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Short communication

Quercetin: A promising drug candidate against the potential SARS-CoV-2-Spike mutants with high viral infectivity

Boyu Pan [a,1], Senbiao Fang [b,1], Liangjiao Wang [a], Zhanyu Pan [c], Min Li [b,*], Liren Liu [a,**]

[a] Department of Molecular Pharmacology, Tianjin Medical University Cancer Institute & Hospital; National Clinical Research Center for Cancer; Key Laboratory of Cancer Prevention and Therapy, Tianjin; Tianjin's Clinical Research Center for Cancer, Tianjin 300060, China
[b] School of Computer Science and Engineering; Central South University, Changsha 410006, Hunan, China
[c] Department of Integrative Oncology; Tianjin Medical University Cancer Institute and Hospital; Key Laboratory of Cancer Prevention and Therapy, Tianjin 300060, China

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ABSTRACT

The emergence of SARS-CoV-2-Spike mutants not only enhances viral infectivity but also lead to treatment failure. Gaining a comprehensive understanding of the molecular binding mode between the mutant SARS-CoV-2-Spike and human ACE2 receptor is crucial for therapeutic development against this virus. Building upon our previous predictions and verifications regarding heightened viral infectivity of six potential SARS-CoV-2-Spike mutants, this study aims to further investigate the potential disruption of the interaction between these mutants and ACE2 by quercetin, a Chinese herbal compound. Molecular docking and dynamics simulations results reveal that the binding sites of quercetin particularly enriched around a specific "cavity" at the interface of Spike/ACE2 complex, indicating a favorable region for quercetin to interfere with Spike/ACE2 interaction. Virus infection assay confirms that quercetin not only attenuates wild-type virus infectivity but also suppresses the infectivity of all six tested SARS-CoV-2-Spike mutants. Therefore, quercetin represents a promising therapeutic candidate against both wild-type and potential future variants of SARS-CoV-2 exhibiting high viral infectivity.

1. Introduction

The development of innovative therapeutics against COVID-19, a global health crisis caused by SARS-CoV-2 infection, remains imperative [1]. SARS-CoV-2 utilizes its Spike protein to bind with ACE2 receptor for viral entry, leading to host infection. Emergence of new SARS-CoV-2 variants, particularly those involving the Spike receptor binding domains (RBDs), not only enhances viral infectivity and transmissibility but also undermines therapeutics targeting this region[2–8]. Disrupting the interaction between SARS-CoV-2 and ACE2 receptor represents one of the most promising approaches for antiviral therapy development. In this regard, a better understanding of this cunning virus through precise prediction of potential mutations within its Spike glycoprotein may facilitate targeted interventions toward novel SARS-CoV-2 variants and significantly increase opportunities for discovering curative therapeutics against COVID-19.

By analyzing the crystal structure of the SARS-CoV-2-Spike/ACE2 interface (RCSB PDB ID: 7CAI) and screening 2681 mutations within the Spike-encoding region deposited in the 2019 Novel Coronavirus Information databank (CNCB-NGDC, https://bigd.big.ac.cn/nCoV/), we recently identified 31 key residues on the Spike glycoprotein that may play a critical role in viral aggressiveness. Subsequently, we derived a total of 589 potential single-mutants from these residues through non-synonymous substitutions. Among them, eight single-mutants significantly enhanced binding affinity to ACE2 (<−65.00 kcal/mol) compared to the wild-type SARS-CoV-2-Spike (−55.07 kcal/mol), as revealed by our molecular dynamics simulation results. Furthermore, random combinations based on these eight single-mutants resulted in a total of 184 potential multiple-mutants (ranging from double- mutants to hepta-mutants). Out of these combinations, sixty multiple-mutants exhibited significant high binding affinity to ACE2 (<−65.00 kcal/mol). To validate our *in silico* analysis findings, we randomly selected six

* Correspondence to: School of Information Science and Engineering, Central South University, Changsha 410006, Hunan, China.
** Correspondence to: Department of Molecular Pharmacology, National Clinical Research Center of Cancer, Tianjin Medical University Cancer Institute & Hospital, Huanhuxi Road, Hexi District, Tianjin 300060, China.
E-mail addresses: limin@mail.csu.edu.cn (M. Li), liuliren@tmu.edu.cn (L. Liu).
[1] The authors contributed equally to this work.

