Kobophenol A Inhibits Binding of Host ACE2 Receptor with Spike RBD Domain of SARS-CoV-2, a Lead Compound for Blocking COVID-19

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ABSTRACT: In the search for inhibitors of COVID-19, we have targeted the interaction between the human angiotensin-converting enzyme 2 (ACE2) receptor and the spike receptor binding domain (S1-RBD) of SARS-CoV-2. Virtual screening of a library of natural compounds identified Kobophenol A as a potential inhibitor. Kobophenol A was then found to block the interaction between the ACE2 receptor and S1-RBD in vitro with an IC50 of 1.81 ± 0.04 μM and inhibit SARS-CoV-2 viral infection in cells with an EC50 of 71.6 μM. Blind docking calculations identified two potential binding sites, and molecular dynamics simulations predicted binding free energies of −19.0 ± 4.3 and −24.9 ± 6.9 kcal/mol for Kobophenol A to the spike/ACE2 interface and the ACE2 hydrophobic pocket, respectively. In summary, Kobophenol A, identified through docking studies, is the first compound that inhibits SARS-CoV-2 binding to cells through blocking S1-RBD to the host ACE2 receptor and thus may serve as a good lead compound against COVID-19.

The new coronavirus, Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2), has quickly spread as the COVID-19 global pandemic. As of December 2020, there were over 68 million active cases and over 1.57 million deaths worldwide. The lack of a specific treatment combined with a large number of associated deaths has raised a global effort to find potential inhibitors of key viral processes. However, developing new drugs is a lengthy process. Natural products possess tremendous structural range and unique chemical diversity, and they continue to serve as excellent starting points for inspiring new drug discovery. The history of the modern pharmaceutical industry includes several examples of how natural products profoundly inspired drug discovery. With the current technological advances, natural products remain potentially transformative drugs for many health conditions. The growing understanding of efficient antiviral drug development has led to the exploration of natural products as an important tactic for identifying effective COVID-19 treatments.

Multiple research efforts have focused upon inhibiting the SARS-CoV-2 main protease (Mpro), also called as 3CLpro, as it plays a key role in mediating viral replication and transcription, specifically as there are no human proteases with a homologue of Mpro. The focus of this study however is on another attractive target, the spike proteins which are involved in the entry of the virion to the host cell.

A large number of glycosylated spike proteins protrude from the envelope of the SARS-CoV-2 virion, and they consist of two subunits S1 and S2. The S1 subunit is the main interaction domain of spike protein, as it consists of a receptor-binding domain (RBD) that interacts with the cell surface angiotensin-converting enzyme 2 (ACE2) receptor of the host cells. A second subunit, S2, facilitates the fusion of the viral membrane into the host cell membrane. A great advance in drug design was made due to the understanding of SARS-CoV-2 entry into the host cell. Cai and co-workers identified the prefusion and postfusion of conformations of full-length spike protein using cryo-electron microscopy. Four distinct conformational states of spike proteins on SARS-CoV-2 were shown by Lu and co-workers. This study shows the movement of activation spike protein from the ground state to the activated state via an intermediate by hACE2 and suggested proteolytic processing of spike accelerates hACE2 dependent activation using single-molecule fluorescence resonance energy transfer (smFRET) imaging. Interestingly, a highly flexible receptor-binding
domain (RBD) of the S-protein was identified to be locked in either “down” or “up” state conformations. These findings suggest the importance of conformation control via rational design for spike protein and can be applied to engineer vaccine against SARS-CoV-2 S proteins.

The spike proteins are crucial for the viral life cycle, and it is believed that they serve as a major target to block the viral entry into the host cells. Recent reports identified that a patient-produced-antibody mediated the blockage of the interaction between ACE2 receptor and the receptor-binding domain of SARS-CoV-2, supporting this interaction as a valid target. To date, there have been a few in silico attempts to find small molecule inhibitors of the interaction between ACE2 and spike S1-RBD. Cao et al., de novo designed sequences of a few amino acids. Raghavan et al., reported ACE2 inhibition by Metadichol. Chowdhury et al., reported the hACE2 inhibitory activity of 15 antiviral peptides containing aromatic nonpolar, and polar amino acids along with their SAR studies. Gutierrez-Villagomez et al. reported the ACE2 inhibitory activity of alkamides and piperamides. Mehranfar and Izadyar et al., designed a sequence of a few amino acids and functionalized with gold nanoparticles as antivirals to prevent SARS-CoV-2 entry into host cells. However, none of these reports have led to actual in vitro potent inhibitors.

