



Tuftsins: A Natural Molecule Against SARS-CoV-2 Infection

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Coronavirus disease 2019 (COVID-19) continuously progresses despite the application of a variety of vaccines. Therefore, it is still imperative to find effective ways for treating COVID-19. Recent studies indicate that NRP1, an important receptor of the natural peptide tuftsins (released from IgG), facilitates SARS-CoV-2 infection. Here, we found 91 overlapping genes between tuftsins targets and COVID-19-associated genes. We have demonstrated that tuftsins could also target ACE2 and exert some immune-related functions. Molecular docking results revealed that tuftsins could combine with ACE2 and NRP1 in stable structures, and their interacted regions cover the binding surfaces of S1-protein with the two receptors. Using surface plasmon resonance (SPR) analysis, we confirmed that tuftsins can bind ACE2 and NRP1 directly. Importantly, using SPR-based competition assay we have shown here that tuftsins effectively prevented the binding of SARS-CoV-2 S1-protein to ACE2. Collectively, these data suggest that tuftsins is an attractive therapeutic candidate against COVID-19 and can be considered for translational as well as clinical studies.

Keywords: tuftsins, SARS-CoV-2, ACE2, NRP1, natural peptide

INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) results in high morbidity and mortality (Zhu et al., 2020; Dai and Gao, 2021). It is known that the spike (S) protein binding to angiotensin-converting enzyme 2 (ACE2) is the core mechanism of SARS-CoV-2 infecting host cells. COVID-19 vaccines have been approved for human use in most countries in the world. However, the concentration of neutralizing antibodies induced by vaccines still needs to be investigated in humans. Another effective way is the neutralizing monoclonal antibodies (mAbs) at present (Renn et al., 2020). However, the production of safe and effective mAbs is complicated, and the duration of effective protection remains to be determined (Su et al., 2021; Taylor et al., 2021). Moreover, continuous mutations of SARS-CoV-2 during the pandemic may cause coronavirus to escape from antibody recognition and reduce the neutralizing activity of mAbs (Du et al., 2021). Therefore, the discovery of a broad-spectrum and effective method for preventing and treating COVID-19 is crucial.

Along with ACE2, neuropilin 1 (NRP1) is another important host factor for SARS-CoV-2 infection (Daly et al., 2020). It has been reported that NRP1 facilitates the entry of SARS-CoV-2 into cells in the presence of ACE2 (Cantuti-Castelvetri et al., 2020). It is worth noting that NRP1 is an important receptor of tuftsins (Vander Kooi et al., 2007; von Wronski et al., 2006). Tuftsins, a natural phagocytosis-stimulating peptide, was found by Najjar and Nishioka (1970). Tuftsins is

released from the Fc fragment of IgG by enzyme cleavage of an endocarboxy-peptidase in the spleen and a leukokininase on the outer membrane of neutrophilic leukocytes (Najjar and Nishioka, 1970; Corazza et al., 1991). Furthermore, tuftsin is a tetrapeptide that consists of Thr-Lys-Pro-Arg, located at amino acid residues 289 to 292 of the heavy chain of IgG (Kozlovskaya et al., 2003). Tuftsin has a broad spectrum of activities mainly associated with immune functions and exerts effects on phagocytic cells, especially macrophages. The function of tuftsin includes cell phagocytosis, motility, immunogenic response, and bactericidal and tumoricidal activities (Najjar, 1983; Fridkin and Najjar, 1989). Spleen is the only organ that produces tuftsin in mammals, and splenic defect caused by spleen injury or diseases often reduces the activity of tuftsin (Chu et al., 2015; Chapman et al., 2022). And it is reported that tuftsin activity is significantly lower in patients with AIDS, cirrhosis, intestinal failure and some infectious diseases than the normal individuals (Corazza et al., 1991; Zoli et al., 1998; Trevisani et al., 2002). Moreover, it was demonstrated that tuftsin has stability and low toxicity *in vitro* and *in vivo* (Amoscato et al., 1983; Fridkin and Najjar, 1989; Siemion and Kluczyk, 1999). It is worth noting that the mutant sequence of tuftsin turns to inactive or inhibitory analogs (Blumenstein et al., 1979; Najjar, 1981). As a natural immune stimulating peptide, tuftsin is an attractive candidate for immunotherapy. We hypothesized that tuftsin could inhibit SARS-CoV-2 infection by interacting with human receptors of coronavirus. The experiments were subsequently performed to test our concepts. In this study, the targets of tuftsin were collected from articles and bioinformatics prediction and the disease-related genes of COVID-19 were mined from database. Next, we selected the intersected proteins for further analyses. According to the theoretical basis and the enrichment results that tuftsin could target ACE2 and NRP1 in the COVID-19 pathway, the possible interactions of tuftsin with the two SARS-CoV-2 entry receptors were determined by molecular docking analyses. Tuftsin is further confirmed to bind the two SARS-CoV-2 entry receptors directly and significantly impair the binding of viral S1-protein to its human ACE2 receptor by using the surface plasmon resonance analyses and competition assays, shedding light on tuftsin-based new drug discovery against COVID-19.