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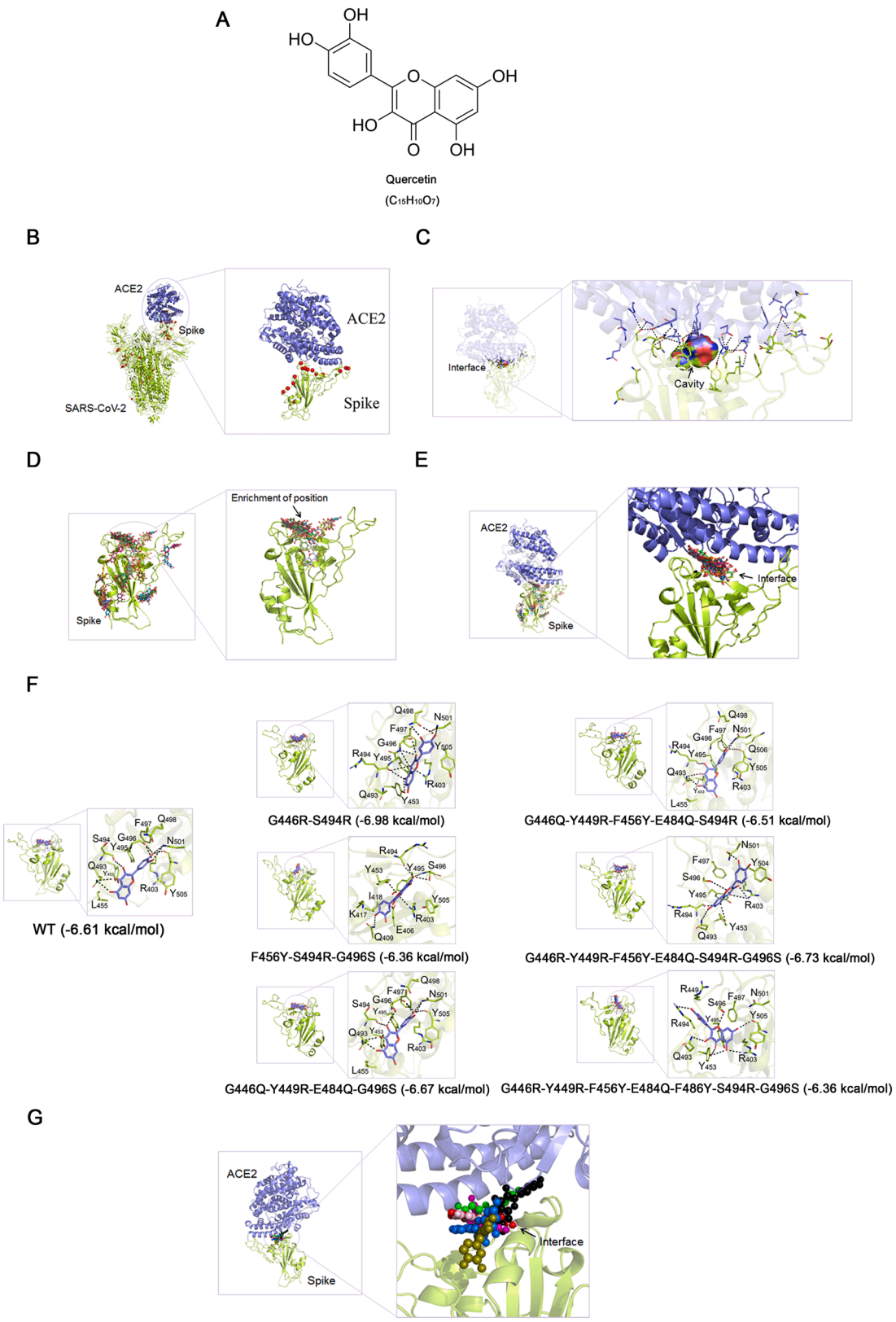

