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Fundamental Research 2 (2022) 562–569

Contents lists available at ScienceDirect

Fundamental Research

journal homepage: http://www.keaipublishing.com/en/journals/fundamental-research/

Article

CEBIT screening for inhibitors of the interaction between SARS-CoV-2 spike and ACE2

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ARTICLE INFO

Article history:
Received 2 December 2021
Accepted 14 January 2022
Available online 3 March 2022

Keywords:
High-throughput screening
SARS-CoV-2
ACE2
Spike protein
Protein-protein interaction

ABSTRACT

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, causing COVID-19, is the most challenging pandemic of the modern era. It has resulted in over 5 million deaths worldwide. To quickly explore therapeutics for COVID-19, we utilized a previously-established system, namely CEBIT. We performed a high-throughput screening of FDA-approved drugs to inhibit the interaction between the receptor-binding domain (RBD) of SARS-CoV-2 spike protein and its obligate receptor ACE2. This interaction is essential for viral entry and therefore represents a promising therapeutic target. Based on the recruitment of interacting molecules into phase-separated condensates as a readout, we identified six positive candidates from a library of 2572 compounds, most of which have been reported to inhibit the entry of SARS-CoV-2 into host cells. Our surface plasmon resonance (SPR) and molecular docking analyses revealed the possible mechanisms via which these compounds interfere with the interaction between RBD and ACE2. Hence, our results indicate that CEBIT is highly versatile for identifying drugs against SARS-CoV-2 entry, and targeting CoV-2 entry by small molecule drugs is a viable therapeutic option to treat COVID-19 in addition to commonly used monoclonal antibodies.

1. Introduction

The world is facing the most challenging pandemic of the modern era, that of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which cause coronavirus disease (COVID-19) and has already resulted in over 437 million cases and 5.96 million deaths worldwide until the publication [1]. Multiple prominent mutant strains have emerged, severely complicating the landscape. More effective therapeutics for COVID-19 are required to end the pandemic and restore normalcy.

Understanding how SARS-CoV-2 enters human cells is a high priority for deciphering its high infectivity and curbing its spread. Its surface spike protein mediates SARS-CoV-2 entry into cells. To fulfill its function, SARS-CoV-2 spike binds to its obligate receptor, angiotensin-converting enzyme 2 (ACE2), which is highly expressed in the heart, kidneys, and lungs [2]. The spike protein binds ACE2 through its receptor-binding domain (RBD) and is proteolytically activated by human proteases [3]. Therefore, the RBD is emerging as the primary target of neutralizing antibodies elicited by natural infection or vaccination [4]. Hence, blockade of the RBD-ACE2 protein-protein interaction (PPI) has the potential to reduce infection efficiency. Unfortunately, RNA viruses, as a further complicating factor, accumulate mutations over time, which yields antibody resistance and requires a more rigorous use of antibody cocktails to avoid mutational escape. Not surprisingly, there is now evidence of the emergence of SARS-CoV-2 mutants for which antibodies against the original strain have diminished activity [2]. Moreover, antibody therapies are also hindered by problems related to their solubility, unsuitability for oral or inhaled administration, long elimination half-lives, and immunogenicity. To this end, small molecule inhibitors, especially in a cocktail with multiple targets, can offer an alternative weapon against the SARS-CoV-2 variants.

Thanks to the development of computational and chemical technologies, the past decade has witnessed the increasing development of small-molecule inhibitors (SMIs) as potential therapeutic medicines and also as useful experimental tools to elucidate pathological mechanisms [5–7]. Encouragingly, the success of small-molecule drugs targeting HIV-1 entry, including Enfuvirtide, Maraviroc, and Fostemsavir, promotes their approval for clinical use [8], and highlights the feasibility of such a strategy for antiviral drug discovery. Therefore, SMI-mediated antiviral therapies are emerging as to be less immunogenic, with better bioavail-

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https://doi.org/10.1016/j.fmre.2022.01.034