MATERIALS AND METHODS

Compound Profiling and Disease-Related Gene Identification

The structure of tuftsin was found in PubChem (<https://pubchem.ncbi.nlm.nih.gov/>). The 3D structure of tuftsin was built using Chem3D. Afterward, the target proteins corresponding to tuftsin screened from the PharmMapper database and PubMed database were standardized in UniProt (<http://www.uniprot.org/>). Finally, Cytoscape 3.8.2 was used to determine the drug-target network. COVID-19-

related genes were mined from the GeneCards database. All of the disease gene targets were normalized with R software using the Bioconductor package when redundancy was deleted (Yu et al., 2012).

Network Establishment

Screening for drug-disease crossover genes was performed. Based on previous steps, two sets of target lists were prepared: drug targets and disease-related genes. The crossover genes were filtered with R software using the Venn Diagram package. The STRING 11.5 database (<http://string-db.org/>) was used to analyse the intersecting protein-protein interactions (PPIs), and the common targets were counted with R software.

Enrichment Analysis

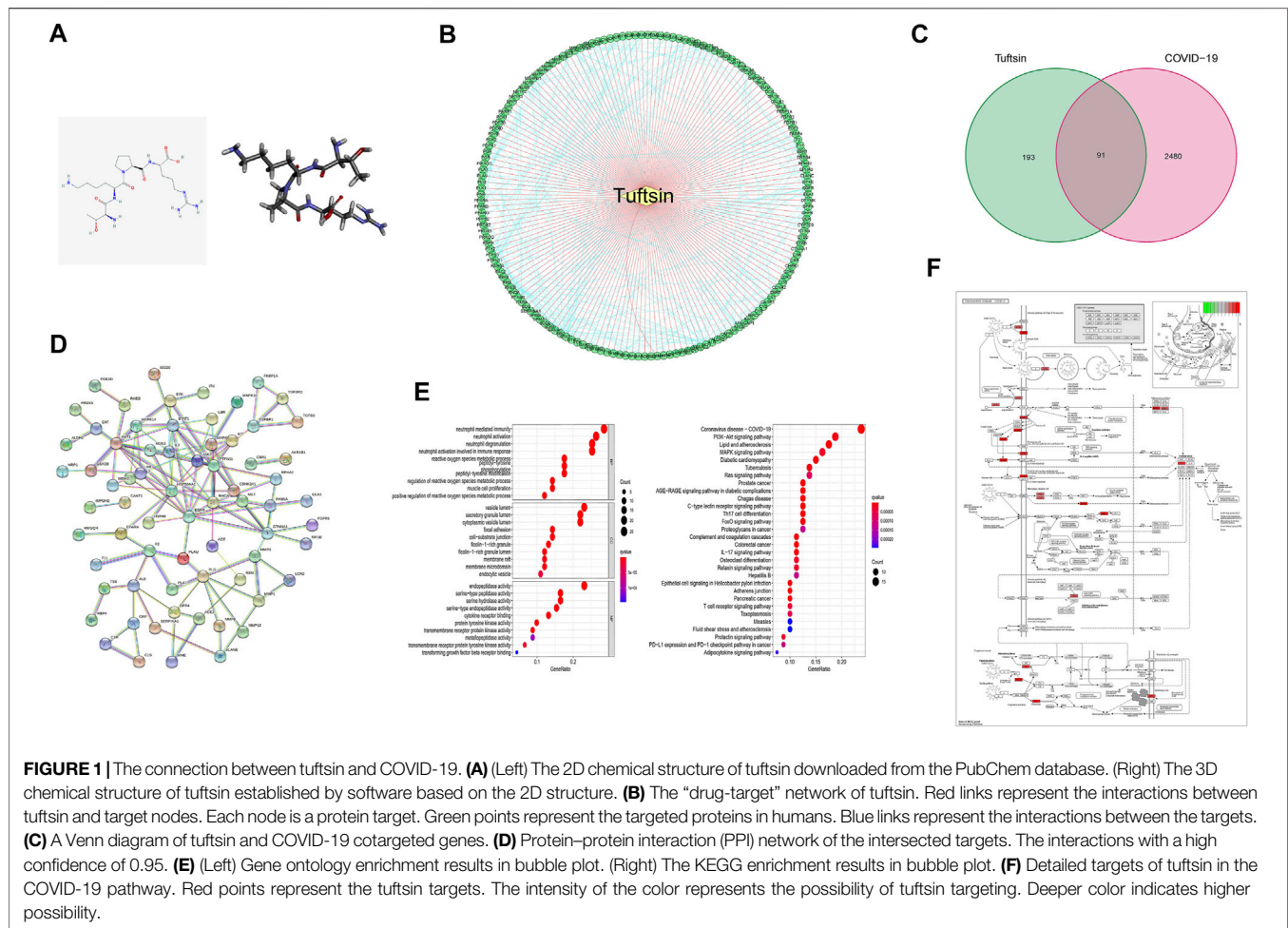
The proteins with overlapping expression patterns were evaluated by bioinformatics annotation with R software using the Bioconductor package, including a panther classification system (<http://www.pantherdb.org/>), a gene ontology (GO) annotation database website (<http://www.geneontology.org/>), and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (<http://www.genome.jp/kegg/>). A $p < 0.05$ was considered statistically significant.

