half-life.[53] However, the mechanisms and the environment in which extracellular vesicles function remain incompletely understood, and the heterogeneity of extracellular vesicles potency caused by distinct generation sources, preparation methods, and other factors could trigger unforeseen side-effects.[56] Therefore, the production and application of extracellular vesicles should be put under strict regulation to ensure acceptable safety and efficacy of EVs-ACE2.[54]

ACE2-mimicking antibodies

The spike protein of SARS-CoV-2, which works as the cell entry mediator, is a natural target for neutralisation antibodies. Neutralisation antibody responses against the RBD of spike protein accounted for approximately 90% of the total response in SARS-CoV-2 convalescent serum samples, suggesting RBD-targeting neutralisation antibodies might serve as a major counterforce against the virus.[57] Accordingly, SARS-CoV-2 neutralisation antibodies that are currently being studied in clinical trials are mainly ones that target the RBD.[58] The widely accepted, structure-featured classification of the RBD proposes four classes of neutralisation antibodies. Class 1 includes ACE2-blocking neutralisation antibodies that bind only and directly to up-conformation RBDs.[59] Although class 1 neutralisation antibodies can potentially have more efficient binding through multivalent viral crosslink,[59] key mutations (eg, K417N/T, E484K, and N501Y) grant SARS-CoV-2 VOCs broad resistance to them.[31] SARS-CoV-2 variants thus become more challenging, invalidating the ongoing antibody strategies through immune evasion capability.[60]

P2C-1F11, a class 1 neutralisation antibody, was effective against SARS-CoV-2 and its VOCs.[31,32] P2C-1F11 shares 11 RBD epitope residues with ACE2, with high clash volumes of approximately 20·48 nm³ whereby P2C-1F11 and ACE2 would bind to the same RBD, indicating a neutralisation mechanism via ACE2 mimicry.[32] More importantly, P2C-1F11 neutralised alpha, beta, and gamma (P.1) variants with better activities than the wild type through ACE2 mimicry.[31] Comparatively, many other class 1 neutralisation antibodies were unable to neutralise these VOCs or exhibited sharply reduced neutralisation ability.[31] Since these VOCs engage in stronger ACE2 binding,[14] the ACE2-mimicking P2C-1F11 becomes a promising antibody candidate for potential vaccines.

S2K146 is another ACE2-mimicking neutralisation antibody that has retained its efficacy against omicron.[33] Compared with wild-type SARS-CoV-2, omicron RBD was reported to bind the ACE2 receptor with higher affinity. Additionally, omicron also featured increased therapeutic antibody evasion.[61] S2K146 shares 18 epitope residues with ACE2 on SARS-CoV-2 RBD and imitates ACE2 via similar electrostatic contacts on the RBD-binding interface.[33] Pseudovirus neutralisation assays revealed that S2K146 had a half-maximal inhibitory concentration (IC_{50}) of 14·2 ng/mL for wild type and 12·6 ng/mL for omicron, outweighing other neutralisation antibodies investigated simultaneously.[34]

Since natural antibodies are not rationally designed to mimic ACE2, the patient-derived ACE2-mimicking class 1 neutralisation antibodies display scarce resemblance to ACE2 compared with synthetic ACE2 peptidomimetics. However, the considerable efficacy of P2C-1F11 and S2K146 neutralising SARS-CoV-2 implies that the competitive binding to RBD against ACE2 affects class I neutralisation antibodies' superiority regarding the viral neutralisation potency and broad-spectrum property.

Peptide or mini-protein mimetics of ACE2

Many ACE2 mimetics take the form of peptides or mini-proteins, typically including the PPI interface's hotspot residues and an optional stabilising scaffold. Their relatively small size makes them well suited for better tissue penetration and cellular transmissibility. Moreover, availability via efficient chemical synthesis and recombinant expression facilitates mass production of these mimetics. The complex structure of ACE2 and SARS-CoV-2 RBD disclosed that most α1 helixes and several residues scattered in α2, β3, and β4 of ACE2 are responsible for binding.[6] Therefore, critical residues of the ACE2 α1 helix are included in most ACE2 mimetics designed as SARS-CoV-2 antagonists.

