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ORIGINAL ARTICLE

# Peimine inhibits variants of SARS-CoV-2 cell entry via blocking the interaction between viral spike protein and ACE2

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## Abstract

Coronavirus disease 2019 (COVID-19) is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Several vaccines against SARS-CoV-2 have been approved; however, variants of concern (VOCs) can evade vaccine protection. Therefore, developing small compound drugs that directly block the interaction between the viral spike glycoprotein and ACE2 is urgently needed to provide a complementary or alternative treatment for COVID-19 patients. We developed a viral infection assay to screen a library of approximately 126 small molecules and showed that peimine inhibits VOCs viral infections. In addition, a fluorescence resonance energy transfer (FRET) assay showed that peimine suppresses the interaction of spike

**Abbreviations:** ACE2, Angiotensin-converting enzyme 2; COVID-19, Coronavirus disease 2019; EUA, emergency use authorization; RBD, receptor-binding domain; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VOCs, variants of concern.

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and ACE2. Molecular docking analysis revealed that peimine exhibits a higher binding affinity for variant spike proteins and is able to form hydrogen bonds with N501Y in the spike protein. These results suggest that peimine, a compound isolated from Fritillaria, may be a potent inhibitor of SARS-CoV-2 variant infection.

**Practical applications**

In this study, we identified a naturally derived compound of peimine, a major bioactive alkaloid extracted from *Fritillaria*, that could inhibit SARS-CoV-2 variants of concern (VOCs) viral infection in 293T/ACE2 and Calu-3 lung cells. In addition, peimine blocks viral entry through interruption of spike and ACE2 interaction. Moreover, molecular docking analysis demonstrates that peimine has a higher binding affinity on N501Y in the spike protein. Furthermore, we found that *Fritillaria* significantly inhibits SARS-CoV-2 viral infection. These results suggested that peimine and *Fritillaria* could be a potential functional drug and food for COVID-19 patients.

**KEYWORDS**

ACE2, Fritillaria, peimine, SARS-CoV-2, *variants of concern*

## 1 | INTRODUCTION

From the end of 2019 to 2021, a novel coronavirus (nCoV), termed "severe acute respiratory syndrome" virus or "(SARS)-CoV-2", has been isolated from patients with coronavirus disease-19 (COVID-19), and it was found to be responsible for the COVID-19 pandemic (Yan et al., 2020). The entry of SARS-CoV and SARS-CoV-2 into host cells is dependent on the presence of the transmembrane spike (S) glycoprotein on the viral surface (Tortorici & Veesler, 2019). The S glycoprotein is composed of two functional subunits—S1 and S2. S1, harboring a receptor-binding domain (RBD), is critical for virus binding to the host cell membrane, whereas S2 is critical for fusion with the host cell membrane (Yan et al., 2020). Angiotensin-converting enzyme 2 (ACE2) was identified as a receptor of SARS-CoV-2 (Li et al., 2003) on the host cell membrane because it directly binds to the viral spike (viral S glycoprotein) protein (Yan et al., 2020). Furthermore, the S2 subunit is critical for the fusion process after cleavage by host cellular proteases that induces irreversible conformational changes, facilitating membrane fusion (Millet & Whittaker, 2015). Overall, SARS-CoV-2 entry is a complex process that requires spike/ACE2 binding and activation of membrane proteases, such as TMPRSS2 (Hoffmann et al., 2020) and furin (Cantuti-Castelvetri et al., 2020), to promote fusion with the cell membrane.

Throughout human medical history, one of the efficient ways to curb viral infection has been through the development of vaccines. The U.S. Food and Drug Administration (FDA) has issued emergency use authorization (EUA) for four COVID-19 vaccines: two first-kind mRNA vaccines and two adenovirus vector vaccines. Notably, mRNA vaccines have demonstrated remarkable effectiveness, of approximately 95%, in preventing SARS-CoV-2 infection in clinical trials (Jackson et al., 2020). The mRNA vaccine is based on a lipid nanoparticle-encapsulated mRNA that encodes the SARS-CoV-2 spike glycoprotein in stabilized conformation. Determination of the strong affinity of the spike glycoprotein for the receptor ACE2 may provide desired targets for the development of vaccines against SARS-CoV-2. It is encouraging that vaccines are finally available from multiple sources. However, the therapeutic efficacy of these vaccines may be affected by variants of SARS-CoV-2 harboring N501Y and E484K mutations (Tanaka et al., 2021). In addition, according to recent studies, the mRNA vaccine (mRNA-1273, Moderna) shows reduced efficacy against the Beta (501Y.V2, B.1.351) variant compared to the original strain (Wang et al., 2021). Another mRNA vaccine (BNT162b2, Pfizer) was found to produce reduced neutralizing titers against the Alpha (B.1.1.7) (Muik et al., 2021), Delta (B.1.617.2) (Planas et al., 2021), and Omicron (B.1.1.529) (Garcia-Beltran et al., 2022) variants in immune sera. Furthermore, the ChAdOx1 (AZD1222, AstraZeneca) SARS-CoV-2 vaccine did not show protection efficacy against the B.1.351 variant (Madhi et al., 2021). In addition, a recent study demonstrated that the mRNA vaccines were less effective against Omicron infection (Abu-Raddad et al., 2022). Thus, small compound inhibitors that directly block the interaction of the spike glycoprotein and ACE2 warrant further study.

In previous studies, herbal medicine has been demonstrated to have anti-inflammatory effects and to inhibit infectious diseases, including hepatitis virus, influenza virus, and coronavirus (Huang et al., 2020; Lau et al., 2005; Lee et al., 2020; Liu et al., 2019; Xiong et al., 2020; Yang et al., 2014). Clinical studies of herbal medicine treatment for patients with SARS-CoV-2 infection have reported significant improvement of symptoms and shortening of the disease course (Ang et al., 2020; Yang, Islam, et al., 2020). In addition, a combination therapy of herbal medicine and Western medicine was able to improve the quality of life and symptoms of patients during the

SARS outbreak in the early 2000s (Liu et al., 2012). These reports suggest that herbal medicine has a beneficial effect in the treatment and prevention of infectious diseases. Research on pure compounds obtained from herbal medicine or their extracts is known to be an important means of developing new drugs (Imran et al., 2020; Yan et al., 2012). To swiftly move bench discoveries into clinical settings, we decided to test whether unbiased drug screening on pure compounds from a library of existing herbal plants can identify potential anti-SARS-CoV-2 drugs.

To test this hypothesis, we screened 126 pure compounds from the Natural Compound Library, a comprehensive drug library, to identify the drugs that harbor antiviral infection activity against SARS-CoV-2 and variants in a cell-based assay. In addition, we analyzed the biophysical properties of the SARS-CoV-2 spike protein binding to ACE2 in an in vitro assay. Our results show that peimine, an active component of *fritillary* species with anti-inflammatory properties, may be a potential therapeutic drug for treating COVID-19 patients.

