Synthetic peptides blocking sars-cov-2 (covid-19), and compositions, methods and uses related thereto

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(54) Title: SYNTHETIC PEPTIDES BLOCKING SARS-COV-2 (COVID-19), AND COMPOSITIONS, METHODS AND USES RELATED THERETO

(57) Abstract: The invention relates to the field of medicine and therapeutically active peptides, more specifically to the design of peptides that antagonise Angiotensin-Converting Enzyme-2 (ACE-2) interaction with the receptor binding spike protein of SARS-CoV-2. Provided is a peptidic compound comprising or consisting of an amino acid sequence selected from the following: IYALLENAEDYNLVN, SRDKHEEHEKENDRGQ, DKFNHEAEDLFYQSSLASWNYNT, IEEQAKTFLDKFQHEVEEIYWQS, QDKHEEDYQMYNKGDKED and IDENARSYIDKFQHDAEEMWYQ, or an amide, an ester or a salt thereof.

Title: Synthetic peptides blocking SARS-CoV-2 (COVID-19), and compositions, methods and uses related thereto.

This invention relates to the field of medicine and therapeutically active peptides. More specifically, it relates to peptides that antagonise Angiotensin-Converting Enzyme-2 (ACE-2) interaction with the receptor binding spike protein of SARS-CoV-2. Also provided are compositions comprising the same and methods and uses related thereto.

The viral spike protein S binds ACE2 on host cells to initiate molecular events that release the viral genome intracellularly. ACE2 inhibits SARS and SARS-2 coronaviruses' entry by acting as a decoy for S binding sites and is therefore an appropriate drug to inhibit the virus from entering human cells, presenting a new modality for therapeutic intervention.

Impairing SARS-CoV-2-RBD binding to ACE2 with appropriate drugs has the potential to inhibit the virus from entering human cells, presenting a modality for therapeutic intervention. Antagonistic peptide drugs represent the best tool to inhibit the RBD:ACE2 interaction, as such peptides combine the best features of antibody approaches (ability to address a large and relatively featureless surface) and small-molecule approaches (improved pharmacokinetics, reduced immune response, ease of production, and cost-of-goods) [1]. This approach is supported by recent publications that have suggested ACE2 based peptides as strong candidates for optimization into therapeutics [2-4]. It may constitute a complementary approach to vaccine development as well as the identification of small-molecule based therapies (novel or repurposed). To affect a timely solution to the current global crisis and possible future events, all avenues should be explored in parallel.

The interaction between the S protein and the ACE2 receptor is the critical route of entry of the virus. Therefore, the S protein is a potential target for drug or vaccine development. Small molecules or peptides can be designed as therapeutics that will disrupt the interaction between the S-protein and the ACE receptor; however, small molecules are not ideal for targeting large protein–protein interactions (PPIs). Peptides, on the other hand, can disrupt the PPIs effectively as they possess a larger surface compared to small molecules and thus specifically bind to the interface-binding region [5-13].

To date, there are at least 39 coronaviruses discovered [4] and scientists have applied various strategies against targeting coronavirus. Unfortunately, no targeted therapy exists for SARS-CoV-2 infections yet.

The present inventors recognized the potential of antagonistic peptide drugs as a promising tool to inhibit the RBD-ACE2 interaction by adequately covering the hotspot residues and loops in the protein contact interface. Therefore, they sought to provide novel inhibitors to destabilize these interactions and block the association of RBD and ACE2.

In an attempt to provide new peptidic compounds having therapeutic efficacy against SARS-CoV-2, they designed, simulated, and synthesized a library of antagonist peptides. To that end, they used a unique approach involving protein contact atlas data and molecular dynamics simulations to locate contact hotspots. Upon the co-crystal structure of ACE2 and SARS-CoV-2-RBD, we observed that the ACE2 α1 helix is an essential portion for binding SARS-CoV-2-RBD. Molecular dynamics simulations emerged that the α-helical peptide preserved its secondary structure and provided a specific and stable binding (blocking) to SARS-CoV-2 in bioluminescence-based assay and Microscale Thermophoresis (MST). Such antagonistic peptide inhibitors could pave an efficient and straightforward therapeutic way against the COVID-19 disease.

This approach resulted in the identification of 6 unique peptides having an amino acid sequence as depicted in Table 1. Synthetic peptides having this sequence were found to efficiently block the interaction of ACE2 with S-RBD. Importantly, the peptides bind to purified S-RBD with nanomolar affinity (see Table 2).

Table 1

ENTRY	SEQUENCE
A1	IEEQAKTFLDKFQHEVEEIYWQS
A2	QDKHEEDYQMYNKGDKED
A3	DKFNHEAEDLFYQSSLASWNYNT
A4	IDENARSYIDKFQHDAEEMWYQ
A5	IYALLENAEDYNLVN
A6	SRDKHEEHEKENDRGQ

Table 2

Entry	Sequence	m/z (monoisotopic)		Kd (nM)	IC50 (nM)
		Theoretical	Experimental		
Peptide 1	H-IEEQAKTFLDKFQHEVEEIYWQS-NH_2	2895,397	2895,477	106 ±1	11 ±5
Peptide 2	H-QDKHEEDYQMYNKGDKED-NH_2	2269,944	2270,011	102 ±6	18 ±2
Peptide 3	H - DKFNHEAEDLFYQSSLASWNYNT-NH_2	2777,225	2777,304	245 ±3	6 ±3
Peptide 4	H-IDENARSYIDKFQHDAEEMWYQ-NH_2	2786,228	2786,308	542 ±5	32 ±2
Peptide 5	H-IYALLENAEDYNLVN-NH_2	1751,862	1751,920	13 ±1	9 ±4
Peptide 6	H-SRDKHEEHEKENDRGQ-NH_2	1991,905	1991,966	46 ±5	10 ±3

Table 2: Amino acid sequence and theoretical and experimental molecular weight of synthetic ACE2-antagonist peptides. Peptides were synthesized on solid phase using the Fmoc strategy have a free N-terminus and are amidated at the C-terminus. Half-maximal inhibitory concentration (IC50) and binding affinities of SARS-CoV-2:ACE2-antagonist peptides, using the Luciferase assay and MST, respectively, are also shown.

Accordingly, in one embodiment the invention provides a peptidic compound comprising or consisting of an amino acid sequence selected from the following:
(a) IYALLENAEDYNLVN (SEQ ID NO:5),
(b) SRDKHEEHEKENDRGQ (SEQ ID NO:6),
(c) DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3),
(d) IEEQAKTFLDKFQHEVEEIYWQS (SEQ ID NO:1),
(e) QDKHEEDYQMYNKGDKED (SEQ ID NO:2), or
(f) IDENARSYIDKFQHDAEEMWYQ (SEQ ID NO:4), or an amide, an ester or a salt thereof.

According to one embodiment, a peptidic compound of the invention has a half-maximal inhibitory concentration (IC50) against the ACE2:RBD interactions of 50 nM or less, preferably of 40 nM or less; more preferably of 35 nM or less, more preferably of 20 nM or less, more preferably of 15 nM or less, more preferably of 12 nM or less, more preferably of 10 nM or less.

Alternatively or additionally, a peptidic compound of the invention has a binding affinity to purified S-RBD protein in the nanomolar range. In a specific aspect, it has a dissociation constant (Kd) of 600 nM or less, preferably of 400 nM or less; more preferably of 200 nM or less, more preferably of 150 nM or less, more preferably of 110 nM or less, more preferably of 60 nM or less, more preferably of 20 nM or less.

In one embodiment, the invention provides an isolated polypeptide consisting of an amino acid sequence selected from the following:

(a) IYALLENAEDYNLVN (SEQ ID NO:5),
(b) SRDKHEEHEKENDRGQ (SEQ ID NO:6),
(c) DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3),
(d) IEEQAKTFLDKFQHEVEEIYWQS (SEQ ID NO:1),
(e) QDKHEEDYQMYNKGDKED (SEQ ID NO:2), or
(f) IDENARSYIDKFQHDAEEMWYQ (SEQ ID NO:4),
or an amide, an ester or a salt thereof.

In one preferred aspect, the peptidic compound comprises or consists of the amino acid sequence IYALLENAEDYNLVN (SEQ ID NO:5) or an amide, an ester or a salt thereof.
Karoyan Philippe *et al.* (Comm. Biol., Vol. 4, no. 1, 12 February 2021) discloses peptides P8-P10, which are derived from the contact residues S19 - L45 of ACE2 which bind to the receptor binding domain on SARS-CoV2 spike protein, and which inhibit said binding and thus viral infection. While these prior art peptides only replace a few amino acids compared to ACE2 19-45, peptide SEQ

ID NO: 5 of the present invention represents a rather complete re-modelling of that peptide, which the skilled person could not have devised on the basis of the prior art teaching.

In another preferred aspect, the peptidic compound comprises or consists of the amino acid sequence SRDKHEEHEKENDRGQ (SEQ ID NO:6) or an amide, an ester or a salt thereof. In yet another preferred aspect, the peptidic compound comprises or consists of the amino acid sequence DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3) or an amide, an ester or a salt thereof.