In this study, an in silico drug design strategy was implemented to examine readily available natural-based compounds, followed by in vitro screening and molecular dynamics studies. The conformational changes and predictions of potential binding sites within the ACE2 receptor and the spike S1-RBD/ACE2 interface were explored. The combined computational and experimental effort suggests that Kobophenol A may disrupt the interaction between ACE2 and the spike protein of SARS-CoV-2.

The spike protein is responsible for the invasion of the virion into the host cell by binding to the cell surface via the ACE2 receptor. This interaction between the receptor-binding domain located in the S1 subunit of the spike protein (S1-RBD) of SARS-CoV-2 and the cell receptor ACE2 is stabilized by salt bridges and hydrogen bonding. Since there are no native natural ligands or organic inhibitors available for this interaction, the X-ray crystal structure of the SARS-CoV-2 S1-RBD bound to ACE2 (PDB ID: 6M0J) was used for a blind docking procedure that utilized a grid consisting of the entire protein. A library of natural compounds was evaluated for initial binding using AutoDock 4.2.

The docking energies of a 25 natural compound library, including some metabolites, are shown in Table 1; for structures, see Table S2 in the Supporting Information. The docking studies found that Kobophenol A effectively binds to the protein in two different regions: (1) At the ACE2/spike interface by establishing a hydrogen bond with residue Gln325 and having docking energy of −11.15 kcal/mol (Figure 1) and (2) at the hydrophobic pocket of the ACE2 domain, where the Kobophenol A docking energy was computed to be −9.98 kcal/mol and hydrogen bonds with Glu375 and Thr347 were formed (Figure 1B).

These in silico predicted interactions inhibit the binding of the SARS-CoV-2 spike protein with host ACE2, presumably by destabilizing the complex formation. Additionally, three metabolites (M1, M2, and M3) of Kobophenol A were also found to bind at the ACE2/spike interface and ACE2 hydrophobic pocket with relatively high favorable docking energies in comparison to the other natural compounds in the library (Table 1). The docking results suggest that Kobophenol A should be the top target for carrying out further in vitro studies targeting the ACE2/spike RBD binding domains.

After the promising in silico results that predicted Kobophenol A could block the interaction between ACE2 and SARS-CoV-2 S1-RBD, the compound was tested in vitro by an enzyme-linked immunosorbent assay (ELISA) (Figure 2). Increasing concentrations of Kobophenol A were placed together with ACE2 in a 96-well plate coated with S1-RBD. The amount of human ACE2 bound to the spike protein was detected by an anti-human HRP antibody; see Experimental Section for more details and Table S1 in the Supporting Information. Kobophenol A was found to inhibit ACE2 binding to SARS-CoV-2 S1-RBD with an IC50 of 1.81 ± 0.04 μM. This inhibition suggests that Kobophenol A may inhibit the viral entry into the host and serve as a lead compound for anti-SARS-CoV-2 treatment.

The findings were further validated by a phenotypic virus-cell based antiviral assay of Kobophenol A against SARS-CoV-2 in VeroE6-EGFP cells. Cells were infected with virus with or without treatment of Kobophenol A, and the increase in the VeroE6 signal yielded a 50% maximum effective concentration (EC50) value of 71.6 μM. This EC50 is similar to values of other FDA approved drugs Indinavir (EC50 = 59.14 μM) and Favipiravir (EC50 = 61.88 μM) and better than those of Penciclovir (EC50 = 95.96 μM) and Ribavirin (EC50 = 109.50 μM).