## 2 | MATERIALS AND METHODS

### 2.1 | Cell lines and culture conditions

Huh-7 and 293T cell lines were obtained from ATCC. The 293T human embryonic kidney cell line, Calu-3 lung cancer cell line, Huh-7 human hepatocellular carcinoma cell line, and Vero E6 African green monkey kidney cell line were maintained in Dulbecco's MEM (Gibco) containing 10% fetal bovine serum (HyClone), and 100 units of penicillin (HyClone), 100 μg of streptomycin (HyClone). In addition, 293T cells stably expressed recombinant human ACE2 (293/hACE2) (Wang, Chen, et al., 2020).

### 2.2 | Small-molecule compound library

The pure compound library, Natural Compound Library (Catalog # L6000, Target Molecule Corp, Inc.) was used to screen for drugs.

### 2.3 | Lentiviral particles pseudotyped with SARS-CoV-2 spike protein infection assay

Lentiviral particles pseudotyped (Vpp) contains SARS-CoV-2 spike protein and luciferase reporter or green fluorescent protein (GFP) gene (Wang, Chen, et al., 2020). SARS-CoV-2 variants were purchased from the National RNAi Core Facility (NRC), Academia Sinica, Taipei, Taiwan. Then, 3–5 μl of supernatant was added to the cells in a 96-well plate (MOI ~ 0.2) in the presence of polybrene (8 μg/ml). The plate was centrifuged at 1,200 μg for 30 min and then returned to the incubator. Twenty-four hours post infection (hpi), the culture supernatants were replaced with a fresh medium. Seventy-two hours post infection, luciferase activity was determined according to the manufacturer's instructions.

### 2.4 | Cell viability assay

Cell survival was measured using WST-8/CCK-8 (Abcam) reagent incubated with cells for 4 hr. The samples were then measured spectrophotometrically at 595 nm using an ELISA plate reader. The percentage of viable and dead cells in each treatment group was calculated by normalization with data of the untreated control group.

### 2.5 | Western blot analysis

Experimental cells were harvested and lysed with RIPA buffer, which included 1 mM PMSF, immediately before use to prepare a modified RIPA buffer, and the lysate proteins were resolved on SDS containing 10% polyacrylamide gel, transferred to PVDF membranes, and probed with specific antibodies against α-tubulin (Sigma, #T5168), TMPRSS2 (Santa Cruz Biotechnology, #sc-515727) and ACE2 (GeneTex, #GTX101395). An enhanced chemiluminescence (ECL) kit was purchased from Bio-Rad.

### 2.6 | Sample preparations

Protein samples of SARS-CoV-2 $M^{pro}$ and the CFP-YFP protein substrate were prepared as previously described (Wang, Yang, et al., 2020).

### 2.7 | Time-resolved fluorescence resonance energy transfer assay

Interruption of the SARS-CoV-2 spike S1 and human ACE2 interaction by peimine was detected using the Time-resolved fluorescence resonance energy transfer (TR-FRET) assay according to the manufacturer's protocol (Catalog #79949-1, BPS Bioscience, Inc.) (Wang, Chen, et al., 2020). Briefly, ACE2 and Spike S1 proteins with or without 100 μM peimine were incubated at room temperature for 1 hr. TR-FRET signals were read at 665 nm.

### 2.8 | LC/MS analysis and quantitative analysis

Quantitative analysis of peimine content was performed with LC/MS. Aqueous extracts of *Fritillaria thunbergii* and *Fritillaria cirrhosa* D. Don were analyzed using a Velos Pro dual-pressure linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an Agilent 1100 Series binary high-performance liquid chromatography pump (Agilent Technologies, Palo Alto, CA). Briefly, the gradient program was 2% buffer B at 2 min to 98% buffer B at 20 min with a flow rate of 50 μl/min, where buffer A was 0.1% formic acid/H2O and buffer B was 0.1% formic acid/acetonitrile. A survey scan was acquired in the mass range m/z 200–2,000. The electrospray voltage was maintained at 4 kV, and the capillary

temperature was set at 275°C. The peimine content in each sample was estimated based on the mass peak-area intensity of the precise molecular weight signal with the exact LC elution time as that in the peimine standard compound.

### 2.9 | Molecular docking

The RBD-ACE2 complex (PDB ID: 6VW1) was used as the template structure for the docking experiment. Initially, we replaced the valine residue at 417 with lysine in 6VW1 to generate the wild-type (WT) docking target using (PS)2V3 protein structure prediction server (Huang et al., 2015). Next, the N501Y mutation was generated to create the B.1.1.7 docking target. The B.1.351 target was established by introducing N501Y, E484K, and K417N mutations into the WT target. L452R and T478K, and K417T, E484K, and N501Y were introduced to generate B.1.617.2 and P.1 variants. The docking target of current major spreading variant, B.1.1.529, was also modeled by making G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H mutations. Docking tasks were performed for the WT, B.1.1.7, B.1.351, B.1.617.2, P.1, and B.1.1.529 variants using iGEMDOCK (Hsu et al., 2011) with the "GA Parameters" settings of Population size = 300, Generation = 80, and Number of solutions = 100. The best docking pose (solution) with the smallest docking score was selected for further representation. The frequency distribution of the docking scores of 100 docking poses found for the WT, B.1.1.7 and B.1.351, B.1.617.2, P.1, and B.1.1.529 targets are depicted. All visualizations of the docking results were visualized using PyMOL (Eriksson et al., 2021).

### 2.10 | Statistical analyses

The statistical significance of a difference between mean values was estimated using the SigmaPlot software package for performing independent Student's *t*-tests. Error bars indicate the *SEM* of technical triplicates. The data are expressed as the means ± *SEM*. *p* values of less than .05 were considered statistically significant. **p* value < .05; ****p* value < .001 compared with control.

## 3 | RESULTS

### 3.1 | Drug screening for anti-SARS-CoV-2 entry

There is an urgent need to find potential therapeutic agents for inhibiting SARS-CoV-2 infection. Huh-7 cells, derived from hepatocellular carcinoma, have been established for the study of viral entry, including entry of influenza virus, rhinovirus, and SARS-CoV-2 (Freymuth et al., 2005; Riva et al., 2020). We performed a virus particle pseudotyping (Vpp) SARS-CoV-2 assay with Huh-7 cells to determine the infectivity of Vpp based on luciferase activity. We collected and identified compounds that suppress SARS-CoV-2 entry. Among the 126 natural compounds, four compounds—sciadopitysin, vanillic acid, peimine, and semagacestat—exhibited potent inhibitory activity. Thus, we selected them as hits using a *z*-score (cutoff of 1.5) in Huh-7 cells (Figure 1). Our findings suggested that pure, natural compounds obtained from herbal plants can be potential anti-SARS-CoV-2 agents.