Molecular Docking Analysis

The flexible docking process between tuftsin and target proteins was conducted by software Discovery Studio 2021 (DS). Briefly, the crystallographic structures of human ACE2 (PDB ID: 1R42) and human NRP1 (PDB ID: 2QQ1) with high resolution were prepared using the Prepare Protein and Minimization module of DS. The active binding site of each protein was defined based on the most representative features of the SARS-CoV-2 interface. Tuftsin was docked into the active binding site of ACE2 and NRP1 using the molecular docking module in DTS.

Surface Plasmon Resonance Analysis

The recombinant human ACE2 protein (Novoprotein, Beijing, China) and recombinant human NRP1 protein were used for surface plasmon resonance (SPR) analysis using a Biacore T200 instrument (Biacore, Uppsala, Sweden). Each target was immobilized onto flow cells in a CM5 sensor chip (GE Healthcare) via the amine-coupling method. Briefly, ACE2 and NRP1 were diluted in 10 mM pH 4.5 acetate to 20 $\mu\text{g}/\text{ml}$. Then, the protein solutions were injected individually on the carboxyl-modified sensor surface to form amine bonds. Both ACE2 and NRP1 immobilized levels were approximately 10,000 RU. Binding analyses were carried out at 25°C and a flow rate of 30 $\mu\text{l}/\text{min}$. Tuftsin diluted in running buffer (1 \times PBS, 0.05% Tween 20 and 5% dimethyl sulfoxide, pH 7.4) was run over each target at gradient concentrations. An empty flow cell without any immobilized protein was used as a deducted reference. The binding curves were analysed using the steady state affinity model supplied with Biacore Evaluation Software (GE Healthcare).



Competition Binding Experiment

For the competition binding experiment, the SARS-CoV-2 S1 protein was immobilized on the CM5 sensor chip via the amine-coupling method. 5 nM ACE2 was injected for negative control. Tuftsin was diluted into a series of solutions with gradient concentrations and fixed with 5 nM ACE2, and then the solutions were injected into the chip. The blocking efficacy was evaluated by comparison of response units with and without tuftsin incubation.