| Sl. no. | compd                          | ACE2/spike interface | ACE2 binding pocket |
|--------|--------------------------------|----------------------|---------------------|
| 1      | Kobophenol A                  | −11.15               | −9.98               |
| 2      | Kobophenol A-M1               | −9.57                | −9.32               |
| 3      | Kobophenol A-M2               | −8.2                 | −9.44               |
| 4      | Kobophenol A-M3               | −8.81                | −8.65               |
| 5      | Bisdemethoxycurcumin (BDC)    | −                    | −8.95               |
| 6      | Tetrahydroncurcumin            | −7.61                | −8.84               |
| 7      | Pallidal                       | −8.17                | −8.38               |
| 8      | Chlorogenic acid              | −6.33                | −8.44               |
| 9      | Curcumin                      | −8.42                | −8.13               |
| 10     | Cis Miyabenol C               | −7.47                | −8.29               |
| 11     | Nitroimidazooxazines (ND)     | −5.09                | −8.28               |
| 12     | Oxyresveratrol                | −8.28                | −8.08               |
| 13     | Artrimisin                    | −                    | −8.01               |
| 14     | Resveratrol                   | −8.0                 | −7.38               |
| 15     | Dimethoxycurcumin             | −7.49                | −8.09               |
| 16     | Scirpusin B                   | −5.98                | −7.56               |
| 17     | Ursodeoxycholic acid (UDCA)   | −6.75                | −7.27               |
| 18     | Vasicinone                    | −                    | −7.17               |
| 19     | Bacoside B                    | −4.22                | −7.05               |
| 20     | Carasinol B                   | −6.13                | −6.83               |
| 21     | Entacapone                    | −6.88                | −6.87               |
| 22     | Vasicine                      | 5.73                 | −6.54               |
| 23     | Bacoside A                    | −                    | −6.18               |
| 24     | Mulberrofuran C               | −5.69                | −6.13               |
| 25     | Eugenol                       | −6.0                 | −6.07               |
| 26     | Limonene                      | −                    | −5.88               |
| 27     | Azadiractin                   | −4.27                | −5.35               |
| 28     | Papain                        | −5.18                | −4.51               |

“−” indicates that the compound did not bind at the ACE2/spike interface.
μM). Both the IC$_{50}$ value of Kobophenol A against recombinant 2019-nCOV Spike(RBD)/hFc protein and EC$_{50}$ value in VeroE6-EGFP cells fit the computational predictions that the compound inhibits the binding of S1-RBD of SARS-CoV-2 to the host ACE2 receptor. The cytotoxicity of Kobophenol A to uninfected cells was examined by MTS assays, and no toxicity was detected up to 100 μM (Figure 3); see an additional EC$_{50}$ curve of Kobophenol A in Figure S4 of the Supporting Information.

Molecular dynamics (MD) simulations were performed to examine any prevalent interactions or conformational changes arising from Kobophenol A binding into the two potential sites, i.e., ACE2/spike interface or ACE2 hydrophobic pocket. Root-mean-square deviations (RMSDs) and root-mean-square fluctuations (RMSFs) of the backbone protein atoms within the ACE2 and S1-RBD regions were examined (Figures 4 and 5). The RMSD calculations provide a sense of the time scale required to stabilize the protein structure after substrate binding. In this study, the RMSD plot was divided into two parts: (i) the ACE2 receptor (residues 19–615) and (ii) the SARS-CoV-2 S1-RBD (residues 333–526). The RMSD plot (Figure 4) found that when Kobophenol A was bound at the ACE2/spike interface, the S1-RBD region (black) rapidly equilibrated, whereas the ACE2 receptor (red) required ∼200 ns to stabilize. Similarly, when Kobophenol A was instead bound in the hydrophobic pocket of the ACE2 domain, the S1-RBD region (green) again quickly equilibrated but the ACE2 receptor (blue) took a more substantial time of ∼350 ns to stabilize (Figure 4). These RMSD calculations suggest that more significant conformational changes may occur within the ACE2 region relative to the S1-RBD regardless of the binding site, the ACE2/spike interface, or the ACE2 hydrophobic pocket.

Figure 1. Illustration of the SARS-CoV-2 spike S1 receptor-binding domain (green) bound with the ACE2 receptor (yellow) and Kobophenol A (blue). The zoomed-in compound binding region is also provided. (A) Location of Kobophenol A predicted at the spike S1-RBD/ACE2 interface and (B) location of Kobophenol A bound within the ACE2 hydrophobic pocket predicted from docking studies.