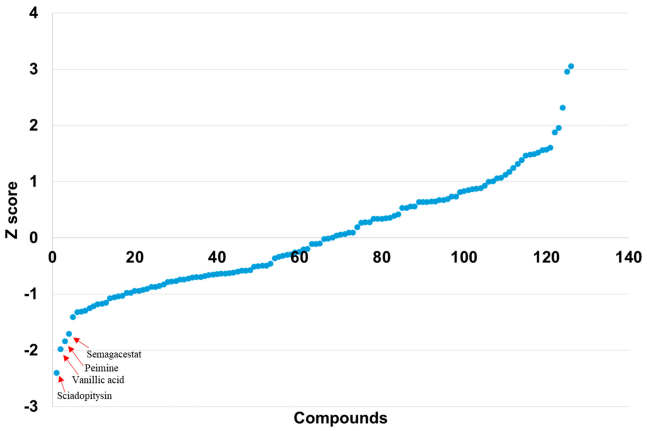


**FIGURE 1** Screening for natural compounds with potential inhibitory activity on SARS-CoV-2 pseudovirus entry. Huh-7 cells were pretreated with 126 natural compounds at a final concentration of 10 μM for 1 hr and then infected with lentivirus particles pseudotyped (Vpp) with SARS-CoV-2 spike protein (MOI ~ 0.1). All viruses and compounds were removed 24 hpi (hours post infection). At 72 hpi, infectivity was assessed based on luciferase activity and is displayed as a *Z*-score chart.

### 3.2 | Peimine inhibits SARS-CoV-2 entry in 293T/hACE2 and Calu-3 cells

We next sought to assess whether the efficacy of the drugs was limited to Huh-7 cells (Freymuth et al., 2005; Riva et al., 2020). We evaluated the pure compound efficacies in an additional human cell line that supports SARS-CoV-2 entry. Specifically, 293T cells were transfected with human ACE2 (hACE2). First, we analyzed the ectopic expression of ACE2 in 293T cells, and the results showed that ACE2 was significantly overexpressed in two subclones of 293T cells—ACE2#1 and ACE2#2 (Figure 2a). Furthermore, we selected the top four candidates—vanillic acid, peimine, sciadopitysin, and semagacestat (Figure 1)—to treat the 293T/hACE2 cells and examine their inhibitory activity. We discovered that cells treated with peimine significantly and consistently inhibited entry of the SARS-CoV-2 pseudovirus (Figure 2b). To further validate the inhibition of virus entry, we performed a dose titration analysis and demonstrated that, at elevated concentrations, peimine inhibited viral infection in the hACE2-overexpressing 293T cell line (Figure 2c) and Calu-3 lung cancer cell line (Figure 2d), which is a common cell line used to examine SARS-CoV-2 entry (Hoffmann et al., 2020). Moreover, the inhibition of peimine was determined based on the expression of the green fluorescent protein (GFP) gene encoding Vpp. Consistent with the detection of luciferase activity, peimine significantly reduced GFP expression caused by Vpp infection in

