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

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Design of an engineered ACE2 as a novel therapeutics against COVID-19

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ABSTRACT

The interaction between the angiotensin-converting enzyme 2 (ACE2) and the receptor binding domain (RBD) of the spike protein from severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) plays a pivotal role in virus entry into the host cells. Since recombinant ACE2 protein has been suggested as an anti-SARS-CoV-2 therapeutic agent, this study was conducted to design an ACE2 protein with more desirable properties. In this regard, the amino acids with central roles in enzymatic activity of the ACE2 were substituted. Moreover, saturation mutagenesis at the interaction interface between the ACE2 and RBD was performed to increase their interaction affinity. The best mutations to increase the structural and thermal stability of the ACE2 were also selected based on B factors and mutation effects. The obtained resulted revealed that the Arg273Gln and Thr445Gly mutation have drastically reduced the binding affinity of the angiotensin-II into the active site of ACE2. The Thr27Arg mutation was determined to be the most potent mutation to increase the binding affinity. The Asp427Arg mutation was done to decrease the flexibility of the region with high B factor. The Pro451Met mutation along with the Gly448Trp mutation was predicted to increase the thermodynamic stability and thermostability of the ACE2. The designed therapeutic ACE2 would have no enzymatic activity while it could bear stronger interaction with Spike glycoprotein of the SARS-CoV-2. Moreover, decreased in vivo enzymatic degradation would be anticipated due to increased thermostability. This engineered ACE2 could be exploited as a novel therapeutic agent against COVID-19 after necessary evaluations.

1. Introduction

The SARS-CoV-2 is a non-segmented positive sense RNA virus which belongs to the genus *Beta Coronavirus* within the *Coronaviridae* family. Its 30 kilo base pairs genome encodes for multiple structural and non-structural proteins. Like SARS and the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), it mainly causes fever, chronic fatigue, dry cough, shortness of breath, and severe respiratory distress (Cabeça et al., 2013; Corman et al., 2018; Enjuanes et al., 2006; Perlman and Netland, 2009; van Boheemen et al., 2012). The envelope-anchored trimeric spike (S) protein at the surface of the virus is considered to be the key protein for virus entry into the host cells. The ACE2 (a homologue of ACE) is reported to be the potent human receptor for SARS-CoV-2 (Othman et al., 2020). This receptor primarily plays physiological roles in the control of vasoconstriction and blood pressure. As a type I membrane protein, ACE2 is found to be expressed in lungs, heart, kidneys and intestine (Donoghue et al., 2000; Zhang et al., 2020; Zhao et al., 2020). It also has been shown that the cardiovascular diseases are associated with reduced ACE2 expression (Crackower et al., 2002). Given these properties, ACE2 has been served as a drug target for cardiovascular and kidney diseases. Although the RBD of the SARS-CoV-2 spike protein contains 6 amino acid substitutions compared to the RBD of the SARS spike protein, existence of highly conserved residues at this region corroborates the ACE2 as the receptor for SARS-CoV-2 entry (Boehm and Nabel, 2002; Donoghue et al., 2000; Ge et al., 2013). To determine whether the SARS-CoV-2 uses ACE2 as the cell entry receptor, Peng Zhou et al. conducted a virus infection study using HeLa cells

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expressing ACE2 protein and cells without ACE2 expression. They have demonstrated that the SARS-CoV-2 can only infect ACE2-containing cells. They also have demonstrated that SARS-CoV-2 does not use other corona virus receptors including aminopeptidase N, dipeptidyl and peptidase 4 (Wu et al., 2020).

Given the crucial role of ACE2 and RBD interaction in the pathogenesis of SARS-CoV-2, inhibitory moieties to halt this interaction would prevent the virus from infecting the host cells. Targeting the interacting interfaces of ACE2 and RBD via antibodies or other therapeutic small molecules has already been practiced in numerous studies (Chen et al., 2017; Coleman et al., 2014; Der Sarkissian et al., 2008; Pang et al., 2020). Developing these kind of conventional therapeutics is inherently time consuming and demands recourses. An alternative approach to interfere with the interaction between ACE2 and RBD is the application of molecules which could compete with the cell membrane ACE2 to bind RBD. A recombinant ACE2 molecule which is designed to interact with RBD with higher affinity, without enzymatic activity and higher thermostability could be a suitable alternative.

Studies shown that specificity, binding affinity, thermostability and immunogenicity of therapeutic proteins can be altered by amino acid substitutions. Since several amino acids at the binding interface of the interacting molecules are responsible for binding affinity and immunogenicity, substitution of such key amino acids can significantly alter the affinity and immunogenicity (Clark et al., 2006; Xu et al., 2009). Such effective amino acids could be determined in vitro or using bioinformatics tools and replaced with more efficient amino acids. Bioinformatics approaches provide the opportunity to assess the effect of various sequence alterations on protein affinity, thermostability and enzymatic activity by reduced research costs and time (Payandeh et al., 2018; Payandeh et al., 2019; Sefid et al., 2019). Given the importance of SARS-CoV-2, its transmission and rapid spread to all countries around the world, it is important to develop new therapeutic approaches to deal with it. Since the ACE2 receptor plays the most important role in transmitting the virus into the pulmonary cells, the purpose of this study is to design a human ACE2 inhibitor that lacks its enzymatic activity and bears higher thermostability and higher affinity towards RBD.

2. Methods

2.1. Sequence and structure preparation

The protein sequences for RBD of the COVID-19 spike protein and the ACE2 protein were searched in UniProt (https://www.uniprot.org/) and NCBI (https://www.ncbi.nlm.nih.gov) databases. The 3D structures of these proteins along with their interacting complexes were obtained from PDB database. The latest structures with highest resolution were used for further analyses.

2.2. Deactivation of enzymatic activity

Since there were no resolved structures for ACE2 and angiotensin 2 (ANG2) complex, the structures of these molecules were docked together using CABSdock (http://biocomp.chem.uw.edu.pl/CABSdock) and ClusPro (https://cluspro.bu.edu/login.php) servers. The complex of ACE2 and ANG2 was used to find the most suitable point mutation candidates.

2.2.1. Generating the two dimensional (2D) interaction plot

The 2D plot of the interactions between the amino acids of ACE2 and ANG2 was generated by LigPlus software (v.2.1). The complex of ACE2 and ANG2 was fed as the input file and the LigPlot tool was employed.

2.2.2. Network based protein analysis for functional and structural key residues

The residue interaction network of ACE2 and ANG2 was extracted from the tertiary structure of the complex of ACE2 and ANG2 by Residue Interaction Network Generator (Piovesan et al., 2016) as provided by http://protein.bio.unipd.it/. Strong and reliable interactions were generated by selecting the strict distance threshold. The network policy for identification of interacting pairs was based on calculating the distance between mass centers of two interacting residues. All types of interactions were considered in this analysis. The generated network further analyzed and visualized by Cytoscape 3.7.2 (Shannon et al., 2003). To investigate the key residues in correct folding and function of the protein, a centrality analysis was carried out by RINspector (Brysbaert et al., 2018) application of Cytoscape. The centrality analysis was based on the change of average shortest path length under removal of each residue (node in the network) as proposed by (del Sol et al., 2006) and embedded in RINSpector. The shortest path length is the minimum number of edges that are required for connecting the first node to the second one in the network. The mean of these numbers, related to all possible pairs of residues in the network of interaction, is defined as average shortest path. The significance of average shortest path length is determined by the calculation of Z-score. The Z-scores greater than 2 are considered as relevant (Brysbaert et al., 2018).

2.2.3. Saturation mutagenesis to check the impact on binding affinity

The complex of ACE2 and ANG2 was fed as input for the mCSM-PPI2 server (http://biosig.unimelb.edu.au/mcsm_ppi2/) to analyze the effects of single-point mutations on protein–protein interactions. All possible mutations were performed for each mutation site to find the most deactivating mutation.

2.2.4. Exerting the mutations and assessing the effects on enzyme substrate interactions

The results of performed analyses along with the results of previous reports were considered to select the key amino acids which their mutation would terminate the enzymatic activity of the ACE2. The candidate mutations were exerted using the protein preparation tool from Molegro VIRTUAL DOCKER (v.6.0). The mutated amino acids and the neighboring amino acids were energy minimized to arrive at proper conformations. The Molegro VIRTUAL DOCKER was employed to determine the cavity sizes within the ACE2 structure before and after exertion of the mutations. The structures of the mutated ACE2 and ANG2 were docked again using CABSdock and ClusPro servers to analyze the consequences of the mutations.

