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A peptide array pipeline for the development of Spike-ACE2 interaction inhibitors

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ABSTRACT

In humans, coronaviruses are the cause of endemic illness and have been the causative agents of more severe epidemics. Most recently, SARS-CoV-2 was the causative agent of the COVID19 pandemic. Thus, there is a high interest in developing therapeutic agents targeting various stages of the coronavirus viral life cycle to disrupt viral propagation. Besides the development of small-molecule therapeutics that target viral proteases, there is also interest molecular tools to inhibit the initial event of viral attachment of the SARS-CoV-2 Spike protein to host ACE2 surface receptor. Here, we leveraged known structural information and peptide arrays to develop an *in vitro* peptide inhibitor of the Spike-ACE2 interaction. First, from previous co-crystal structures of the Spike-ACE2 complex, we identified an initial 24-residue long region (sequence: STIEEQAKTFLDKFNHEAEDLFYQ) on the ACE2 sequence that encompasses most of the known contact residues. Next, we scanned this 24-mer window along the ACE2 N-terminal helix and found that maximal binding to the SARS-CoV-2 receptor binding domain (CoV2-RBD) was increased when this window was shifted nine residues in the N-terminal direction. Further, by systematic permutation of this shifted ACE2-derived peptide we identified mutations to the wildtype sequence that confer increased binding of the CoV2-RBD. Among these peptides, we identified binding peptide 19 (referred to as BP19; sequence: SLVAVTAAQSTIEEQAKTFLDKFI) as an *in vitro* inhibitor of the Spike-ACE2 interaction with an IC_{50} of 2.08 ± 0.38 μM. Overall, BP19 adds to the arsenal of Spike-ACE2 inhibitors, and this study highlights the utility of systematic peptide arrays as a platform for the development of coronavirus protein inhibitors.

1. Introduction

In humans, seven coronaviruses (CoVs) are known to cause respiratory disease of varying severity. Thus far, four of these CoVs (229E, NL63, OC43, HKU1) are known to be endemic in the human population and cause mild disease [1]. Furthermore, SARS-CoV and MERS-CoV are the causative agents of severe disease and were responsible for major outbreaks that occurred in 2002 and 2012, respectively [2,3]. Most notably, the recent COVID-19 pandemic was due to the emergence of SARS-CoV-2 and increasingly diverse variants [4]. Besides the development of vaccines, targeted therapeutics are a research and development area of interest as means to combat COVID-19 [5]. Thus, it is of outmost importance to identify and develop therapeutic interventions for COVID-19, as well as create pipelines for these purposes.

'Coronavirus' is primarily used as an umbrella term for the *Coronavirinae* subfamily. Within this subfamily, SARS-CoV-2 belongs to the *betacoronavirus* genera in lineage 'B' [4]. Most CoVs in this subfamily have the same general architecture, a single-stranded RNA genome packaged into a spherical ~120 nm envelope composed of a lipid membrane and 4 structural proteins: Nucleocapsid, Membrane, Envelope, and Spike proteins [6]. Of particular interest is the Spike glycoprotein protein as it assembles into a trimeric 20-nm projection on the viral surface and is responsible for attachment to the host cell. Further, those CoVs belonging in the *betacoronaviruses* genera in lineage 'A' have an additional structural protein known as hemagglutinin esterase, which dimers to form an 8-nM projection on the viral particle. Besides these structural proteins, the CoV genome also encodes for the two polyproteins which are processed into individual non-structural proteins

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(NSPs) that are essential for viral replication, as well as several accessory proteins that influence host protein function and cellular signaling pathways [7,8]. Thus, multiple drug target avenues are available for the development of targeted therapeutics.