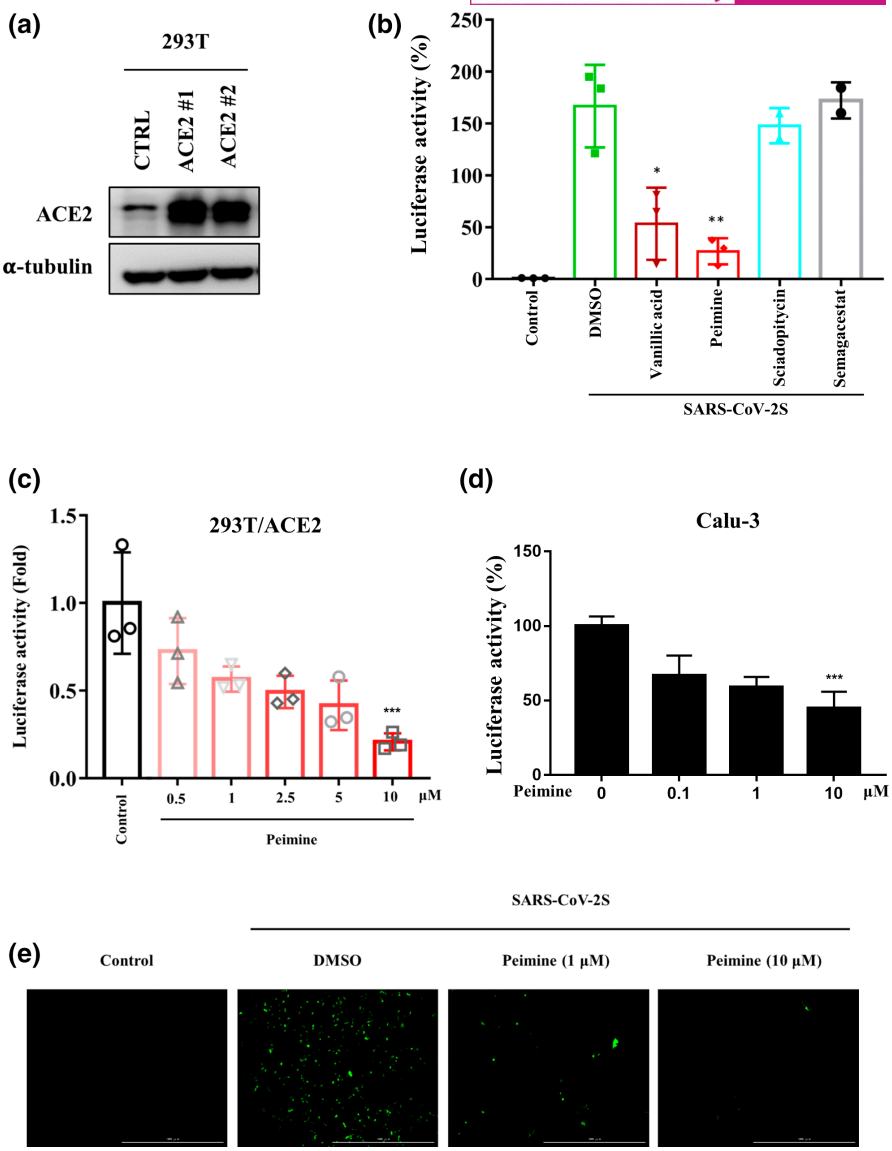


FIGURE 2 Peimine can be an effective natural compound for blocking SARS-CoV-2 entry. (a) Human ACE2 (hACE2) was overexpressed in 293T cells, and the proteins were extracted from the cell lysates. Tubulin: loading control. (b) 293T/hACE2 cells were pretreated with DMSO only, vanillic acid (10 μM), peimine (10 μM), sciadopitysin (10 μM), or semagacestat (10 μM) for 2 hr and then inoculated with lentivirus particles pseudotyped (Vpp) with SARS-CoV-2 spike protein for 24 hr. (c,d) 293T/hACE2 and Calu-3 cells were preincubated with different doses of peimine for 2 hr. The cells were lysed 24 hr later, and Vpp transduction was measured. Experiments were performed in triplicate. Error bars indicate the *SEM* of technical triplicates. (e) Vpp backbones incorporate green fluorescent protein (GFP) that is expressed upon infection into target cells. Fluorescence was recorded 24 hr post infection. Magnification, 4×. Scale bar: 1,000 μm. Statistical significance was calculated using Student's *t*-test. **p* value < .05; ***p* value < .01; ****p* value < .001 compared with the control.

hACE2-overexpressing 293T cells (Figure 2e). In addition, peimine has been shown to play a role in anticancer activity (Tan et al., 2020). Therefore, to further understand the safety and toxicity of peimine in 293T/hACE2 cells, cell viability was evaluated after the cells were treated with peimine. Interestingly, peimine treatment up to 1,000 μM did not affect cell viability compared to hydroxychloroquine (HCQ) (Supporting Information Figure S1a,b). HCQ has been reported to have several side effects, such as retinotoxicity, neuromyotoxicity, and cardiotoxicity, in patients (Nord et al., 2004). Therefore, clinical studies on COVID-19 have failed due to safety concerns over HCQ (Stevenson et al., 2020). The data presented in Figure 2 are consistent with those of other reports (Liu et al., 2020) showing that HCQ exhibits toxicity at high concentrations. Taken together, these results indicate that peimine can inhibit SARS-CoV-2 entry in multiple cell lines.

### 3.3 | Peimine inhibits variants of SARS-CoV-2 infection in 293T/ACE2 and Vero E6/furin cells

Furin is a calcium-dependent protease that recognizes and cleaves the specific sequence motif "RRAR" and enhances viral fusion to host

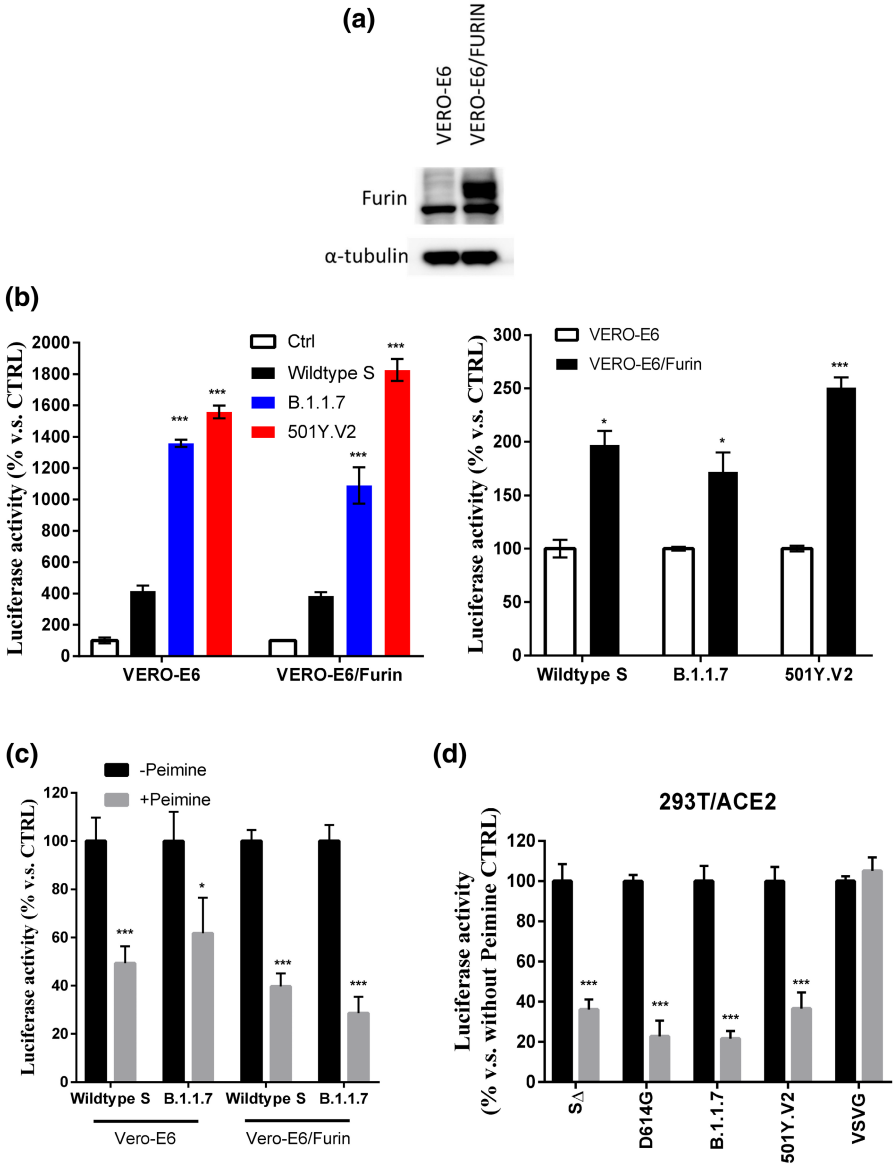


FIGURE 3 Peimine inhibits variants of SARS-CoV-2 infection in furin-overexpressing cells. (a) Human furin was overexpressed in Vero E6 monkey kidney epithelial cells, and proteins were extracted from the cell lysates. Tubulin: loading control (b) Vero E6 cells were transfected with and without furin expression and then inoculated with the B.1.1.7 (United Kingdom) and 501Y.V2 (South African) variants of lentivirus particles pseudotyped (Vpp) with SARS-CoV-2 mutant spike protein for 24 hr. (c,d) Vero E6 cells with and without furin expression and 293T/hACE2 cells were preincubated with 10 μM peimine for 2 hr and then infected with wild type, B.1.1.7, or 501Y.V2, D614G or VSV-G Vpp. The cells were lysed 24 hr later, and Vpp transduction was measured. Experiments were performed in triplicate. Error bars indicate the *SEM* of technical triplicates. Statistical significance was calculated using Student's *t*-test. **p* value < .05; ****p* value < .001 compared with the control.