Fig. 1. ***Molecular docking analyses and experimental verification.*** **(A)** The 2D-chemical structure of quercetin. **(B)** The binding pattern between ACE2 and SARS-CoV-2 (PDB: 7CAI). Red dots: mutation sites. **(C)** The schematic diagram of "cavity" position. **(D)** To freely explore the potential action pocket of quercetin on the SARS-CoV-2-Spike region. **(E)** The schematic diagram of "interface" position between the SARS-CoV-2-Spike domain and ACE2. **(F)** Results of molecular docking studies of quercetin in the active sites of SARS-CoV-2-Spike proteins (WT and six multiple-mutants). **(G)** The schematic diagram of "the common binding pocket" position. Dots with different colors represent different conformations.

out of sixty multiple-mutants, including G446R-S494R, F456Y-S494R-G496S, G446Q-Y449R-E484Q-G496S, G446Q-Y449R-F456Y-E484Q-S494R, G446R-Y449R-F456Y-E484Q-S494R-G496S and G446R-Y449R-F456Y-E484Q-F486Y-S494R-G496S, for virus infection assay using pseudo-virus containing wild-type Spike or six multiple-mutant variants. Remarkably, all tested mutants showed enhanced viral infectivity compared to the wild-type pseudo-virus (For detailed results, please refer to another back-to-back research article we have simultaneously submitted to this journal - Collaborative effects of 2019-nCoV-Spike mutants on viral infectivity; Supplementary Table 1).

The understanding of the potential impact of novel Spike variants on therapeutics is crucial, as it plays an undeniable role in facilitating the development of effective drugs for COVID-19. In a previous study utilizing a network pharmacology approach, we have identified quercetin (Chem CID:5280343, Fig. 1A) as a promising compound with high binding affinity to both SARS-CoV-2 Spike protein and ACE2 receptor. Furthermore, our SPR-based competition assay has validated that quercetin significantly affects the binding between the wild-type Spike and ACE2. These findings present a compelling basis for exploring the potential inhibitory effects of quercetin on the emerging SARS-CoV-2 Spike mutants in this study.

2. Material and methods

2.1. Protein structure construction

Crystal structures of SRAS-CoV-2-Spike trimer domain and SARS-CoV-2 spike/ACE2 were retrieved from the RCSB PDB database (PDB ID: 7CAI and 6M0J) resolved at low resolution 3.49 Å and 2.45 Å, respectively. Specifically, we utilized the structure of the SARS-CoV-2 S trimer bound with two RBDs in an open state and complexed with two H Fab (RCSB PDB ID: 7CAI) to extract the trimer structure of SARS-CoV-2. Subsequently, we superimposed the crystal structure (RCSB PDB ID: 6M0J) of SARS-CoV-2 spike/ACE2 onto this extracted trimer structure to obtain a complete sequenced ACE2-bound form of the SARS-CoV-2 S trimer. Six multiple-mutants of SRAS-CoV-2-Spike/ACE2 were constructed based on the wild-type monomer complex (PDB ID: 6M0J) by using Mutagenesis Wizard tool on PyMol software.

2.2. Molecular docking & dynamics simulations

The procedures of molecular docking have been described previously [9]. In brief, a flexible docking process between chemical compounds and proteins was conducted using AutoDock 4.2 software. Then, a total of 30 docking conformations were clustered and ranked based on their docking energy. Finally, the top-ranked conformation with the lowest binding energy within the largest cluster was selected for further analysis of binding modes.

In order to check the binding modes of wild-type and six mutated SRAS-CoV-2-Spike/ACE2 systems with quercetin, molecular dynamics simulations[10] were carried out by AMBER software 20 to optimize the complexes. AMBER ff99sb force field were setting for each protein. Hydrogen atoms were added to the initial proteins using the leap module, setting ionizable residues as their default protonation states at a neutral PH value. The complex were solvated in a cubic periodic box of explicit TIP3P water model that extended a minimum 10.0 Å distance from the box surface to any atom of the solute. The particle mesh Ewald (PME) method for simulation of periodic boundaries was used to estimate the long-range electrostatic interactions with a cutoff of 10.0 Å. All bond lengths were constrained using the SHAKE algorithm and integration time step was set to 2 fs using the Verlet leapfrog algorithm.