2.3. Increasing ACE2 affinity toward RBD

2.3.1. ACE2 mutation analyses at its interface with RBD

The structure of ACE2 and RBD complex was extracted from the previously resolved protein complex under the PDB ID of 6M17. This complex was fed as the input for the mCSM-PPI2 server (http://biosig.unimelb.edu.au/mcsm_ppi2/) to analyze the effects of single-point mutations on protein–protein interactions. The Saturation Mutagenesis tool of this server was employed to mutate all residues at the interaction interface to all 19 standard amino acids. The effect of each mutation is reported as predicted affinity change ($\Delta\Delta G^{affinity}$) (positive values correspond to affinity increase and vice versa). The LigPlus software was employed to draw the 2D plot of the interactions between the amino acids of ACE2 and RBD at their interaction interface.

2.3.2. Mutation of ACE2 to increase its RBD binding affinity

The mutations with highest possibility to increase the binding affinity between the ACE2 and RBD were exerted in the ACE2 structure by Molegro VIRTUAL DOCKER. The mutated amino acids and the neighboring amino acids were energy minimized to arrive at proper conformations. The effects of the mutation were also analyzed by LigPlus software by drawing the 2D plots of ACE2 and RBD interactions after each mutation.

2.4. ACE2 mutation to increase thermal stability

Average B factor for each residue was calculated by Chimera software for both 1R42 and 6M17. The results for 19 possible mutations at the residue with highest B factor were done by iStable2.0 software (http://ncblab.nchu.edu.tw/iStable2/result.php). The PoPMuSiC and HotMuSiC (https://soft.dezyme.com/) were respectively used for the prediction of protein thermodynamic stability changes upon single-site mutations and the prediction of protein thermostability changes upon single-site mutations. A systematic prediction was performed to have the prediction results for 19 mutations per ACE2 residue. The best mutations to enhance the thermal stability were exerted and optimized by Molegro VIRTUAL DOCKER.

2.5. Flexibility simulation

The CABSflex (http://biocomp.chem.uw.edu.pl/CABSflex2) server was employed for or fast simulation of protein structure flexibility. This tool generates protein dynamics at highly reduced (3 orders of magnitude) computational cost, although with some decrease in resolution. The ACE2 structure with 6 mutations was fed as input for flexibility simulation.

2.6. Molecular dynamics (MD) analysis

The MD analysis was used to investigate the stability of the ACE2 protein following the exerted mutations. This method allows the protein atoms to move freely and find the best location based on the applied force-fields, while placed at a specific temperature and pressure conditions. If the mutated protein continues to maintain its structure and stability after the completion of the molecular dynamics process, the structure could be considered as stable. The GROMACS software version 4.6.5 and the GROMOS96-54 A7 force field were used to perform the MD simulation. The complex was put into a cube box and filled with water using the TIP3 model. If required, the system was neutralized by using Na+ or Cl− ions. Finally, the MD simulation was performed for 30 ns. The Root Mean Square Deviation (RMSD) and radius of gyration (Rg) was used to evaluate the stability of the mutated ACE2 protein.

2.7. Molecular docking analyses for mutant ACE2

The predicted mutations were exerted in the structure of the ACE2 from 1R42. The interaction orientation for mutant ACE2 and RBD was reanalyzed by molecular docking. The docking analysis was performed using HADDOCK server (https://wenmr.science.uu.nl/haddock2.4/) (Van Zundert et al., 2016). The results of the docking analyses were superimposed on the original ACE2 and RBD complex and the RMSD value was calculated using Chimera software (v.1.10.2).

2.8. Immunological analyses

The sequences of wild type and mutant ACE2 proteins were analyzed by Bepipred linear epitope prediction tool (http://tools.iedb.org/bcell/) of the IEDB database. The glycosylation sites of the wild type and mutant ACE2 proteins were analyzed by NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/) server.

3. Results

3.1. Prepared sequences and structures

The sequences for RBD of the SARS-CoV-2 spike protein and the ACE2 protein were found under the NCBI accession numbers of QHD43416 and NP_001358344, respectively. The 3D structure for interacting complex of the RBD from spike protein and the ACE2 protein was stored under the PDB ID of 6M17. The ACE2 structure was obtained under the PDB ID of the 1R42. The extra protein chains and the non-protein moieties were removed from both 6M17 and 1R42 structures. The RBD of the SARS-CoV-2 spike protein was also extracted from the 6M17 as a separate structure for further analyses.

3.2. Deactivation of enzymatic activity

The molecular docking analyses between the ACE2 and ANG2 proteins indicated that the ANG2 could form a tight interaction with the ACE2 protein within the expected central cleavage of the enzyme. Correct deployment of the ANG2 within the ACE2 active site could be interpreted as the suitability of the employed docking method.

3.2.1. The 2D interaction plot

The 2D interaction plot (Fig. 1) of the ACE2 and ANG2 complex shows that a number of amino acids are involved in hydrophobic interactions and hydrogen bonds. Amongst the involved amino acids, the Arginine 273 and the Threonine 445 amino acids of the ACE2 molecule are involved with hydrogen bonds. Since hydrogen bonds are highly important for the stability of protein-peptide interaction, these amino acids should have significant roles in the stability of the ACE2 and ANG2 complex.

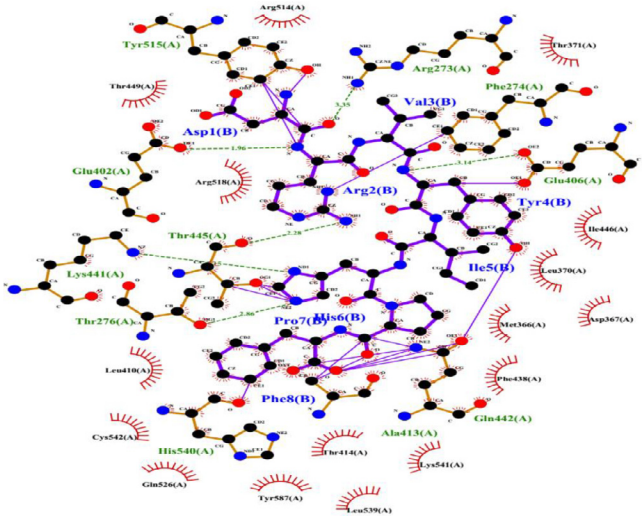

Fig. 1. The 2D plot of the ACE2 and ANG2 interaction (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the ligand atoms they contact, purple lines are either covalent bonds between protein and ligand, or "elastic" bonds within the ligand). The ANG2 is located at the center of the plot, while the ACE2 residues are around it. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