cells (Papa et al., 2021). Mechanistically, after interacting with ACE2, the spike protein is cleaved into S1 and S2 by furin (Lan et al., 2020). Recently, it was shown that the transmission rate of variants of SARS-CoV-2, such as B.1.1.7 and B.1.351, has become much faster due to different genetic changes in the RBD of the spike protein at the furin cleavage site (Ali et al., 2021). Therefore, identifying an effective inhibitor to block the entry of different variants of SARS-CoV-2 is an urgent need. To determine whether the efficacy of peimine is furin dependent, we established stable furin transfectants in Vero E6 cells (Figure 3a), and these transfected cells were infected with different SARS-CoV-2 variants. As expected, the B.1.1.7 and 501Y.V2 (B.1.351) variants had a better entry rate in furin-stable transfectants than in wild-type transfectants (Figure 3b left). Furthermore, overexpression of furin-enhanced WT and variants of Vpp infection in Vero E6 cells (Figure 3b right). To further investigate the efficacy of peimine on SARS-CoV-2 variant infection, furin-overexpressing Vero E6 and ACE2-overexpressing 293T cells were pretreated with peimine and then infected with the WT and Vpp variants. The results demonstrated that peimine significantly inhibited B.1.1.7 and 501Y.V2 infection in parental and furin-overexpressing Vero E6 cells (Figure 3c). As shown in Figure 2a, ACE2-overexpressing 239T cells also showed high effectiveness in inhibiting Vpp variant infection

TABLE 1 Selectivity index (SI) of peimine

| Peimine (μM) | CC50 | EC50 | SI |
|---|---|---|---|
| Wild type | 19,099 | 0.4497 | 42,470.53 |
| UK (B.1.1.7) variant | 19,099 | 0.4222 | 45,236.85 |
| South Africa (501Y.V2) | 19,099 | 0.4318 | 44,231.13 |

after peimine treatment (Figure 3d). These results suggest that, although furin promotes Vpp variant infection, peimine still significantly inhibits variant infection. Furthermore, we showed that peimine has a preferential selectivity index (SI) for different COVID-19 variants (Table 1). Taken together, these data showed that peimine is a promising, effective agent against not only WT SARS-CoV-2 but also the B.1.1.7 and 501Y.V2 variants, which have been identified as variants of concern (VOCs) by the WHO.

### 3.4 | Peimine inhibits interactions between variant SARS-CoV-2 spike proteins and ACE2

SARS-CoV-2 is required for binding to ACE2 on the cell membrane and fusing with the cell membrane through the expression of TMPRSS2 to enter host cells (Li et al., 2003). Therefore, to further test whether peimine blocks SARS-CoV-2 infection via inhibition of ACE2 and TMPRSS2 protein expression, Huh-7 and 293T/hACE2 cells were treated with a range of 1.25 μM to 20 μM peimine. The results showed that peimine did not affect the expression of ACE2 or TMPRSS2 in either Huh-7 or 293T/hACE2 cells (Supporting Information Figure S2). Next, we asked whether peimine may inhibit the binding activity of the spike protein to host ACE2. By using a fluorescence resonance energy transfer (FRET)-based assay, we found that peimine significantly inhibited the binding activity of the SARS-CoV-2 spike protein and ACE2 (Figure 4a). Furthermore, to identify the binding sites of peimine on spike and ACE2, molecular docking analysis revealed that peimine forms three hydrogen bonds (H-bonds) with the main chain of Q388 and sidechain of K353 and R393 of human ACE2 and four H-bonds with the main chain of S494 and G496 and sidechain of Y453 of the SARS-CoV-2 spike RBD (Figure 4b). Therefore, we investigated whether peimine binds to ACE2 and blocks the interaction of spike and ACE2. Calu-3 cells were pretreated with peimine for 2 hr, washed with PBS, and incubated for another 2 hr. Next, we inoculated the cells with Vpp for 24 hr, and the results showed no difference between the wash and not wash cells (Supporting Information Figure S3). The results suggested that the effects of peimine are not reversible under washing conditions. Furthermore, peimine is a major bioactive alkaloid extracted from *Fritillaria thunbergii* Miq, *Fritillaria cirrhosa* D. Don, *Fritillaria unibracteata* Hsiao (Tan et al., 2020), and the most popular *Fritillaria cirrhosa*-containing herbal preparations in Taiwan, *Nin Jiom Chuanbei Pipa Gao* (NJCPG; Nin Jiom Medicine Manufactory, Hong Kong) (Guo et al., 2020). Therefore, it would be interesting to know the percentage of peimine in different types of *Fritillaria* and NJCPG. *Fritillaria*

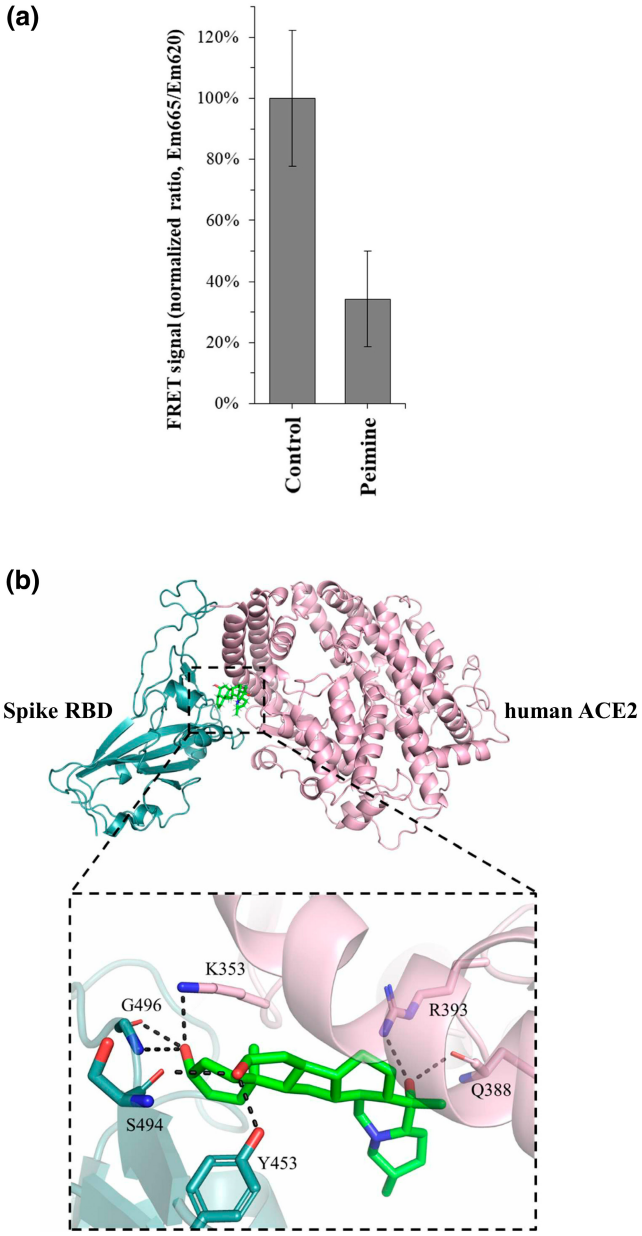


FIGURE 4 Peimine blocks SARS-CoV-2 spike protein binding ACE2. (a) Time-resolved FRET assay of the binding between SARS-CoV-2 Spike S1 and human ACE2 with or without 100 μM peimine. (b) Interaction between peimine and the spike RBD-ACE2 complex. Overview of the optimal pose of the interaction between potent compounds, peimine, and the spike RBD-ACE2 complex (PDB: 6VW1). Peimine is shown as green sticks. Spike RBD (light teal) and ACE2 (light pink) are shown in the cartoon. An enlarged view of the binding mode of peimine showing the hydrogen bonds as black dashed lines.