To eliminate possible bumps between the solute and the solvent, the entire systems was minimized in two steps. Firstly, the complex was restrained with a harmonic potential of the form k $(\Delta x)^2$ with a force constant k = 100 kcal/mol^{-1} Å^{-2}. The water molecules and counter ions were optimized using the steepest descent method of 2500 steps, followed by the conjugate gradient method for 2500 steps. Secondly, the entire system was optimized by using the first step's method without any constraint. These two minimization steps were followed by annealing simulation with a weak restraint (k = 100 kcal/mol^{-1} Å^{-2}) for the complex and the entire system was heated gradually in the NVT ensemble from 0 to 298 K over 500 ps. After the heating phase, a > 100 ns MD simulation was performed under 1 atm. The constant temperature was selected at 298 K with the NPT ensemble. Constant temperature was maintained using the Langevin thermostat with a collision frequency of 2 ps^{-1}. The constant pressure was maintained employing isotropic position scaling algorithm with a relaxation time of 2 ps. RMSD values were tested to monitor the conformation fluctuations of protein-ligand complex. Based on the final 100 ns MDs trajectory, 3000 snapshots were extracted from the last 6 ns trajectory for the final average structures of wild-type and six mutated SRAS-CoV-2-Spike/ACE2 complexes. The binding free energies of SRAS-CoV-2-Spike to ACE2 or Quercetin were computed using the MM-PBSA module implemented as script (MMPBSA.py) in the AMBER software [11].

2.3. Reagent, cell lines and plasmids

Quercetin standard (Q111273) was purchased from Aladdin Company (China). The HEK-293 T and Huh7 cell lines were purchased from ATCC and the HEK-293 T-hACE2 cell line was generated by stably transfection of hACE2 gene into HEK-293 T cells. HEK-293FT, HEK-293FT-hACE2, and Huh-7 cell lines were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Cat #C11995500BT) supplemented with 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES, GIBCO, Cat#15630080), 1% of Penicillin Streptomycin solution (GIBCO, Cat #15140163) and 10% fetal bovine serum (FBS, Gibco, Cat #10091148). Above all cells were cultured at 37 ℃ with 5% CO_2, and cell passage were performed using 0.25% EDTA trypsin every 2–3 days.

The codon-optimized full-length coding sequences of WT or mutated Spike gene of SARS-CoV-2 (Wuhan-Hu-1 strain, GenBank NC_045512.2) were synthesized and respectively cloned into pcDNA3.1-myc-HisA vector. Plasmids were transformed into DH5α E.coli and extracted using QIAGEN Plasmid Maxi Kit (QIAGEN, Cat #12163).

2.4. Infection and functional assay

The procedures for production, titration and quantification of pseudotyped virus particles incorporated with S proteins from SARS-CoV-2 using a protocol reported in a recent study[12]. Pseudotyped viral particle numbers in the same range were obtained and normalized to the same amount by using quantitative RT-PCR module. Next, pseudotyped virus pre-coincubation treatment (37 ℃ and 3 h) with different concentrations of quercetin (50, 100 and 200 μM) at a volume ratio of 1:1 were interpolated to the Huh7 cells in 48-well cell culture plate after normalization. The plates were then incubated at 37 ℃ in a humidified atmosphere with 5% CO_2. The cells were trypsin-digested 24 h post infection, and flow cytometry and inverted microscopy were performed to account the rate of infected cells.

The flow cytometry experiment was conducted as follows: Huh-− 7 cells were rinsed twice with warm PBS, followed by a 5-minute digestion with 0.25% EDTA-trypsin. Subsequently, the cells were collected into 1.5 mL tubes and washed twice with PBS to remove trypsin. Clumps of cells were removed by filtration using a 70 μm cell strainer (BD Falcon, USA). The final cells were placed on ice in a dark chamber for further analysis. Flow cytometry analyses were performed using CytoFLEX instrument (Beckman Coulter, USA). To optimize the voltage settings for FSC, SSC, and FITC detectors and quantify the positive or negative particles within cell populations, VSV△G* -G viruses or negative control were utilized. A minimum of 5000 total cells were analyzed for each infection assay.