Figure 2. Representative dose-dependent inhibition curve of soluble hACE2 binding to SARS-CoV-2 RBD in the presence of Kobophenol A as determined by ELISA using anti-human HRP antibody. Data are presented as the mean of OD$_{450}$ ± SEM (n = 2) produced by the HRP with TMB.
The effects of Kobophenol A binding upon protein dynamics can be further explored using a RMSF analysis of the positional deviations over time relative to a reference structure (Figure 5). For example, Figure 5A provides the RMSF for the S1-RBD region of the protein (residues 333–533) over the entire simulation when Kobophenol A was bound at both of the proposed binding sites; greater fluctuations were computed when Kobophenol A was located at the ACE2/spike interface (black) as compared to the ACE2 hydrophobic pocket (green). The binding of Kobophenol A at the ACE2/spike interface produced large fluctuations, particularly within residues ranging from 435 to 460 (brown) and 475 to 515 (pink), which constitutes the receptor binding motif (RBM) of S1 in the system (Figure 5B). Interestingly, the residues located in the ACE2 domain were computed to have similar fluctuations for both binding motifs, although binding Kobophenol A at the ACE2/spike interface (red) gave somewhat larger absolute fluctuation distances compared to binding within the ACE2 hydrophobic pocket (blue) (Figure 5C). Overall, the RMSF analysis suggests that the binding location of Kobophenol A has a bigger effect on the structural conformation of the S1-RBD region, whereas a much smaller conformational difference was noted for the ACE2 receptor region.

A crystal structure of the SARS-CoV-2 spike receptor-binding domain bound to the ACE2 receptor was recently reported that highlighted the potential importance of 17 hydrogen bonds and two salt bridges occurring between S1-RBD and ACE2. The electrostatic interactions reported between the S1-RBD and ACE2 receptors were: N487–Q24, K417–D30, Q493–E37, Y505–E37, Y505–D38, Y449–D38, T500–Y41, N501–Y41, G446–Q42, Y449–Q42, Y489–Y83, N487–Y83, N487–Q325, N487–E329, N487–N330, G502–K353, Y505–R393, and K417–D30. These intermolecular interactions were monitored in the current MD simulations to examine the extent to which these favorable interactions may have been altered as a response to Kobophenol A binding in comparison to the unbound (i.e., Apo) system. Figure S1 in the Supporting Information provides the hydrogen bonding percent occupancy for each interaction present over the course of the 100–500 ns trajectory. The differences in computed hydrogen bonding interactions for the Apo system and the systems with Kobophenol A bound at either pocket were minor.
Interestingly, over half of the hydrogen bond interactions that were present in the crystal structure were completely eliminated for all three systems. This suggests that a significant number of the favorable electrostatic interactions present in the crystal structure may not be required to bind the ACE2 receptor to the S1-RBD and may possibly be attributed to the conditions of crystallization. Instead those residues are forming hydrogen bonds with other residues located within the individual proteins themselves, *i.e.*, S1-RBD-to-S1-RBD residues or ACE2-to-ACE2 residues. Of the original crystal structure hydrogen bonds identified between the two proteins, five hydrogen bonds remained intact: N487-Q24, Q493-E35, Y449-D38, N487-Y83, and G502-K353, regardless of where Kobophenol A was bound. In addition, two more hydrogen bonds interactions, *i.e.*, Y505-E37 and T500-Y41, were preserved when Kobophenol A was bound solely in the ACE2 hydrophobic pocket. A salt bridge reported between K417-D30 also remained regardless of substrate binding location.

New hydrogen bonds were computationally identified between ACE2 and the S1-RBD receptor (T500-D355, G502-D355, Y495-K353, and Q493-K31) that were not.

Figure 5. (A) Root-mean-square fluctuations (RMSFs) by residue relative to the lowest energy structure for the S1-RBD backbone atoms (C, Cα, and N) with Kobophenol A bound at the ACE2/spike interface (black) and the ACE2 hydrophobic pocket (green). (B) Illustration of highly fluctuating residues, *i.e.*, residues ranging from 435 to 460 (brown) and 475 to 515 (pink) in SARS-CoV-2 spike 1 receptor-binding domain (S1-RBD). (C) RMSF plot by residue for ACE2 receptor backbone atoms (C, Cα, and N) with Kobophenol A bound at the ACE2/spike interface (red) and the ACE2 hydrophobic pocket (blue) relative to the lowest energy structure.