Literature review shows that peptides have been previously designed and proposed as SARS-COV-2 spike RBD inhibitors. See Table 3 for an overview of prior art peptides.

Table 3

	Sequence	Kd (nM)	IC50 (nM)	ref
1	IEEQAKTFLDKFNHEAEDLFYQS TFLDKFNHEAED	47 -	-	4
2	Inhibitor1:IEEQAKTFLDKFNHAEDLFYQSSLASWNY NTNIT Inhibitor2: (1) IEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITE ENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEI (2) WDLGKGDFR Inhbitor3: (1) IEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITE ENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEIQA LTVKLQLQALQQNGS (2) MTQGFWENSMLTDPGNVQKAVCHPTAWDLGKGD FRILMCT Inhibitor4: (1) IEEQAKTFLDKFNHEAEDLFYQSSLASWNYNTNITE ENVQNMNNAGDKWSAFLKEQSTLAQMYPLQEIQA LTVKL (2) DPGNVQKAVCHPTAWDLGKGDFRILMCTKVTMDD FLTAHHEMGHIQYDMAYAAQPFLLRNGANEGF	-	-	5

3	SLDQINVTFLDLEYEMKKLEEAIKKLEESYIDLKEL		270±0.01	10
4	TWLATRGLLRSPGRYVYFSPSASTWPVGIWTTGEL VLGCDAAL GCASRCKAKCAGRRCKGWASAFRGRCYCKCFRC	-	-	10
5	LPDPLKPTKRSFIEDLLFNKVTLADAGFMKQYG ASANLAATKMSECVLGQSKRVDFCGKGYH QILPDPSKPSKRSFIEDLLFNKVTLADAGFIK ASANLAATKMSECVLGQSKRVDFCGKGY KMSECVLGQSKRV LLFNKVLTA	1.80E-06 ± 1.1E-06M - - 5.20E-06 ± 1.7E-06M 4.00E-06 ± 2.2E-06M 2.40E-06 ± 1.7E-06M	- - - - 30-35nM	11
6	LAKILKEKYGLD EILDKSKEKTSFD LKESKDLV VPKHLKKGLSKEEAESLKKQLEEV	-	-	12
7	PeplCovid19 database (http://aleph.upf.edu/pepicovid19/)	-	-	13
8	NGAICWGPCPTAFRQIGNCGHFKVRCCKIR NGAICWGPCPTAFRQIGNCGRFRVRCCRIR NGAICWGPCPTAFRQIGNCGHFKVRCCKIRDED NGAHSWHPNETHFRQIHNSGRHRVRSHRIR		2.4 (μM) 0.9 (μM)	14

CN111471088A discloses a polypeptide of the sequence FLKDENHEAEDLLDK that can be specifically bound with an RBD region of an S protein of SARS-COV-2 to block combination of viruses and cell receptors and further effectively restrain virus infection from the source. It also relates to a composition for restraining the SARS-COV-2, and a purpose of the composition.

However, peptidic compounds comprising an amino acid sequence as herein disclosed are not taught or suggested in the art. The ACE2 antagonist peptides of the present invention are unique among all discovered peptides in that they exhibit a favourable entropy, an affinity increase associated largely with a faster on-rate, and enhanced specificity for the critical residues which placed on alpha helix 1 and 2. Without wishing to be bound by theory, it is conceivable that these effects may indicate that the two α-helices are closely joined on one side, they

stabilize each other and would be enough to preserve the structure as well as function. In this scenario, the interactions could lead to both a faster on-rate and reduced entropic penalty associated with binding.

The term "peptidic compound" is intended to encompass peptide analogues, peptide derivatives and peptidomimetics that mimic the chemical structure of a peptide composed of a given sequence of naturally-occurring amino acids. Examples of peptide analogues include peptides comprising one or more non-natural amino acids. Examples of peptide derivatives include peptides in which an amino acid side chain, the peptide backbone, or the amino- or carboxy-terminus has been derivatized (e.g. peptidic compounds with methylated amide linkages). Examples of peptidomimetics include "inverso" peptides in which all L-amino acids are substituted with the corresponding D-amino acids, and compounds having at least a proteinaceous part.

In one aspect, the invention provides a C-terminally amidated polypeptide consisting of an amino acid sequence selected from the following:

(a) IYALLENAEDYNLVN-NH_2 (SEQ ID NO:11),
(b) SRDKHEEHEKENDRGQ-NH_2 (SEQ ID NO:12),
(c) DKFNHEAEDLFYQSSLASWNYNT-NH_2 (SEQ ID NO:9),
(d) IEEQAKTFLDKFQHEVEEIYWQS-NH_2 (SEQ ID NO:7),
(e) QDKHEEDYQMYNKGDKED-NH_2 (SEQ ID NO:8), or
(f) IDENARSYIDKFQHDAEEMWYQ-NH_2 (SEQ ID NO:10).

A peptidic compound or isolated polypeptide can be readily (chemically) synthesized by a conventional method for those skilled in the art to prepare polypeptides. For example, it is prepared by Fmoc polypeptide synthesis.

One major hurdle for the clinical development of therapeutic peptides is their sensitivity to proteases which reduce their half-life upon systemic administration. A peptidic compound of the invention may be modified to

enhance the in vivo stability. The most common structural modifications adopted to enhance peptide stability are chemical modifications in the N- and C-termini of the peptide backbone (e.g., acetylation of the N-terminus, amidation of the C-terminus), introduction of D-amino acids, unnatural (for example, nonproteogenic) amino acids and peptide backbone cyclization. Engineering peptides by introducing dextrorotary (D)-amino acids instead of levorotatory (L) forms is an effective strategy to avoid proteolytic degradation by proteases. D-amino acids produce conformational changes in the proteins that make them less recognizable by L-protein enzymes such as proteases. In addition, D-amino acids residues are not very common in nature, which makes them immune-inert when entering the host organism.

In addition to unnatural amino acids, peptides can be cyclized to enhance half-life. This can be done by different modalities such as the establishment of disulfide bonds between two cysteines, by introducing a lanthionine-bridge, by adding an amide bond between the C- and N-terminus of the peptide (head-to-tail cyclization), or between the side chains of natural (such as lysine and aspartic acid/glutamic acid) and another types of cyclization such as ring-closing metathesis for unnatural amino acids.

A peptidic compound may comprise a combination of two or more of the above sequences, for example two distinct amino acid sequences selected from Table 1 covalently attached by a linker moiety. Also, two or more copies of the same amino acid sequence can be present. In one embodiment, it is a monomeric compound comprising one of the polypeptide sequences herein disclosed. In another embodiment, it is a dimeric or tandem compound, comprising two copies of the same or a distinct peptide connected via a linker moiety, e.g. peptideA-linker-peptideA or peptideA-linker-peptideB. The linker moiety can contain proteinaceous and/or lipid components. In one embodiment, the linker moiety is a proteinaceous linker. In another embodiment, peptides are linked by a lipid moiety.

The peptidic compound may contain non-proteinaceous elements. For example, the peptidic compound may contain one or more structural modification(s) that can avoid or delay proteolytic cleavage and thus mitigate immune system recognition and immunogenicity. For example, pegylation is one of the most used strategies to prevent immunogenicity of protein therapeutics. Polyethylene glycol (PEG) itself is immune-inert and in aqueous media prevents the access of proteases or peptidases to the protein/peptide by steric hindrance.

In a specific aspect, the peptidic compound is a lipoprotein comprising a peptide part and a lipid part (lipid "tag"). Suitable lipid tags include cholesterol (Chol), and , optionally combined with fatty acids like lauric acid) one or more PEG molecules, and combinations thereof. In one aspect, the peptidic is a tandem lipopeptide, comprising a dimeric cholesterol-conjugated (linked) PEG-tagged peptide ([peptideA]-$PEG_n)_2$-Chol, wherein n is 2-30, preferably 4-26. In a preferred embodiment, the tandem lipopeptide comprises at least one of the sequences IYALLENAEDYNLVN (SEQ ID NO:5), SRDKHEEHEKENDRGQ (SEQ ID NO:6), and DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3).

The invention further provides a pharmaceutically active peptidic compound comprising or consisting of one or more of the sequences of as depicted herein above, and a pharmaceutically acceptable carrier, vehicle or diluent. A "pharmaceutically active peptidic compound" is intended to refer to a peptidic compound that exhibits pharmacologic activity, either in its present form or upon processing in vivo (i.e., pharmaceutically active peptidic compounds include peptidic compounds with constitutive pharmacologic activity and peptidic compounds in a "prodrug" form that have to be metabolized or processed in some way in vivo following administration in order to exhibit pharmacologic activity.

The composition can be formulated for any type of drug dosage form, including any one of oral preparations, nasal sprays, injection preparations, tablets, capsules, granules, pills, emulsions, solutions and suspensions. Emulsions, solutions and suspensions can inhibit the invasion of viruses in mucosal sites.