Notably, standalone ACE2 α1 exhibited neither significant helical secondary structure in solution nor evident binding of S1 RBD, accompanied by unexpected oligomerisation tendency.[62] This disadvantage, possibly attributed to absent backing scaffolds like α2, prevents

standalone ACE2 α1 from clinical application. To exploit ACE2 α1's potential to neutralise SARS-CoV-2, researchers widely use in-silico computational design before experimental verifications. Molecular dynamics simulation has revealed recombinant ACE2 α1 and α1–α2 peptides' potential for effective binding of S1 RBD[63] and binding affinity enhancement by some mutations in α1 residues—eg, F8, K11, Q22, and L25.[64] ACE2 α1 helix was also incorporated in silico into helical bundles to generate a mini-protein inhibitor AHB2, with both S1 RBD binding K_D and SARS-CoV-2 neutralisation IC_{50} at nanomolar concentrations.[35] Alternatively, iterative computation has provided insight into maintaining a high helical folding propensity of standalone ACE2 α1 and has introduced strategic mutations to achieve a peptide named P10 with S1 RBD binding K_D <0·03 nM.[36]

Meanwhile, short peptides are expected to surpass medium-sized mini-proteins in synthesis efficiency and are considered potent candidates for drug development. A hexapeptide spike S1-interacting domain of ACE2 receptor was extracted from the ACE2 binding interface of SARS-CoV-2 S1 RBD and was shown to suppress SARS-CoV-2 infection and its associated inflammation in cell and mouse experiments.[37] Computation-based sequential optimisation and targeted evolution strategies were used to design an N-terminus and C-terminus-capped heptapeptide SI5α-b that had a half maximal effective concentration (EC_{50}) of 1·52 μM on Vero E6 cells treated with model coronavirus GX_P2V.[38] Reorganisation of ACE2 α1 and β4 residues resulted in the creation of peptides Pep12 and Pep15, both showing nanomolar affinity to SARS-CoV-2 S1 RBD and, therefore, being practical components of a colorimetric bioassay against SARS-CoV-2.[39]

Modifying the conventional conformation of peptides might enable superior, naturally uncommon properties.[65] For example, D-peptides—ie, peptides synthesised with D-amino acids—typically have reduced immunogenicity and increased proteolytical stability, compensating for common L-peptides' high biodegradability.[66] A D-peptide was designed and found capable of neutralising wild-type SARS-CoV-2 and alpha and beta VOCs with micromolar IC_{50} values.[40] Noticeably, chemical modifications designed to force peptides to adopt bioactive conformations might give unfavourable results. As disulphide bonds help impose strong conformational restrictions and improve peptide structural stability, a synthesised chimera peptide X2 consisting of disulphide-bond-anchored ACE2 α1 and α2 helices was expected to inhibit SARS-CoV-2.[41] However, X2 did not exhibit any substantial antiviral activity in vitro.[41]

Similarly, peptide stapling strategies are thought to promote drug metabolism and pharmacokinetic properties of peptides.[67] To regain helical conformation of ACE2 α1 in solution for proper ACE2–RBD interaction, i, i+4 or i, i+7 single-hydrocarbon-stapled ACE2 peptidomimetics were developed, but found incapable of inhibiting SARS-CoV-2 infection.[68] Curreli and colleagues[42] designed i, i+4 double-hydrocarbon-stapled peptides based on the ACE2 α1 sequence and achieved submicromolar K_D and IC_{50} values and a modest proteolytic half-life of more than 289 min. In 2021, Maas and colleagues[69] discovered that i, i+4 lactam-based, single-stapled ACE2-based peptides have better SARS-CoV-2 inhibition (IC_{50} of 3·6 μM and K_D of 2·1 μM) than those studied by Curreli and colleagues. These discoveries could provide insight into how chemical modifications affect ACE2 mimics' inhibition efficiency.