Notably, those CoV proteins that are involved in key steps of the viral life cycle have been the main targets for drug development. One of such determining steps is the autoproteolytic processing of the polyproteins by viral proteases into individual NSPs. This step is essential for formation of the viral replicase-transcriptase complex (RTC) that is responsible for making copies of the viral RNA genome and transcribing viral mRNAs. In fact, the SARS-CoV-2 main protease (Mpro) is the target of Pfizer's paxlovid drug for treatment of COVID-19 [9]. Besides Mpro, inhibition of Spike-receptor protein-protein interactions, through targeting of the Spike protein, is a feasible avenue to prevent initial CoV attachment to the host cell. Thus far, four of the human CoVs use a known protein receptors for cell entry. DPP4 and APN receptors are recognized by MERS-CoV and 229E, respectively [10,11]. Furthermore, the ACE2 receptor is used by NL63, SARS-CoV, and SARS-CoV-2 [12–14]. Disruption of the interaction of these CoVs Spike protein, with their respective receptor, has been explored with a several types of drugs; of the many examples this includes small-molecule inhibitors for disruption of the SARS-CoV-2 Spike protein [15]. Furthermore, peptide-based inhibitors have been explored, such as for the disruption of SARS-CoV and SARS-CoV-2 cell entry [16,17].

Disruption of CoV internalization with the use of peptides is an active area of research and development. Numerous peptides have been the developed to interfere with different stages of the internalization process; receptor binding and membrane fusion [16,17]. Many approaches aiming to interfere with the former stage postulate the use of receptor-derived peptides as 'mimics' of the endogenous human receptor. In this manner, and analogous to treatments with soluble receptor proteins, the exogenous receptor-derived peptide would directly compete with the endogenous receptor for binding to the CoV Spike protein. The crystal structure of the SARS-CoV-2 Spike protein receptor-binding domain (RBD) in complex with the human ACE2 receptor demonstrated that most of the interacting residues on ACE2 reside in the N-terminal helix [18–20]. Further, peptides have been designed based on this region and were demonstrated to block SARS-CoV-2 infection of human pulmonary cells [21]. Further, *in silico* tools such as docking and molecular dynamic simulations, of peptides derived from this region of ACE2, have been used as a method to identify and optimize these ACE2-derived peptides [22–25]. Besides computational methods to optimize protein- and peptide-protein interactions, several *in vitro* methods could also be used to screen and develop receptor-derived peptide inhibitors of CoV Spike proteins.

Protein- and peptide-protein interactions can be studied in a medium- to high-throughput manner with the use of peptide arrays [26]. More specifically, in 1992, SPOT synthesis was developed and enables the parallel synthesis of hundreds of short peptides on a solid cellulose membrane support [27,28]. Thus, a protein of interest can be assessed for binding to libraries of peptides in parallel. In this manner, peptide-protein interactions have may be studied in a systematic manner, such as in experiments involving permutations, truncations, and 'walking'/scanning of a given protein or peptide sequence [26]. Given that mutations to ACE2 have been found to augment SARS-CoV-2 Spike binding [29,30], we reasoned that peptide arrays would provide a platform to streamline these endeavors. Here, we used systematically designed peptide arrays to improve SARS-CoV-2 Spike binding to ACE2-derived peptides. Further we found that one of the optimized binding peptides was able to outcompete ACE2 binding to the SARS-CoV-2 RBD *in vitro*. Thus, peptide arrays provide a useful tool to directly screen binding of hundreds of peptides to a given CoV protein for inhibitor development.

2. Materials and methods

2.1. Peptide synthesis

Synthesis, of both free and immobilized synthetic, peptides was performed using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry on an automated ResPep SL peptide synthesizer (Intavis) following procedures essentially as described previously [31]. All Fmoc-amino acid derivatives were purchased from P3 BioSystems. Briefly, free peptides were synthesized at a scale of 5 μmol on Rink-NH2 resin. For quantification, peptides were synthesized with a C-terminal tryptophan, separated by a 6-aminohexanoic acid (6-ahx) flexible linker. After synthesis, peptides were liberated from the resin and protecting groups were cleaved using an acidic cleavage solution (95 % trifluoroacetic acid, 3 % tri-isopropylsilane, 2 % water). Cold (−20 °C) diethyl ether was used to precipitate and wash the peptides. Peptides were dried, dissolved in 1X PBS with 4% acetic acid, were adjusted to pH 7 with 10 M NaOH, and stored in − 20 °C.