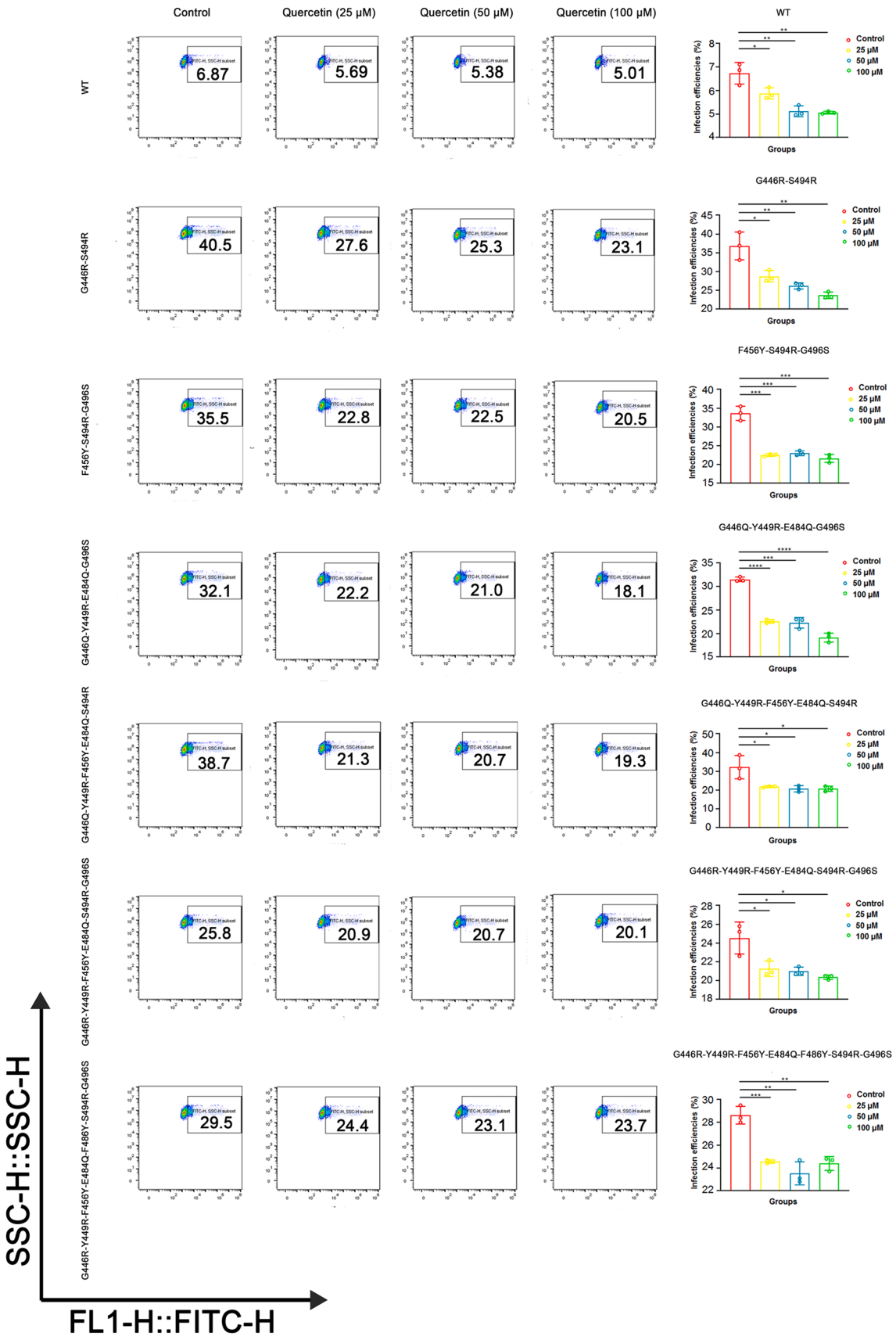

Fig. 2. ***The proportion of green fluorescent protein-positive cells were evaluated by flow cytometry.*** Statistical analyses of the abundance of the green fluorescent protein-positive cells. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$ and* ** * $p < 0.0001$.