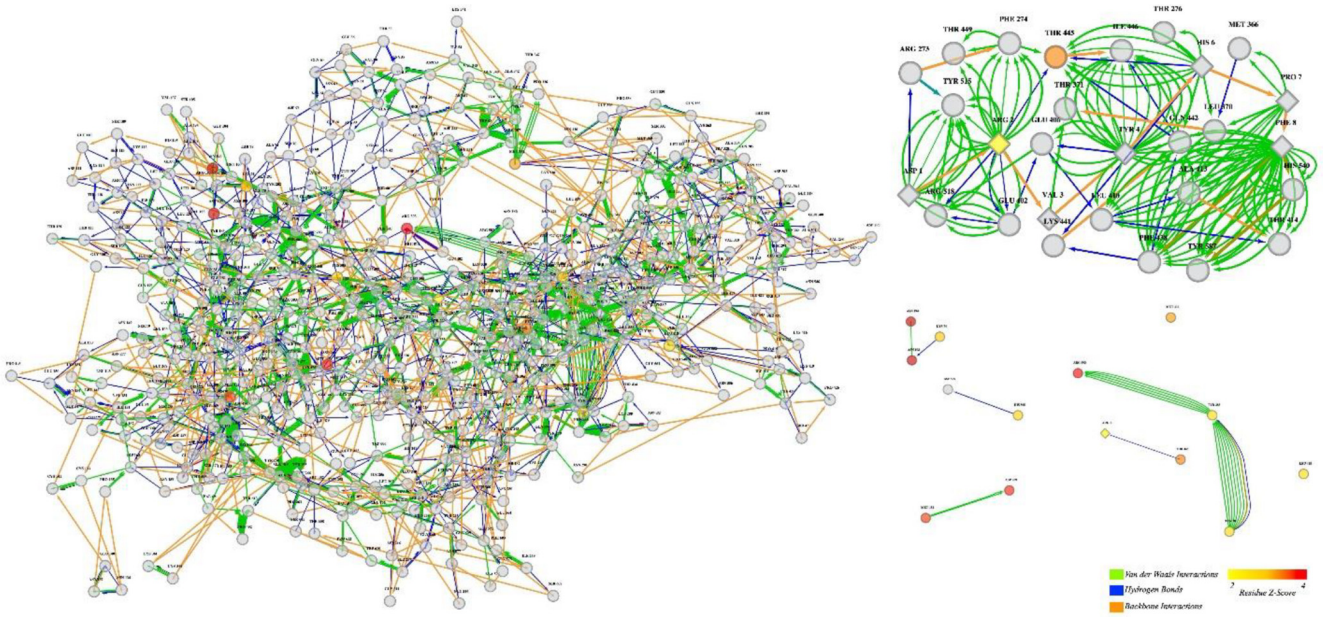

Fig. 2. The residue interaction network of ACE2-ANG2 complex. Left panel: The interaction network of whole complex. Upper right panel: The interaction network of active pocket of the enzyme. Lower right panel: the significant central residues within whole network. Each node represents a residue in the protein structure; ellipses are related to ACE2 (chain A), diamonds are related to AGE2 peptide (chain B). The fill color of central residues is based on the Z-score (yellow = 2 to red = 5.3, the nodes of greater Z-scores are colored brown: Asn103 and Asn194). The edges that connect each node represent the type of interaction (see the lower right color key). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.2. Network analyses of the complex

To determine the functional residues in the protein, a centrality analysis was performed on the residue interaction network of the ACE2-ANG2 complex. As expected, the residue interaction network is somehow a complex network (Fig. 2, left panel). To have a more clear view, the active pocket of the enzyme (ACE2), the interface residues between ACE2 (Chain A) and ANG2 were extracted from the whole network (Fig. 2, upper panel and Table 1). Moreover, the significant residues in the term of centrality were separated and illustrated in Fig. 2 lower panel. The Arginine 273 and the Threonine 445 of the ACE2 are among the amino acids attributed with high and significant Z-scores. Moreover, the Threonine 445 is shown to have a hydrogen bond with the Arginine 2 of the ANG2 molecule, while the Arginine 273 is shown to have a hydrogen bond with Aspartate 1 of the ANG2 molecule. The Arginine 2 of ANG2 is determined to be the central residue of ANG2. The increased level of Z-score indicates the increase in the average shortest path length upon node removal. When a significant Z-score assigned to a node (residue), it can be inferred that the related node may play an important role in the communication of the network. Therefore, the subjected residue may be an important structural or functional one. The illustration of active pocket is represented in protein structure to get a better insight about the native structure of the enzyme (Fig. 3).

3.2.3. Saturation mutagenesis for selected mutation sites

The Arginine 273 and the Threonine 445 of the ACE2 molecule were mutated to all 19 possible amino acids. The results of the

Table 1
The effect of Arginine 273 and the Threonine 445 saturation mutation on binding affinity ACE2 and ANG2 complex.

Wild-type	Res-number	Mutant	Mcsm-ppi2-prediction ($\Delta\Delta G^{affinity}$) Kcal/mol	Affinity	Wild-type	Res-number	Mutant	Mcsm-ppi2-prediction ($\Delta\Delta G^{affinity}$) Kcal/mol	Affinity
ARG	273	GLY	−1.548	Decreasing	THR	445	GLY	−1.344	Decreasing
ARG	273	MET	−1.517	Decreasing	THR	445	PRO	−1.238	Decreasing
ARG	273	ALA	−1.51	Decreasing	THR	445	ALA	−0.854	Decreasing
ARG	273	CYS	−1.464	Decreasing	THR	445	CYS	−0.709	Decreasing
ARG	273	PRO	−1.414	Decreasing	THR	445	MET	−0.701	Decreasing
ARG	273	VAL	−1.362	Decreasing	THR	445	VAL	−0.65	Decreasing
ARG	273	LEU	−1.229	Decreasing	THR	445	LEU	−0.448	Decreasing
ARG	273	ILE	−1.205	Decreasing	THR	445	ILE	−0.431	Decreasing
ARG	273	THR	−1.153	Decreasing	THR	445	PHE	−0.36	Decreasing
ARG	273	ASN	−1.123	Decreasing	THR	445	TRP	−0.176	Decreasing
ARG	273	SER	−1.073	Decreasing	THR	445	LYS	−0.105	Decreasing
ARG	273	ASP	−1.045	Decreasing	THR	445	GLN	−0.002	Decreasing
ARG	273	HIS	−1.019	Decreasing	THR	445	TYR	0.023	Increasing
ARG	273	LYS	−0.889	Decreasing	THR	445	HIS	0.11	Increasing
ARG	273	PHE	−0.754	Decreasing	THR	445	ARG	0.13	Increasing
ARG	273	TYR	−0.752	Decreasing	THR	445	SER	0.341	Increasing
ARG	273	TRP	−0.594	Decreasing	THR	445	ASN	0.394	Increasing
ARG	273	GLU	−0.449	Decreasing	THR	445	GLU	2.02	Increasing
ARG	273	GLN	1.724	Increasing	THR	445	ASP	2.331	Increasing

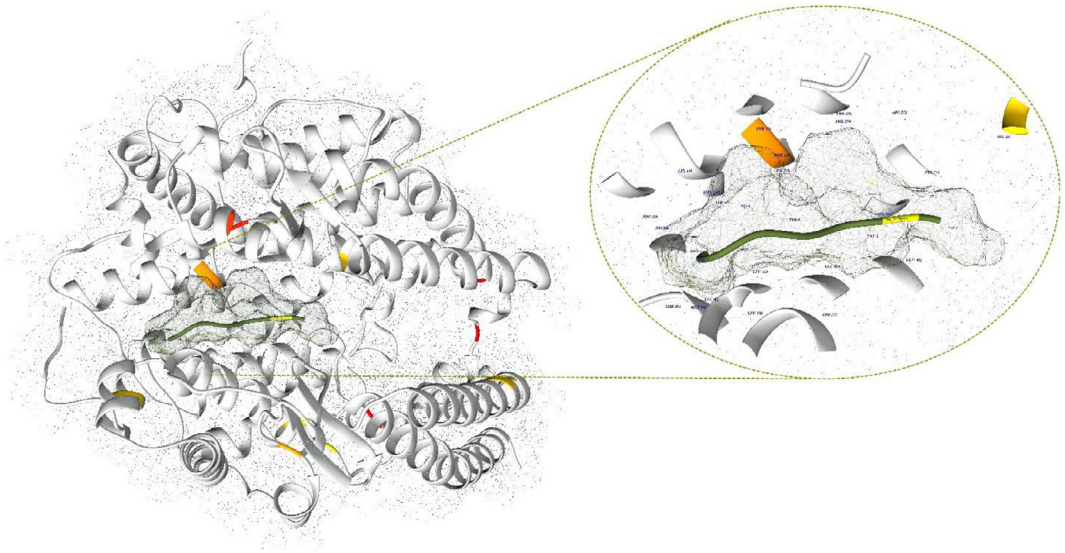

Fig. 3. Cartoon illustration of ACE2-ANG2 complex. The ACE2 enzyme is presented as white ribbons; the surface of ACE2 is presented as dots. ANG2 peptide is colored dark olive green with mesh surface. Other colors are adopted to the Z-score and synchronized by relative nodes in the network (the color scheme is the same as Fig. 2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mutagenesis analyses for both selected amino acids have indicated that the mutation to Glycine is the best mutation to decrease the binding affinity between the ACE2 and the ANG2 molecules (Table 1). Given the previously reported experimental evidences, mutating the Arginine 273 residue to Glutamine would drastically change the active site interactions with the ANG2 protein. Therefore, we preferred to mutate the Arginine 273 residue to Glutamine and the Threonine 445 residue to Glycine. Choosing the Glutamine and the Glycine residues to substitute the original amino acids would prevent drastic structural changes due to their similar sizes to the original residues.

3.2.4. Mutation effects on ACE2 and ANG2 interaction

Reanalysis of the molecular docking between the mutated ACE2 and ANG2 molecule indicates that following the exerted mutations the ANG2 molecule loses its ability to make a stable interaction with the active site of the mutated enzyme. The cavity search within the structures of ACE2 molecule before and after mutations shows that the volume of central cavity of the ACE2 (active site) is not drastically affected from the performed mutations (4764.67 A $^{\circ 3}$ before mutations and 4819.46 A$^{\circ 3}$ after mutations). The mutant ACE2 interacts with the ANG2 in a significantly different orientation (Fig. 4). These results indicate that the performed mutations would most likely terminate the enzymatic activity of the ACE2 by removing highly stabilizing bonds which were provided by original amino acids at positions 273 and 445.