The formulation for injection can directly pass through the blood to reach the infected tissue, preventing the spread of the virus. In a preferred aspect, the pharmaceutical composition is formulated for administration in the upper and/or lower respiratory tract. In one embodiment, the composition is a nasal spray, preferably comprising a lipopeptide compound as herein disclosed.

The pharmaceutical composition may comprising one or more further therapeutic agents, preferably selected from protease inhibitors and antiviral replication inhibitors.

The invention also relates to a peptidic compound as described herein above for use as medicament. In particular, it provides the use thereof as inhibitor of the interaction between SARS-CoV-2 (COVID-19) Receptor Binding Domain and the human ACE2 protein. For example, it is used as inhibitor of SARS-CoV-2 spike protein-mediated infection of human ACE2-expressing cells.

In a preferred aspect, it relates to a peptidic compound according to the invention for use in a method of treating or preventing COVID-19 in a subject, preferably a human subject. In one embodiment, the invention provides a method of treating or preventing COVID-19 in a subject, comprising administering a therapeutically effective dose of a peptidic compound or a pharmaceutical composition as herein disclosed.

LEGEND TO THE FIGURES

Fig. 1: Interaction of ACE2 with SARS-CoV-2 Spike protein.
(A) Surface representation of the complex between the receptor binding (RBD) domain of SARS-CoV-2 Spike protein (yellow) and the human ACE2 receptor (pink). Interfacing residues are drawn in green. The green portion of the ACE2 domain including main interacting residues of helices H1, H2, H3, and beta sheets B3 and B4 are drawn in blue.

(B) The closer view displays the interacting residues at the interface site. The hotspots are shown as surfaces.

Fig. 2 : Stick representation of residues involved in the interprotomer interaction of RBD. A side view of the surface representation of the interactions within three protomers is shown. A, B, C, D, E, and F are represented by Peptide 1, 2, 3, 4, 5, and 6 respectively. Residues involved in the subunits interaction are labeled with stick representations (green; the cartoon transparency is set to 40 %). Four contact regions are boxed and enlarged. Figure created by PyMOL.

Fig. 3: Alpha-carbon RMSF analysis for the peptide-S-RBD systems. a) alpha carbon RMSF profiles of all studied peptides, apo-S-RBD and ACE2 are presented for comparison. b) RMSF structural representation of apo-S-RBD and ACE2:S-RBD. c) structural representation of peptide:S-RBD complexes including the binding of the peptides across 10 representative snapshots. Normalized scale for Peptides1-5, Peptide6 is presented with its own scale.

Fig. 4: Molecular interaction from the MD analysis of the designed peptides and S-RBD including polar (hydrogen bonds, water bridges, salt bridges, cation-π, and π-π staking) and non-polar interactions (hydrophobic). B) A representative frame taken from MD displaying the most frequent interaction between the peptides and S-RBD.

Figure 5: Luminescence based assay of ACE2:RBD interaction. 293T cells were transfected with the ACE2 or RBD expression constructs. 48 hours post-transfection, luciferase assays were performed on 20 µg total protein from cell lysates using FMZ as a substrate. (n=3, mean ± SD; one-way ANOVA, *** $p < 0.005$ relative to smBiT-ACE2 alone, Dunnett's correction for multiple comparisons). Panels a, b, c, d, e, and f represent Peptide 1, 2, 3, 4, 5, and 6 respectively.

Fig. 6: Binding analysis for the interaction between Spike RBD and ACE2. The concentration of RBD is kept constant at 50 nM, while the ligand concentration varies from 12.5 µM to 0.19 nM. The serial titrations result in measurable changes in the fluorescence signal within a temperature gradient that can be used to calculate the dissociation constant (Kd). The curve is shown as ΔFnorm (change of Fnorm with respect to the zero ligand concentration) against RBD concentration on a log scale. Panels a, b, c, d, e, and f represent Peptide 1, 2, 3, 4, 5, and 6 (see Table 2) respectively

EXPERIMENTAL SECTION

Materials and Methods

1- Protected amino acids, coupling agents (HATU, Oxyma) used for peptide synthesis were purchased from Sigma-Aldrich (Milan, Italy) and Fmoc-Rink Amide MBHA LL resin was purchased from Novabiochem (Milan, Italy). Synthesis products, including acetonitrile (CH_3CN), dimethylformamide (DMF), N,N′-Diisopropylcarbodiimide (DIC), tri-isopropylsilane (TIS), trifluoroacetic acid (TFA), sym-collidine, di-ethyl-ether, diisopropylethylamine (DIPEA), piperidine, were from Sigma-Aldrich (Milan, Italy).

2- Peptide synthesis and characterization

Peptides were assembled on solid phase (Rink-Amide LL resin) with a substitution of 0.40 mmol/g, using a standard Fmoc peptide protocol with Oxyma-DIC and HATU-collidine as coupling reagents, as previously reported [Caporale et al. Peptides, 2018, 102, pp. 38–46]. The cleavage of peptides from the solid support was performed by treatment with a TFA/TIS/H_2O (95:2.5:2.5, v/v/v) mixture for 3 h at room temperature. Crude peptides were precipitated in cold di-ethyl-ether, dissolved in a H_2O/ CH_3CN (75:25, v/v) mixture and lyophilized. Purifications were performed at 15 mL/min using a Jupiter C18 (5

µm, 300 Å, 150 x 21,2 mm ID) column applying a linear gradient of 0.1% TFA in CH_3CN from 1% to 80% over 15 min, and monitoring the absorbance at 210 nm. ESI-TOF-MS analyses of crude and purified peptides were performed with an Agilent 1290 Infinity LC System coupled to an Agilent 6230 time-of-flight (TOF) LC/MS System (Agilent Technologies, Cernusco Sul Naviglio, Italy). The liquid chromatograph Agilent 1290 LC module was coupled with a photodiode array detector (PDA) and a 6230 time-of-flight MS detector, along with a binary solvent pump degasser, column heater and auto-sampler. The characterizations were performed at 0.2 mL/min using a XBridgeTM C18 column (5 µm, 50 x 2,1 mm ID) applying a linear gradient of 0.1% TFA in CH_3CN from 1% to 80% over 10 min, and monitoring the absorbance at 210 nm.

3- Plasmid construction

Inserts were ordered from GeneScript. Biosensors were cloned into the BamH*I*/Not*I* sites of pcDNA3.1 to generate mammalian expression constructs.

4- Cell culture

293T (ATCC® CRL-3216™) were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% FBS, and 1% penicillin/streptomycin (Invitrogen).

5- In vitro NanoLuc assay

293T cells (3×10^5 cells) were plated in 12 well plates in triplicate 24 h before transfection. Five hundred nanograms of the biosensor constructs were transfected using PolyJet transfection reagent (SignaGen Laboratories). After 48 h, supernatant or cells were collected. Cells were lysed using passive lysis buffer (Promega), and NanoLuc luciferase assays were performed using one of two substrates: furimazine, FMZ(Nano-Glo Cell Reagent, Promega) or native coelenterazine, CTZ (3.33 µM final concentration) (Nanolight Technologies – Prolume Ltd., Pinetop, AZ, USA). Synergy Microplate Reader (BioTek, Winooski, VT, USA) was used to measure luminescence. Results are presented as RLU

(Relative Luminescence Unit) normalized to control. The data presented are the mean of three independent experiments.

6- Microscale thermophoresis (MST):

The binding affinity of peptide to its cognate receptor was measured by Microscale thermophoresis (MST) on a Nanotemper Monolith NT.115 instrument (Nanotemper Technologies GmbH).32 Commercial Spike RBD was freshly labeled with the Monolith Lys-Tag RED-tris-NTA labeling dye according to the supplied protocol (Nanotemper Technologies, GmbH). The labeled protein was concentrated using a PES centrifugation filter (3 kDa cutoff; VWR). Measurements were done in MST buffer (50 mM Tris, 250 mM NaCl, pH=7) in standard capillaries (K002; Nanotemper Technologies GmbH). The final concentrations of either labeled protein in the assay were 50 nM. The ligands (ACE2 peptides) were titrated in 1:1 dilution following manufacturer's recommendations and starting from 12.5 μM. All binding reactions were incubated for 5 min on ice followed by centrifugation at 20,000 g before loading into capillaries. Then, samples were loaded into standard glass capillaries (Monolith NTCapillaries, Nano Temper Technologies) and the MST analysis was performed (settings for the light-emitting diode and infrared laser were 80%). All measurements were performed in triplicate using automatically assigned 20% LED and 50% MST power; Laser On-time was 30 sec and Laser Off time was 5 sec.

7-Statistical analysis

All graphs and statistical analyses were generated using Excel or GraphPad Prism v.8. Means of two groups were compared using two-tailed unpaired Student's t-test. Means of more than two groups were compared by one-way ANOVA with Dunnett's or Tukey's multiple comparisons correction. Alpha levels for all tests were 0.05, with a 95% confidence interval. Error was calculated as the standard deviation (SD). Measurements were taken from distinct samples. For all analyses, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001; n.s. = not significant. Data was reproduced by two different operators.