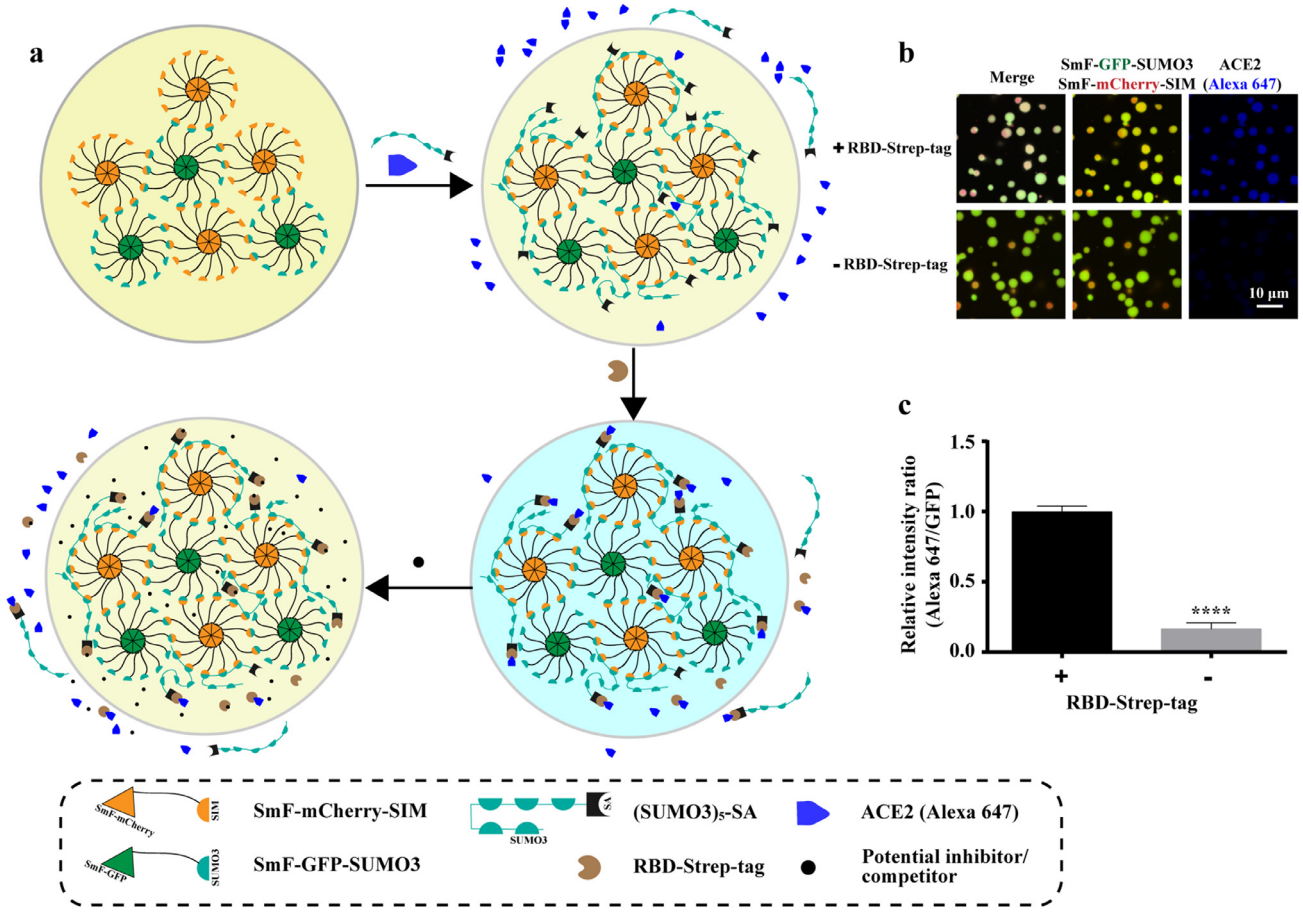

Fig. 1. Validation of CEBIT-based system for detecting RBD-ACE2 interaction. (a) Schematic diagram showing the strategy of SmF-based phase separation system. The condensates formed by SmF-mCherry-SIM and SmF-GFP-SUMO3 serve as the "reactor" for PPI recruitment (top left). With the addition of $(SUMO3)_5$-SA and RBD-Strep-tag in turn (top right and bottom right), ACE2-His was recruited into the condensates via its interaction with RBD-Strep-tag. ACE2-His is fluorescently tagged to monitor its recruitment (bottom right). Disruption of the RBD-ACE2 interaction by an inhibitor or competitor evicts ACE2 from the condensates (bottom left). (b) Microscopy analysis of the recruitment of ACE2 labeled by Alexa 647 into the condensates formed by SMF-mCherry-SIM and SmF-GFP-SUMO3. (c) Relative fluorescence intensity ratio of ACE2 (Alexa 647) versus condensates (GFP).

ability (i.e., suitable for oral or inhaled administration), and broader activity (i.e., less strain- and mutation-sensitive compared to acquired resistance to neutralizing antibodies or enzyme inhibitor treatments) [9,10].

The pace of development of therapeutic approaches to modulate PPIs has grown and led to some visible success in the pharmaceutical industry. In this process, various high-throughput screening (HTS) approaches play a vital role. In our previous work, we developed a protein-protein interaction (PPI)-based biomolecule recruitment system, called Condensate-aided Enrichment of Biomolecular Interactions in Test tubes (CEBIT), as a sensitive and versatile tool for *in vitro* screening of compounds which disrupt the interaction between proteins [11]. The CEBIT system offers a subtle blend of reconstituted biomolecular condensates and biomolecular interactions, with the recruitment of nanometer-sized molecules into micrometer-sized condensates as the readout.

Here, we tailored CEBIT to measure the ACE2-RBD interaction and to identify specific SMIs. By utilizing this recruitment system, we successfully performed an HTS of 2572 compounds and identified several SMIs which inhibit the ACE2-RBD interaction *in vitro*. Those SMIs include Methylene blue, Varenicline tartrate, Sennoside A, Quercetin, Quinacrine-2HCl, and Sunitinib malate, some of which have already been reported to be potent against SARS-CoV-2 in biological assays [12–21]. Overall, our data emphasize the robustness and versatility of CEBIT, and open up a new opportunity for efficient discovery of SMIs against SARS-CoV-2 and other severe diseases.

2. Materials and methods

2.1. Cell culture

Expi293F (Thermo Fisher Scientific) cells were grown in SMM 293-TII expression medium (Sino Biological Inc.). Cells were cultured in sterile flasks, vented, with a baffled bottom in an Orbital Shaker at 120 r.p.m. at 37 °C and 5% CO_2.

2.2. Protein expression and purification

The recombinant proteins were overexpressed in *E. coli* BL21 (DE3). After overnight induction by 0.1 mM isopropyl β-D-thiogalactoside (IPTG) at 16 °C in LB medium, cells were harvested and suspended in buffer (40 mM Tris-HCl, pH 8.0; 500 mM NaCl; 2mM phenylmethylsulfonyl fluoride). After cell lysis and centrifugation, the recombinant proteins were purified by Ni-NTA (GenScript) affinity chromatography. Ion exchange columns and gel-filtration chromatography (SD200, Superdex200 Increase 10/300, GE Healthcare) were used for further purification.

SARS-CoV-2 RBD wild-type proteins (with an N-terminal signal peptide and a C-terminal TwinStrep tag or 8 × His tag or TwinStrep-KKETPV tag) were expressed in Expi293F (Thermo Fisher Scientific) cells at 37 °C in a humidified 5% CO_2 incubator rotating at 120 r.p.m. Transfections were performed using PEI MAX (Polysciences) at a DNA:PEI ratio of 1:3.

