Rapid de novo discovery of peptidomimetic affinity reagents for human angiotensin converting enzyme 2

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Rapid discovery and development of serum-stable, selective, and high affinity peptide-based binders to protein targets are challenging. Angiotensin converting enzyme 2 (ACE2) has recently been identified as a cardiovascular disease biomarker and the primary receptor utilized by the severe acute respiratory syndrome coronavirus 2. In this study, we report the discovery of high affinity peptidomimetic binders to ACE2 via affinity selection-mass spectrometry (AS-MS). Multiple high affinity ACE2-binding peptides (ABP) were identified by selection from canonical and noncanonical peptidomimetic libraries containing 200 million members (dissociation constant, \( K_D = 19-123 \) nM). The most potent noncanonical ACE2 peptide binder, ABP N1 (\( K_D = 19 \) nM), showed enhanced serum stability in comparison with the most potent canonical binder, ABP C7 (\( K_D = 26 \) nM). Picomolar to low nanomolar ACE2 concentrations in human serum were detected selectively using ABP N1 in an enzyme-linked immunosorbent assay. The discovery of serum-stable noncanonical peptidomimetics like ABP N1 from a single-pass selection demonstrates the utility of advanced AS-MS for accelerated development of affinity reagents to protein targets.
The discovery of high affinity reagents is a critical initial step in drug discovery, diagnostic development, and proteome profiling. High affinity ligands are under constant development for the modulation of activity or function of target proteins, including challenging protein–protein interactions (PPIs) and intracellular targets. Diagnostics inform clinical decision making and require sensitive and selective detection within highly complex media often containing a broad range of other protein concentrations (e.g., plasma). Lastly, affinity reagents have been crucial for understanding the proteome, facilitating the compilation of a knowledge base for protein expression profiles and localization across normal and disease tissue (e.g., Human Protein Atlas). Yet, the vast majority of known proteins have no corresponding affinity reagent. Overall, each of these fields relies on the rapid discovery and development of selective, high affinity binders to specific protein targets.

Current methods to discover high affinity reagents against protein targets vary in production speed and chemical diversity, ranging from antibody production to panning fully synthetic libraries. While they are the ‘gold standard,’ antibodies require a long production timeline, demonstrate low tissue penetration, and can exhibit batch variability. Thus, several non-antibody systems including DNA-encoded libraries, aptamers, and peptide discovery platforms, have been developed to discover specific, high affinity binders. Most ‘hit’ discovery techniques rapidly isolate and enrich binders from libraries based on their high affinity to the target protein, although high-throughput screening of individual compounds has also been used. The design and diversity of these libraries greatly affect the rate of discovery against a novel target. Larger molecular scaffolds can bind broader portions of protein surfaces, enabling the efficient targeting of PPIs. The chemical and structural diversity of the curated library can improve discovery success. Thus, an ideal discovery platform should combine high chemical diversity and rapid responsiveness to new clinically relevant targets.

Much research has been devoted to the discovery and engineering of peptidomimetic binders because of their broad access to diverse chemical spaces, amenability to rapid synthesis, and availability of multiple rapid discovery platforms. The use of noncanonical amino acids, macrocyclization, and chemical stapling, in particular, have been proven to be useful in promoting cell uptake, proteolytic stability, and improved pharmacokinetics. Discovery via selection using genetically-encoded techniques including phage display and mRNA display samples vast libraries up to 10^13 members, being amenable for de novo discovery of high affinity reagents. However, these techniques are not well suited to the incorporation of highly noncanonical library members, even in cell-free systems. Thus, following the initial identification of high affinity peptides, further development is required via iterative synthetic cycles of derivatization and screening.