Statistical Analysis

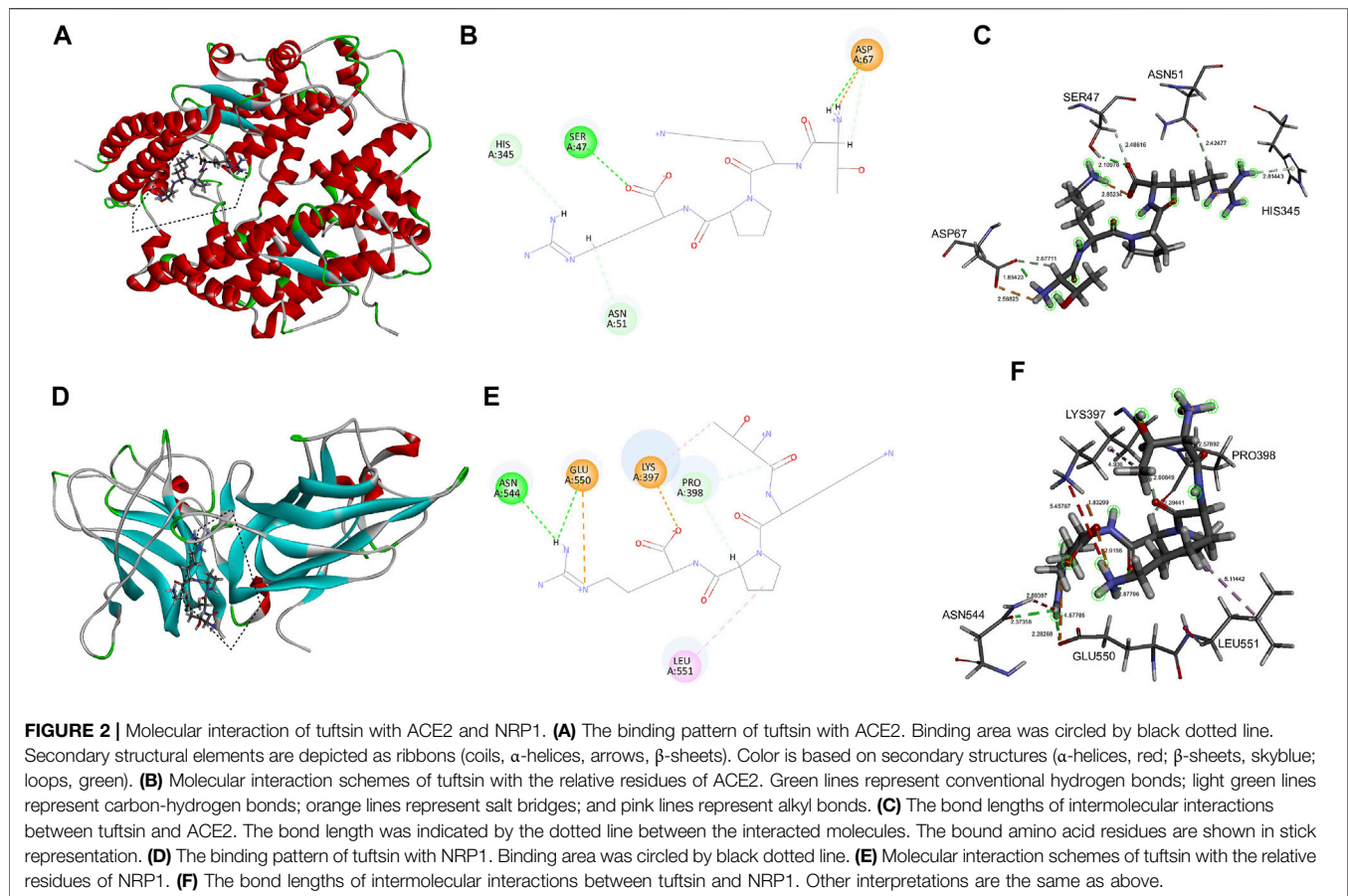
Partial results were analysed using chi-square test with SPSS software and R 4.1.0.

RESULTS

Bioinformatics Analyses Revealed the Connection Between Tuftsin and COVID-19

The 2D structure of tuftsin was obtained from the PubChem database (Compound CID: 156080), and the most stable 3D structure was built based on the 2D structure through a molecular simulation assay (Figure 1A). In addition to the