3.3. Increasing the binding affinity

3.3.1. Determination of suitable mutations across the ACE2 and RBD interface

The results of the Saturation Mutagenesis analyses were obtained and sorted according to their effects to increase the binding affinity between ACE2 and RBD. The best possible mutations

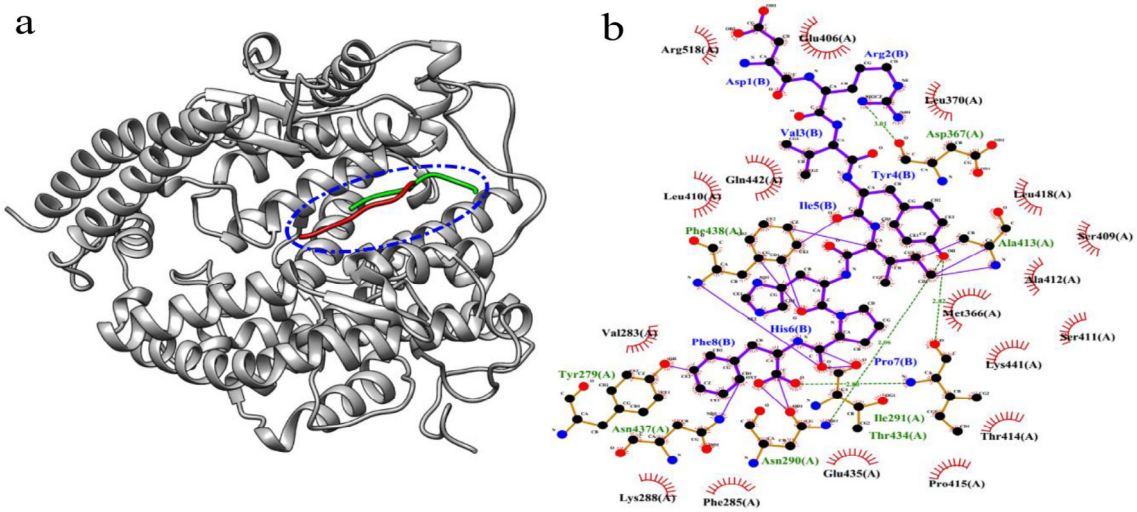

Fig. 4. The interaction between the ACE2 and ANG2. The structure of the ACE2 (grey protein) in complex with ANG2 (blue dashed circle) before (red peptide) and after (green peptide) active site mutations (a). The 2D plot of the ACE2 and ANG2 interaction after exertion of mutations (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the ligand atoms they contact, purple lines are either covalent bonds between protein and ligand, or "elastic" bonds within the ligand). The ANG2 is located at the center of the plot, while the ACE2 residues are around it (b). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

are listed in Table 2. The mCSM-PPI2 models the effects of variations on the inter-residue non-covalent interaction network using graph kernels, evolutionary information, complex network metrics and energetic terms to better assess the molecular mechanism of the mutation. The employed graph-based structural signatures called mCSM has been shown to be an accurate and high-throughput approach to predict the impact of mutations on protein structure and function, and was one of the first methods capable of assessing the impact of mutations on protein interaction binding affinity (Rodrigues et al., 2019). The important amino acids which are involved in the interaction between ACE2 and RBD were also plotted by LigPlus software. The amino acids which are involved in hydrogen bonds or hydrophobic interactions are depicted in Fig. 5.

3.3.2. Exerting the mutations at ACE2 and RBD interface

Analyses of various single or multiple mutations were performed using their 2D interaction plots. The mutations which were able to conserve the original interacting amino acids at the ACE2 and RBD interface and increasing the number of involved amino acids were considered as suitable mutations. The obtained results demonstrated that the threonine 27 to Arginine mutation is the only mutation which met the selection criteria (Fig. 6). Obeying these criteria would grantee the preservation of the ACE2 and RBD interactions in their original orientation.

3.4. Mutations to increase thermal stability

Five ACE2 residues with highest average B factors are listed in Table 3. The aspartate residue at position 427 has been among top 5 residues with high B factor. The region with highest B factor is depicted in Fig. 7. These high B factor regions are more flexible regions which could be affected by protease activity. The results of mutation analyses have shown that the mutation form aspartate 427 to arginine would increase the thermal stability of the ACE2. The Proline 451 to methionine mutation (PoPMuSiC prediction) and the glycine 448 to tryptophan mutation (HotMuSiC prediction) were predicted to increase the thermodynamic stability and thermostability of the ACE2. These mutations were exerted and optimized using Molegro VIRTUAL DOCKER.

3.5. Protein flexibility simulation

Analyzing the RMSF plot of the perfumed simulation indicated that the flexibility of mutated amino acids during the simulation is not high (Fig. 8a). This means that there are no large-scale con-

Table 2
Mutation candidates to increase the affinity. The mutations with highest capability to increase the binding affinity between the ACE2 and RBD are listed in the table.

Wild-type	Res-number	Mutant	Mcsm-ppi2-prediction ($\Delta\Delta G^{affinity}$) Kcal/mol	Affinity
THR	27	ARG	1.371	Increasing
LYS	31	GLU	1.311	Increasing
ASP	38	VAL	1.27	Increasing
THR	27	GLU	1.26	Increasing
PHE	28	TRP	1.152	Increasing

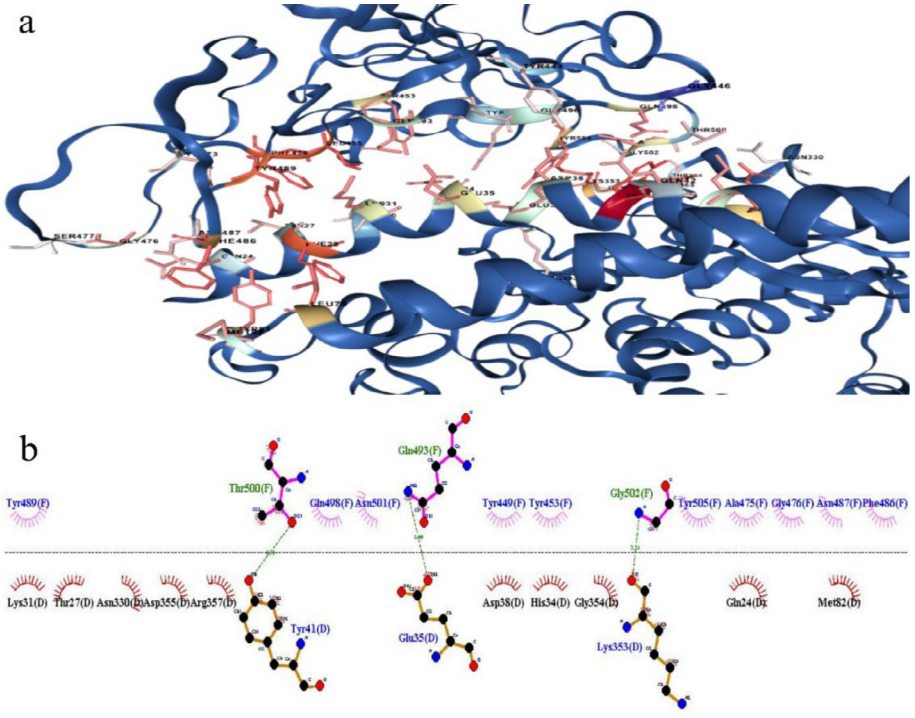

Fig. 5. The amino acids involved in ACE2 and RBD interaction. The structural visualization of the interactions across the interface is depicted in part (a). The 2D interaction plot of the interactions across the interface is depicted in part (b). The horizontal dashed line represents the interface. The amino acids located above the line belong to RBD and the amino acids located below the line belong to ACE2 (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the other atoms they contact).