High affinity peptidomimetic binders can also be identified by affinity selection-mass spectrometry (AS-MS), enabling the straightforward use of entirely noncanonical synthetic libraries without expending rapid discovery. AS-MS generally functions through the enrichment and identification of peptidomimetic binders to the target protein through a single enrichment step since it cannot be genetically amplified. Thus, discovery efforts with AS-MS have generally been limited to small combinatorial libraries (10^3–10^6 members), which were biased toward the target protein in a ‘focused’ or structure-based manner. AS-MS of these focused libraries remains a reliable way to rapidly identify key binding ‘hot-spot’ residues and combinatorially introduce noncanonical amino acids. Recent advancements made by our group in the MS/MS sequencing of complex peptidomimetic mixtures and optimized AS-MS selection conditions have enabled de novo discovery of high affinity binders from fully randomized peptidomimetic libraries up to 10^8 members. Thus, AS-MS can enable rapid discovery across highly diverse libraries. With these methods, we set out to perform de novo discovery with synthetic highly noncanonical peptidomimetic libraries against recently identified clinically relevant targets.

Angiotensin converting enzyme 2 (ACE2) has been identified as an important plasma biomarker for cardiovascular disease-induced events and death in a global, population-based study. Thus, ACE2 is ubiquitously known as the receptor utilized for cell entry by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) beta-coronavirus. Thus, high affinity reagents for the specific serum detection of ACE2 are increasingly important. Here we demonstrate rapid discovery of high affinity peptidomimetic binders to ACE2 through a single-pass AS-MS experiment utilizing fully randomized canonical and noncanonical libraries. By comparing selection results between the canonical library and a noncanonical library over a standard canonical library quantitatively and qualitatively, we highlight that the noncanonical binders exhibit improved proteolytic serum stability. In further tests, our noncanonical peptidomimetic ACE2 binder, ABP N1, demonstrated ACE2 binding specificity in a serum pulldown experiment and as low as picomolar detection in an enzyme-linked immunosorbent assay (ELISA), highlighting the development as promising diagnostic tools.

**Results**

AS-MS identifies low-nanomolar affinity canonical and noncanonical peptide binders to ACE2. We recently optimized in-solution affinity selection combined with nano-liquid chromatography-tandem mass spectrometry (nLC-MS/MS) sequencing to enable the identification of high affinity binders from fully randomized synthetic libraries with a diversity of up to 10^9 members, a 100-fold increase over the standard practice. Noncanonical amino acids can be extensively used in the preparation of the synthetic libraries utilized in AS-MS, provided there is no isobaric monomer mass overlap and sufficient tandem sequencing fidelity. Thus, we sought to compare the results of our selections against human ACE2 protein using a standard, canonical-L library, and a noncanonical-L library, each containing 200 million members (Fig. 1a).

The two libraries share the same design. 12 variable positions followed by an amidated C-terminal lysine to facilitate sequence identification and filtering. The monomer set utilized in the canonical library (Library 1) is fully proteogenic, except Cys because it could form intra- or intermolecular disulfides and 1le because it is isobaric in mass with Leu, to give 18 amino acids total (Fig. 1b). For side-by-side comparison and exploration of noncanonical chemical space, another library (Library 2), composing 17 non-proteogenic monomers out of 21 amino acids total (81% noncanonical, significantly more than the available genetically encoded techniques) was synthesized (Fig. 1b, c). Library 2 was designed to sample an entirely new chemical space, while still capturing similar chemical properties of the natural amino acid set. While nearly all the canonical monomers were replaced with non-proteogenic analogs, the number of positively and negatively charged residues at physiological pH was kept constant at 2 each, respectively. Nonstandard backbones including β-amino acids and achiral linkers were included to diversify structural availability and improve proteolytic stability. The final monomers were selected based on considerations of balanced chemical diversity, mass uniqueness, library solubility,
stereochrmary, and compatibility with Fmoc solid-phase peptide synthesis (SPPS).

Following the workflow depicted in Fig. 1a, we performed the affinity selection using both the canonical and noncanonical peptide libraries against human ACE2 in parallel with 12ca5 as an unrelated control protein, and the enriched peptides were eluted and analyzed by nLC-MS/MS. The sequences were decoded using the PEAKS software and filtered to isolate peptidomimetic sequences that matched the original library design. The peptidomimetics were sorted to reveal those that were unique to ACE2 in comparison to the off-target control protein and are reported in Supplementary Tables 2 and 3. From both the canonical and noncanonical selections, we observed a preferred N-terminal motif, indicating a potential new class of ACE2 binders (see Supplementary Note 1 and Supplementary Figs. 1 and 2). For the canonical L-peptides, leucine (L) and valine (V) were preferred at the N-terminus position followed by glutamine (Q) and asparagine (N) with some cationic or ionizable residues nearby (H, K, and R, see Supplementary Fig. 1). Similarly, the noncanonical L-peptidomimetics discovered preferred cyclopropylalanine (Cpa, C) and isoleucine (Ile, I) at the N-terminal position followed by 3-(4-thiazolyl)-alanine (Tha, T, see Supplementary Fig. 2). The observation of a preferred motif added confidence in the discovery of a class of peptidomimetic binders to ACE2.