reported receptors of tuftsin, the potential targets of tuftsin in human body were predicted through the PharmMapper database. As a result, 284 targets of tuftsin were selected (Figure 1B and Supplementary Data S1), and 2,572 disease-associated genes of COVID-19 were excavated from the GeneCards database (Supplementary Data S2). It is surprised to find that there exists 91 intersecting proteins between tuftsin targets and COVID-19-associated gene-coded proteins through intersection analysis, and the overlapping proteins account for nearly one-third of tuftsin targets (Figure 1C). The protein–protein interaction network of the overlapping proteins was established, and the result indicated that JAK2, STAT1 and AKT1 are core molecules in the network (Figure 1D). The 91 intersecting genes were further studied by enrichment analysis. GO and KEGG annotation revealed that the expressed tuftsin-COVID-19 crossover proteins are mainly associated with immune functions such as neutrophil activation, neutrophil-mediated immunity and cytokine receptor binding, and the COVID-19 pathway is most significantly enriched. In addition, many target genes were strongly associated with some immune pathways, such as Th17 cell differentiation, the IL-17 signaling pathway and the immune checkpoint pathway (Figure 1E). In the COVID-19 pathway, the SARS-CoV-2 receptors ACE2 and NRP1, some immune molecules such as IL-2, STAT1, and some

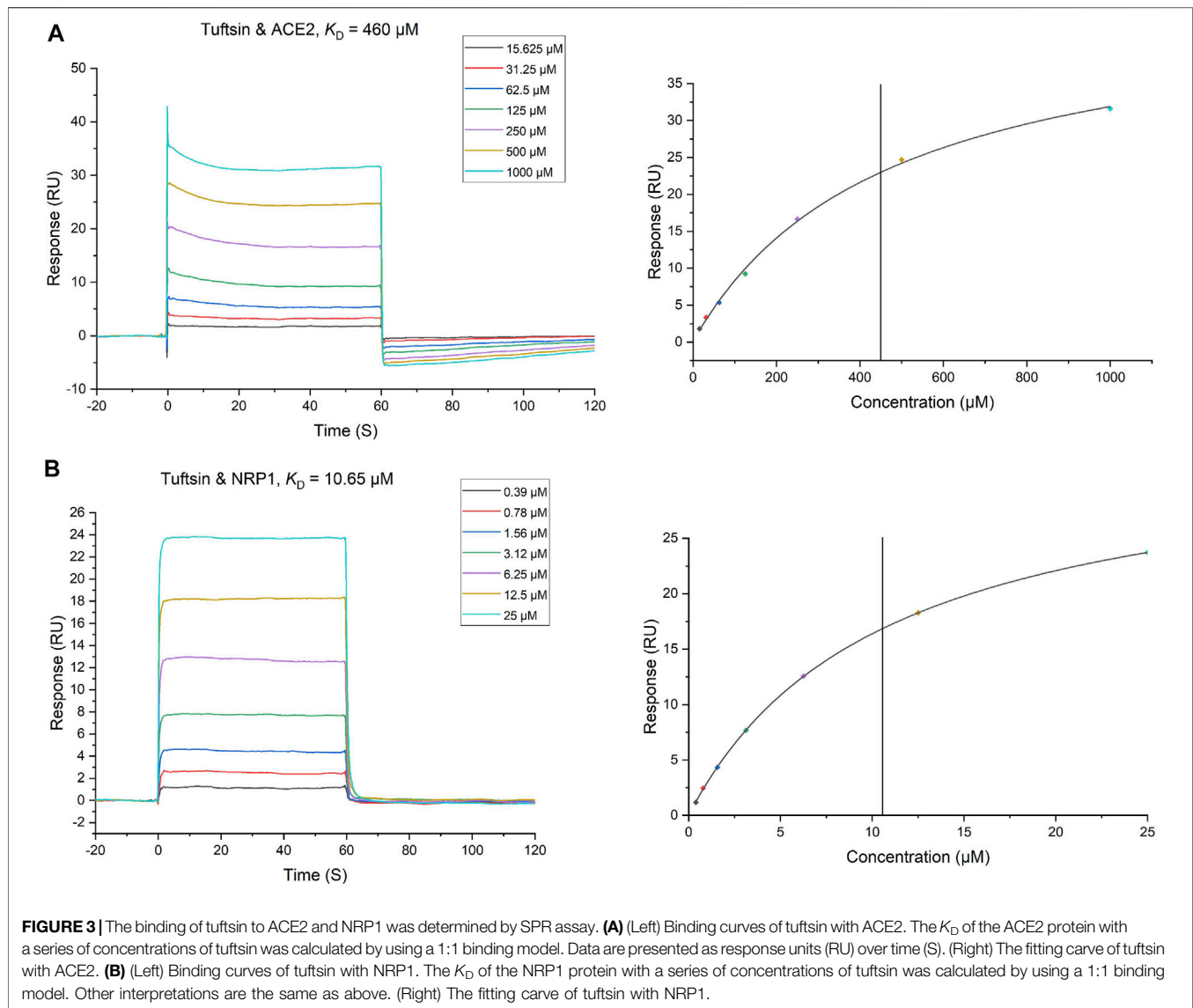


complement molecules are the targets of tuftsin (Figure 1F). These results suggest that tuftsin is a promising candidate against COVID-19 owing to its multifaceted pharmacological activities.