For mini-protein inhibitors, assembly extension-mediated potency enhancement is an alternative option. For example, the ACE2-mimicking mini-binder AHB2 mentioned above was reutilised to construct a self-assembled homotrimer TRI2-2.[43] TRI2-2 had multivalent interaction with all three RBDs of the spike trimer, and IC_{50} values of 35 pM for SARS-CoV-2 reference strain and 73 pM for the omicron strain in the live-virus neutralisation assay. Another multivalent tetramer inhibitor, Kansetin, with two ACE2-extracted sequences within each monomer, likewise displayed substantial neutralisation of live virus (with an IC_{50} of 108·6 pM for reference strain, 121·9 pM for omicron BA.1, and 134·0 pM for omicron BA.2).[44,45] These mini-binders share advantages over the monoclonal antibodies and feature better availability via large-scale production under *Escherichia coli*[43] or the cell-free expression system.[44]

The peptide and mini-protein mimetics of ACE2 and their derived constructs with specific assembly extensions indicate a direction in which guided ACE2 segmentation towards SARS-CoV-2 inhibitor design is more than possible. As mentioned previously, easy biosynthesis and considerable bio-transmissibility make peptide and mini-protein mimetics of ACE2 a powerful weapon to fight SARS-CoV-2. However, the small size of the two helices α1 and α2 in ACE2 restricts the standalone use of the peptide mimetics without loss of efficacy or stability unless proper modifications or extended scaffolds are applied. The structural resilience of the binding interface needs to be maintained for removed parts to continue functioning properly.

De-novo-designed peptides

If ACE2 mimetics aim to exploit the high native binding of ACE2 to SARS-CoV-2 RBD, de-novo-designed SARS-CoV-2 antagonists might aim for residue-wise elevation of PPI magnitude by rational design. De-novo protein design focuses on creating new proteins with low sequential relevance to known proteins in nature on the basis of fundamental biophysical principles and is expected to accomplish new functions or better activities in structurally tuned, novel proteins with computational aids.[70] A study from 2022 showed that only a little information (ie, information on the target protein structure) is required to design binding

proteins.[71] Regarding COVID-19, de-novo-designed peptide antagonists were expectedly complementary to regular therapeutics.

Several mini-protein inhibitors with picomolar affinity against the spike protein were de-novo designed without referring to the known RBD-binding interactions of ACE2.[35] These inhibitors, named from LCB1 to LCB8, were found more capable of binding SARS-CoV-2 spike RBD than the ACE2-derived AHB2. Live-virus neutralisation assays also revealed the vigorous neutralisation activity of LCB1 to LCB3 with picomolar IC_{50} values. Furthermore, Khatri and colleagues[46] adopted LCB1 as a starting point and developed a truncated, dimeric helix-hairpin that effectively neutralises SARS-CoV-2 through an RBD dimerisation strategy. However, the potency of the renowned LCB1 deteriorated against VOCs, with beta and gamma variants displaying complete immune evasion.[72]

ACE2 binding specificity might be one area for potential RBD evolution, which would at least partly address the innate weakness of de-novo proteomimetics. To address the concerns of SARS-CoV-2 evasion mutations, Case and colleagues[47] created LCB1v1.3 by introducing seven polar mutations into LCB1. Tested against recombinant SARS-CoV-2, which contains the key mutations—ie, E484K, N501Y, and D614G—present in beta and gamma, LCB1v1.3 displayed nanomolar EC_{50} values. The compensating adaptations on LCB1 suggested that de-novo peptidyl inhibitors might require multi-step optimisations for sufficient VOC neutralisation.

Alternatively, Linsky and colleagues[48] designed de-novo RBD inhibitors by combining minimal replication of hACE2's binding interface and directed evolution of the supporting scaffold. Sequence optimisation generated a monomeric, ultra-stable protein decoy, CTC-445.2, which exhibited a nanomolar RBD affinity and IC_{50} value, and resilience to SARS-CoV-2 RBD mutational evasion. Further trimerisation granted the decoy a picomolar RBD affinity. Moreover, even in a de-novo design strategy, few residual elements of the wild-type ACE2 interface could help maintain the broad-spectrum neutralisation ability against rapidly emerging VOCs.