An additional refinement step was taken to analyze the extracted ion chromatogram (EIC) of the observed ion from each MS-identified peptide. This EIC-based refinement provides an additional level of confirmation that each peptide was uniquely selected against ACE2 or if it binds nonspecifically to the selection matrix (e.g., magnetic beads or streptavidin). A comparable number of sequences was identified as EIC-selective between the two libraries (48 noncanonical sequences and 60 canonical sequences), indicating that sequencing and identification of noncanonical binders can robustly be achieved with a similar throughput to canonical peptides on our AS-MS platform using Orbitrap nLC-MS/MS. However, the noncanonical selection showed a higher primary MS baseline that obscured more peptidomimetics from being discovered (Supplementary Table 3; Supplementary Fig. 17). This ambiguity could be explained by a higher level of baseline binding from the noncanonical peptidomimetics library or poorer ionization of the eluted library members. Lastly, both selections showed a low rate of nonspecific binder recovery (Supplementary Fig. 18).

With the discovered sequences in hand, nine canonical and five noncanonical binders were chosen for synthesis and validation efforts based on their high average local confidence (ALC) scores. Each binder was re-synthesized and purified individually (see Supplementary Notes 2 and 3) and their binding affinities (dissociation constant, $K_D$) to ACE2 were measured using biolayer interferometry (BLI, see Supplementary Note 4). As a result, low-nanomolar ACE2-binding affinities were determined for the noncanonical binders ABP N1 ($K_D = 19 \text{nM}$, Fig. 2a), ABP N4 ($K_D = 123 \text{nM}$, Fig. 2b), and ABP N6 ($K_D = 33 \text{nM}$, Fig. 2c), and for the canonical binders ABP C3 ($K_D = 35 \text{nM}$, Fig. 2d), ABP C7 ($K_D = 26 \text{nM}$, Fig. 2e), and ABP C8 ($K_D = 36 \text{nM}$, Fig. 2f). All other peptides either presented lower affinity or no binding to ACE2 (Fig. 2g), indicating that approximately half of the ACE2-selective sequenced binders following AS-MS were non-binders by BLI.

To determine whether the observed binding is sequence-specific, two scrambled variants of ABP N1 (Scrm N1.1 and Scrm N1.2) were synthesized and their binding to ACE2 was tested by BLI. Under these conditions, no binding to ACE2 was observed (Fig. 2g; Supplementary Fig. 21). We also observed minimal binding to an unrelated protein (12ca5) for ABP N1, ABP N4, and ABP N6 (Supplementary Fig. 22). However, the binding of 500 nM of ACE2...
Fig. 2 Nanomolar affinity binders were identified from both canonical and noncanonical libraries. a–c ACE2-binding traces, measured by bio-layer interferometry, for noncanonical binders ABP N1, ABP N4, and ABP N6, respectively. d–f ACE2-binding traces, measured by bio-layer interferometry, for canonical binders ABP C3, ABP C7, and ABP C8, respectively. g A summary of all individually synthesized peptides. Column headers: ‘ID’, the peptide identifiers; ‘Sequence, X12K’, the peptide sequences with a lysine at the C-terminus; ‘ALC’, the exported average local confidence score from sequence decoding; ‘Error, ppm’, the mass error (in ppm) between the precursor and assigned sequence; ‘K_D, obs, nM’, the apparent dissociation constant, in nM, measured by bio-layer interferometry. Cyan highlights the canonical (ABP C7) and noncanonical (ABP N1) peptides with the highest binding affinity.
to ABP N1 results in ~1.3 nm BLI response signal, compared to 0.25 nm signal from 500 nM of the off-target protein (12ca5), translating to ~5-fold higher signal and indicating selectivity toward ACE2.