Peptide arrays were assembled via synthesis of peptides directly on aminated cellulose membranes (Cellulose-NH2; made in house) at a scale of approximately 2 nmol per spot. Peptides were synthesized such they were separated from the membrane by a C-terminal 6-ahx linker. After synthesis, side chain protecting groups were cleaved with a mild acidic cleavage solution (51% water, 47.5% trifluoroacetic acid, 1.5% tri-isopropylsilane). The first sliding window array of the ACE2 N-terminal helix (UniProt ID: Q9BYF1) started from a 24-mer initial window (sequence: STIEEQAKTFLDKFNHEAEDLFYQ) comprising contacts found in the co-crystal structure of ACE2 in complex with the SARS-CoV-2 Spike protein RBD (PDB: 6M0J). The window was iteratively shifted one residue at a time in the N-terminal and C-terminal direction, to a maximum of nine residues, while maintaining the length of the peptide. Permutation of the 9^N ACE2-derived peptide involved individually substituting each residue position with the other 19 natural amino acids, while leaving the remainder of the sequence unchanged.

2.2. Protein array probing

Peptide arrays were rehydrated in anhydrous ethanol, washed in dH_2O, then equilibrated in 1X Tris Buffer Saline with 0.05 % Tween-20 (TBST; 50 mM Tris-Cl, 350 mM NaCl, 0.05% Tween20). The arrays were blocked in 1X TBST containing 5 % w/v Non-Fat Skim Milk. After blocking, the array was equilibrated with peptide binding buffer (PPB; 50 mM Tris-Cl, 350 mM NaCl, 10 % glycerol, 0.5 mM DTT and 0.05% Tween 20). The array was then incubated with 0.3 μM of His-Spike S1 Protein (13–685 aa; rCoV2-RBD, Abclonal RP01262) in PPB overnight at 4 °C with rocking. Unbound protein was washed away by three consecutive 10 min washes with TBST. Each array was then incubated with HisProbe™-HRP Conjugate (Thermo Scientific) (1:5000) in TBST for 1 h. Binding was then detected using chemiluminescence on a Bio-Rad GelDoc XR+ Imaging System. To control for any non-specific interaction between binding peptides and the detection probe, unbound arrays were also probed alone with the His-reactive probe (1:5000) in TBST for 1 hr and treated as described above. The signal intensities observed were subjected to densitometry analysis using ImageLab software.

2.3. Enzyme-linked immunosorbent assay (ELISA)

The SARS-CoV-2 Spike-ACE2 Interaction Inhibitor Screening Assay Kit (Cat. No. 502050) was used to evaluate inhibition of the ACE2-Spike interaction by the binding peptides (BPs). The ELISA was performed according to the manufacturer's instructions. For BP19, the final peptide concentration in the binding reaction ranged from 1 to 128 μM and all conditions were assessed in triplicates. IC_{50} was determined after curve fitting with a non-linear regression using the equation *[Inhibitor] vs. normalized response – Variable slope* in GraphPad Prism.

2.4. Docking

Docking of BP19 with the CoV2-RBD was performed with HPEPDOCK Server [32]. The BP19 sequence (SLVAVTAAQSTIEEQAKTFLDKFI) was provided as the 'Peptide Input'. For the 'Receptor Input' the structure of CoV2-RBD, in PDB format, was uploaded from the Spike-ACE2 crystal structure (PDB: 6M0J) after removing the ACE2 structure. The highest ranked model was used to depict the interaction (Fig. 3B).