Figure 6. Hydrogen bonding percent occupancy for the interactions between the ACE2 and S1-RBD regions when Kobophenol A was bound at the ACE2/spike interface (black) and ACE2 hydrophobic pocket (red) in comparison to Apo system (brown) from the MD simulations.
Table 2. MM/PBSA Terms Including the Free Energy of Binding, \(\Delta G_{\text{bind}}\) (in kcal/mol) for the Binding of Kobophenol A at the ACE2/Spike Interface and the ACE2 Hydrophobic Pocket

| complex                     | \(E_{\text{vdW}}\) | \(E_{\text{el}}\) | \(G_{\text{pol}}\) | \(G_{\text{np}}\) | \(\Delta G_{\text{bind}}\) |
|-----------------------------|--------------------|-------------------|-------------------|-------------------|------------------|
| ACE2/spike interface        | −39.6 ± 6.4        | −6.2 ± 5.9        | 31.5 ± 10.7       | −4.7 ± 0.6        | −19.0 ± 4.3      |
| ACE2 pocket                 | −56.3 ± 5.7        | −15.3 ± 5.4       | 53.1 ± 7.5        | −6.3 ± 0.5        | −24.9 ± 6.9      |

\(E_{\text{vdW}}\) = van der Waals energy, \(E_{\text{el}}\) = electrostatic energy, \(G_{\text{pol}}\) and \(G_{\text{np}}\) = polar and nonpolar contributions to the solvation free energies, respectively.

Scheme 1. Metabolite of Kobophenol A, Identified in Rat Feces after Oral Administration

observed in the crystal structure (Figure 6). Of these new interactions, the Y495-K353 hydrogen bond between the hydroxy group of Y495 in S1-RBD and the nitrogen atom of K353 in ACE2 domain was particularly interesting as it was observed in Apo simulation for 57% of time, but was completely eliminated when the Kobophenol A was bound in either pocket. This Y495-K353 interaction is located at the core center region of the interface-pocket formed between ACE2 and S1-RBD and may help stabilize the interaction between both domains (Figure S2). A distance analysis over the entire MD trajectory of the Apo system found that the O=H···NH₂ interaction between Y495 and K353 maintained an average distance of 2.95 Å (Figure S3) and its elimination upon ligand binding may suggest the origin of inhibition.

The molecular mechanics energies combined with the Poisson–Boltzmann surface area continuum solvation (MM/PBSA) method were utilized to estimate the free energy of binding of Kobophenol A from the MD simulations. The binding affinity of Kobophenol A to the ACE2/spike interface region and the ACE2 hydrophobic pocket was computed to be −19.0 ± 4.3 and −24.9 ± 6.9 kcal/mol, respectively, over the course of the 500 ns trajectory (Table 2). To understand the substantial preference for Kobophenol A in the ACE2 hydrophobic pocket, the individual energy contributions to the binding affinity were examined. As shown in Table 2, the van der Waals energy contribution (\(E_{\text{vdW}}\)) and the polar contribution to the solvation free energies (\(G_{\text{pol}}\)) nearly cancel themselves out. This suggests that the electrostatic energy contribution of the ACE2 hydrophobic pocket that is more than double of that of the ACE2/spike interface, i.e., −15.3 versus −6.2 kcal/mol, may be a major contributor to the ACE2 pocket preference in the net binding free energy calculation.

Kobophenol A is a natural oligomeric stilbenediol isolated from Caragana genus and is a tetramer of resveratrol. Compounds containing Kobophenol A have been isolated from Caragana sinica for preventing and treating West Nile virus infection. Externally, the anti-infective activity of Kobophenol A has been found to have antiviral activity by inhibiting neuraminidase, and thus, inhibiting the influenza virus suggests action on an infection step successive to the cell surface recognition (Patent Application No.: KR20200026550A). In addition, Kobophenol A is known to inhibit acetyl cholinesterase activity, to exhibit neuroprotection, and to be used for neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. Furthermore, Kobophenol A strongly stimulates the proliferation of osteoblasts cultured in vitro, and a pharmaceutical composition of Kobophenol A was used as a cosmetic for effective skin whitening by inhibiting the biosynthesis of melanin in melanocytes. Moreover, Kobophenol A was used as a cardioprotective agent against sodium nitroprusside induced cardiac cell death. Kobophenol A exhibits anti-inflammatory activity by regulating NF-kB nuclear translocation in J774A.1 Cells. Caragana extract along with Kobophenol A is also used for chronic bronchitis in the form of a daily decoction, without any toxicity. SARS-CoV-2 can trigger an excessive immune response known as cytokine storm, which can lead to multiple organ failure and death because of the inflammation of the organs. The additional anti-inflammatory, bronchodilator, cardioprotective, and antioxidant activities of Kobophenol A can be an additive health benefit for COVID-19 patients and may potentially help to reduce mortality in COVID-19 patients. Overall, Kobophenol A has great potential to serve as a therapeutic lead compound or as an additive for COVID-19 patients.