The Interaction of Tuftsin With ACE2 and NRP1 Analysed by Molecular Docking

Having proved by the above pharmacology analysis of tuftsin, it is possible that ACE2 is a target of tuftsin. Therefore, the potential binding sites and binding affinity of tuftsin with the SARS-CoV-2 receptors ACE2 and NRP1 were further determined by molecular docking analysis. The interaction interface of SARS-CoV-2 S1 receptor-binding domain (RBD) with ACE2 was defined as the active sites of ACE2. These interface sites in ACE2 include Q24, M82, N330, and R393 residues, which are mainly located in the N-terminal peptidase domain of ACE2 (Lan et al., 2020). The docking region of ACE2 is a sphere containing the defined ACE2 active sites (Supplementary Figure S1A). The docking results showed that the affinity of tuftsin with ACE2 was -6.9 kcal/mol, demonstrating that they could combine spontaneously (Figure 2A). Furthermore, tuftsin could form strong hydrogen bonds to Ser47 and Asp67, hydrogen bonds to His345, Asp67 and Asn51, and salt bridges to Asp67 residues of ACE2 (Figure 2B). In addition, the bond lengths of the interacted sites are that 2.110 Å of Ser47, 1.854 Å of Asp67, 2.814 Å of His345 and 2.425 Å of Asn51

(Figure 2C). It is worth mentioning that the binding sites were adjacent to the interactional sites of S1-RBD and ACE2 (Lan et al., 2020), indicating that tuftsin could inhibit S1 binding to ACE2 by covering their binding sites. It is reported that the extracellular b1b2 domain of NRP1 combines with S1 CendR peptides (Plein et al., 2014; Daly et al., 2020), therefore the b1b2 domain of NRP1 was prepared for docking. The interactional sites of S1-RBD with b1b2 proteins were defined as the active sites of NRP1 b1b2 domain, including D320, E348, Y353 residues and so on (Daly et al., 2020). Subsequently, the docking region was a sphere containing these defined active sites in the b1b2 domain of NRP1 (Supplementary Figure S1B). The docking results showed that tuftsin and NRP1 b1b2 domain have a high binding affinity of -8.1 kcal/mol. In addition, tuftsin fits solidly into a binding pocket on NRP1 b1b2 domain (Figure 2D). Furthermore, tuftsin could form a salt bridge to Lys397 and a carbon-hydrogen bond to Pro398 residues of NRP1 (Figure 2E), which are near the interactional sites of S1-RBD with NRP1 b1b2 domain. The shortest bond lengths of these interacted sites are that 1.833 Å of Lys397 and 2.394 Å of Pro398 (Figure 2F). Moreover, the binding region of tuftsin and NRP1 overlapped with the binding area of NRP1 with S1-RBD in space. Collectively, these results demonstrated that tuftsin could bind ACE2 and NRP1, and inhibit the SARS-CoV-2 S1 binding to ACE2 and NRP1 by covering their interactional sites.



Tuftsin Binds ACE2 and NPR1 Directly, as Confirmed by Surface Plasmon Resonance (SPR) Analyses

The interactions of tuftsin with ACE2 and NRP1 were further evaluated by real-time biomolecular interaction analysis with SPR. The steady state affinity of the binding reaction were determined by injecting different concentrations of tuftsin over recombinant human ACE2 immobilized on one half of the chip surface, and over recombinant human NRP1 immobilized on another half of the chip surface. The results showed that tuftsin can bind ACE2 with an equilibrium dissociation constant (K_D) of 460 $\mu\text{mol/L}$ (Figure 3A). Moreover, the K_D fitting curve of tuftsin with ACE2 is gradually stable when the concentrations of tuftsin increased gradually, indicating that the interaction of tuftsin and ACE2 is specific (Figure 3A). Tuftsin can also bind NRP1 strongly with a higher binding affinity with K_D value calculated as 10.65 $\mu\text{mol/L}$ (Figure 3B). The K_D fitting curve of tuftsin with NRP1 is

gradually stable when the concentrations of tuftsin increased gradually, conforming to the characteristic of specific binding (Figure 3B). As SPR is the gold standard for detecting drug-target interactions, these results demonstrate that tuftsin binds ACE2 and NRP1 directly and specifically with ideal affinities, and accord with the accuracy of the previous results of bioinformatics analyses and molecular docking assays.