Discussion

From the initial attempt of applying clinical hrsACE2 in COVID-19 treatment to the later modifications of ACE2, its fragments, or its residues, many ACE2 derivatives have been designed and produced as potential alternatives to conventional SARS-CoV-2 inhibitors. Although hazardous SARS-CoV-2 sublineages might emerge, various studies indicate the dependence of variants on the ACE2-involved cell entry and, consequently, the sensitivity to ACE2-mimicking decoys. Furthermore, the RBD of SARS-CoV-2 spike protein, especially the ACE2-binding surface, is less immunogenic than other regions of the spike protein, explaining the low neutralisation activity of spike-targeting antibodies from COVID-19 patients.[73] Therefore, therapeutic uses of ACE2-mimicking decoys or antibodies are expected to counteract the immune recognition shift. These factors play a large role in encouraging the application of ACE2-based decoy peptides or proteins as candidates for COVID-19 therapies. In addition to relying on simply reusing the clinically available hrsACE2 products, there is promising room for advances in ACE2-based drug development as therapeutic options aim to improve pharmacokinetic performance, target specificity, and safety properties.

Stable affinity to VOC spike proteins is characteristic of ACE2-mimetics adopting the strategy of binding hotspot replication. During the long-term evolutionary path of SARS-CoV-2, recombinant peptides and proteins that bear a sequential and structural resemblance to native hACE2, like Kansetin, were observed to retain their neutralisation efficacy on VOCs. Moreover, these auxiliary-enhanced ACE2 derivatives outperform antibodies in large-scale expression efficiency and long-term preservability, suggesting extensive clinical uses. This native-ACE2-mimicking strategy is expected to help protect people from potentially dangerous COVID-19 mutants in the future. In contrast, saturated or directed hotspot engineering on the original RBD-binding interface of ACE2 could enable specialised binding of a specific VOC target at the expense of broad neutralisation. The de-novo peptide design heavily exploits the hotspot engineering strategy, and needs periodic upgrades to optimise neutralisation efficacy against the evolving nature of the pandemic. Considering the demand for both fast-acting suppression of emerging regional outbreaks brought by VOC emergence and long-term elimination of COVID-19 in a global sense, a balance between the two strategies instead of choosing between them could be a better action guide for developing ACE2-derived drugs.

Despite hrsACE2's advantage of low immunogenicity, systemic delivery of hrsACE2 by intravenous injection might result in insufficient drug concentration at local infection sites and an unsatisfactory efficacy,[74] which could be attributed to its short plasma half-life of approximately 10 h.[20] Consequently, multi-faceted approaches to improve the utility of ACE2 derivatives have been reported (eg, bioavailability-friendly IgG-Fc fusion,[75] and extracellular vesicle-based protein[26] and aerosol formulation enabling high-efficient inhalation delivery to lungs).[51] Kansetin, one of the peptidyl ACE2 mimetics with extra fusion scaffolds for tetramerisation, also showed no observable toxicity via oral and inhalation intake,[44] suggesting controllable low toxicity of ACE2 derivatives with proper recombinant modifications. Alternatively, specific secondary-structured epitopes are needed for antibodies to function in some cases,[76] and are favourable for reduced immunogenicity of smaller ACE2-mimicking peptides. Altogether, it is expected that ACE2 mimetics will achieve feasible bioavailability in the future, benefiting the clinical use of these SARS-CoV-2 inhibitors.

Nevertheless, appropriate tests should be completed to assess and minimise medical risks, and clinical factors such as delivery routes and pharmacokinetic parameters should be closely studied to ensure optimal treatment.

ACE2 derivatives inhibit viral infection by competing with human membrane-bound ACE2 to bind spike RBDs, indicating a possible effect on their efficacy by different human ACE2 expressions affected by disease severity. In 2022, the elevation of ACE2 concentrations within patients with severe COVID-19 was reported,[77] and circulating ACE2 activity, strongly correlating with COVID-19 severity, was claimed to predict the prognosis and mortality of patients with COVID-19.[78] ACE2 derivative-mediated viral neutralisation should be maximised when endogenous soluble ACE2 is not sufficiently released to promote viral cell entry blockage; thus, patients with less severe COVID-19 or at an early infection stage are expected to benefit more from ACE2 mimetics administration. The ACE2 derivative hrsACE2 has been administered to patients in the early stages of COVID-19 to prevent hospitalisation and disease progression, similar to antivirals such as nirmatrelvir–ritonavir, which targets viral replication. Notably, the use of hrsACE2 in patients with severe COVID-19 is followed by an improvement in their clinical condition.[49] In treatment of patients with severe COVID-19, therapeutic efficacy was also attributed to hrsACE2-induced renin-angiotensin-aldosterone system down regulation.[50] These results imply possible application of ACE2-based therapies to patients with late-stage COVID-19. More solid clinical and mechanistic evidence is needed for use of ACE2-based therapies in patients with COVID-19 who do not fall into these categories.