EXAMPLE 1: Molecular docking and computational modelling of ACE2:S-RBD antagonistic peptides

To identify SARS-CoV-2 S-RBD:ACE2 complex hotspot residues, protein-protein docking was performed using HADDOCK and binding interfaces was predicted using PiPreD, Rossetta, protein contact atlas, PeplCovid19 database and DALI server. Additionally, we performed a structural analysis aiming to identify the unique amino acids at specific positions of the SARS-CoV-2 S-RBD energetically favour contacts with the ACE2 receptor by stabilizing a number of important interactions. The peptide-protein docking results have demonstrated that peptides have a stronger interaction with ACE2 than intact SARS-CoV-2 S-RBD. The molecular interaction profile allowed us to identify the most frequent contacts between the peptides and SARS—CoV-2 S-RBD, suggesting these peptides might be an option to block the S-RBD-ACE2 axis by direct binding and conformational change induction on RBD (Fig.2).

Previous structural reports identified that 14 positions are key for binding of SARS-CoV SB to hACE2 (Li et al., (2005) Science 309, 1864–1868) and revealed critical amino acid residues at the contact interface between SARS-CoV-2-S receptor-binding domain (RBD) and full-length human ACE2 receptor and provide valuable structural information that can be leveraged for the development of disruptors specific for the SARS-CoV-2/ACE2 protein-protein interaction (PPI). Analysis of the 144 SARS-CoV-2 genome sequences available from the Global Initiative on Sharing All Influenza Data (GISAID) (Elbe and Buckland-Merrett, Global Challenges. https://doi.org/10.1002/gch2.1018 (2017)) shows that 8 out of these 14 positions are strictly conserved in SARS-CoV-2 SB (Table 4).

In the crystal structure of ACE2 and S-RBD of SARS-CoV-2 (PDB: 6M0J and 6M17), we first analyzed the interacting amino acids at the ACE2 and S-RBD interface using PDBePISA (Baskaran, K. et al. BMC Struct Biol. 1422. doi:10.1186/s12900-014-0022-0 (2014)). In total, 15 residues out of 23 residues

(21-43) located on the α1 helix of ACE2 interact with SARS-CoV-2 S-RBD. These residues are clearly located in the crystal structure and include Q24, T27, D30, K31, H34, E35, E37, D38, Y41, and Q42 from helix α1, one residue (residue M82) from helix α2, residues K353, G354, D355, and R357 from the linker between ß3 and ß4. Most of the interacting residues are located in α1 (Fig.1 and Fig. 2).

Table 4

SARS-COV2	ACE2	SARS-COV	ACE2
N487, A475	Q24	R426	Q24
Y489, A475, F456	T27	Y436	T27
Y489	F28	Y440	F28
K417	D30	Y442	K31
Q493, Y489, F456	K31	L443	H34
Q493, L455, Y453	H34	L472	E37
Q493	E35	N473	D38
Y505	E37	Y475	Y41
Y449	D38	N479	Q42
N501,T500,Q498	Y41	G482	L45
Q498,Y449,G446	Q42	Y484	L79
F486	L79	T486	M82
F486	M82	T487	Y83
F486, N487	Y83	G488	Q325
T500	N330	I489	E329
Y505	K353	Y491	N330
G502, Y505	G354		K353
T500	D355		G354
K534	E430	K534	E430
K534	D431	K534	D431
E536	K419	E536	K419

Using the results from protein:protein molecular docking and the structural analysis, we assembled peptide 1, peptide 3, and peptide 4 from helix α1 alone (Table 1 and 2). The design strategy of peptide 2 is as a discontinuous peptide that includes some critical interacting amino acids from α1 and some key amino acids from residues between ß3 and ß4 (349 to 357), as shown in Fig.1, Fig. 2B. However, a number of amino acids were identified as passenger residues and replaced with appropriate amino acids to preserve the binding energy (Fig. 1B,

Fig. 2). Peptide 5 was also designed around helix α1, but to additionally include main interacting amino acids from helix α2 (Y83, L79, E75, A71, D67, N63, V59) (Fig.1B, Fig. 2e (Peptide 5)). Finally, we designed a highly discontinuous peptide (Peptide 6) interfacing residues from α1, α2, α3-helices and ß4 (Fig.1B, Fig. 2F).

Interestingly, as shown in Table 5, peptides 1, 3, and 4 are closest in binding energies because of being structurally similar and derived from helix α1 alone. Of note, peptide 2, 5, and 6 have higher binding strength of the α-1 helix alone in which the van der Waals and electrostatic interactions make a significant difference.

Table 5 : Energetic calculations with HADDOCK. Effect; The measured biochemical effect on the interaction with the S-RBD. A-No effect on interaction with S-RBD; B-Slightly inhibits interaction with S-RBD; C-Strongly inhibits interaction with S-RBD.; D-Abolishes interaction with S-RBD

Entry	Electrostatic energy score (arbitrary units of energy)	van der Waals energy score (arbitrary units of energy)	Score	Buried Surface Area (A^{-2})	Effect
Peptide 1	-284.989±2.3	-113.23±1.1	-94.364± 7.2	927.565± 32	B
Peptide 2	-386.163±1.7	-127.31±2.2	-104.789± 4.3	1024.23±21	C
Peptide 3	-272.940±2.4	-111.43±0.17	-93.334±9.6	995.967±34	B
Peptide 4	-265.347±1.1	-105.61±0.9	-87.56±3.5	1021.25±29	B
Peptide 5	-388.163±4.2	-190.20±1.4	-155.53±9.2	1123.57±37	D
Peptide 6	-383.163±3.3	-187.36±1.9	-143.034±10.13	1017.77± 41	D

Aiming to characterize the binding of the designed peptides against SARS-CoV-2 S-RBD, we performed molecular docking experiments using the peptides and a model of SARS-CoV-2 S-RBD. In all docking experiments, the peptides were found to bind at the S-RBD surface in a manner similar to that of the α1 helix of ACE2.

Taken together, these data suggest that the six designed peptides could bind to SARS-CoV-2 S-RBD and induce a conformational change. Additionally, the MD interaction analysis suggested that the designed peptides, apart from Peptide 3, could bind with a high affinity to S-RBD. As a consequence, we decided to synthesize and evaluate their ability to antagonise the S-RBD:ACE2 interaction and their binding to a recombinant SARS-CoV-2 S-RBD.

EXAMPLE 2: Synthetic peptides efficiently block the interaction of ACE2 with S-RBD

The various ACE2 antagonist peptides identified above were synthesized and assessed for their ability to inhibit the SARS-CoV-2 S-RBD interaction with ACE2 using a luciferase assay. To study this, Taha *et al.* (Molecular Therapy (2021) doi.org/10.1016/j.ymthe.2021.02.007) developed a novel bioluminescence-based sensor reporter system - the reassembly of SmBiT and LgBiT into NanoBiT when S-RBD and ACE2 interact- to probe antagonism of the protein:protein interaction, which could thereby perform a structure-function analysis of critical amino acids of the SARS-CoV-2 S-RBD that modulate its interaction with ACE2. This sensitive yet robust assay, developed for the discovery of neutralizing antibodies, allowed us to rapidly test peptide-based hypotheses for their ability to antagonize the S-RBD:ACE2 interaction.

Initially, we measured infection inhibition and toxicity following exposure to 9 concentrations (0.38nM - 25 μM) of peptides, performed in triplicate. We then used ACE2-antagonistic peptides to determine whether these peptides could efficiently compete for the SARS-CoV-2 S-RBD:ACE2 interaction in a cell-based

system. The reported half-maximal inhibitory concentration (IC50) of our peptides against the ACE2:S-RBD interactions demonstrates dose-dependent inhibition, with measured IC50s of 11 ±5, 18 ±2, 6 ±3, 32 ±2, 9 ±4, and 10 ±3 nM against Peptides 1 to 6, respectively (Table 2 & Fig. 4). At the highest concentrations measured (25 µM), we observed that all peptides completely inhibited SARS-CoV-2 S-RBD binding to ACE2. At a concentration of 0.39 µM, Peptides 2 and 4 exhibited up to ~95% inhibition and Peptides 1, 3, 5 and 6 exhibited a statistically significant inhibition of S-RBD binding at concentrations as low as 0.09 µM (Fig. 4).

EXAMPLE 3: Synthetic peptides bind to purified S-RBD with nanomolar affinity.

Microscale thermophoresis (MST) of the interaction of purified SARS-CoV-2 S-RBD with the six designed peptides was performed on a NanoTemper Monolith NT.115 (Nano Temper Technologies, Germany) and the results are shown in Figure 5. To perform these experiments, pure SARS-CoV-2 S-RBD protein was labelled and incubated with a concentration series of peptides (12.5-0.00019 µM or 0.125-0.00000019 mM) in PBS-Tween (0.01%) + PEG8000 1% (w/v). The addition of Tween and PEG8000 was necessary, as previous experiments in the absence of these reagents resulted in a high degree of aggregation of the peptide in the MST experiments.