Tuftsin Impairs the Binding of SARS-CoV-2 S1 to ACE2

Whether tuftsin could affect the binding of S1 protein with ACE2 was further determined by SPR-based competition assay. The binding affinity of the S1 protein with ACE2 was firstly determined by SPR assay, which unsurprisingly showed a high affinity. A suitable concentration ACE2 solution was injected over the immobilized SARS-CoV-2 S1 protein as a control. A series of gradient concentrations of tuftsin solutions containing

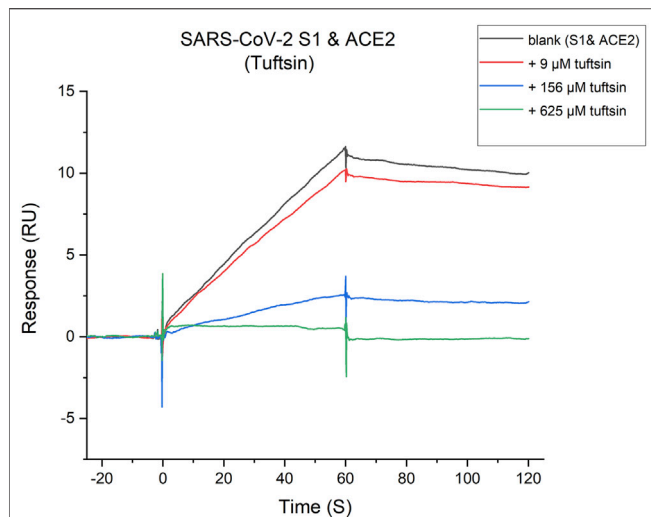


FIGURE 4 | Tuftsin inhibits the SARS-CoV-2 S1 binding to ACE2. The binding activity of SARS-CoV-2 S1-protein to ACE2 in the presence of increasing concentrations of tuftsin. The gray “blank” line represents the binding response of S1-protein with ACE2 alone for negative control. Then, multiple concentrations of tuftsin were added based on the “blank” for comparison. Intensive concentrations of tuftsin showed enhanced inhibitory effects.

equal concentrations of ACE2 were injected over the immobilized SARS-CoV-2 S1 protein for comparison. As a result, 9 $\mu\text{mol/L}$ tuftsin has a mild inhibitory effect. It is worth noting that the addition of 156 $\mu\text{mol/L}$ tuftsin significantly attenuates the response signal about two-thirds compared to that of ACE2 alone over the immobilized S1. Notably, a substantial decrease in the response signal was observed with increasing concentrations of tuftsin. The response signal is declined approximately to zero when the added concentration of tuftsin was 625 $\mu\text{mol/L}$. This result indicates that the interaction between S1 and ACE2 is completely abrogated in the presence of 625 $\mu\text{mol/L}$ tuftsin (Figure 4). The experiment was repeated three times independently. In conclusion, the competition binding experiment revealed that tuftsin effectively impairs the binding of SARS-CoV-2 S1 to ACE2 in a dose-dependent manner.

DISCUSSION

Vaccination is widely used to prevent COVID-19 at present (Forni et al., 2021). The costs of production of vaccines and neutralizing antibodies for COVID-19 are relatively high, and it is reported that the effectiveness of the SARS-CoV-2 vaccines has declined significantly since 2021 (Cohn et al., 2021). Nonetheless, some collaborative methods can be applied to work with vaccines. It is demonstrated that peptides could be a promising approach to combat COVID-19 (Schütz et al., 2020). In this study, a human natural peptide holds great potential against SARS-CoV-2 virus is reported the first time. It is found that tuftsin could target many disease-related proteins of COVID-19 by intersection analysis. The COVID-19 pathway and some immune-related functions are highly relevant to these intersecting targets indicated by enrichment analysis. ACE2