3. Results

3.1. Structure-guided selection Spike-binding peptides

The interaction between the Spike-RBD and mammalian surface receptors has been extensively studied. Specifically, the co-crystal structure of the SARS-CoV-2 Spike-RBD and human ACE2 has been determined by multiple groups and specific contact residues have been resolved [18–20]. Contact residues belonging to ACE2 occur throughout the different regions of the primary structure, though after folding are in closer spatial proximity. We found that most of those contact residues (n = 12 residues; S19, Q24, T27, F28, D30, K31, H34, E35, E37, D38, Y41, Q42), determined by other groups [18,19], occur in the human ACE2 N-terminal helix and can be encompassed on a 24-mer peptide (Fig. 1A). Given that peptides derived from the ACE2 N-terminal helix have been shown to inhibit the Spike-ACE2, we reasoned that this initial window (IW) of $ACE2_{S19\text{-}Q42}$ encompassing an extensive number of contact residues may serve as a template for developing peptide-based inhibitors of the ACE2-Spike interaction.

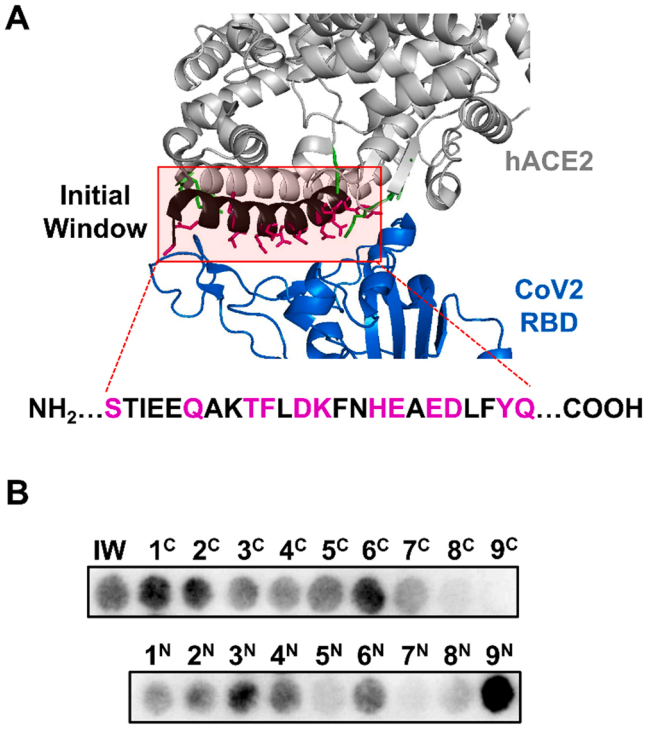

Fig. 1. Structure-guided development of ACE2-derived Spike-binding peptides. (A) Crystal structure (PDB: 6M0J) of human ACE2 N-terminal peptidase domain (S19 – D615; grey) bound to the SARS-CoV-2 RBD (R319 – F541; blue). ACE2 contact residues residing in the N-terminal helix have side chains displayed as pink and more distal contact residues are shown as green. The part of the N-terminal helix used as an initial window (IW) peptide is shown as black with the sequence underneath. (B) Monitoring binding of rCoV2-RBD towards 24-mer peptides representing a sliding window of the IW peptide + /- 9 residues along the hACE2 sequence.

To assess binding of recombinant CoV2-RBD to multiple ACE2-derived peptides, we used a peptide array system. In this manner, synthetic peptides were synthesized directly on a solid aminated cellulose support and separated from the array by a flexible linker. Besides testing the 24-mer IW, which contains most of the known contacts, we reasoned that the residues flanking this window may be important for the binding of ACE2 to CoV2-RBD. Further, the co-crystal structure of this interaction used truncated ACE2 beginning at the S19 residue. Thus, we used a sliding window approach to test binding of the CoV2-RBD to the IW and other 24-mer peptides where the IW was slid nine residues in the N- or C-terminal direction, one residue at a time (Fig. 1B). Interestingly, the highest binding signal observed among all peptides was the window slid nine residues N-terminally (9^N; SLVAVTAAQSTIEEQAKTFLDKFN). Among all the peptides tested, the 9^N ACE2-derived peptide (i.e., $ACE2_{S10\text{-}N33}$) bound the CoV2-RBD the strongest and was used for downstream development.