*thunbergii* and *Fritillaria cirrhosa* D. Don were chosen for analysis. To characterize the aqueous extracts of *Fritillaria* and NJCPG, we separated them using LC/MS. The results showed that the percentage of peimine was much higher in *Fritillaria thunbergii* (Supporting Information Table S1). Thus, the percentage of peimine varies widely among different species of *Fritillaria*. Furthermore, we investigated the efficacy of *Fritillaria thunbergii*, *Fritillaria cirrhosa* D. Don, and

NJCPG in SARS-CoV-2 infection. The results demonstrated that different species of *Fritillaria* and CBPPG blocked SARS-CoV-2 Vpp infection in ACE2-overexpressing 293T cells (Supporting Information Figure S4). In sum, these results demonstrated that not only peimine but also *Fritillaria* can inhibit SARS-CoV-2 viral infection.

### 3.5 | Peimine molecular docking analysis reveals the potential inhibitor for wild type and variants of SARS-CoV-2

We already showed that peimine can interrupt the interaction of ACE2 and spike protein (Figures 4a). Therefore, to understand how peimine interferes and blocks the binding between the spike proteins and ACE2. In Figure 5a,b, we showed the docking scores of 100 different peimine docking poses associated with the target SARS-CoV-2_RBD-ACE2 complex. A smaller negative docking score (DS) represents more stable binding to peimine. We tested SARS-CoV-2 RBD of wild type and five variants including B.1.1.7, B.1.351, B.1.617.2, P.1, and B.1.1.529, all the docking potential can be converged less than 100 docking iterations. When comparing peimine binding stability among WT, B.1.1.7, B.1.351, B.1.617.2, P.1, and B.1.1.529 variants, we can find WT has the best and B.1.1.529 has the least binding stability to peimine. The stability of docking peimine to WT, B.1.617.2, and P.1 (DG) is similar. In addition, we can find the sidechain conformations of the variants' RDB residues change not much comparing to the wide types. The docked poses of peimine are similar among wild type, B.1.1.7 and B.1.617.2 (Pose 1), and between B.1.351 and P.1 (Pose 2). The peimine docked pose for B.1.1.529 (Pose 3) is very different compared with Pose 1 and Pose 2 (Figures 5c). Furthermore, peimine formed polar contact with the N501Y mutant spike protein, and N501Y is one of the common mutations in B.1.1.7, B.1.351, P1 lineage (Brazil), and B.1.1.529 (Omicron) variants (Kazybay et al., 2022; Liu et al., 2022). Moreover, in electrostatic potential view, we can observe the peimine molecule is located in a hydrophobic pocket whatever WT or variants (Figure 6a). Furthermore, to demonstrate the inhibition activity of peimine on variants of SARS-CoV-2 and confirm the results of molecular docking. Similarly, peimine significantly inhibited all VOCs of SARS-CoV-2 viral infection, including the Omicron variant, the major variant that causes pandemics in the world. Furthermore, a recent study demonstrated that peimine significantly increases the Ca2+ concentration in cancer cells (Tan et al., 2020). However, the Ca2+ promotes SARS-CoV-2 entry by facilitating membrane fusion of the spike protein (Singh et al., 2022). Therefore, we confirmed whether the efficacy of peimine could be counteracted by Ca2+. And, the results showed that the effects of peimine could not be counteracted under the Ca2+ treatment (Supporting Information Figure S5). In summary, the results showed that peimine could inhibit the binding activity of the wild type and variants of SARS-CoV-2 spike protein to ACE2 on host cells and inhibited VOCs viral infection in cell model.

In this study, we utilized an herbal medicine library and found that peimine blocks WT and variants of SARS-CoV-2 pseudovirus entry. Moreover, the FRET assay also showed that peimine decreases the binding activity of the SARS-CoV-2 spike protein and ACE2 and contains binding sites on N501Y of mutant spike proteins, which is consistent with our hypothesis.

## 4 | DISCUSSION

Numerous research laboratories and clinical trials have been initiated to identify potential therapeutic drugs against the COVID-19 pandemic. Previously, remdesivir is supported by a clinical trial on COVID-19; however, the data show that remdesivir treatment does not significantly improve the symptoms of patients with COVID-19 (Hordijk & Patnaik, 2020). In addition, several repurposing drugs used in antiviral therapies have been used in clinical investigations, including anti-HIV-1 lopinavir/ritonavir (Lipsitch et al., 2020) and anti-hepatitis C virus danoprevir (Chen et al., 2020). Recently, FDA approved molnupiravir for the therapy of COVID-19 patients. Molnupiravir is a nucleoside analog and significantly reduces the mortality rate in patients with SARS-CoV-2 infection (Jayk Bernal et al., 2022). In addition, results from our study provide evidence that peimine could be a potential inhibitor for SARS-CoV-2 variants by blocking Spike and ACE2 interaction.