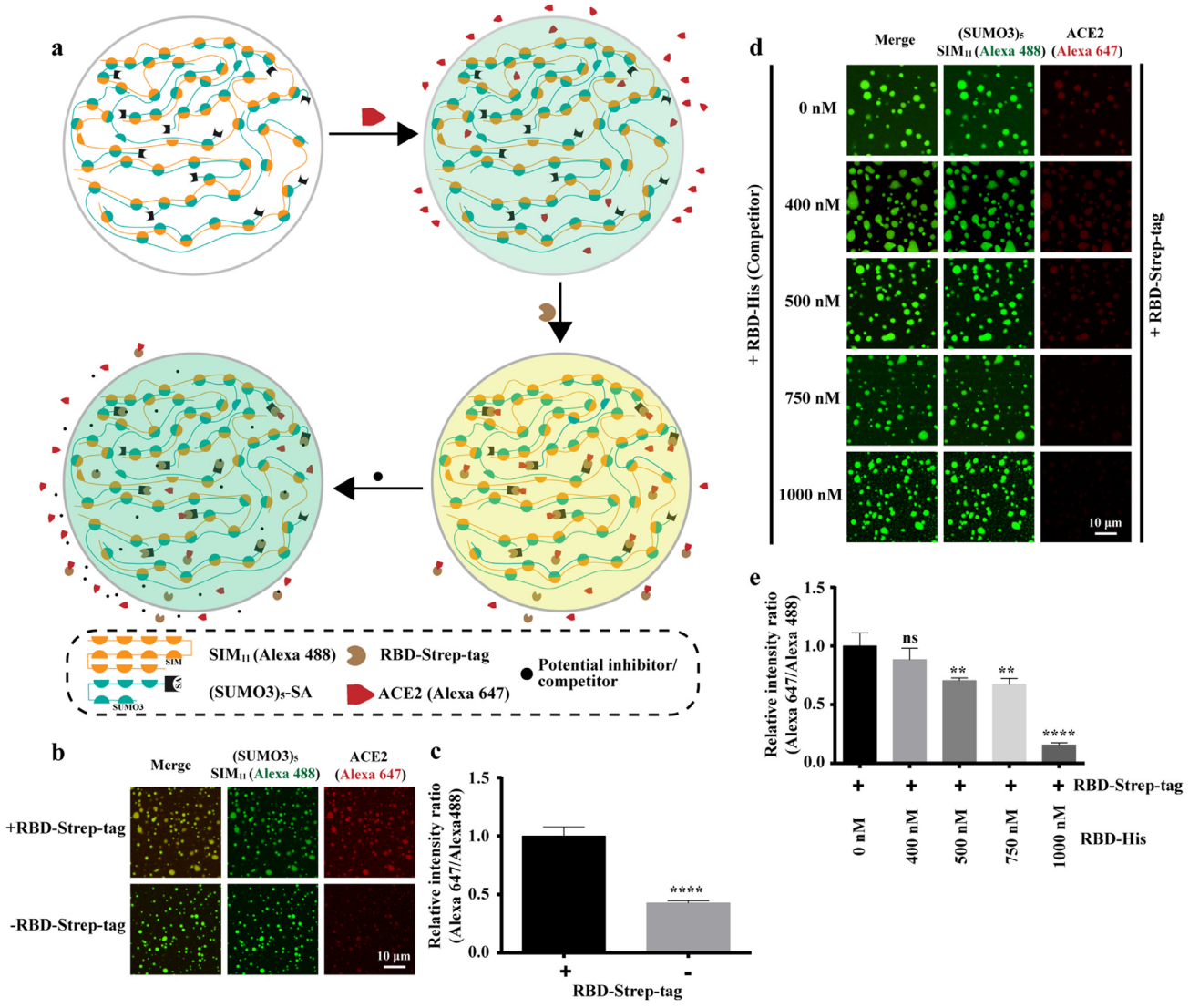

Fig. 2. Optimization of a multivalent recruitment system driven by polySUMO-polySIM. (a) Schematic diagram showing the strategy for the phase separation system mediated by polySUMO-polySIM. The scaffolds used here to mediate the formation of condensates are $(SUMO3)_5$-SA and SIM_{11}. The colocalization of the client protein ACE2-His with the condensates was observed in the presence of the bridging protein RBD-Strep-tag. (b) Microscopy analysis of the recruitment of ACE2 labeled by Alexa 647 into the condensates formed by $(SUMO3)_5$-SA and SIM_{11} (Alexa 488). (c) Relative fluorescence intensity ratio of ACE2 (Alexa 647) versus condensates (Alexa 488). (d-e) Competitive binding assay between RBD-His and RBD-Strep-tag for ACE2 protein.

After cultivation for 96 h the supernatants were collected and purified by Ni-NTA (GenScript) affinity chromatography or Strep-Tactin® XT (IBA Lifesciences) according to the manufacturer's protocol. Ion exchange columns and gel-filtration chromatography (SD200, Superdex200 Increase 10/300, GE Healthcare) were used for further purification. Recombinant hACE2 protein was produced as described previously with some modifications [22].

2.3. *Protein labeling*

Recombinant proteins without a fluorescent tag were labeled by incubating with a 1:1 molar ratio of Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647 carboxylic acid (succinimidyl ester) (Thermo Fisher Scientific) for 1 h at room temperature with continuous stirring. Free dye was removed by illustra™ Microspin G-50 Columns (Cytiva).

2.4. In vitro *phase separation assay*

All phase separation assays were performed in a final salt concentration of 150 mM.

For the SmF-mCherry-SIM/SmF-GFP-SUMO3 phase separation assay, the condensates formed by 2 μM SmF-mCherry-SIM and 1 μM SmF-GFP-SUMO3 served as the "reactor" for PPI recruitment. With the addition of 100 nM $(SUMO3)_5$-SA and 100 nM RBD-Strep-tag in turn, 50 nM ACE2-His labeled by Alexa 647 was recruited into the condensates via its interaction with RBD-Strep-tag.