Triplicates of the thermophoretic progress curves are reported as the median of Kd posterior distribution for peptide 1 to peptide 6, showing values of 106 ±1, 102 ±6, 245 ±3, 542 ±5, 13 ±1 and 46 ±5 nM, respectively. Overall, all six peptides showed a sigmoid binding curve with Kd in the low nanomolar range, which indicate a strong binding of these peptides to the protein in a short incubation time (5 minutes). The results shown are mean values ± SD of 3 measurements and are in close correlation with those of the luciferase assay (Fig. 4), in which peptides 5 and 6 also demonstrated the strongest antagonism (Fig. 4 e,f)). Given the affinity between S-RBD and ACE2 has been reported as 44.2 nM [36] and 94 nM [37] by SPR and ITC, respectively, these peptides can

serve to effectively inhibit viral cell entry in *an in vivo* settling. In summary, in contrast to neutralizing antibody therapies and other approaches that seek to target the virus, we established as first decoy peptide-based antiviral technology to compete with viral binding to RBD and thus inhibit viral infection.

5

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CLAIMS

1. A peptidic compound comprising or consisting of an amino acid sequence selected from the following:

(a) SRDKHEEHEKENDRGQ (SEQ ID NO:6),
(b) IYALLENAEDYNLVN (SEQ ID NO:5),
(c) DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3),
(d) IEEQAKTFLDKFQHEVEEIYWQS (SEQ ID NO:1),
(e) QDKHEEDYQMYNKGDKED (SEQ ID NO:2), or
(f) IDENARSYIDKFQHDAEEMWYQ (SEQ ID NO:4),
or an amide, an ester or a salt thereof.

2. Compound according to claim 1, being an isolated polypeptide consisting of an amino acid sequence selected from the following:

(a) SRDKHEEHEKENDRGQ (SEQ ID NO:6),
(b) IYALLENAEDYNLVN (SEQ ID NO:5),
(c) DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3),
(d) IEEQAKTFLDKFQHEVEEIYWQS (SEQ ID NO:1),
(e) QDKHEEDYQMYNKGDKED (SEQ ID NO:2), or
(f) IDENARSYIDKFQHDAEEMWYQ (SEQ ID NO:4),
or an amide, an ester or a salt thereof.

3. Compound according to claim 1 or 2, being an isolated polypeptide consisting of an amino acid sequence selected from the following:

(a) SRDKHEEHEKENDRGQ-NH_2 (SEQ ID NO:12),
(b) IYALLENAEDYNLVN-NH_2 (SEQ ID NO:11),
(c) DKFNHEAEDLFYQSSLASWNYNT-NH_2 (SEQ ID NO:9),
(d) IEEQAKTFLDKFQHEVEEIYWQS-NH_2 (SEQ ID NO:7),
(e) QDKHEEDYQMYNKGDKED-NH_2 (SEQ ID NO:8), or

(f) IDENARSYIDKFQHDAEEMWYQ-NH_2 (SEQ ID NO:10).

4. A pharmaceutical composition comprising one or more peptidic compound(s) according to any one of claims 1-3, and a pharmaceutically acceptable carrier, vehicle or diluent.

5. The pharmaceutical composition of claim 4, comprising one or more further therapeutic agents, preferably selected from protease inhibitors and antiviral replication inhibitors.

6. The pharmaceutical composition of claim 4 or 5, formulated for administration in the upper and/or lower respiratory tract.

7. A peptidic compound according to any one of claims 1-3, for use as medicament.

8. The use of a peptidic compound according to any one of claims 1-3, as inhibitor of the interaction between SARS-CoV-2 (COVID-19) Receptor Binding Domain and the human ACE2 protein.

9. A peptidic compound according to any one of claims 1-3, for use in a method of treating or preventing SARS-CoV-2 spike protein-mediated infection of human ACE2-expressing cells.

10. A peptidic compound according to any one of claims 1-3, for use in a method of treating or preventing COVID-19 in a subject, preferably a human subject.

11. A method of treating or preventing COVID-19 in a subject, comprising administering a therapeutically effective dose of a peptidic compound according to any one of claims 1-3, or a pharmaceutical composition according to any one of claims 4-6.