Additionally, we investigated whether known binders to ACE2 inhibited the binding of the ABPs, including the native ACE2 substrate angiotensin 2 (AngII) and the ACE2 inhibitor MLN-4760, an ACE2 substrate AngII and MLN-4760, and the receptor binding domain (RBD) of SARS-CoV-2 (see Supplementary Notes 5 and 6). First, we performed competitive binding experiments on all binders discovered (ABP N1, N4, N6, C1, C2, C3, C4, C7, and C8) versus AngII and MLN-4760 (Supplementary Figs. 27–35). We observed that the native ACE2 substrate AngII did not inhibit the binding of any of our ABPs, indicating that the binding sites of ABPs and AngII likely do not overlap. However, MLN-4760 was able to partially block the binding of ABP N1 and C8 at the 10-fold excess of MLN-4760 mixed with ACE2, indicating that the binding sites likely overlap with the active site of the ACE2 enzyme. Secondly, we performed a competitive BLI experiment to determine if any of the discovered binders could disrupt the interaction between ACE2 and SARS-CoV-2 spike protein receptor binding domain (RBD). We did not observe any competition by our peptides on the binding of ACE2 to RBD (Supplementary Figs. 23–26) at 5- or 50-fold excess, indicating these ABPs do not bind at the RBD site of interaction.

The noncanonical binder ABP N1 demonstrates enhanced serum stability. A significant limitation to the development of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Thus, the use of noncanonical amino acids, macrocyclization, and chemical stapling have each grown as approaches to improve their stability. Thus, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Therefore, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Thus, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Therefore, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Thus, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Therefore, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Thus, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Therefore, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Thus, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. Therefore, the use of canonical L-peptides is their susceptibility to enzymatic degradation within physiological environments. The ability of selective binding and isolation of ACE2 from a complex biological matrix indicates a promising diagnostic application of ABP N1.

ABP N1 pulls down ACE2 from human serum selectively. To further investigate the ACE2-binding capacity of the identified high affinity reagents within a biological matrix and demonstrate binding selectivity, we performed a human serum pull-down experiment using the most potent noncanonical binder ABP N1. As depicted in Fig. 4a, the biotinylated ABP N1 was immobilized onto streptavidin-coated magnetic beads and then incubated with ACE2 (represented in orange from PDB: 6M17) in human serum. Bound ACE2 was eluted for subsequent analysis. The SDS-PAGE image with samples showing from left to right lanes: (1) molecular weight standard; (2) purified ACE2 protein (1.5 µg) as a loading control; (3) normal human serum as a control; (4) normal human serum mixed with ACE2 (1.5 µg); (5) elution of the bound fraction from the magnetic beads. NHS, Normal Human Serum (5%).
Picomolar ACE2 can be detected with ABP N1 via ELISA. Human ACE2 was recently identified as the top biomarker for cardiovascular disease and an elevated level of plasma ACE2 significantly associates with death, heart failure, stroke, and myocardial infarction. To demonstrate the utility of our non-canonical ACE2 binders as detection probes of the plasma ACE2 level, we developed an ELISA-based detection assay (Fig. 5a).

After immobilization on an ELISA plate, the picomolar concentration of ACE2 was detected by biotinylated ABP N1 in a dose-dependent manner (Fig. 5b). As a negative control, no binding was observed from biotinylated ABP N8 at the tested concentration of ACE2 (10 pM–100 nM in Fig. 5b), which is consistent with the non-binding observation as demonstrated in Fig. 2g. Interestingly, when a similar ELISA experiment was performed with the plate being coated with a mixture of normal human serum and exogenous ACE2, the dose-dependent ACE2 detection using ABP N1 was retained (Fig. 5c). However, we observed a decreased detection sensitivity presumably due to the decreased amount of ACE2 being immobilized on the plate with many other serum proteins present. As a summary, with an ELISA-based approach, we demonstrated that ABP N1 could selectively detect soluble ACE2 from the human serum at concentrations ranging from picomolar to low nanomolar levels.