For the SIM_{11}/$(SUMO3)_5$-SA phase separation assay, the scaffolds were 20 μM $(SUMO3)_5$-SA and 10 μM SIM_{11} labeled by Alexa 488 to mediate the formation of condensates. The colocalization of 250 nM client protein ACE2-His labeled by Alexa 647 with the condensates was observed in the presence of 500 nM bridging protein RBD-Strep-tag. For the competition experiment, RBD-His proteins at different concentrations (400, 500, 750, and 1000 nM) were used as the competitor protein to block the interaction between ACE-His and RBD-Strep-tag.

For the HP1α-PDZ phase separation assay, except for the addition of 5% PEG, other reaction conditions were the same as the assays mentioned above. The interaction of 250 nM ACE2-His labeled by Alexa 546 with 500 nM RBD-KKETPV mediated their partition into the condensates formed by 5 μM HP1α-PDZ labeled by Alexa 647. Similar competition

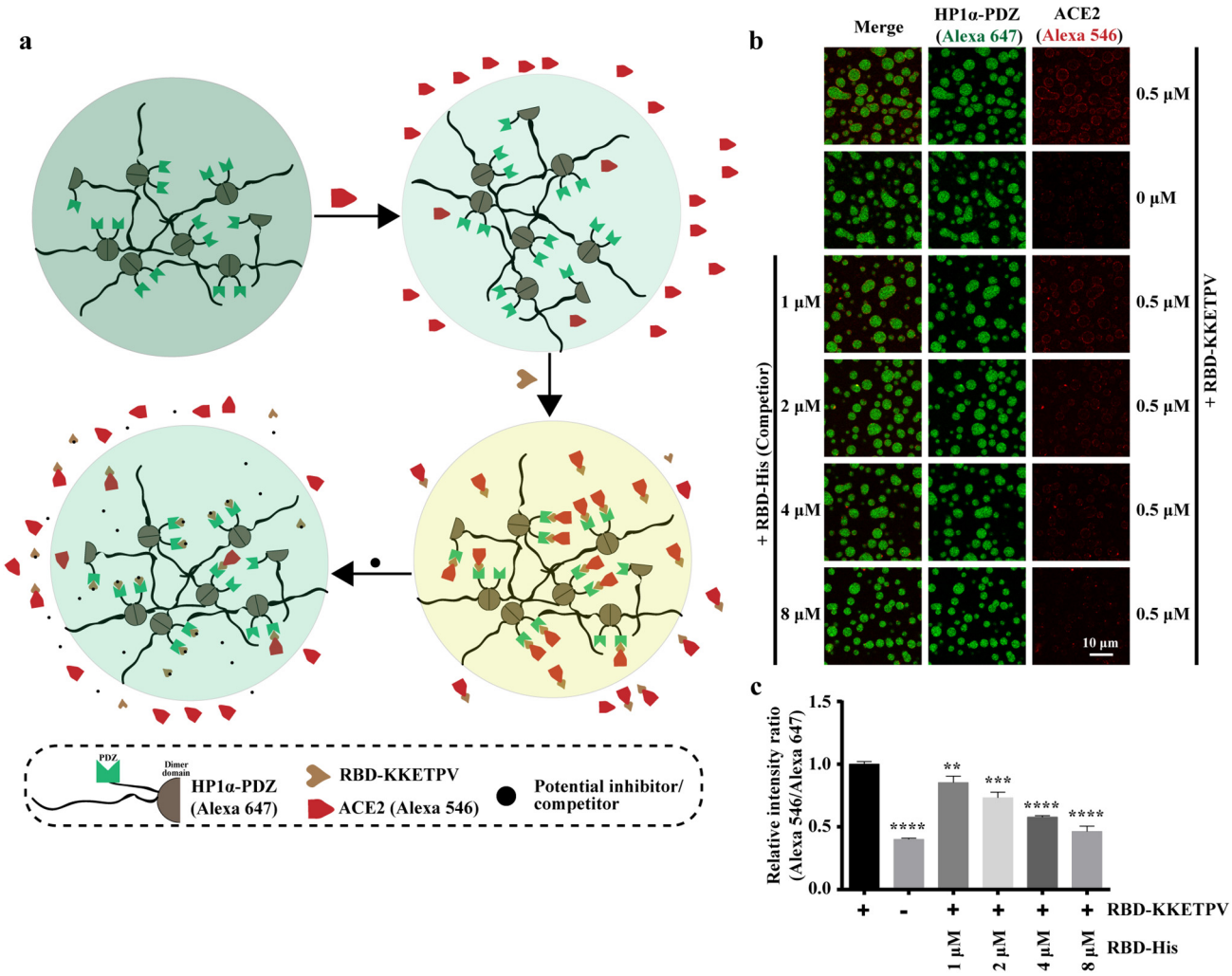

Fig. 3. Optimization of a multivalent recruitment system driven by HP1α. (a) Schematic diagram showing the strategy for the HP1α-driven phase separation system. Partitioning of ACE2-His into the HP1α-PDZ-driven condensates is mediated by its interaction with RBD- KKETPV. (b) Microscopy analysis of the recruitment of ACE2 labeled by Alexa 546 into the condensates of HP1α-PDZ (Alexa 647), and the competitive binding assay by RBD-His protein. (c) Analysis of the relative fluorescence intensity ratio of ACE2 (Alexa 546) versus condensates (Alexa 647).

assays were performed using RBD-His proteins at different concentrations (1, 2, 4, and 8 μM).

2.5. Confocal capture

All the phase separation experiments were performed on 384-low-binding multi-well 0.17 mm microscopy plates (Cellvis). For *in vitro* phase separation assays, laser scanning confocal microscopy (Nikon A1R HD25) was used for capturing images and NIS-Element AR5.2 software was used for analyzing the data.