One focus of the scientific community is to develop a vaccine against SARS-CoV-2 infection to control the COVID-19 pandemic. The WHO has listed more than 200 COVID-19 vaccines in different stages of clinical trials (Haynes et al., 2020). Currently, two mRNA vaccines and one adenovirus vector vaccine against COVID-19 have been approved by the USA for EUA. A phylogenetic cluster (named “lineage B.1.1.7”) was detected in early December of 2020 by the COVID-19 Genomics UK Consortium (Shen et al., 2021). The B.1.1.7 lineage accumulates 17 mutations in its genome. Eight of these mutations are located in the gene encoding the spike glycoprotein localized on the viral surface (Shen et al., 2021). The B.1.1.7 lineage phenotype has also been proven to be more transmissible than the original SARS-CoV-2 lineages (Fratev, 2020). N501Y, which is located in the spike gene, has been identified in the B.1.1.7, B.1.351, and P1 and other lineages, covers the critical contact amino acid residues within the spike RBD, and increases the binding affinity for human ACE2 (Starr et al., 2020). Alarmingly, a report showed that another variant, B.1.351, and the P1 lineage harboring the E484K mutation on the spike protein show greater spike RBD and ACE2 affinity than that of N501Y (Tanaka et al., 2021). In addition, the Omicron (B.1.1.529) variant has spread rapidly around the world and has already become the dominant variant in many countries (Abu-Raddad et al., 2022). In the Omicron variant, 15 of the mutations are located in the RBD of spike protein (Cui et al., 2022). A recent study showed that the Omicron spike enhances the ability of viral attachment with the host cell and induces viral

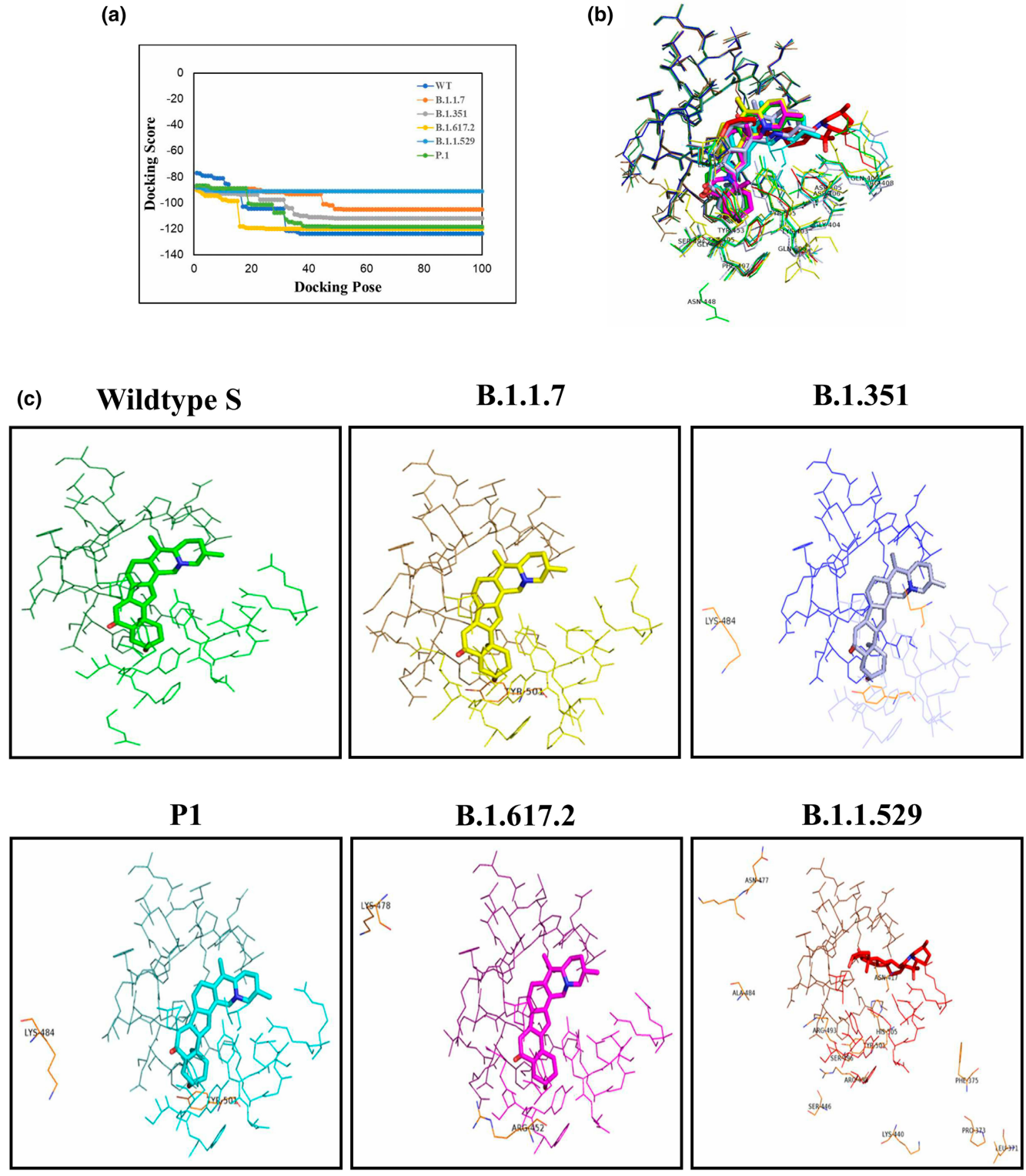


FIGURE 5 Peimine docking analysis for wild type and variants of SARS-CoV-2. (a) One hundred docking poses of disulfiram were analyzed for the targets of wild type (WT), B.1.1.7, B.1.1.529, B.1.351, B.1.617.2, and P.1 variants of SARS-CoV-2_RDB-hACE2 complex. (b) The binding pocket of SARS-CoV-2_RDB-hACE2 complex with peimine. The light colors represent SARS-CoV-2_RDB residues, whereas dark colors represent residues of human ACE2. The green, yellow, red, light blue, magenta, and cyan lines indicate residues of wild type, B.1.1.7, B.1.1.529, B.1.351, B.1.617.2, and P.1 variants, respectively. (c) The binding poses of peimine docked with the binding pocket of SARS-CoV-2_RDB-hACE2 for different SARS-Cov-2 variants. The poses of peimine docked to WT, B.1.1.7, B.1.351, B.1.617.2, P.1, and B.1.1529 are shown. The RBD mutations revealed in each variant are shown in orang lines with labels.