Search strategy and selection criteria

We searched PubMed, Google Scholar, ClinicalTrials.gov, WHO.int, and Web of Science for literature, clinical registrations, and reports without restrictions on language. A combination of the keywords "COVID-19", "SARS-COV-2", "Omicron", "variants of concern", "VOC", "sublineage", "pathogenesis", "mechanism", "symptom", "stage", "severity", "therapy", "prevention", "treatment", "cell entry", "evolution", "mutation", "receptor", "spike", "RBD", "receptor binding domain", "structure", "inhibition", "neutralization", "immune evasion", "ACE2", "angiotensin converting enzyme 2", "mediate", "binding", "interaction", "interface", "recombinant", "soluble", "strategy", "EV", "extracellular vesicle", "delivery", "route", "mimetic", "mimic", "analog", "derivative", "peptide", "protein", "mini-protein", "antibody", "mAb", "monoclonal antibody", "epitope", "de novo", "rational design", "modification", "optimization", "fusion", "bioavailability", "immunogenicity", "administration", and "expression level" were used in the search string to select relevant studies published between Jan 1, 2000, and Jan 1, 2023.

Concerns about virus resistance to therapeutic ACE2 mimetics might arise. Since similarities between ACE2 derivatives and native ACE2 are high regarding the spike-binding interface, whether other routes should substitute ACE2-mediated entry under evolutionary pressure introduced by ACE2-based therapies is worth consideration. Notably, the SARS-CoV-2 omicron variant uses ACE2 as the main cell entry receptor,[79] and omicron RBD binds ACE2 with a higher affinity than the wild-type SARS-CoV-2 RBD.[61] Accumulation of key mutations, such as N501Y in spike proteins, enables omicron to bind ACE2 more efficiently for cell entry,[80] suggesting an ongoing viral evolution guided by ACE2 binding enhancement. Other SARS-CoV-2 cell entry mechanisms beyond ACE2, such as the integrin α5β1 route, have been reported.[81] However, regarding newly emerging SARS-CoV-2 variants, evidence supporting the replacement of ACE2-based cell entry routes by other routes is still scarce. Before such evidence appears, therapists and researchers might prefer to focus on ACE2-based entry-blockage therapies whose potential against COVID-19 already has evidence backing.

The potential of ACE2 derivatives can be expanded with the additional participation of other SARS-CoV-2 targeting drugs. For instance, antivirals are one of the mainstream treatment options for COVID-19, and some of them (eg, remdesivir) have retained viral neutralisation efficacy due to the high conservation of their target protein during SARS-CoV-2 evolution.[82] Thus, these non-ACE2-targeting antivirals are complementary to the ACE2 mimetics serving as SARS-CoV-2 inhibitors underlying different molecular mechanisms. Hence, the concomitant use of ACE2 derivatives and orthogonal antivirals under co-ordinated or sequential coadministration could empower improved COVID-19 therapeutics capable of subduing the SARS-CoV-2 VOCs. However, caution should be taken and rigorous pre-clinical and clinical trials should be conducted to avoid possible side-effects of co-administration.

In the wake of the first outbreak of COVID-19, hrsACE2 and its derivatives—initially tools to overcome angiotensin 2-dependent hypertension—were once again found to be effective and useful, showing efficacy against SARS-CoV-2. Beyond SARS-CoV-2, other coronaviruses (eg, SARS-CoV) have ACE2 binding mechanisms,[4] suggesting that ACE2 derivatives could have a broader applicability against certain viruses. We hope that our Review helps improve understanding of the design and application of hrsACE2 and its derivatives and hopefully promotes enlightenment on developing practical coronavirus-aiming therapies.

Contributors
All authors contributed to all aspects of this Review.

Declaration of interests
We declare no competing interests.

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