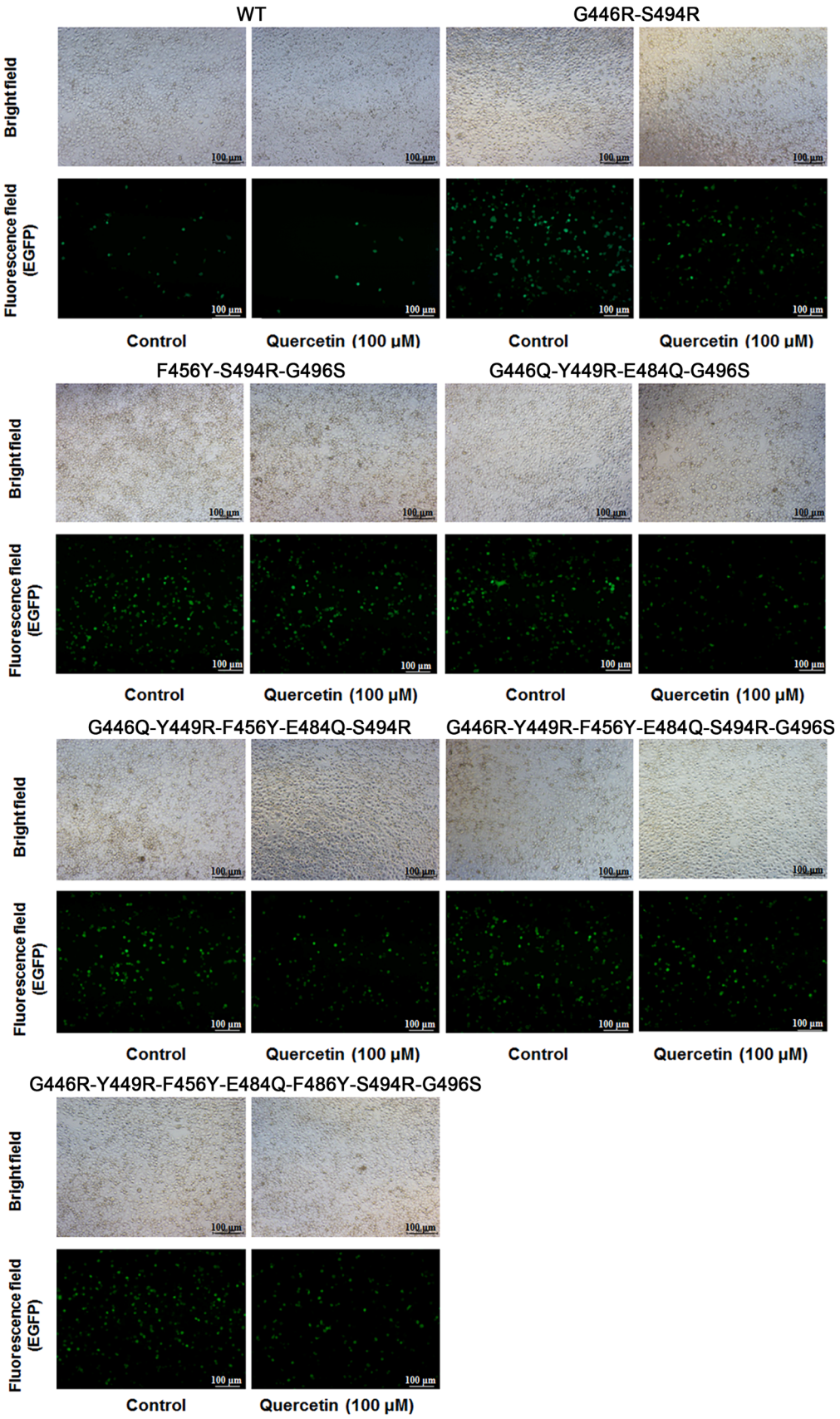

Fig. 3. The proportion of green fluorescent protein-positive cells were evaluated in fluorescence field by inverted microscopy.

2.5. Statistical analysis

The experimental data were analyzed by the Graphpad Prism 8.0 software (USA). Data are shown as mean±SEM. The Student's *t*-test was used to evaluate between-group differences. * $p < 0.05$ denoted significance.