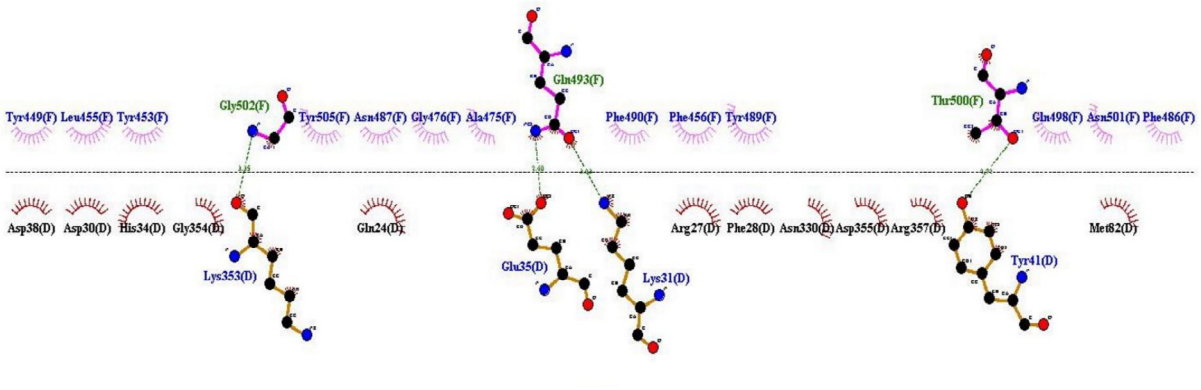

Fig. 6. The 2D interaction plot of the interactions across the interface. The horizontal dashed line represents the interface. The amino acids located above the line belong to RBD and the amino acids located below the line belong to ACE2 (hydrogen bonds are indicated by dashed lines between the atoms involved, hydrophobic contacts are represented by an arc with spokes radiating towards the other atoms they contact).

Table 3
The top 5 positions with highest average B factors for 6M17 and 1R42 structures (the B factors are normalized to the scale of 0 to 100 $A^{\circ 2}$).

6M17		1R42	
Position	Normalized B factor	Position	Normalized B factor
427	100	86	100
426	99	338	99
425	99	427	99
428	95	85	98
429	95	339	96

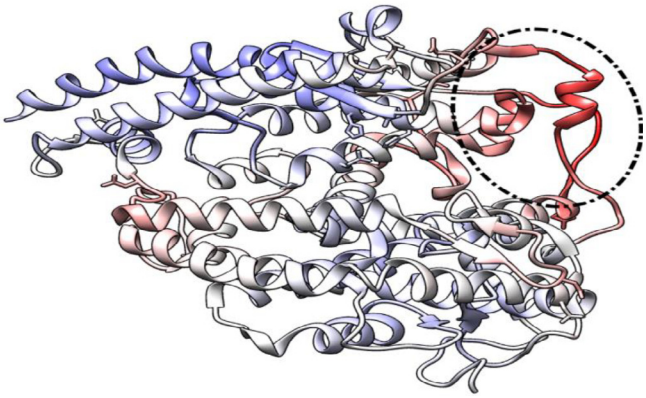

Fig. 7. The ACE2 structure colored by B factor change. The structure is colored blue to red as a function of B factor increase. The region with highest B factors is circled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

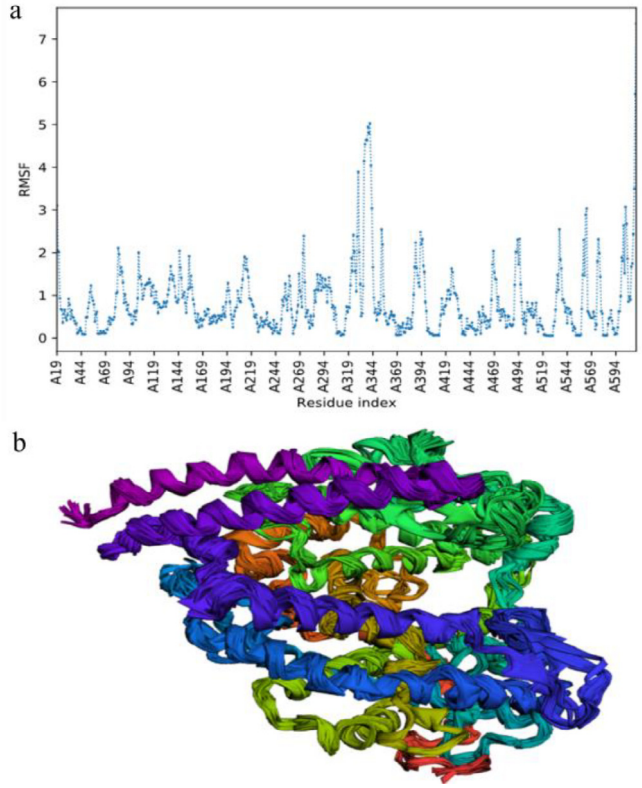

Fig. 8. The flexibility simulation. The RMSF (A°) per residue of the mutated ACE2 is depicted in part (a). The superimpose view of 10 ACE2 structures modeled during the simulation (b).

formational transitions of protein system at the mutations sites. The superimposed structures of 10 protein models during the simulation has also indicated the low structural fluctuation (Fig. 8b)

3.6. The stability of mutated ACE2 through MD analysis

The RMSD diagram of the mutated was plotted as a function of time. The diagram shows the deviation of the protein structure at different simulation times compared to the pre-simulation structure. The results showed that the enzyme reached stability after 10 ns. The fluctuations of the RMSD graph should be due to the amino acid changes. However, at the end of the simulation time the system reached stability (Fig. 9a). The R_g is another important factor for protein stability analysis. The lower the R_g, the more folded and stable is the protein. As shown, the radius of gyration of the mutant protein decreased during the simulation, which means that its stability is increased during the simulation (Fig. 9b).

3.7. Molecular docking of mutant ACE2 and RBD

The results of the molecular docking analyses indicated that the mutant ACE2 is capable of interacting with RBD with proper orientation (Fig. 10). This interaction orientation could include all original interactions across the ACE2 and RBD interface. The low RMSD (0.8 A°) between the original and the mutated ACE2 proteins indicates that the exerted mutation does not make a drastic changes in

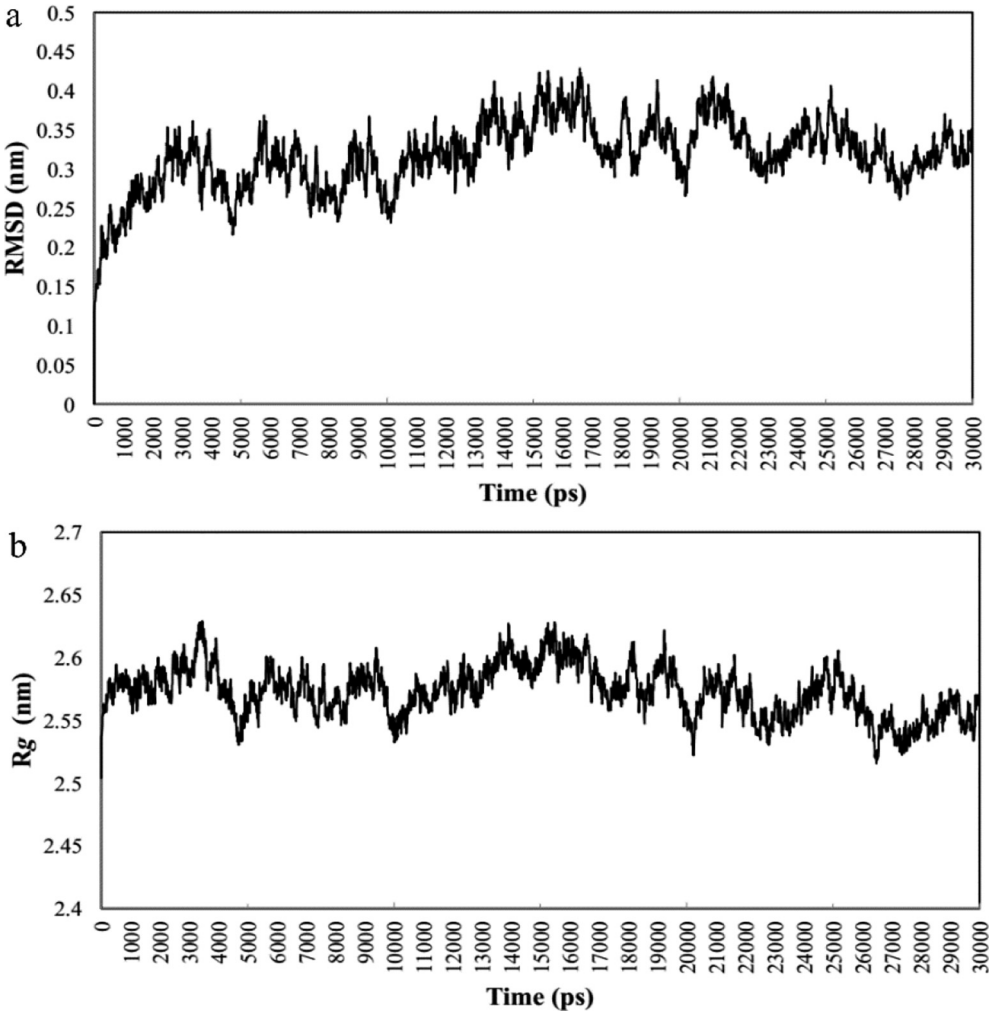

Fig. 9. The RMSD (a) and Rg (b) plots of the mutant ACE2 as a function of time.