Discussion

In this study, we report rapid discovery of high affinity peptidomimetic binders to ACE2 via AS-MS. After screening two ultra-large synthetic libraries, a series of ACE2-binding peptides with low-nanomolar affinities ($K_D$ from 19 to 123 nM) were identified. We showed that the most potent noncanonical peptide binder, ABP N1, demonstrated enhanced serum stability in comparison with the most potent canonical binder, ABP C7. Furthermore, ABP N1 demonstrated a high selectivity to ACE2 over human serum proteins, indicated by a serum pull-down experiment. Lastly, in an ELISA-based format, picomolar to low nanomolar ACE2 concentrations in human serum could be detected using ABP N1. The de novo discovered high affinity reagents offer specific serum detection of ACE2 and represent a promising diagnostic tool of related diseases.

We chemically synthesized both canonical and noncanonical peptide libraries (each containing 200 million peptides) and subjected them to a single-pass affinity selection experiment as a side-by-side comparison. Of note, a comparable number of putative binders was observed from both canonical and noncanonical library selections, with a similar portion of binders from both the canonical and noncanonical selections, indicating a potential preferred binder class to ACE2. Therefore, even though the binders were identified as ACE2-selective with high sequencing confidence, individual synthesis and validation of the putative hits are necessary to confirm ACE2-specific interaction. We observed an N-terminal motif from both the canonical and noncanonical selections, indicating a potentially preferred binder class to ACE2 (Supplementary Figs. 1 and 2). However, after individual validation, binders containing such a motif do not warrant ACE2-binding capability (Fig. 2g), indicating that the other amino acids within the sequence also play an important role in binding. Notably, all of the discovered binders are linear, whereas previous phage display efforts to discover linear ACE2 binding peptides were unsuccessful and only discovered macrocyclic binders.

Even though the canonical or noncanonical binders discovered here are linear, they do not have a clear similarity to other known binders of ACE2, including the substrate angiotensin 2 (AngII, Sequence: DRVYIHPF) or the ACE2 inhibitor MLN-4760. The design of both libraries is similar in size to AngII. Yet, the lack of peptidomimetics discovered that are similar to AngII could be due to the libraries’ carboxamide C-terminus interfering with the substrate binding site or because of ACE2 enzymatic activity.

Fig. 5 Picomolar ACE2 concentration was detected by ABP N1 by ELISA. a Schematic representation of the ELISA workflow. b ACE2 in 1× PBS at different concentrations (100 nM–10 pM) was immobilized on an ELISA plate. c ACE2 in human serum at different concentrations (100 nM–10 pM) was immobilized on an ELISA plate. The plate was incubated with ABP N1 or control peptide, followed by streptavidin-HRP and TMB substrate. Absorbance was measured at 450 nm. The experiment was performed in technical triplicates ($n=3$). Each data point, the mean signal as a bar, and error bars from experimental standard deviation, and statistical significance calculated with the unpaired Student’s t-test are shown.
cleaving peptides closely bound to the AngII active site. Cleaved peptides would be filtered out during analysis.42 Moreover, ACE2 receptor blockers (ARBs) also do not appear similar to the discovered binders here, though peptidomimetic ARBs have been a source of inspiration for their small-molecule design33. With the use of the ARB MLN-4760, competitive BLI binding experiments were able to identify that ABP N1 and C8 are partially inhibited by MLN-4760, suggesting N1 and C8 bind near the active site and could be further developed. Natively, ACE2 is a counter-regulator of the renin-angiotensin hormonal cascade. However, elevated levels of ACE2 in plasma have been found to be the highest-ranked predictor of death, heart failure, stroke, and myocardial infarction compared to several established risk factors47,55. As the poorly understood, ACE2 plasma concentrations are a measurable indicator of renin-angiotensin system dysregulation47. As the several established risk factors47,55 have been found to be the highest-ranked predictor of death, hormonal cascade. However, elevated levels of ACE2 in plasma were able to identify that ABP N1 and C8 are partially inhibited. Cleaved peptides closely bound to the AngII active site. Cleaved solution was washed with dry ice-cold diethyl ether and followed by centrifugation at 4000 rpm for 3 min to precipitate the crude peptides. The obtained solids were briefly dried and dissolved in water/acetonitrile (50:50, v/v) before freezing and lyophilization.