and NRP1 are outstanding in these intersecting targets. ACE2 is a major receptor of SARS coronavirus’ spike (S) protein, and facilitates the subsequent membrane fusion (Jackson et al., 2022). NRP1 was confirmed to bind with S1 CendR motif and the furin-cleaved substrates, and significantly potentiates SARS-CoV-2 infectivity (Cantuti-Castelvetri et al., 2020; Daly et al., 2020). Subsequently, the combined constructions and binding affinity of tuftsin with ACE2 and NRP1 were determined by molecular docking analysis. As a result, tuftsin could combine with ACE2 and NRP1 in stable structures, with binding free energy of -6.9 kcal/mol and -8.1 kcal/mol, respectively. These interacted molecules are mainly linked with hydrogen bonds, carbon-hydrogen bonds and salt bridges. More importantly, it was found that the bond energy of these binding forces are high with low bond lengths ranging from 1.8 Å to 2.9 Å, indicating the complexes of tuftsin with ACE2 and NRP1 are stable. The binding sites of tuftsin with ACE2 and NRP1 were next to the binding sites of SARS-CoV-2 S1-RBD with the two receptors, such as Ser47, His345, Asp67 residues of ACE2 and Lys397, Pro398 residues of NRP1. These results indicate tuftsin could inhibit SARS-CoV-2 binding to ACE2 and NRP1 by covering their interacted regions competitively.

To further confirm the inhibiting properties of tuftsin, we conducted the surface plasmon resonance (SPR) analysis. Tuftsin shows a moderate binding affinity to ACE2 with a K_D at 460 $\mu\text{mol/L}$, and shows an even higher binding affinity to NRP1 with a K_D value of 10.65 $\mu\text{mol/L}$, which are actually very good values for an unmodified natural peptide. More importantly, it was found that the viral S1 protein binding to ACE2 receptor was almost abrogated in the presence of tuftsin at 625 μM . These indicate tuftsin plays important roles in inhibiting SARS-CoV-2 by impairing the binding of S1 protein with ACE2 significantly, which is an important way of preventing virus infection. Tuftsin as a natural and unmodified tetrapeptide which exists in human bodies, originates from a special fraction of the parent carrier IgG (Siebert et al., 2017). Therefore, tuftsin may have lower toxicity and side effects than other synthetic and exogenous drugs (Catane et al., 1983). It is reported that approved drugs which main ingredient is tuftsin have satisfactory clinical efficacy at present, in addition, tuftsin incorporates specific molecules such as tuftsin-phosphorylcholine and tuftsin-bearing liposomes, showing greater immunogenicity and specific targeting (Shakya et al., 2012; Croci et al., 2019; Khan, 2021). Tuftsin can be produced on a large scale at low cost (Siebert et al., 2017), which allows tuftsin to be widely applied for against COVID-19. These It has been reported that the amount of IgG induced by vaccines is mainly focused on the lower respiratory tract (Krammer, 2020). Therefore, tuftsin could be applied as spray form to protect the upper respiratory which suffers coronavirus infection. It is worth noting that there exists a group of asymptomatic carriers or mild symptoms’ individuals during the pandemic (Schijns and Lavelle, 2020; Boyton and Altmann, 2021). As tuftsin has anti-infection and anti-inflammatory properties (Blok-Perkowska et al., 1984; Thompson et al., 2018), it is possible that asymptomatic individuals have higher content of tuftsin.

In conclusion, a natural peptide, tuftsin, shows high potency of treating COVID-19 along with the immunomodulation effect, and exhibits satisfactory ACE2 and NRP1-targeting ability. More importantly, tuftsin is demonstrated to impair the interaction

between S1-protein and ACE2. This study provided direct biophysics evidences associated with molecular mechanisms of possible clinical use of tuftsin for prevention and treatment of COVID-19. In addition, this study demonstrated the utility of human natural peptide has good potency in treating COVID-19. Therefore, tuftsin is a promising anti-SARS-CoV-2 drug candidate.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

YW, JH, and MC conceptualized and designed this study. JH performed the bioinformatic analysis. JH and ZW performed the molecular docking. JH performed the SPR experiments with assistance from JW and JH processed the data. JH drafted, edited the manuscript. YW, CM, and YL administered the

project. All authors have read and agreed to the published version of this manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmolb.2022.859162/full#supplementary-material>

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