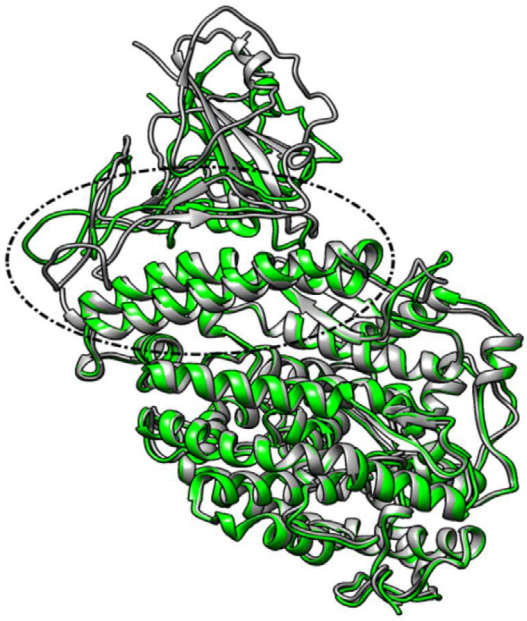

Fig. 10. Superimposition of original (light grey) and mutated (green) ACE2 and RBD complexes. The circled region is the interface for ACE2 (lower molecule) and RBD (upper molecule) interaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the protein structure. These results indicate that the mutant ACE2 could compete with the cell membrane ACE2 to bind to the RBD of the Spike protein.

3.8. Immunological analyses

The immunological analyses indicated that the mutant ACE2 does not contain any newly formed epitopes as the consequence of the exerted mutations. Moreover, none of the glycosylation sites were omitted or added to the glycosylation pattern of the ACE2 protein following the mutations. The 55, 92, 105, 324, 424 and 548 positions were predicted to be the glycosylation sites at both wild type and mutant ACE2 protein. The intact pattern of the glycosylation would help the mutant ACE2 to be considered as a self-protein upon clinical administration.

4. Discussion

There is still no definitive drug or effective vaccine to fight against COVID-19. Due to its pivotal role in SARS-CoV-2 infection, the interaction between ACE2 and RBD could be targeted for drug or vaccine development (Prompetchara et al., 2020). Vaccines are considered to be one of the most cost-effective healthcare tools in the prevention of infectious diseases. As expected, various strategies have been adapted to develop effective vaccines against RBD of SARS-CoV-2 (Chen et al., 2020a). This is while alternative

competitive molecules to interrupt this interaction could be as effective (Molina et al., 2020). ACE2 could be the alternative target for drug or vaccine design efforts against SARS-CoV-2. This enzyme is expressed in variety of tissues with profound physiological roles (Der Sarkissian et al., 2008; Rentzsch et al., 2008; Tipnis et al., 2000; Wong et al., 2007). Therefore, ACE2 targeted treatments bear the risk to have negative effects on its inherent physiological roles. Given these circumstances, a recombinant ACE2 molecule which lacks its enzymatic activity with higher thermostability and affinity towards RBD would compensate for the challenges of targeting the cell membrane ACE2. In this regard, Changhai et al. have recently reported a recombinant ACE2-Ig with potential applications for diagnosis, prophylaxis, and treatment of COVID-19 (Lei et al., 2020). Moreover, Montiel et al. have demonstrated that recombinant ACE2 can significantly block early stages of SARS-CoV-2 infections (Monteil et al.). However, their study does not focus on enhancing the binding affinity and thermostability of the ACE2. It should be noted that recombinant ACE2 has already been tested in phase 1 and phase 2 clinical trials and its administration is reported to be well tolerated by healthy human subjects (Haschke et al., 2013; Khan et al., 2017).

Two mutations were performed at the active site of the ACE2 enzyme according the results of network analysis, 2D interaction plot and previously reported studies. One of the mutations at the active site of the ACE2 protein has already been shown to abolish the enzymatic activity of this protein via site directed mutagenesis (Guy et al., 2005). This study has corroborated the accuracy of mutation candidates introduced by the employed approach. Although the saturation mutation results proposed the Arginine 273 to Glycine mutation, we have preferred the experimentally approved Arginine to Glutamine mutation. It seems that this mutation could omit the hydrogen bond between Arginine 273 and Aspartate 1 of the ANG2 molecule, while the active site cavity would be occupied by the bulky side chain of the Glutamine. The Threonine 445 to Glycine mutation does the same regarding the hydrogen bond omission and preservation of the occupied volume by an equivalent sidechain. Replacing the selected residues with amino acids of similar size would limit the structural changes to a minimum. These mutations had been shown to have a significant effect on binding orientation and affinity of the ANG2 against the active site of the ACE2. The cavity size of the ACE2 active site has slightly become larger after the introduction of the mutations. This increase in cavity volume seems expected due to the slightly smaller size of the replaced amino acids. These changes would inhibit the ACE2 enzyme to perform its carboxypeptidase function due to the loss of key enzyme and substrate interactions and the lack of proper lock between enzyme and substrate in a correct orientation. Reduced enzymatic activity of ACE2 would bring about reduced physiological consequences upon its prescription.

Aside from reduced enzymatic activity, the engineered ACE2 needs to have higher affinity toward the RBD in comparison to the membrane attached ACE2 of the target cells. This property would give the engineered ACE2 to have a winning chance to interact with RBD and compete with membrane attached ACE2. Since the ACE2 and RBD molecules are complexed in a certain orientation (Chen et al., 2020b; Yan et al., 2020), the mutations to enhance the binding affinity should preserve this orientation. Our results indicate that the threonine 27 to Arginine mutation does not have a drastic effect on the interaction orientation and the interacting amino acids. However, new amino acids with extra hydrogen bonds and hydrophobic interactions are engaged at the interaction interface. These results could be construed as stronger interactions between ACE2 and RBD with similar interaction orientation following the threonine 27 to Arginine mutation.

Recombinant ACE2 has already been used for severe acute lung injury, acute ANG2-induced hypertension, ANG2-induced heart hypertrophy, cardiac dysfunction, adverse myocardial remodeling, renal oxidative stress, inflammation, and fibrosis (Chen et al., 2016; Gu et al., 2016; Huentelman et al., 2005; Imai et al., 2005; Zhong et al., 2011; Zou et al., 2014). However, according to the pharmacokinetic studies the half-life of recombinant ACE2 is only hours in humans and mice due to its fast clearance rates (Haschke et al., 2013; Wysocki et al., 2010). Resistance against proteolytic degradation is one of the most important factors which could determine the protein half-life. Prior studies involving L-asparaginase, β-galactosidase, λ-repressor, Clostridium difficile toxin A-specific antibodies, and Bacillus subtilis lipase have shown that increasing a protein's thermostability may also increase its resistance to protease digestion (Ahmad et al., 2012; Daniel et al., 1982b; Hussack et al., 2011; Parsell and Sauer, 1989). Thermal and structural stability of proteins are important determinants of proteolytic stability (Akasako et al., 1995; Daniel et al., 1982a; Parsell and Sauer, 1989). Proteins are resistant against proteolytic degradation in their native conformation. However, they are highly susceptible to proteases after unfolding. The accessible and flexible loop regions of proteins could be attacked by unspecific proteases even in the native conformation of the highly compact and thermostable proteins (Hubbard, 1998; Sarath et al., 1989). A number of strategies have been harnessed to extend the low half-life of therapeutic proteins. Amino acid manipulation, post translational modification, bio-conjunction and carrier mediated delivery are the main categories of technologies reported for half-life extension (Zaman et al., 2019). In order to increase the thermostability of the ACE2 protein we have used the amino acid manipulation strategy. The first approach involved the mutation of amino acids with highest B factors (aspartate 427 to arginine). The diffusion of atomic electron densities with respect to their equilibrium positions due to thermal motion and positional disorder is represented by B factor. Thus, B factor shows the extent of mobility for individual amino acids. Replacing an amino acid with high B factor would increase the protein rigidity (Reetz et al., 2006; Xie et al., 2014). On the other hands, two other mutations were performed to increase the thermodynamic stability and thermostability of the ACE2. The mutations were selected at the regions which are far from the RDB binding site to avoid any destructive effects on the binding affinity. The obtained MD results have also corroborated the stability of the mutated ACE2 structure. We believe that increased thermodynamic stability and thermostability of the ACE2 would enhance the protein half-life. It should be noted that ACE2 amino acid manipulation could be accompanied with other half-life extension strategies like bio-conjugation.