The half-life of Kobophenol A was found to be 0.68 h when administered i.v. and 5.78 h when administrated orally in rats. Kobophenol A metabolites [koboquinone A (M1), koboquinone B (M2), and koboquinone C (M3)] (Scheme 1) were isolated from rats feces after oral administration. The three metabolites M1, M2, and M3 were considered as part of the in silico studies and were found to bind well with good docking energies of −9.73, −10.48, and −10.27 kcal/mol, respectively.

To date, there is no proper gold standard treatment available for curing SARS-CoV-2 infection. Vaccines and other antiviral agents are currently under various clinical phases or administration; however, the clinical benefits are still being assessed. Herbal supplements either as core ingredients or in combination may achieve synergistic action in treating COVID-19 patients. The present investigation strongly suggests that the natural based, oligomeric stilbenediol Kobophenol A from C. sinica effectively suppressed the interaction between the ACE2 receptor and S1-RBD domain of SARS-CoV-2. A measured in vitro IC₅₀ value of 1.81 μM for Kobophenol A against recombinant 2019-nCOV spike(RBD)/hFc protein and an EC₅₀ value of 71.6 μM from a phenotypic
virus cell based antiviral assay with SARS-CoV-2 in VeroE6 cells support this assessment. Moreover, no cytotoxicity was found for Kobophenol A with a CC50 value of more than 100 μM.

Docking calculations identified two potential binding sites for Kobophenol A, i.e., the ACE2 hydrophobic pocket and the spike1/ACE2 interface. Molecular dynamics simulations further elucidated the origin of Kobophenol A inhibitory action. The MD simulations found that binding the substrate in either pocket eliminated a central core interaction, Y495-K353, found between the ACE2 and S1-RBD interface pocket. Computed free energies of binding for Kobophenol A at the spike/ACE2 interface and the ACE2 hydrophobic pocket using MM/PBSA calculations yielded values of −19.0 ± 4.3 and −24.9 ± 6.9 kcal/mol, respectively. The electrostatic energy contribution of the ACE2 hydrophobic pocket was more than double of that of the ACE2/spike interface when binding Kobophenol A, which may explain the preference.

In summary, Kobophenol A was computationally identified as a good lead compound effective against SARS-CoV-2 infection, which was then validated experimentally to inhibit the binding of S1-RBD from SARS-CoV-2 to the host ACE2 receptor. The obtained results suggested that Kobophenol A may be further developed as a safe and effective drug without toxicity for SARS-CoV-2 infection.

## EXPERIMENTAL SECTION

Kobophenol A was purchased from ChemFaces, China with a purity of ≥98% (catalog no. CFN92530). We also acquired the following: SARS-COV-2 S1-RBD (Novateinbio, USA, catalog no. PR-nCOV-2), ACE2 receptor protein (Novateinbio, catalog no. PR-nCOV-4), and goat anti-human IgG-Fc-HRP conjugate. Kobophenol A was dissolved in DMSO and stored at −20 °C. Molecular docking studies were carried out by using AutoDock ver. 4.2 on Windows. All the molecular dynamics simulations were carried out using the GPU-enabled Amber18 pmemd engine, and cpptraj was utilized for analyzing the trajectory.

The crystal structure of SARS-CoV-2 was retrieved from rcsb.org (PDB ID: 6M0J) and used to generate initial 3D coordinates of the spike S1-RBD-ACE2 complex. Cocrystallized water molecules were deleted. Polar hydrogens were added, and Gasteiger charges were computed. The structures of selected natural compounds were superimposed against the prelocked ligand in the PDB, and the latter was then removed to generate initial conformation of natural compound at the active site of SARS-CoV-2. As the natural compounds were not available in the X-ray crystal structure of S1-RBD bound with ACE2, a grid box was generated by considering the whole protein and blind docking was performed (PDB ID: 6M0J). Finally, both Autogrid and AutoDock were run with the default parameters as described in following references: refs 45–47. Top scoring molecules were evaluated for their interactions.