2.6. High throughput screening

The HTS screening was carried out using the HP1α-PDZ-driven phase separation system for PPI recruitment. The system was comprised of 5 μM HP1α-PDZ, 500 nM RBD-KKETPV, and 250 nM ACE2 in a total volume of 10 μl in 384-well microplates (Cellvis). A total of 2572 small molecules from the Selleck library of FDA-approved drug library were the resource for HTS. In each well, a small molecule with a concentration of 100 μM was added using the Echo550 Acoustic Liquid Handler (Labcyte). After incubation at room temperature for 1 h, images were collected and analyzed by an Opera Phenix Plus High-Content Screening System (PerkinElmer) combined with Harmony® Imaging and Analysis software.

2.7. Surface plasmon resonance (SPR)

SPR was performed using a Biacore T200 system (GE Healthcare). Briefly, purified RBD was pre-immobilized onto a CM5 sensor chip. Serially diluted small molecules were flowed over the chip to evaluate the interaction between SMIs and RBD protein. Kinetic analysis was conducted to determine the affinity of binding (equilibrium dissociation constants, K_D). The curve-fitting process was carried out using a 1:1 binding model by Biacore Evaluation software (GE Healthcare).

2.8. Molecular docking

The crystal structures of SARS-CoV-2 spike RBD and human ACE2 (PDB ID: 6m0j) were obtained from the protein databank (https://www.rcsb.org/). ChemBioDraw Ultra 14.0 was used to draw the two-dimensional structure of the small molecules. The structures were then converted into PDB format using OpenBabel-3.1.1. Molecular docking between RBD and small molecules was performed by AutoDock, in which RBD was regarded as rigid macromolecule while the small molecules were considered as flexible ligands. MGLTools1.5.6 was used for further analyses. After docking, the ACE2 structure was fitted to the docked complexes. The chimeric complex (after insertion of ACE2) was viewed in PyMOL-2.4.0.

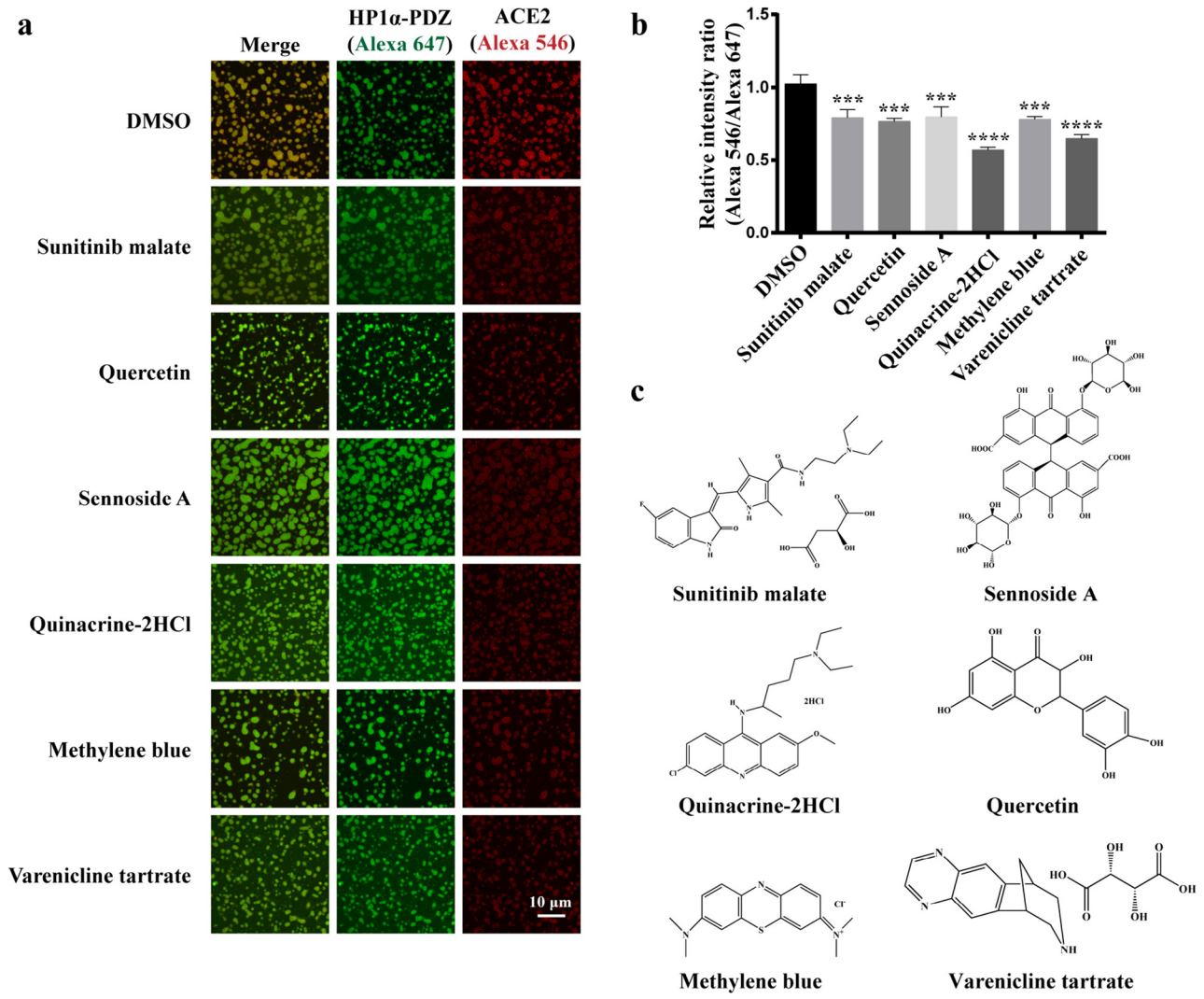

Fig. 4. High throughput screening for inhibitors of the RBD-ACE2 interaction. (a) Microscopy analysis of the inhibition activity of positive compounds. (b) Quantitative analysis of the relative ratio of fluorescence intensities of ACE2 (Alexa 546) and HP1α-PDZ (Alexa 647). (c) Schematic draws of the two-dimensional structures of the positive compounds.