Protein engineering is a powerful tool to design therapeutic proteins with desired properties. Bioinformatics tools are contemporary widely used for protein engineering (Khalili, 2017a,b). These tools could save a lot of time and effort in the case of diseases like COVID-19 which time is essential. Using these tools an engineered ACE2 protein has been designed which is expected to bind RBD with higher affinity while it is more thermostable and lacks enzymatic activity. Since ACE2 is reported to be the main RBD binding receptor, its application as an inhibitory agent provides an evolutionary advantage over other conventionally employed therapeutics. Moreover, using the extracellular domain of ACE2 reduces the possible adverse effects and immunological responses against this therapeutic molecule. In vitro an in vivo examination of the engineered protein would unveil its true potentials.

CRediT authorship contribution statement

Zahra Payandeh: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization.

Mohammad Reza Rahbar: Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Abolfazl Jahangiri:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Zahra Sadat Hashemi:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Alireza Zakeri:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Moslem Jafarisani:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Mohammad Javad Rasaee:** Methodology, Validation, Formal analysis, Investigation, Resources, Writing - review & editing, Visualization. **Saeed Khalili:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Ahmad, S., Kumar, V., Ramanand, K.B., Rao, N.M., 2012. Probing protein stability and proteolytic resistance by loop scanning: a comprehensive mutational analysis. Protein Sci. 21, 433–446.
Akasako, A., Haruki, M., Oobatake, M., Kanaya, S., 1995. High resistance of Escherichia coli ribonuclease HI variant with quintuple thermostabilizing mutations to thermal denaturation, acid denaturation, and proteolytic degradation. Biochemistry 34, 8115–8122.
Boehm, M., Nabel, E.G., 2002. Angiotensin-converting enzyme 2—a new cardiac regulator. N. Engl. J. Med. 347, 1795–1797.
Brysbaert, G., Lorgouilloux, K., Vranken, W.F., Lensink, M.F., 2018. RINspector: a Cytoscape app for centrality analyses and DynaMine flexibility prediction. Bioinformatics 34, 294–296.
Cabeça, T.K., Granato, C., Bellei, N., 2013. Epidemiological and clinical features of human coronavirus infections among different subsets of patients. Influenza Other Respir. Viruses 7, 1040–1047.
Chen, L.-J., Xu, Y.-L., Song, B., Yu, H.-M., Oudit, G.Y., Xu, R., Zhang, Z.-Z., Jin, H.-Y., Chang, Q., Zhu, D.-L., 2016. Angiotensin-converting enzyme 2 ameliorates renal fibrosis by blocking the activation of mTOR/ERK signaling in apolipoprotein E-deficient mice. Peptides 79, 49–57.
Chen, W.-H., Strych, U., Hotez, P.J., Bottazzi, M.E., 2020a. The SARS-CoV-2 vaccine pipeline: an overview. Curr. Tropical Med. Rep., 1–4
Chen, W.-H., Chag, S.M., Poongavanam, M.V., Biter, A.B., Ewere, E.A., Rezende, W., Seid, C.A., Hudspeth, E.M., Pollet, J., McAtee, C.P., 2017. Optimization of the production process and characterization of the yeast-expressed SARS-CoV recombinant receptor-binding domain (RBD219-N1), a SARS vaccine candidate. J. Pharm. Sci. 106, 1961–1970.
Chen, Y., Guo, Y., Pan, Y., Zhao, Z.J., 2020b. Structure analysis of the receptor binding of 2019-nCoV. Biochemi. Biophys. Res. Commun.
Clark, L.A., Boriack-Sjodin, P.A., Eldredge, J., Fitch, C., Friedman, B., Hanf, K.J., Jarpe, M., Liparoto, S.F., Li, Y., Lugovskoy, A., 2006. Affinity enhancement of an in vivo matured therapeutic antibody using structure-based computational design. Protein Sci. 15, 949–960.
Coleman, C.M., Liu, Y.V., Mu, H., Taylor, J.K., Massare, M., Flyer, D.C., Glenn, G.M., Smith, G.E., Frieman, M.B., 2014. Purified coronavirus spike protein nanoparticles induce coronavirus neutralizing antibodies in mice. Vaccine 32, 3169–3174.
Corman, V.M., Muth, D., Niemeyer, D., Drosten, C., 2018. Hosts and sources of endemic human coronaviruses. Adv. Virus Res. Vol. 100.
Crackower, M.A., Sarao, R., Oudit, G.Y., Yagil, C., Kozieradzki, I., Scanga, S.E., Oliveira-dos-Santos, A.J., da Costa, J., Zhang, L., Pei, Y., 2002. Angiotensin-converting enzyme 2 is an essential regulator of heart function. Nature 417, 822–828.
Daniel, R.M., Cowan, D.A., Morgan, H.W., Curran, M.P., 1982. A correlation between protein thermostability and resistance to proteolysis. Biochem. J 207, 641.
del Sol, A., Fujihashi, H., Amoros, D., Nussinov, R., 2006. Residues crucial for maintaining short paths in network communication mediate signaling in proteins. Molecular systems biology 2.
Der Sarkissian, S., Grobe, J.L., Yuan, L., Narielwala, D.R., Walter, G.A., Katovich, M.J., Raizada, M.K., 2008. Cardiac overexpression of angiotensin converting enzyme 2 protects the heart from ischemia-induced pathophysiology. Hypertension 51, 712–718.
Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., 2000. A novel angiotensin-converting enzyme–related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. Circ. Res. 87, e1–e9.
Enjuanes, L., Almazán, F., Sola, I., Zuñiga, S., 2006. Biochemical aspects of coronavirus replication and virus-host interaction. Annu. Rev. Microbiol. 60, 211–230.
Ge, X.-Y., Li, J.-L., Yang, X.-L., Chmura, A.A., Zhu, G., Epstein, J.H., Mazet, J.K., Hu, B., Zhang, W., Peng, C., 2013. Isolation and characterization of a bat SARS-like coronavirus that uses the ACE2 receptor. Nature 503, 535–538.
Gu, H., Xie, Z., Li, T., Zhang, S., Lai, C., Zhu, P., Wang, K., Han, L., Duan, Y., Zhao, Z., 2016. Angiotensin-converting enzyme 2 inhibits lung injury induced by respiratory syncytial virus. Sci. Rep. 6, 19840.
Guy, J.L., Jackson, R.M., Jensen, H.A., Hooper, N.M., Turner, A.J., 2005. Identification of critical active-site residues in angiotensin-converting enzyme-2 (ACE2) by site-directed mutagenesis. FEBS J. 272, 3512–3520.
Haschke, M., Schuster, M., Poglitsch, M., Loibner, H., Salzberg, M., Bruggisser, M., Penninger, J., Krähenbühl, S., 2013. Pharmacokinetics and pharmacodynamics of recombinant human angiotensin-converting enzyme 2 in healthy human subjects. Clin. Pharmacokinet. 52, 783–792.
Hubbard, S.J., 1998. The structural aspects of limited proteolysis of native proteins. Biochim. Biophys. Acta 1382, 191–206.
Huentelman, M.J., Grobe, J.L., Vazquez, J., Stewart, J.M., Mecca, A.P., Katovich, M.J., Ferrario, C.M., Raizada, M.K., 2005. Protection from angiotensin II-induced cardiac hypertrophy and fibrosis by systemic lentiviral delivery of ACE2 in rats. Exp. Physiol. 90, 783–790.
Hussack, G., Hirama, T., Ding, W., MacKenzie, R., Tanha, J., 2011. Engineered single-domain antibodies with high protease resistance and thermal stability. PLoS ONE 6, e28218.
Imai, Y., Kuba, K., Rao, S., Huan, Y., Guo, F., Guan, B., Yang, P., Sarao, R., Wada, T., Leong-Poi, H., 2005. Angiotensin-converting enzyme 2 protects from severe acute lung failure. Nature 436, 112–116.
Khalili, Saeed et al., 2017a. Structural analyses of the interactions between the thyme active ingredients and human serum albumin. Turkish Journal of Biochemistry 42 (4), 459–467.
Khalili, Saeed et al., 2017b. In silico Prediction and in vitro Verification of a Novel Multi-Epitope Antigen for HBV Detection. Molecular Genetics, Microbiology and Virology 32 (4), 230–240.
Khan, A., Benthin, C., Zeno, B., Albertson, T.E., Boyd, J., Christie, J.D., Hall, R., Poirier, G., Ronco, J.J., Tidswell, M., 2017. A pilot clinical trial of recombinant human angiotensin-converting enzyme 2 in acute respiratory distress syndrome. Crit. Care 21, 234.
Lei, C., Fu, W., Qian, K., Li, T., Zhang, S., Ding, M., Hu, S., 2020. Potent neutralization of 2019 novel coronavirus by recombinant ACE2-Ig. BioRxiv.
Molina, R., Oliva, B., Fernandez-Fuentes, N., 2020. A collection of designed peptides to target SARS-Cov-2–ACE2 interaction: PepI-Covid19 database. bioRxiv.
Monteil, V., Kwon, H., Prado, P., Hagelkrüys, A., Wimmer, R. A., Stahl, M., Leopoldi, A., Garreta, E., del Pozo, C. H., Prosper, F., Inhibition of SARS-CoV-2 infections in engineered human tissues using clinical-grade soluble human ACE2.
Othman, H., Bouslama, Z., Brandenburg, J.-T., Da Rocha, J., Hamdi, Y., Ghedira, K., Srairi-Abid, N., Hazelhurst, S., 2020. Interaction of the spike protein RBD from SARS-CoV-2 with ACE2: similarity with SARS-CoV, hot-spot analysis and effect of the receptor polymorphism. Biochem. Biophys. Res. Commun.
Pang, J., Wang, M.X., Ang, I.Y.H., Tan, S.H.X., Lewis, R.F., Chen, J.-I.-P., Gutierrez, R.A., Gwee, S.X.W., Chua, P.E.Y., Yang, Q., 2020. Potential rapid diagnostics, vaccine and therapeutics for 2019 novel Coronavirus (2019-ncoV): a systematic review. J. Clin. Med. 9, 623.
Parsell, D., Sauer, R., 1989. The structural stability of a protein is an important determinant of its proteolytic susceptibility in Escherichia coli. J. Biol. Chem. 264, 7590–7595.
Payandeh, Z., Rajabibazl, M., Mortazavi, Y., Rahimpour, A., Taromchi, A.H., 2018. Ofatumumab monoclonal antibody affinity maturation through in silico modeling. Iranian Biomed. J. 22, 180.
Payandeh, Z., Rajabibazl, M., Mortazavi, Y., Rahimpour, A., Taromchi, A.H., Dastmalchi, S., 2019. Affinity maturation and characterization of the ofatumumab monoclonal antibody. J. Cell. Biochem. 120, 940–950.
Perlman, S., Netland, J., 2009. Coronaviruses post-SARS: update on replication and pathogenesis. Nat. Rev. Microbiol. 7, 439–450.
Piovesan, D., Minervini, G., Tosatto, S.C., 2016. The RING 2.0 web server for high quality residue interaction networks. Nucl. Acids Res. 44, W367–W374.
Prompetchara, E., Ketloy, C., Palaga, T., 2020. Immune responses in COVID-19 and potential vaccines: lessons learned from SARS and MERS epidemic. Asian Pac. J. Allergy Immunol.
Reetz, M.T., Carballeira, J.D., Vogel, A., 2006. Iterative saturation mutagenesis on the basis of B factors as a strategy for increasing protein thermostability. Angew. Chem. Int. Ed. 45, 7745–7751.
Rentzsch, B., Todiras, M., Iliescu, R., Popova, E., Campos, L.A., Oliveira, M.L., Baltatu, O.C., Santos, R.A., Bader, M., 2008. Transgenic angiotensin-converting enzyme 2