3.2. Evolution of CoV2-RBD binding peptides by permutation library

SPOT synthesis on solid cellulose supports has enabled studying of protein-peptide interactions using medium- to high-throughput peptide libraries [33,34]. Particularly, peptide permutation libraries (i.e., substitutional matrices) have been used to for antibody epitope mapping using short linear peptides. To name one of many examples of this application, the binding specificity of an anti-myc antibody has been studied using a peptide permutation array of the myc-tag [35]. Analogously, we applied this approach to semi-quantitatively discover amino acid substitutions to the 9^N ACE2-derived peptide which may increase binding of the rCoV2-RBD. In this manner, each residue within the 9^N ACE2-derived peptide was individually substituted to all other 19 naturally occurring amino acids while maintaining the wild-type 9^N sequence at the remaining positions (Fig. 2). It was observed that binding of the CoV2-RBD was maintained with most of the mutated 9^N peptides. Further, equal peptide spotting was confirmed after synthesis with bromophenol blue staining of the peptide array, thus differences in binding signal intensity are not due to variance in peptide spot abundance (Fig. S1). To develop a peptide inhibitor of the natural Spike-ACE2 interaction, we were most interested in the mutations which

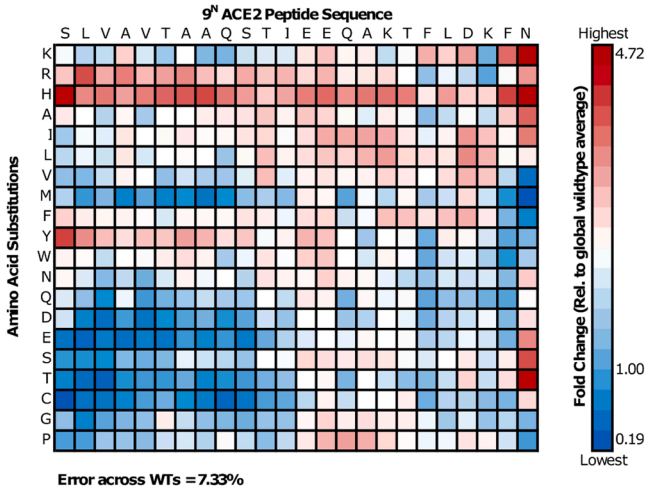

Fig. 2. Binding profile of rCoV2-RBD to a permutation array of the 9^N ACE2-derived peptide. Binding of 0.3 μM rCoV2-RBD was assessed towards the 24-mer permutation array (n = 480 peptides) overnight at 4 °C and detected by chemiluminescence with anti-HisProbe. Binding signal was quantified by densitometry with ImageLab. Individual binding signals were normalized to the average wildtype peptide signal to visualize Fold Changes. Deviation of the binding signal between all individual wildtype peptides (n = 24 peptides) is shown. The wildtype 9^N sequence and individual amino acid substitutions are shown on the horizontal and vertical axes, respectively.

conferred the highest binding intensity. A list of mutated peptide sequences yielding at least 3-fold greater binding, relative to the wildtype 9^N peptide, is provided (Table S1). Among these binding peptides (BP), we were only able to observe inhibition of the Spike-ACE2 by BP19, which was also found to be dose-responsive when using a competitive ELISA for ACE protein interaction (Fig. 3A). The IC_{50} of BP19 was 2.08 ± 0.38 μM. Further, we determined that the BP19 peptide was competing with approximately 13.48 μM of ACE2. Thus, BP19 which represents $ACE2_{S10\text{-}N33}$-N33I, was able to outcompete the natural ACE2 at lower than equimolar concentrations. Further, docking of BP19 with the CoV2-RBD predicted that this peptide binds in a similar region as the ACE2 protein (Fig. 3B).