3. Results

3.1. *Evaluation of CEBIT in identifying RBD-ACE2 interaction* in vitro

Our ultimate goal was to use CEBIT to identify inhibitors of the interaction between the SARS-CoV-2 S protein RBD and ACE2, since this interaction is an essential first step for viral entry of this novel, highly pathogenic coronavirus. To begin, we explored the feasibility of establishing a PPI-based biomolecule recruitment assay using a similar design as in our previous works [11]. We used two fluorescently tagged fusion proteins consisting of SmF, a *Saccharomyces cerevisiae*-derived protein prone to forming a stable tetradecameric complex [23], and SUMO3 or the SUMO3-interacting motif (abbreviated to SIM) [24], to serve as the scaffold proteins to trigger robust multivalent interaction-mediated phase separation (Fig. 1a). To assess the RBD-ACE2 interaction, we prepared two client proteins. The first is RBD-Strep-tag, consisting of RBD fused to Strep-tag, an eight-residue minimal peptide sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) which exhibits intrinsic affinity toward streptavidin (SA). The second client is ACE2 labeled with Alexa 647. To connect the scaffold proteins with the client proteins, we introduced a replaceable mediator protein, $(SUMO3)_5$-SA, consisting of SUMO3 fused to SA. SA in the mediator binds to RBD-Strep-tag, and SUMO3 in the mediator binds to SIM in the scaffold. Moreover, the involvement of the mediator ensures that the recruitment of ACE2 depends on its specific interaction with RBD but not its indiscriminate links with scaffolds (Fig. 1b). Corresponding quantitative analysis, based on the fluorescence intensity ratio, confirmed that this ternary design can be applied to measure the RBD-ACE2 interaction (Fig. 1c). Our results indicate that this refined CEBIT method can yield specific and robust output for measuring RBD-ACE2 interaction *in vitro* and thus it works as the backbone for subsequent studies.

3.2. *Optimization of a multivalent recruitment system for RBD-ACE2 interaction*

With a growing body of evidence establishing a link between client valency and recruitment for multivalent interaction pairs such as poly-SUMO3 and poly-SIM [24], it seemed rational to construct a more streamlined phase separation system without the extra addition of SmF. For this reason, we constructed different tandem repeats of SIM and finally determined that the optimum valency of polySIM was eleven repeats (Fig. 2a). *In vitro* phase separation assays were performed using SIM_{11}, the $(SUMO3)_5$-SA adaptor, and the clients RBD-Strep-tag and ACE2 labeled with different Alexa fluorophores. ACE2 was specifically recruited into the condensates based on its interaction with RBD-Strep-tag (Fig. 2b and c). Further competitive binding analysis, using purified