In Vitro Spike S1-RBD and ACE2 Inhibitory Activity of SARS-CoV-2 by Enzyme-Linked Immune Sorbent Assay (ELISA). To test whether Kobophenol A inhibits the interaction between ACE2 and S1-RBD of SARS-CoV-2, a 96-well plate was coated with recombinant 2019-nCoV S1-RBD (catalog no. PR-nCOV-2, Novatein Biosciences, USA) at 0.1–0.4 μg/mL overnight. Plates were washed 3× with PBS pH 7.2 (without Ca2+ and Mg2+) with 0.05% Tween-20 and blocked with 1% BSA in PBS. ACE2 receptor protein (catalog no. PR-nCOV-4), 0.1–0.2 μg/mL, was added in the presence or absence of Kobophenol A at various concentrations. For data sheets of human ACE2, spike RBD, and Kobophenol A, see Supporting Information Figure S5. Samples were incubated for 1–2 h in the binding buffer (0.1% BSA in PBS, pH 7.2). Plates were washed, and anti-human Fc-antibody-HRP 1:20,000 in binding buffer was added. After three washes 3,3′,5′,5′-tetramethylbenzidine (TMB) was added for a signal; after stopping the reaction with an acidic solution, the plates were read at 450 nm. IC50 values were calculated by using GraphPad (https://www.graphpad.com/support/faq/how-to-determine-an curing50sub50sub50sub50sub/). The ELISA assay was performed twice by Novatein Biosciences, USA.

Cell Culture. VeroE6-EGFP cells were from Tibotec “VeroE6-EGFP CI-21 #2 02-sep-2003”. Cells were propagated in growth medium which was prepared by supplementing DMEM (Gibco cat. no: 41965-039) with 10% v/v heat-inactivated FCS and 5 mL of sodium bicarbonate 7.5% (Gibco cat. no. 25080-060). Cells were cultured in T150 flasks and split 1:4 twice a week. Pen-strep was added directly to the T150 flask at a 1:100 dilution. The assay medium was prepared by supplementing DMEM (Gibco cat. no: 41965-039) with 2% v/v heat-inactivated FCS and 5 mL of sodium bicarbonate 7.5% (Gibco cat. no: 25080-060).

Compound Dilution. A volume of 100 μL of medium was added to columns 1−12 of a Greiner Bio One 655090 plate. Then 100 μL of medium was added to column 12, 50 μL of medium was added to columns 11 and 2, and 50 μL of medium was added to column 2. Compound was added to column 2, rows B–G and further diluted over the plate. Cell Suspension. A T150 cell culture flask containing a confluent cell monolayer was washed with DPBS, after which 10 mL of trypsin/EDTA was added. The trypsin was left on the cells for 1 min, ascertaining the full monolayer has been in contact by gently tilting the cell culture flask. A volume of 8 mL of the liquid was removed, leaving 2 mL on the cell monolayer. The cell culture was incubated for 15 min at 37 °C, after which the cells were resuspended in 10 mL of assay medium (DMEM with 2% FCS and 5 mL of sodium bicarbonate, no penicillin/streptomycin). To remove cell clumps, the cell suspension was passed through a Cell Strainer. The amount of harvested cells was quantified by analyzing three samples of a 10 μL cell suspension in 10 mL of isotonic buffer using a Coulter Counter. A cell suspension with a density of 25 000 cells/50 μL was prepared in assay medium. Then 50 μL of this cell suspension was seeded to each well of the plate. The plates were incubated overnight (37 °C/5% CO2).

Adding Virus. To prepare the virus to an appropriate dilution in assay medium, SARS2 stock SARS2_Belgium_20200414, was prepared in a 1:50,000 dilution (Final dilution in the plate is 200,000). This virus stock has a titer of 2 × 107 TCID50/mL. The final titer in the experiment was therefore 100 TCID50/mL = 20 TCID50/well, with 25,000 cells/well the MOI = 0.001 TCID50/cell. A volume of 50 μL of this virus preparation was added to columns 1–10. Plates were incubated at 37 °C and 5% CO2. On day 4, the plates were transferred to a high-content imager for determination of the Green Fluorescent Protein (GFP) signal using high-content imaging. The number of fluorescent pixels above threshold was used as the readout. The percentage of inhibition was calculated by subtracting the background (number of fluorescent pixels in untreated/infected control wells) and normalizing to control wells without virus (also background
subtracted). The cytotoxicity assay was identical to the antiviral assay with the difference that the assay medium without virus was added instead of assay medium with virus.