Peptide purification. Peptides with crude purity >85% were purified using a Biogate Select® flash purification system and peptides with crude purity >85% were purified using reverse-phase high-performance liquid chromatography (HPLC). 1) Reverse-Phase HPLC. Crude peptides with purity below 85% were dissolved in water with 0.1% TFA and then purified by semipreparative reverse-phase HPLC using an Agilent 1260 HPLC system (Agilent Zorbax SB-C3 column: 9.4 × 250 mm, 5 μm; or Agilent Zorbax SB-C18 column: 9.4 × 250 mm, 5 μm). The gradient used was 10 to 61% acetonitrile over 60 min with a 4.0 mL/min flow rate. HPLC fractions were analyzed by LC-MS and pure fractions were combined and lyophilized. 2) Flash chromatography purification. Synthetic peptides with the estimated LC-MS crude purity above 85% were purified with a Biogate Select® instrument using Biotage Sfär C18 D column (Duo 100 Å 30 Å, 12 g) under a 5–60% acetonitrile gradient over 12 column volumes, and the flow rate was set to 12 mL/min. Fractions were collected based on absorbance at 214 nm and then subjected to LC-MS analysis before combining the pure fractions and lyophilization.

Analytical high-performance liquid chromatography (HPLC). Analytical HPLC was performed on an Agilent 1200 series system with UV detection at 280 nm. The column used was Phenomenex Kinetex (100 × 2.1 mm, 2.6 μm, 100 Å silica) with a flow rate of 0.75 mL/min. Solvents used are acetonitrile with 0.08% TFA additive (solvent B) and water with 0.1% TFA additive (solvent A). A linear gradient: 2% B hold 2% B, 2–32% B gradient from 2 to 17 min, 32–65% B gradient from 17 to 17.5 min, hold 65% B from 17.5 to 19 min. A final 6 min hold was performed with 2% B. The total method time was 25 min. HPLC purities were determined by manual integration of all signals in the area of 2–13 min.

LC-MS analysis. Crude synthetic peptides or peptide fractions from the purification steps were analyzed by LC-MS (Agilent 6545 or 6650 ESI Q-TOF) using an Agilent Zorbax 300SB-C3 or Phenomenex Luna C18 column. Total ion chromatograms and integrated MS over the main peak are provided in the supplementary information.

Condition 1. Analysis was performed on an Agilent 1290 Infinity HPLC coupled to an Agilent 6545 ESI Q-TOF mass spectrometer. MS was run in positive ionization mode, extended dynamic range (2 GHz), and standard mass range (m/z in the range of 300–3000 a.m.u.). The solvent mixtures used for LC-MS chromatography were: A = water + 0.1% formic acid (LC-MS grade), B = acetonitrile + 0.1% formic acid (LC-MS grade). The following conditions were used for peptide analysis. Column: Zorbax 300SB-C3 (5 μm, 150 × 2.1 mm, 300 Å silica); Flow Rate: 0.8 mL/min; Gradient: 1% B 0–2 min, linearly ramp from 1% B to 61% B to 11 min, 61% B to 95% B 11 to 12 min. Post time is 1 B for 3 min. MS data were acquired from 4 to 11 min.

Condition 2. Analysis was performed on an Agilent 1290 Infinity HPLC coupled to an Agilent 6650 Q-TOF with Dual Jet Stream ESI ionization and iFunnel. MS was run in positive ionization mode, extended dynamic range (2 GHz), and low mass...
range (m/z in the range of 100–1700 a.m.u.). The solvent mixtures used were as above. Column: Phenomenex C18 (3 μm, 150 × 1 mm, 100 Å silica); Flow Rate: 0.1 mL/min; DMD: 0.1% B in 100% A (ammonium aceto- nitride), and DMF (1% B, for histidine; 3% for all other amino acids) were each added to individual portions of resin. Couplings were allowed to proceed for 20 min, and resin portions were recombined and washed with DMF for deprotection. Fmoc removal step was carried out by treatment of the resin with 20% piperidine in DMF (1 × flow wash; 2 × 5 min batch treatments). The resin was washed again with DMF before splitting. For the canonical library preparation, splitting was performed by suspending the resin in DMF and evenly dividing among 18 plastic fritted syringes on a manifold (18 canonical amino acids except for isoleucine and cysteine). For the noncanonical library preparation, 21 plastic fritted syringes were used (4 canonical amino acids and 17 non-proteogenic amino acids). The cycle was then continued with the next coupling until the completion of all random positions.