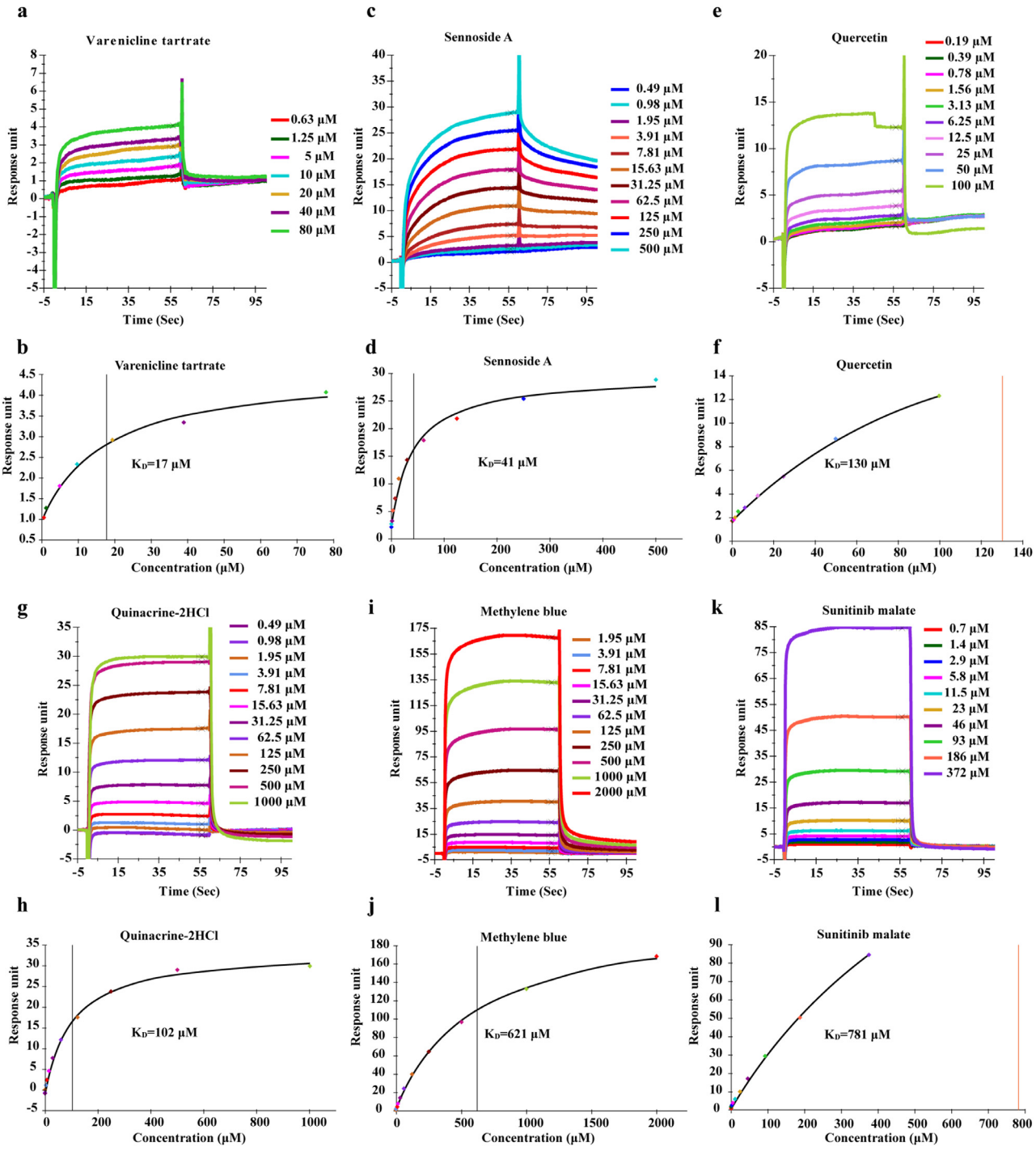

Fig. 5. SPR analysis of the binding of the positive compounds to RBD. (a, c, e, g, i, k) Sensorgrams of the multicycle interaction between the indicated compounds and RBD. (b, d, f, h, j, l) Fitting analysis of the concentration-dependent response in a 1:1 equilibrium model.

RBD-His protein without Strep-tag, attenuated the ACE2/RBD-Strep-tag intensity ratio in a concentration-dependent manner (Fig. 2d and e). This shows that the RBD-ACE2 interaction is required for enrichment of ACE2 inside the condensates.

To make the system simpler, we tried to further decrease the number of elements triggering phase separation by replacing the polySUMO/polySIM pair with heterochromatin protein 1α (HP1α), which is competent for phase-separation in higher-order oligomerization [25,26]. We also used a new bridging combination, KKETPV/PDZ (Fig. 3a), in which PDZ is the third PDZ domain of human PSD-95 and KKETPV is a synthetic PDZ ligand [27]. KKETPV was fused to RBD and PDZ was fused to HP1α. In the presence of RBD-KKETPV, ACE2 was recruited into the HP1α-PDZ-driven condensates (Fig. 3b). And, this strategy performed a relatively weaker competition binding by RBD-His protein with a higher concentration (Fig. 3c).

3.3. *High-throughput screening of SMIs against RBD-ACE2 interaction*

Employing the established HP1α recruitment system, we screened a total of 2572 small molecules. The readout was reduced ACE2 enrichment in RBD-containing condensates. Six compounds that showed over 20% inhibition were selected for further validation (Fig. 4). The compound showing the strongest activity, i.e., Quinacrine 2HCl, is a mem-

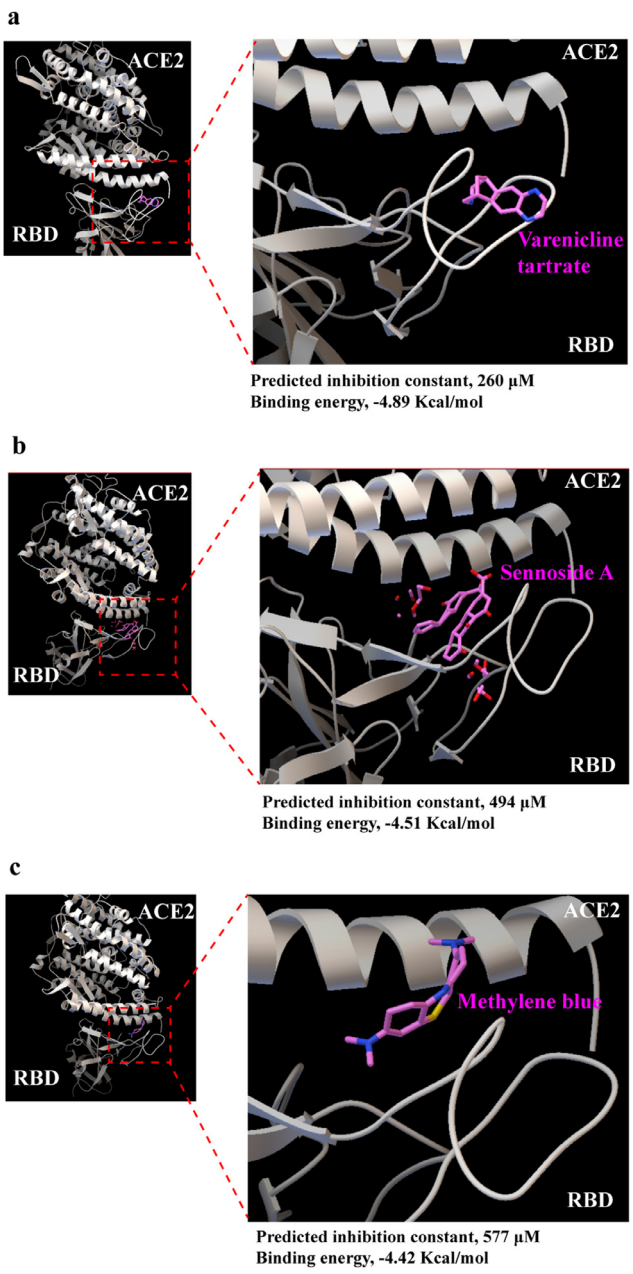

Fig. 6. Molecular docking analysis of RBD complexed with selected compounds. (a) Molecular docking results of RBD in complex with Varenicline tartrate. The binding energy is -4.89 Kcal/mol and the predicted inhibition constant is 260 μM. (b) Molecular docking results of RBD in complex with Sennoside A. The binding energy is -4.51 Kcal/mol and the predicted inhibition constant of 494 μM. (c) Molecular docking results of RBD in complex with Methylene blue. The binding energy is -4.42 Kcal/mol and the predicted inhibition constant of 577 μM. The RBD and ACE2 proteins are depicted using ribbon structure while the compounds are depicted with stick models.

ber of the family of cationic amphiphilic drugs (CADs), which function as host-directed antimicrobial drugs with potential broad-spectrum efficacy against viruses [28]. This screen also identified Methylene blue, a phenothiazine dye approved by the FDA for the treatment of methemoglobinemia. Methylene blue was recently found to be a potent SMI of the SARS-CoV-2 Spike-ACE2 interaction in a screen using a cell-free ELISA, with Fc-conjugated ACE2 coated on the multi-well plate and His-tagged RBD in the solution [13].