**Molecular Dynamics Simulations.** A crystal structure of SARS-CoV-2 spike receptor-binding domain bound with ACE-2 receptor (PDB: 6M0J) was utilized to provide the starting Cartesian coordinates. The AmberTools18 leap module was used to add missing hydrogen atoms to the enzyme. The protein and ligand complexes were solvated explicitly using a TIP3P orthorhombic water box that extended 10 Å away from the protein in each direction, and the overall charge of the system was neutralized by adding a suitable number of sodium cations. The topology file for the protein was created using the ff14SB force field, the generalized Amber force field (GAFF) was used to parametrize the ligands, and all NAG molecules were parametrized using the GLYCAM_06j-1 force field. The IOD parameters were used to describe the Zn(II) ion bound at the active site of ACE2 domain, the co-crystallized Cl⁻ ion at the ACE2 domain, and the Na⁺ ions that were used to neutralize the system. All simulations were carried out using the GPU-enabled Amber18 pmemd engine.

The water molecules and anions were minimized solely using the conjugate gradient (CG) method for 3000 steps followed by 10,000 steps of CG minimization for the entire system. The system was then gradually heated from 0 to 300 K using a constant NVT ensemble over 50 ps with a Berendsen thermostat and temperature coupling value set to 2.0 fs. The system was then switched back to the NVT ensemble and further equilibrated for 500 ps. Following the minimization and equilibration phase, a 500 ns NVT production run was carried out. Analysis was performed with the cpptraj and ptraj programs available in the AmberTools18 suite.

The first 100 ns of the data was discarded with an aim to ensure only an equilibrated trajectory analysis.

The MM/PBSA (Molecular Mechanics/Poisson–Boltzmann Surface Area) methodology began with reports from Kollman and co-workers, but more recent developmental efforts have provided an important tool for studying ligand binding on various biological systems. It is one of the robust methods that has been successfully applied to estimate the free energy of binding for inhibitors with small or large differences in the principle scaffold, chiral compounds, and small peptides. In this work, the binding affinity of Kobophenol A in the ACE2 pocket and the ACE2/spike interface was calculated using the MM/PBSA approach available within the Amber package (eqs 1-6),

\[
\Delta G_{\text{binding}} = \Delta G_{\text{complex}} - \Delta G_{\text{receptor}} - \Delta G_{\text{ligand}}
\]

\[
\Delta G_{\text{solvated}} = \Delta G_{\text{gas}} + \Delta G_{\text{solvation}} - T \Delta S_{\text{solute}}
\]

\[
\Delta G_{\text{gas}} = E_{\text{bonded}} + E_{\text{nonbonded}}
\]

\[
E_{\text{bonded}} = E_{\text{stretching}} + E_{\text{bending}} + E_{\text{torsional}}
\]

\[
E_{\text{nonbonded}} = E_{\text{vdW}} + E_{\text{electrostatic}}
\]

\[
\Delta G_{\text{solvation}} = G_{\text{pol}} + G_{\text{np}}
\]

where \(-\Delta G_{\text{gas}}\) is the standard energy term in molecular mechanics for bonded and nonbonded integrations, and \(G_{\text{pol}}\) and \(G_{\text{np}}\) are the polar and nonpolar contribution states of the solvation free energy (\(\Delta G_{\text{solvation}}\)), respectively. By solving the PB equation, the value of \(G_{\text{pol}}\) can be derived and the solvent accessible surface area (SASA) method can be used to determine \(G_{\text{np}}\). Alternatively, \(G_{\text{pol}}\) can be derived from the generalized Born (GB) equation, which is an equivalently popular approach. In addition, \(T\) is the absolute temperature and \(S\) is the entropy. The dielectric constant for the interior of the protein and implicit solvent was 1 and 80, respectively. The free energy calculation utilized the MD trajectory from the production run. Using a single trajectory protocol, all the necessary ensembles for the bound, unbound, and ligand where extracted. The remaining parameters were set to AMBER default values, and the binding free energy entropic contributions were not included.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.0c03119.

Percent of hydrogen bonding occupancy over the MD simulation of the interactions between the ACE2 and S1-RBD regions when Kobophenol A was bound; illustration of the initial conformation of Y495 of S1-RBD and K353 of ACE2 domain in the reported crystal structure (PDB ID: 6M0J); distance computed from MD simulations between the O of the hydroxy group in Y495 in S1-RBD and N of the ammonium group for K353 in the ACE2 domain of the complex; light absorption at 450 nm, representing the amount of human ACE2 bound to the spike protein in the ELISA assay; additional EC_{50} curve of Kobophenol A antiviral activity in cell infected with SARS-CoV-2; chemical structure of natural compounds of Table 1; data sheets of human ACE2, spike RBD, and Kobophenol A (PDF)

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Notes
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