overexpression in vessels of SHRSP rats reduces blood pressure and improves endothelial function. Hypertension 52, 967–973.
Rodrigues, C.H., Myung, Y., Pires, D.E., Ascher, D.B., 2019. mCSM-PPI2: predicting the effects of mutations on protein–protein interactions. Nucl. Acids Res. 47, W338–W344.
Sarath, G., De La Motte, R., Wagner, F., 1989. Proteolytic enzymes: a practical approach. Oxford UK 25.
Sefid, F., Payandeh, Z., Azamirad, G., Abdolhamidi, R., Rasooli, I., 2019. In silico engineering towards enhancement of bap–VHH monoclonal antibody binding affinity. Int. J. Pept. Res. Ther. 25, 273–287.
Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., Ideker, T., 2003. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 13, 2498–2504.
Tipnis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G., Turner, A.J., 2000. A human homolog of angiotensin-converting enzyme cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. 275, 33238–33243.
van Boheemen, S., de Graaf, M., Lauber, C., Bestebroer, T.M., Raj, V.S., Zaki, A.M., Osterhaus, A.D., Haagmans, B.L., Gorbalenya, A.E., Snijder, E.J., 2012. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. MBio 3, e00473–e512.
Van Zundert, G., Rodrigues, J., Trellet, M., Schmitz, C., Kastritis, P., Karaca, E., Melquiond, A., van Dijk, M., De Vries, S., Bonvin, A., 2016. The HADDOCK2. 2 web server: user-friendly integrative modeling of biomolecular complexes. J. Mol. Biol. 428, 720–725.
Wong, D.W., Oudit, G.Y., Reich, H., Kassiri, Z., Zhou, J., Liu, Q.C., Backx, P.H., Penninger, J.M., Herzenberg, A.M., Scholey, J.W., 2007. Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. Am. J. Pathol. 171, 438–451.
Wu, A., Peng, Y., Huang, B., Ding, X., Wang, X., Niu, P., Meng, J., Zhu, Z., Zhang, Z., Wang, J., 2020. Genome composition and divergence of the novel coronavirus (2019-nCoV) originating in China. Cell Host Microbe.
Wysocki, J., Ye, M., Rodriguez, E., González-Pacheco, F.R., Barrios, C., Evora, K., Schuster, M., Loibner, H., Brosnihan, K.B., Ferrario, C.M., 2010. Targeting the degradation of angiotensin II with recombinant angiotensin-converting enzyme 2: prevention of angiotensin II–dependent hypertension. Hypertension 55, 90–98.
Xie, Y., An, J., Yang, G., Wu, G., Zhang, Y., Cui, L., Feng, Y., 2014. Enhanced enzyme kinetic stability by increasing rigidity within the active site. J. Biol. Chem. 289, 7994–8006.
Xu, J., Song, J., Yan, F., Chu, H., Luo, J., Zhao, Y., Cheng, X., Luo, G., Zheng, Q., Wei, J., 2009. Improving GPX activity of selenium-containing human single-chain Fv antibody by site-directed mutation based on the structural analysis. J. Mol. Recognit. 22, 293–300.
Yan, R., Zhang, Y., Li, Y., Xia, L., Guo, Y., Zhou, Q., 2020. Structural basis for the recognition of the SARS-CoV-2 by full-length human ACE2. Science.
Zaman, R., Islam, R.A., Ibnat, N., Othman, I., Zaini, A., Lee, C.Y., Chowdhury, E.H., 2019. Current strategies in extending half-lives of therapeutic proteins. J. Control. Release.
Zhang, H., Kang, Z., Gong, H., Xu, D., Wang, J., Li, Z., Cui, X., Xiao, J., Meng, T., Zhou, W., 2020. The digestive system is a potential route of 2019-nCov infection: a bioinformatics analysis based on single-cell transcriptomes. BioRxiv.
Zhao, Y., Zhao, Z., Wang, Y., Zhou, Y., Ma, Y., Zuo, W., 2020. Single-cell RNA expression profiling of ACE2, the putative receptor of Wuhan 2019-nCov. BioRxiv.
Zhong, J., Guo, D., Chen, C.B., Wang, W., Schuster, M., Loibner, H., Penninger, J.M., Scholey, J.W., Kassiri, Z., Oudit, G.Y., 2011. Prevention of angiotensin II–mediated renal oxidative stress, inflammation, and fibrosis by angiotensin-converting enzyme 2. Hypertension 57, 314–322.
Zou, Z., Yan, Y., Shu, Y., Gao, R., Sun, Y., Li, X., Ju, X., Liang, Z., Liu, Q., Zhao, Y., 2014. Angiotensin-converting enzyme 2 protects from lethal avian influenza A H5N1 infections. Nat. Commun. 5, 1–7.