Affinity selection from both canonical and noncanonical libraries. HEK293 cell-expressed biotinylated ACE2 was obtained from AcroBiosystems (Catalog: AC2-H82E6). ACE2 protein was immobilized onto streptavidin-coated magnetic beads (Thermo Fisher, Catalog number 65001) in the presence of 10% FBS in 1 × PBS on a rotating mixer for 4.0 h at 4 °C. Subsequently, functionalized beads were washed with 1 × PBS (3 × 1 mL) and then incubated with peptide library (typically, the concentration was at 10–20 μM/membrane and the reaction volume was 1.0 mL × 1 × PBS containing 10% FBS) on a rotating mixer for 1.0 h at 4 °C. The beads were then washed with PBS (3 × 1 mL) on a magnetic separation rack. Bound peptides were eluted with 100 μL of 6 M guanidine hydrochloride, 200 mM sodium phosphate, pH 6.8. Elution volume was concentrated using C18 ZipTip® pipette tips. After lyophilization, powders were resuspended in water (0.1% formic acid) and submitted for nLC-MS/MS analysis.

Nano LC-MS/MS (nanoLC-MS/MS) sequencing. Peptide sequence analysis was performed on an EASY-nLC 1200 (Thermo Fisher Scientific) nano-liquid chromatography handling system with an Orbitrap Fusion Lumos Tribrid Mass-Spectrometer (Thermo Fisher Scientific). The sample was previously described.43 Samples prepared from the library selection steps were run on a PepMap RSLC C18 column (2 μm particle size, 15 cm × 50 μm ID; Thermo Fisher Scientific, P/N ES801). A nanoViper Trap Column (C18, 3 μm particle size, 10 μm pore size, 20 mm × 75 μm ID; Thermo Fisher Scientific, P/N 164946) was used for desalting. The standard nano-LC method was run at 40 °C and a flow rate of 300 nL/min with the following gradient: 1% solvent B in solvent A ramping linearly to 61% B at 10 min for 30 min at 4 °C. Subsequently, functionalized beads were washed with 1 × PBS (3 × 1 mL) and then incubated with peptide library (typically, the concentration was at 10–20 μM/membrane and the reaction volume was 1.0 mL × 1 × PBS containing 10% FBS) on a rotating mixer for 1.0 h at 4 °C. The beads were then washed with PBS (3 × 1 mL) on a magnetic separation rack. Bound peptides were eluted with 100 μL of 6 M guanidine hydrochloride, 200 mM sodium phosphate, pH 6.8. Elution volume was concentrated using C18 ZipTip® pipette tips. After lyophilization, powders were resuspended in water (0.1% formic acid) and submitted for nLC-MS/MS analysis.

Buffer-only and protein-only conditions (concentration at 1000 nM) were used as references for background subtraction. The association and dissociation curves were fitted with the ForteBio Biosystems Data Analysis Software under 5 exponential conditions (n = 5, global fitting algorithm, binding model 1:1) to calculate the apparent dissociation constant (Kd).

Serum stability study. Following our previous protocol,44, the normal human serum (NHS) (Sigma Catalog number H4522) was thawed, centrifuged at 14,000 rpm for 10 min and the supernatant was pre-warmed in a 37 °C water bath. 500 μL of tested peptides were placed in a 1.7 mL Eppendorf tube. Five hundred μL of either 10% NHS (diluted in 1 × PBS) or 1 × PBS for the control, was added into the tube (5% NHS after dilution), and immediately the tubes were vortexed. Assay tubes were placed at 37 °C water bath for 3.0 h, and 200 μL was transferred to a fresh microfuge tube with 40 μL of 15% trichloroacetic acid (TCA). Tubes were then placed on ice for 15 min and centrifuged at 14,000 rpm for 10 min. Supernatant from each tube was collected and subjected to LC-MS analysis.