To examine the ability of each drug to block the SARS-CoV-2 spike protein from binding ACE2, we conducted surface plasmon resonance (SPR) analyses using immobilized RBD. As a positive control, recombinant ACE2 was shown to bind RBD with high affinity. The equilibrium dissociation constant, K_D, was 10 nM (data not shown), in accordance with the reported K_D of 15 nM for the SARS-CoV-2 spike protein-ACE2 interaction [29]. Further SPR studies revealed distinct affinities of the six compounds for RBD, ranging from 17 μM (Varenicline tartrate) to 781 μM (Sunitinib malate) (Fig. 5). Notably, Methylene blue was reported to inhibit the Spike-ACE2 interaction in a concentration-dependent manner with a low micromolar half-maximal inhibitory concentration (IC_{50} = 3 μM) [29]. It shows low affinity (K_D = 621 μM) with RBD in our SPR analyses. When we performed molecular docking analysis of certain representative SMIs as examples (Varenicline tartrate, Sennoside A and Methylene blue), the hierarchy of predicted affinities was the same as determined by SPR (Fig. 6).

4. Discussion

Despite tremendous global efforts, COVID-19 remains a serious health concern. In the pursuit of effective strategies to combat SARS-CoV-2, pharmacological approaches modulating a direct receptor-ligand interaction involved in viral entry are promising alternatives to other therapies. In the early 2000s, the emergence of SARS-CoV provided an impetus to discover SMIs that target various early steps in the cell invasion process [30,31]. To this end, the Spike-ACE2 PPI is an attractive target. Host ACE2 was shown to be a specific receptor for Spike protein more than a decade ago [23]. The discovery that Spike proteins bind directly to ACE2, and the discovery of the importance of their interaction in mediating viral entry, encouraged efforts to develop SMIs to block coronavirus (CoV) invasion. Even though if certain compounds showed evidence of inhibiting CoV infection, no approved preventive or curative therapy is currently available for CoV diseases mainly due to the unsuitability of the compounds for clinical translatability. Therefore, methods for screening and repurposing clinical drugs in a more effective manner are of primary importance.

Results obtained here confirmed again the robustness and usefulness of our CEBIT *in vitro* system [11] to identify potent SMIs for PPI inhibition. Here, we explored the potential of this system and achieved considerable progress in targeting the PPI between ACE2 and SARS-CoV-2 spike protein as a viable therapeutic strategy. By screening a library of FDA-approved compounds, we identified several promising SMIs, which partially overlap with SMIs from other screening systems. Varenicline tartrate, which showed the strongest affinity to RBD in our study, is a known nicotinic AChR partial agonist approved by FDA as a prescription medication for treating smoking addiction. Intriguingly, Varenicline tartrate has shown potent antiviral activity against wildtype SARS-CoV-2 and even its variants, i.e. alpha and beta, by directly binding RBD to impair the interaction with ACE2 [14]. Notably, another positive candidate, Methylene blue, has been listed as an Essential Medicines by WHO for its application in treating malaria [21,32]. Other clinical uses of Methylene blue include treatment of methemoglobinemia, ifosfamide-induced neurotoxicity and vasoplegic syndrome [32–34]. With resurgent interest, there is also recent evidence that Methylene blue has *in vitro* virucidal activity against SARS-CoV-2 [35,36]. Regarding clinical applications to COVID-19, one promising indication comes from a Phase 1 clinical trial (NCT04370288) in Iran as part of a new combination drug regimen, Methylene blue-vitamin C-N-acetyl cysteine [36].

Likewise, another cationic compound Quinacrine 2HCl, which is well-known for its antimalarial activity, has also been reported as an inhibitor of SARS-CoV-2 replication independent of its binding with RBD [18]. In common with this study, our data showed that Quinacrine 2HCl interacted with RBD with a particularly weaker affinity, which suggests that more optimization is needed to improve its performance. Regarding Quercetin, this natural plant flavonoid has been shown to induce mitophagy, apoptosis and protective autophagy through stimulating SIRT1 or inhibiting PI3K, resulting in a wide range of health benefits [16]. Moreover, recent studies have demonstrated that Quercetin has antiviral activity by binding both RBD and ACE2 to disrupt their interaction

[15]. For Sunitinib and Sennoside A, there is no in-depth data to confirm the relevance of their ability to inhibit RBD-ACE2 interaction inhibition, but it is intriguingly that both have been validated for reducing SARS-CoV-2 infection [19].

Considering the continued disruption caused by COVID-19, the quick screening of therapeutic agents may be a highly practical approach. Although previously considered "undruggable", PPIs are becoming the focus of the researchers with the development of screening methodologies, and there is increasing evidence that small molecules are competent to modulate the “hot spots” regions of PPI interfaces. Technological innovation is absolutely the primary requirement for progress in this area. To this end, CEBIT has been proved as a simple and efficient system for PPI identification and drug screening *in vitro*. With continual improvement, CEBIT will be of great help in developing more effective and specific therapeutics.

5. Conclusion

In summary, the current work utilized a refined method, namely CEBIT, for identifying small molecule inhibitors of the PPI between SARS-CoV-2 spike protein and its cognate receptor ACE2. Most of the six candidates have been reported as showing promising inhibition of the entry of SARS-CoV-2 in a concentration-dependent manner. While detailed activities and specificities require further verification and optimization, our results provide clear proof-of-principle evidence that CEBIT is highly versatile for identifying drugs that block SARS-CoV-2 entry, thus providing alternative therapeutic options to prevent and treat COVID-19.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest in this work.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (Grants No. 32100990 and 31800637), and the National Key Research and Development Program of China (Grant No. 2019YFA0508403).

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