3. Results

3.1. In silico prediction analyses

Here, we adopted the crystal construct of SARS-CoV-2-Spike trimer with RBD domain (RCSB PDB ID: 7CAI) resolved at 3.49 Å and extracted the monomer structure containing the Spike region and ACE2 for the analyses (Fig. 1B). Intriguingly, our result revealed the presence of a "cavity" at the binding interface between the Spike protein and ACE2 receptor (Fig. 1C). To ascertain whether this "cavity" could potentially serve as a binding pocket for quercetin, we employed molecular docking and molecular dynamics simulation analysis to comprehensively explore all potential interaction sites between quercetin and the entire Spike protein. In these analyses, Spike protein was considered as the receptor, while quercetin served as the ligand. RMSD values showed that all systems were relatively stable at about 3.0–10.0 Å (Supplementary Figure 1). Our findings revealed that the binding sites of quercetin were distributed throughout the surface of Spike protein, with a particular enrichment around that specific "cavity" at the binding interface, suggesting that this region may offer a potential binding site for quercetin to disrupt the interaction between the Spike protein and ACE2 receptor (Fig. 1 D & E). Furthermore, we observed that quercetin exhibited a binding affinity to the Spike protein of − 6.61 kcal/mol, which was comparable to that of the 6 selected mutants (Fig. 1F). Notably, despite containing multiple mutations within the interface region, all six mutants displayed relatively conservative conformation in terms of their 3D-structure within this "cavity", allowing quercetin to fit into it with similar binding affinities (Fig. 1G).

3.2. Virus infection assays

To verify our *in silico* results, we generated pseudotyped viruses carrying the wild type SARS-CoV-2 or six mutated Spike proteins co-expressing GFP fluorescent protein. These pseudotyed viruses were pre-incubated with different concentrations of quercetin (50, 100 and 200 μM), at a volume ratio of 1:1 at 37 ℃ for 3 h prior to infecting human Huh-7 cells. Flow cytometry analysis indicated a dose-dependent reduction in the percentage of green fluorescent-positive cells by approximately 40% (ie. from 40.5% to 25.3% with G446R-S494R mutant) following quercetin treatment (Fig. 2). Additionally, fluorescence observation using inverted microscopy demonstrated a significant decrease in the number of green fluorescent-positive Huh-7 cells after quercetin treatments (Fig. 3). Hence, these results provide evidence supporting the notion that quercetin attenuates the infection by these pseudotyped viruses through impairment of viral spike binding to ACE2 receptor.

4. Discussion

The emergence of new variants of SARS-CoV-2 has raised concerns about their increased transmissibility and potential resistance to current treatments or vaccines. Therefore, understanding how these mutants affect viral infectivity and exploring potential effective strategies against them is crucial[4,5]. We have recently predicted and verified the high viral infectivity of six SARS-CoV-2-Spike mutants. In this study, we further determined the effectiveness of quercetin in impairing the binding of these mutants with ACE2.

Quercetin, a natural compound found in various herbs, fruits and vegetables renowned for its antioxidant properties, has been previously suggested to possess antiviral activity against other coronaviruses such as SARS-CoV-1[13]. Studies have shown that quercetin possesses inhibitory effects on viral replication by interfering with key steps in the viral life cycle. It can inhibit viral entry host cells by blocking specific receptors or proteins required for attachment and fusion[14,15]. Furthermore, quercetin exhibits potent anti-inflammatory properties that may help mitigate the excessive immune response often observed during viral infections[15]. Moreover, quercetin has been reported enhance immune function by modulating various signaling pathways involved in immune cell activation and cytokine production[9]. By a balanced immune response, quercetin may contribute to reducing disease severity and improving overall survival from respiratory infections caused by coronaviruses.

The findings from this study will contribute valuable insights into both understanding the behavior of emerging SARS-CoV-2 variants and exploring alternative treatment strategies beyond traditional antiviral drugs or vaccines. By investigating the interaction between these mutant strains and quercetin, we aim to shed light on whether quercetin can serve as a viable option for inhibiting their binding to ACE2. Indeed, our findings demonstrate that quercetin effectively impairs the binding ability of these mutant strains, thereby presenting an attractive therapeutic option for managing COVID-19 cases caused by specific variant strains.

5. Conclusions

The compound quercetin shows promise as a therapeutic candidate not only against the wild-type SARS-CoV-2, but also against future emerging variants with highly infectious potential.

Author contributions

Liren Liu and Min Li designed and supervised the study and finalized the manuscript. Boyu Pan, Senbiao Fang, Liangjiao Wang and Zhanyu Pan contributed to the study design, performed the molecular docking & molecular dynamics simulations analyses and biological experiments and drafted the manuscript. All the authors approved the version to be published.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgement

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2023.10.029.

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