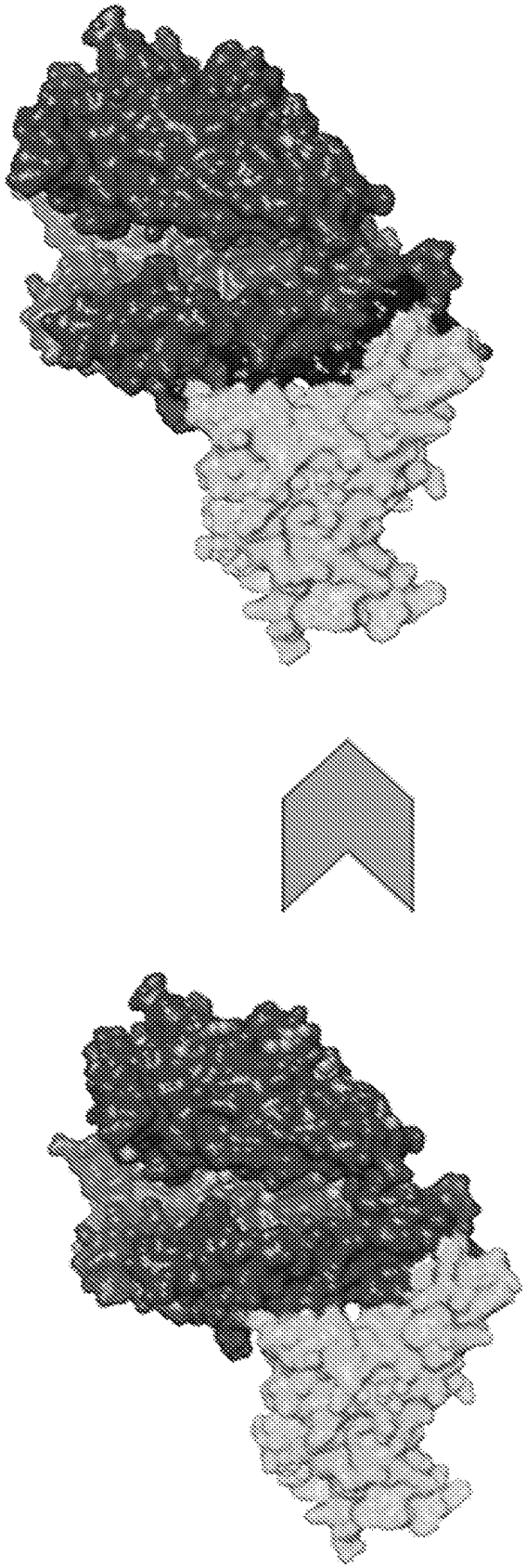


Fig. 1A

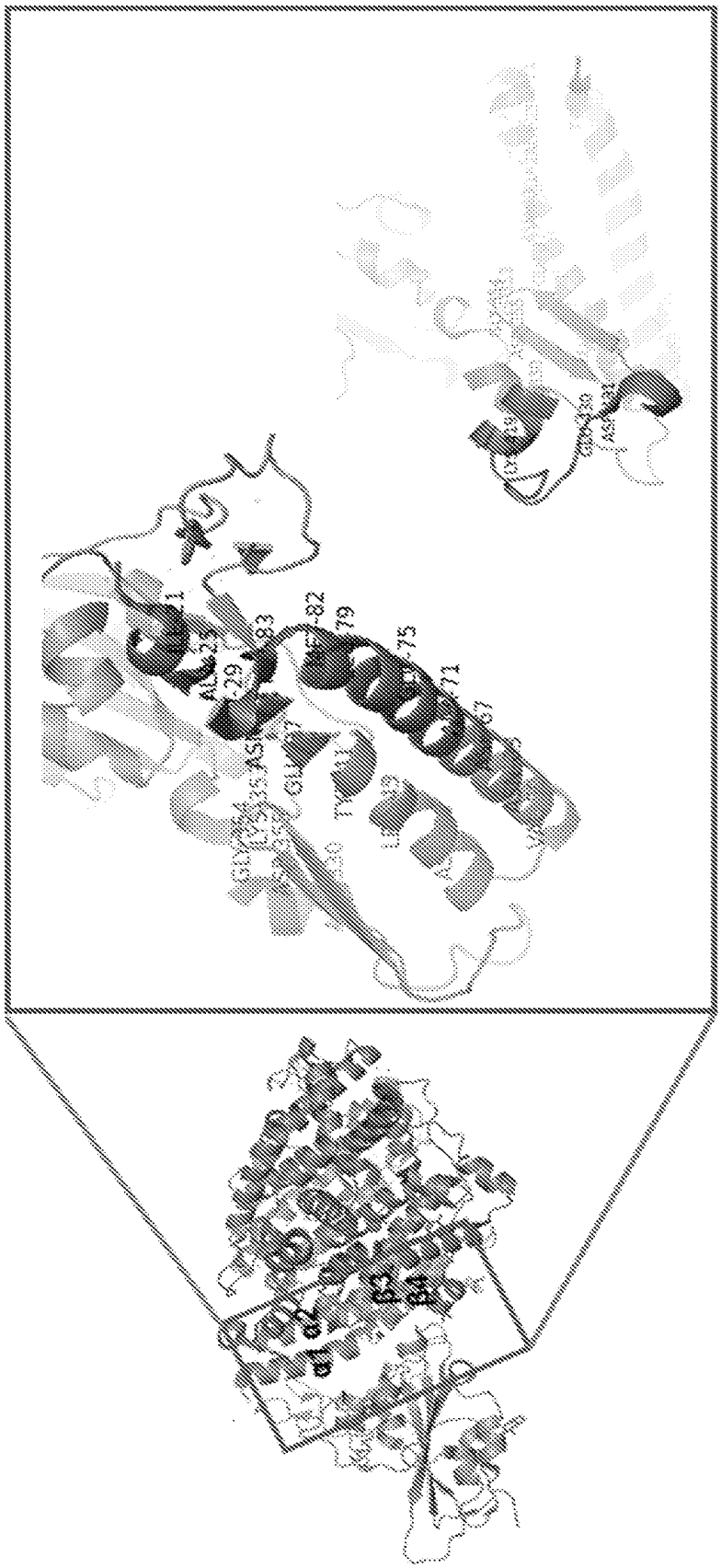


Fig. 1B

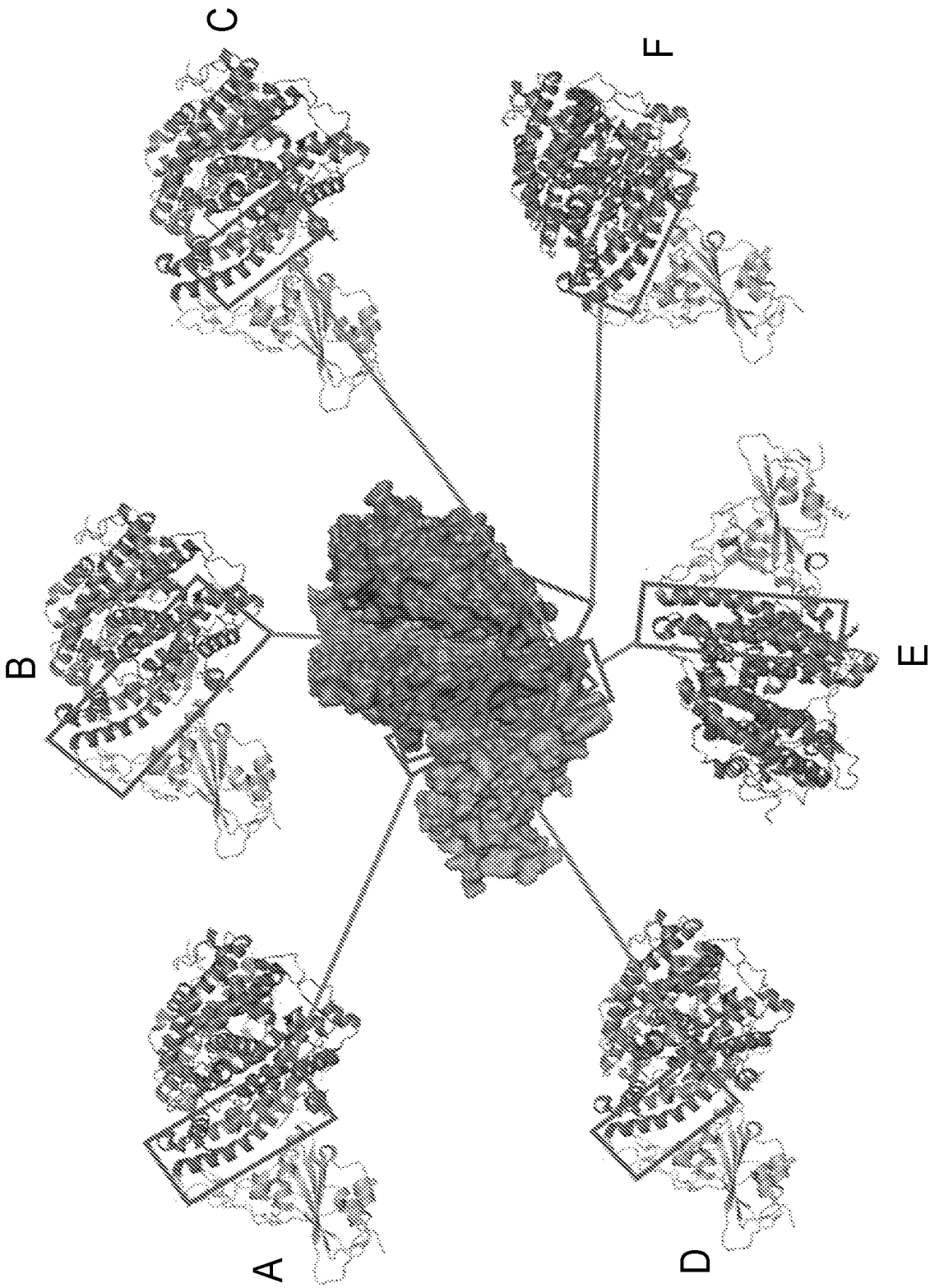


Fig. 2

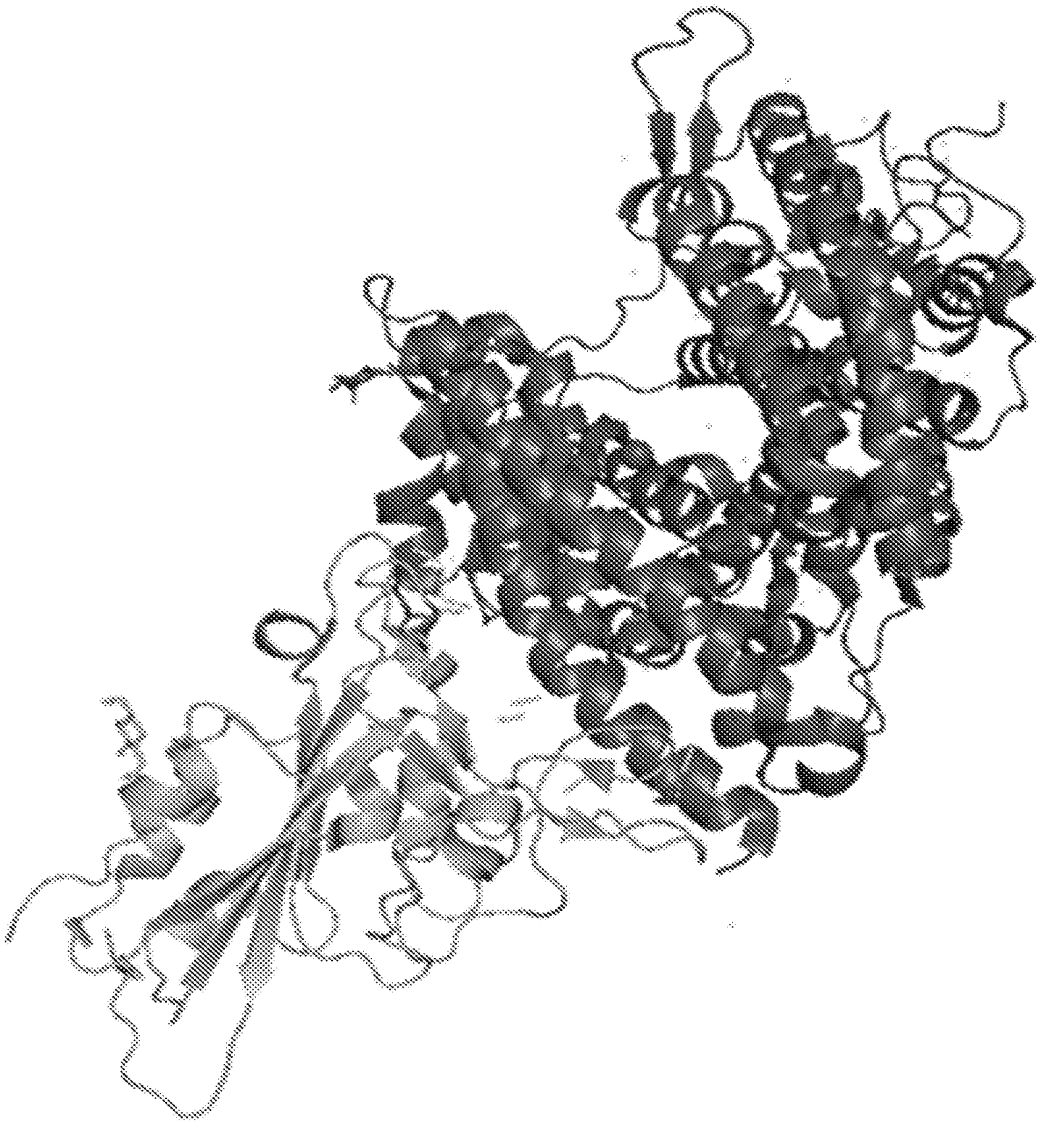


Fig. 3A

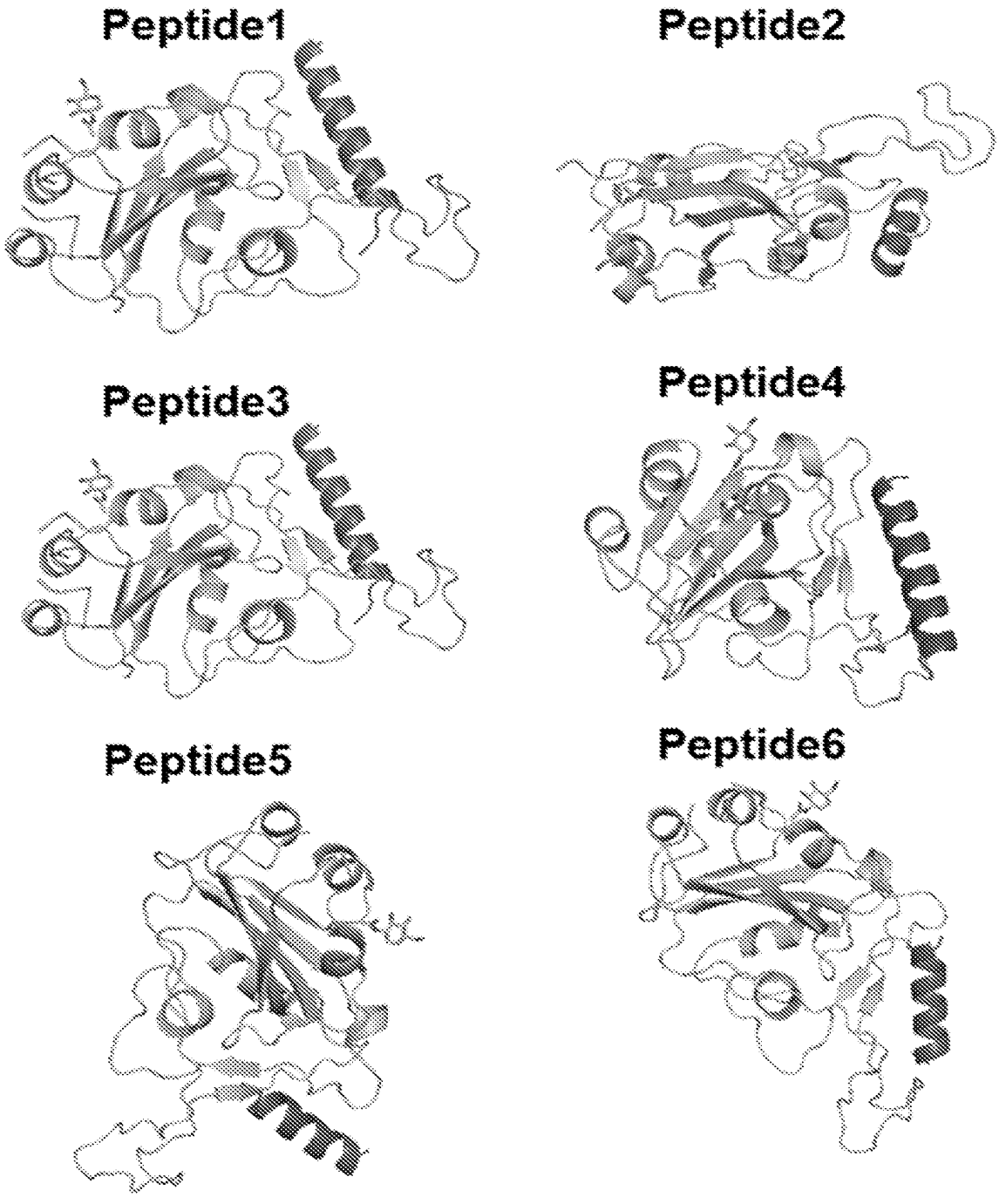