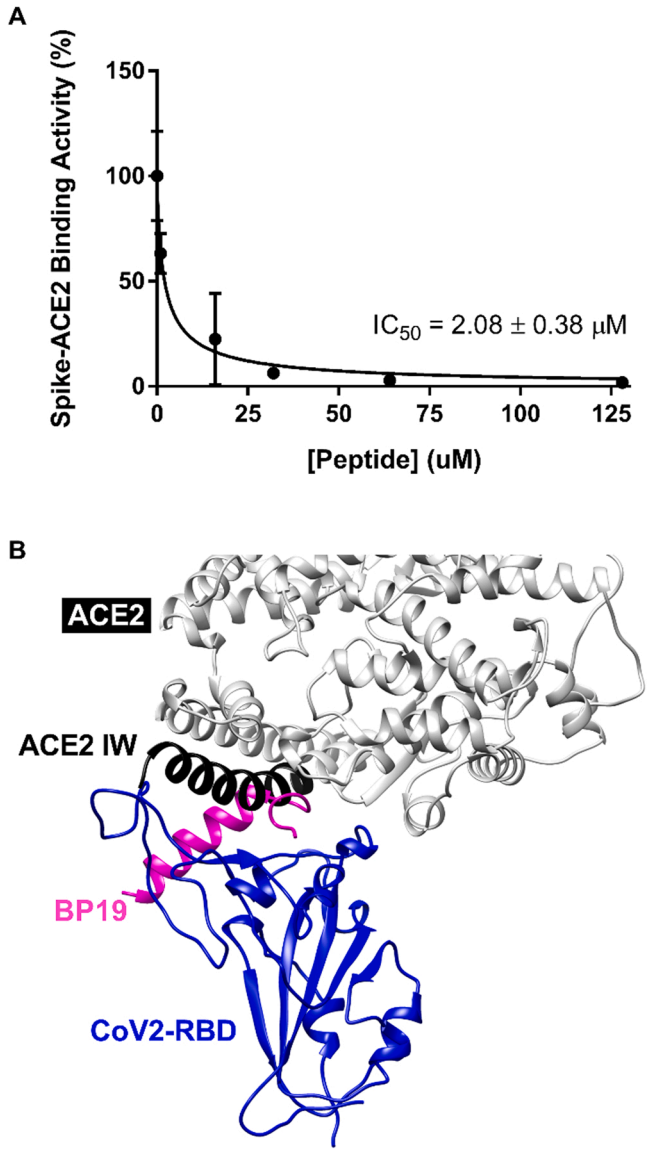

Fig. 3. Dose-responsive inhibition of the *in vitro* ACE2-Spike interaction by BP19. (A) The binding reactions of Spike and ACE2 occurred in the presence of either no peptide and or a concentration range of BP19 (1–128 μM). Binding activity between Spike and ACE2 was monitored by ELISA and shown as a percentage compared to that without BP19. Half-maximal inhibitory concentration (IC50) is shown within figure. (B) Docking of BP19 (magenta) with the CoV2-RBD (blue) using HPEPDOCK Server. The ACE2 protein (grey) from the original crystal structure is shown, and the initial window peptide within this protein is colored black.

4. Discussion

Several CoV proteins are attractive options for the development of targeted therapeutics for the treatment of CoV infection. Blocking the cell entry of CoVs is an active area of research, specifically to inhibit the initial attachment of CoVs to their corresponding human receptor. 'Receptor trapping' is a concept used to mitigate the interaction between extracellular ligands and endogenous human receptors [36]. This was first described for blocking of the human immunodeficiency virus type-1 to the endogenous CD4 receptor, with the use of soluble derivatives of this receptor [37]. Extending this concept to human CoV infection, soluble versions of engineered ACE2 have been shown as a promising tool to prevent SARS-CoV-2 infection, as well as the other human CoVs utilizing ACE2 as their entry receptor [29,30]. Further, mutations that increase binding to the human receptor have been either predicted computationally or screened for *in vitro*. Here, we demonstrate the use of peptide arrays to screen for alterations in receptor-derived peptides for that would optimize binding to CoV proteins.