ACE2 pulldown from human serum. Following a previous protocol,44 streptavidin-coated magnetic beads (100 μL, at 10 mg/mL) were pre-washed with blocking buffer (1 × PBS with 0.05% Tween-20, pH 7.4) and captured on a magnetic separation rack. The beads were then re-suspended in 1.0 mL of the same blocking buffer. Biotinylated ABP N1 peptide (25 μL, 0.1 μM) was then added to the beads. After 30 min of incubation at 4 °C, the supernatant was removed and the beads were washed with 1 × PBS (3 × 1.0 mL) before incubation with the protein mixture. Soluble ACE2 (5 μL, 0.62 mg/mL) was mixed with 50 μL normal human serum in 1 × PBS (pH 7.4) to a final volume of 100 μL. Biotinylated magnetic beads displaying immobilized ABP N1 were then added into the protein complex. After 1.0 h incubation at 4 °C with gentle rotation, the supernatant was removed and the beads were washed with 1 × PBS (3 × 1.0 mL) and captured on a magnetic separation rack. The captured protein was eluted with 20 μL of 6 M urea solution, followed by SDS-PAGE gel analysis. A precast polyacrylamide gel, Bolt™ Bis-Tris Plus gel (Invitrogen, Catalog number NW04120BOX), was used for an optimal separation under denaturing conditions with a running voltage of 165 V for 36 min. The SeeBlue™ Plus2 pre-stained protein standard (Thermo Fisher, Catalog number LC5925) was used as the molecular weight reference, and Bolt™ LDS Silver Stain Buffer (Thermo Fisher, Catalog number B0007) was used to prepare and load the protein samples.

Enzyme-linked immunosorbent assay (ELISA). A serial dilution of soluble ACE2 was coated onto an ELISA plate (96-well format) overnight at 4 °C. The next day, the plate was blocked with 5% BSA in 1 × PBS supplement with 0.05% Tween-20 (PBSt, pH 7.4) for 2–3 h at room temperature. After a brief wash, 100 μL solution of biotinylated peptide ABP N1 (100 nM in PBSt) was added to the wells and incubated for 1.0 h at room temperature. Control groups are biotinylated peptide ABP N8 and PBSt alone. The supernatant was then removed, and the wells were washed with PBSt (3× 20 μL). Streptavidin-HRP conjugate in PBSt (0.25 μg/mL, 100 μL) was added to each well and incubated for 30 min at room temperature. The plate was washed twice (PBSt-washed again) and 100 μL of 3,3′,5,5′-Tetramethylbenzidine (TMB) solution was added to each well (20 μL) and incubated at 37 °C for 10–15 min. Finally, the absorbance at 450 nm was measured on a microplate reader (BioTek) for all treated wells. The same procedure was repeated when the ELISA plate was coated with a mixture of ACE2 and normal human serum. Three replicates were used throughout.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
All data generated during this study are available either in the main text or supplementary materials. The raw data underlying Figs. 2a–f, 3a–d, 5c, b and Supplementary Figs. 21 and 22 are provided as source data in the Supplementary Data 1 file.

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Bio-layer interferometry. Peptide binding validation was carried out using the ForteBio Octet RED96 system. The chamber temperature was kept constant at 30 °C ± 0.1 °C at a plate speed of 1000 rpm. Briefly, streptavidin (SA)-coated biosensor tips were dipped into 200 μL of 1.0–2.0 μM biotinylated peptide solution (in a kinetic buffer (K.B.): 1 × PBS with 0.1% BSA and 0.02% Tween-20) for peptides immobilization. Once loaded with peptides, the tips were then moved into solutions containing various concentrations (1000, 500, 250, 125, and 62.5 nM) of recombinant ACE2 protein (purchased from Sino Biological, Catalog number 10108-H808) in the K.B. to observe the association curve. After the 180 association step, the tips were moved back into the K.B. to obtain the dissociation curve.

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Competing interests
B.L.P. is a co-founder of Amide Technologies and Resolute Bio, companies focusing on the development of protein and peptide therapeutics. All other authors declare no competing interests.

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