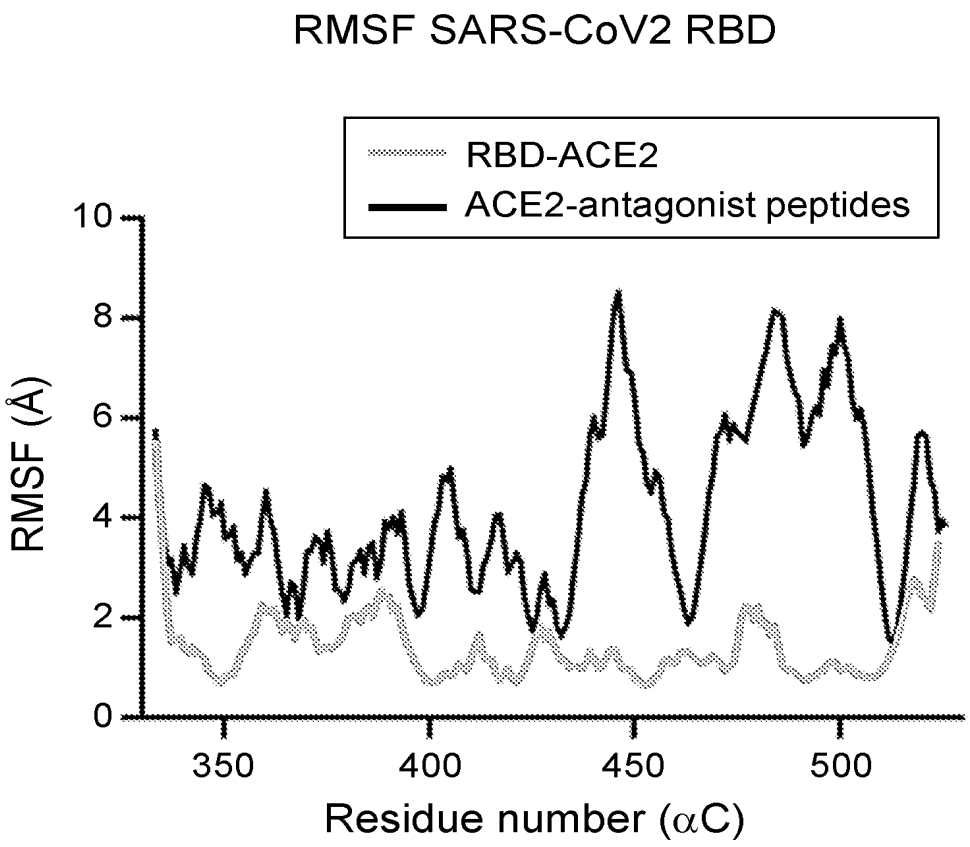


Fig. 3B


RMSF SARS-CoV2 RBD
RBD-ACE2
ACE2-antagonist peptides
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Residue number (αC)

Fig. 3C

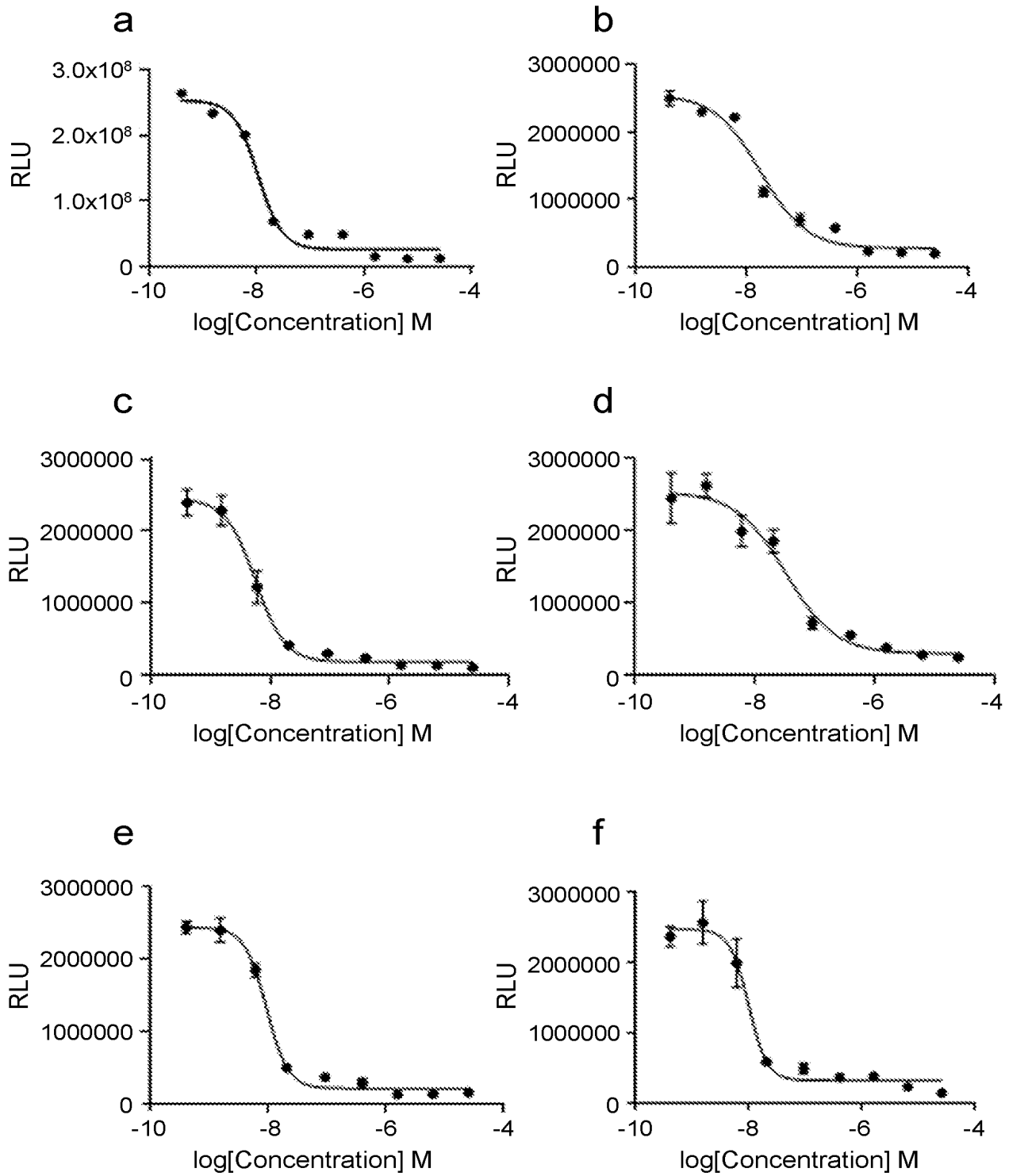
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Fig. 4