We decided to apply peptide arrays to screen for binding peptides of the SARS-CoV-2 Spike RBD, and in this manner potentially identify an inhibitor of the Spike-ACE2 interaction. Analogous to other studies that have identified or engineered soluble ACE2-derived peptides or soluble ACE2 variants, we began from an ACE2-derived peptide from the region known to be at the interface of the Spike-ACE2 interaction. The initial window chosen, residing on the ACE2 N-terminal helix, encompassed most of the known contact residues that confer this interaction (Fig. 1A) [18,19]. Further, there is overlap in the peptide sequence used compared with other Spike RBD-binding peptides shown in literature [21]. To assess whether the initial window chosen was optimal, the window was shifted along the ACE2 N-terminal helix region. The 9^N ACE2-derived peptide bound the CoV2-RBD with the greatest intensity (Fig. 1B). Co-crystal structures of the Spike-ACE2 use a truncated ACE beginning at the Serine-19 residue, thus any contacts of these additional N-terminal nine residues are not previously known. Interestingly, this region of ACE2, closer to the N-terminus, is known to be a signal peptide for the migration of ACE2 to the cell membrane [38]. However, we speculate that the greater binding exhibited by the 9^N ACE2-derived peptide could be attributed to either additional contacts in the N-terminal end of the peptide or the loss of certain residues in the C-terminal end of the initial window. Nonetheless, we identified 9^N ACE2-derived peptide as optimal for binding to the SARS-CoV-2 Spike RBD.

As mentioned, the binding of receptor-derived peptides, such as ACE2-derived peptides, has been improved by the introduction of mutations to the wild-type sequence and these mutations have been selected for using both computational predictions and *in vitro* screening [22–25]. Here, we used SPOT peptide synthesis to create a permutation array of the 9^N ACE2-derived peptide and identify mutations that may confer stronger binding to the CoV2-RBD. In this manner, 456 individual mutations were screened in parallel for binding to the CoV2-RBD. Visually, the CoV2-RBD can tolerate a wide range of mutations (Fig. 2). More specifically, 261/456 mutations conferred equal to or greater binding than the wild-type 9^N ACE2-derived peptide. This is not surprising given that the CoV2-RBD can tolerate sequence differences between ACE2 orthologs across many species [39]. We were interested those mutations conferring the highest-fold, at least 3-fold, improvement in binding (Table S1). Among these peptides we identified BP19 as inhibitory of the Spike-ACE2 interaction *in vitro* by a standard ELSIA (Fig. 3A). BP19 outcompeted 13.48 μM of ACE2 for binding to the CoV2-RBD, displaying an IC_{50} of 2.08 ± 0.38 μM. Therefore, it is unsurprising that docking of BP19 predicted that this peptide binds to the CoV2-RBD at the interface of the interaction with ACE2 (Fig. 3B).

Several open questions remain to be investigated: (1) Does BP19 confer inhibition of the CoV2-RBD in a more physiological setting such as tissue culture models. (2) What is the necessary length of the BP19 peptide? That is, can the peptide be truncated from the N- or C-terminal end and yet still confer inhibition? (3) How, if at all, is the region of

ACE2 prior to the S19 residue involved in binding to the CoV2-RBD? (4) What is the crystal structure of the BP19-CoV2-RBD interaction?

In summary, using peptide arrays, we have identified a new inhibitory peptide of the Spike-ACE2 interaction. Besides the general workflow shown here, starting from the wild-type ACE2 sequence, this system may also be used to further optimize existing CoV inhibitors by mutational analysis or screen for a given set of beneficial mutations predicted by computational methods. Thus, peptides arrays provide a beneficial tool to streamline CoV inhibitory peptide development endeavors.

CRediT authorship contribution statement

Anand Chopra: Conceptualization, Methodology, Investigation, Validation, Data curation, Formal analysis, Visualization, Writing-Original draft preparation. **Ali H. Shukri**: Investigation, Validation, Data curation, Writing- Original draft preparation. **Hemanta Adhikary**: Investigation, Validation. **Valentina Lukinović**: Investigation. **Matthew Hoekstra**: Investigation. **Michael Cowpland**: Project administration, Funding acquisition. **Kyle K. Biggar**: Conceptualization, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.peptides.2022.170898.

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