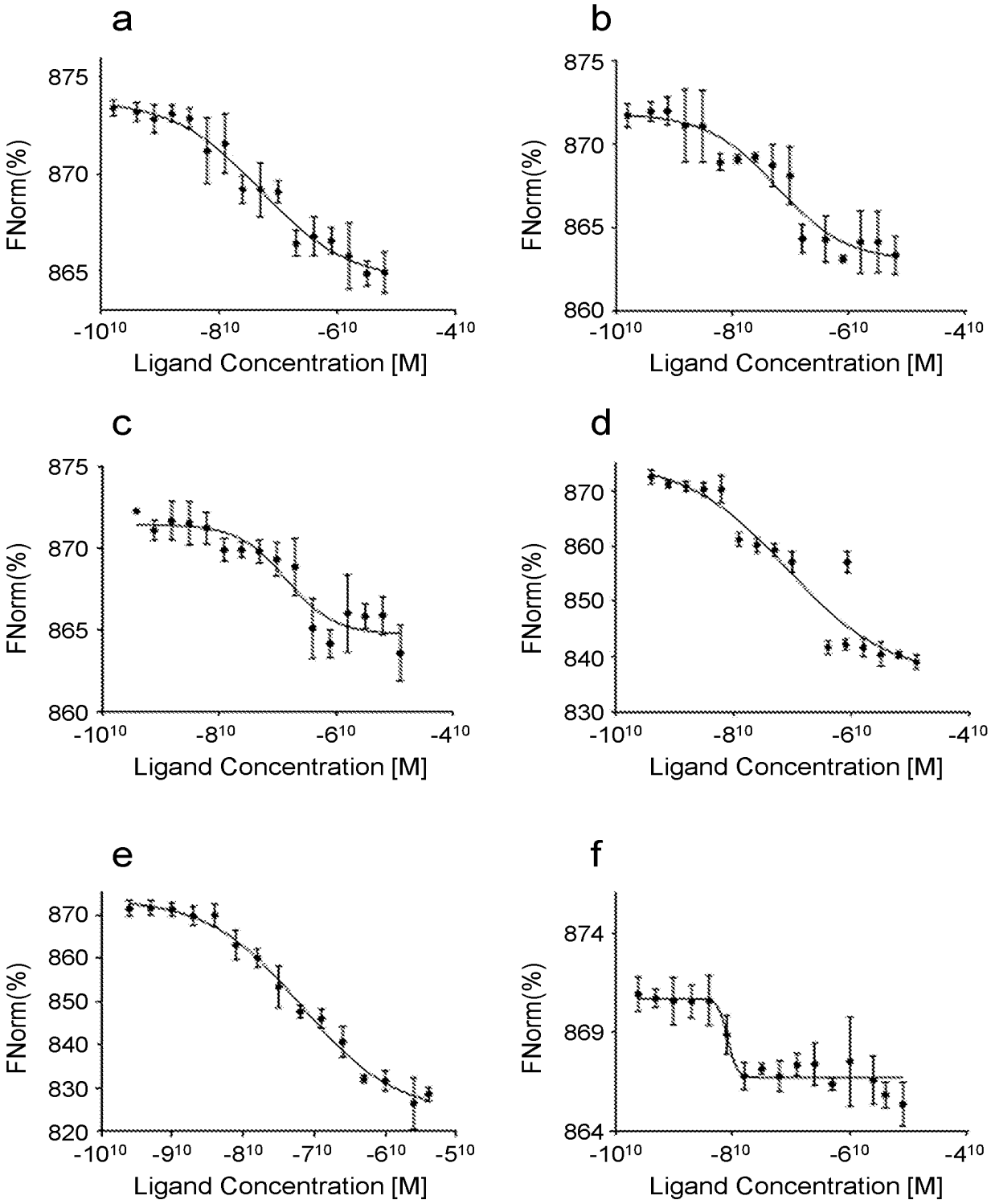
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Fig. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2022/050112

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K38/08 A61P31/14 C07K14/47
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CN 111 471 088 B (BEIJING ZHONGKE WEIDUN BIOTECHNOLOGY CO LTD) 9 February 2021 (2021-02-09) cited in the application claim 1; examples 1-3; table 1	1-11
A	CN 112 321 686 A (UNIV PEKING SHENZHEN GRADUATE SCHOOL ET AL.) 5 February 2021 (2021-02-05) examples 1-6	1-11

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[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2022	22/07/2022

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Hars, Jesko

3

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2022/050112

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	G. Zhang ET AL: "Abstract", bioRxiv, 30 March 2020 (2020-03-30), XP055759391, DOI: 10.1101/2020.03.19.999318 Retrieved from the Internet: URL:https://www.biorxiv.org/content/10.1101/2020.03.19.999318v1.full.pdf [retrieved on 2020-12-11] cited in the application figures 1,2	1-11
A	KAROYAN PHILIPPE ET AL: "Human ACE2 peptide-mimics block SARS-CoV-2 pulmonary cells infection", COMMUNICATIONS BIOLOGY , vol. 4, no. 1 12 February 2021 (2021-02-12), pages 1-9, XP055821752, DOI: 10.1038/s42003-021-01736-8 Retrieved from the Internet: URL:https://www.nature.com/articles/s42003-021-01736-8.pdf figure 1	1-11
X	WO 2008/021542 A2 (CELL SIGNALING TECHNOLOGY INC [US]; HORNBECK PETER [US] ET AL.) 21 February 2008 (2008-02-21) page 12; sequence 35	1,4-11
X,P	SADREMOMTAZ AFSANEH ET AL: "Synthetic Peptides That Antagonize the Angiotensin-Converting Enzyme-2 (ACE-2) Interaction with SARS-CoV-2 Receptor Binding Spike Protein", JOURNAL OF MEDICINAL CHEMISTRY, vol. 65, no. 4, 30 July 2021 (2021-07-30), XP055921590, US ISSN: 0022-2623, DOI: 10.1021/acs.jmedchem.1c00477 Retrieved from the Internet: URL:https://pubs.acs.org/doi/pdf/10.1021/acs.jmedchem.1c00477> page 2837	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2022/050112

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).

      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2022/050112

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;; it is covered by claims Nos.:
1-11 (partially)

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-11(partially)

A peptidic compound comprising or consisting of the amino acid sequence SRDKHEEHEKENDRGQ (SEQ ID NO:6), or an amide, an ester or a salt thereof.
Also SEQ ID NO: 12. Compositions comprising the compound, and uses thereof.

2-6. claims: 1-11(partially)

A peptidic compound comprising or consisting of an amino acid sequence selected from the following (invention number in brackets):
(b) IYALLENAEDYNLVN (SEQ ID NO:5) or SEQ ID NO: 11 (invention 2),
(c) DKFNHEAEDLFYQSSLASWNYNT (SEQ ID NO:3) or SEQ ID NO: 9 (invention 3),
(d) IEEQAKTFLDKFQHEVEEIYWQS (SEQ ID NO:1) or SEQ ID NO: 7 (invention 4),
(e) QDKHEEDYQMYNKGDKED 10 (SEQ ID NO:2) or SEQ ID NO: 8 (invention 5), or
(f) IDENARSYIDKFQHDAEEMWYQ (SEQ ID NO:4) or SEQ ID NO: 10 (invention 6),
or an amide, an ester or a salt thereof.
Compositions comprising the compound, and uses thereof.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2022/050112

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CN 111471088 B	09-02-2021	NONE	
CN 112321686 A	05-02-2021	NONE	
WO 2008021542 A2	21-02-2008	US 2010092992 A1	15-04-2010
		WO 2008021542 A2	21-02-2008