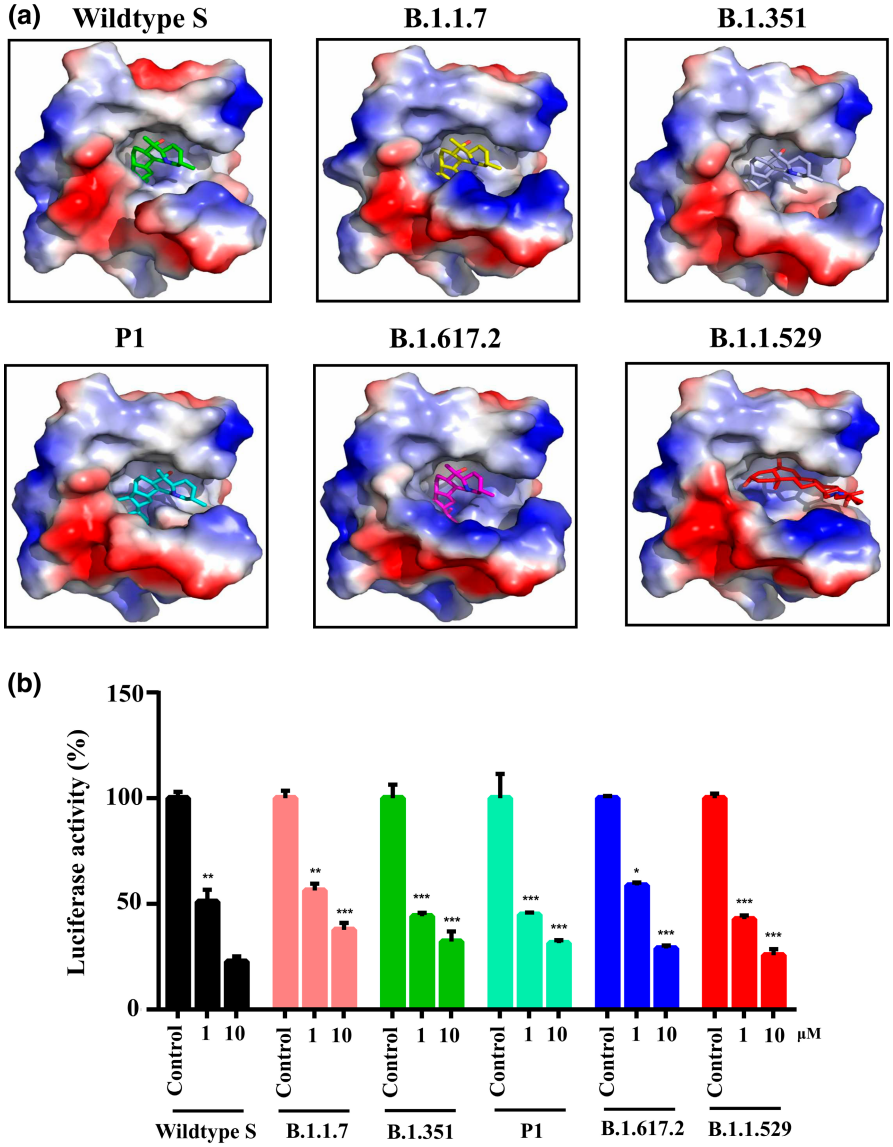


FIGURE 6 The contact potential of the binding pocket of SARS-CoV-2_RDB-hACE2 for different SARS-CoV-2 variants in complex with peimine. (a) The red, blue, and white colors represent negative charge, positive charge, and hydrophobic area, respectively. The environment of the binding cavity is mainly hydrophobic. The binding affinity of peimine predicted for WT is −10.6 kcal/mol, −11.2 kcal/mol for B.1.1.7, −11.0 kcal/mol for B.1.351, −11.1 kcal/mol for P.1, −10.2 kcal/mol for B.1.1.529, and −11.4 kcal/mol for B.1.617.2. The binding affinity values were computed using PRODIGI platform. (b) Calu-3 cells were preincubated with 1 or 10 μM peimine for 2 hr and then infected with wild type, B.1.1.7, B.1.351 (501Y.V2), P1, B.1.617.2, or B.1.1.529 lentiviral particles pseudotyped (Vpp), respectively. The cells were lysed 24 hr later, and Vpp transduction was measured. Experiments were performed in triplicate. Error bars indicate the *SEM* of technical triplicates. Statistical significance was calculated using Student's *t*-test. **p* value < .05; ***p* value < .01; ****p* value < .001 compared with the control.

fusion. Moreover, the mutations on the spike of Omicron disturb the neutralizing antibody recognition (Cui et al., 2022). In addition, the clinical study also showed that some therapeutic monoclonal antibodies have lower neutralizing activity against Omicron than against other VOC strains (Takashita et al., 2022). Furthermore, accumulating evidence has shown that an mRNA vaccine (BNT162b2) shows reduced vaccine effectiveness against VOCs (Kustin et al., 2021; Xie, Liu, et al., 2021). Thus, the efficacy of vaccines against SARS-CoV-2 variants in humans still needs to be improved. Therefore, it is important to seek other alternative means to target the virus or create a synergistic effect with a vaccine to prevent variant SARS-CoV-2 infections, and it would be interesting to examine whether peimine can inhibit infection by the variants of concern. In addition to mutations, there are other ways to enhance viral infectivity. Recent studies have shown that furin, a class of proprotein convertases (PCs), cleaves the Arg-Arg-Ala-Arg (R-R-A-R) C-terminal sequence at the S1/S2 junction in the SARS-CoV-2 spike glycoprotein to enhance virus fusion with cells (Daly et al., 2020). Moreover, after cleavage by furin, the C-end rule (CendR) motif is exposed and binds to the surface neuropilin 1 (NRP1) receptor to significantly induce SARS-CoV-2 infectivity (Cantuti-Castelvetri et al., 2020).

Furthermore, based on our FRET assay and molecular docking analysis, we know that peimine can block the interaction of the spike glycoprotein and ACE2. Moreover, our results suggested that peimine inhibits viral infection in furin-overexpressing cells. Therefore, peimine may be a potential drug for inhibiting virus infection in furin/NRP1-dependent cells.

Peimine, also known as verticine, is a major pure compound in several species of *Fritillaria*. In addition, peimine has been demonstrated to play an important role in treating human diseases, including analgesic, antiasthmatic, antitussive, and expectorant effects (Tan et al., 2020). Previous studies also showed that peimine can serve as an anticancer drug in a variety of cancer models with different types of cancer cells, such as prostate cancer and colon cancer (Chen et al., 2016; Tan et al., 2020). In this study, we provide a plausible explanation for the antiviral activity of peimine by blocking interactions between the viral spike protein and the host ACE receptor on the cell surface. Thus, peimine can act as an antiviral agent against COVID-19.

## 5 | CONCLUSIONS

We screened an herbal medicine library containing 126 pure compounds and found that peimine significantly inhibits viral entry by WT and VOCs including B.1.1.7, 501Y.V2, P1, B.1.617.2, and B.1.1.529 variants using a SARS-CoV-2 pseudovirus entry assay. In addition, we used the FRET assay to demonstrate that peimine inhibits the binding activity of the SARS-CoV-2 spike protein and ACE2 but does not inhibit the protein expression of ACE2 or TMPRSS2. Furthermore, peimine contains binding sites for both the WT and mutant spike proteins of SARS-CoV-2 and ACE2, as determined through molecular docking analysis. Peimine at relatively high concentrations did not exert toxicity in the cells. Taken together, the data suggest that peimine may be a relatively safe and novel drug that inhibits SARS-CoV-2 infection by blocking the interaction between the spike protein and ACE2.

### AUTHOR CONTRIBUTIONS

**Mien-Chie Hung:** conceptualization, funding acquisition, supervision, and writing - review & editing, **Wei-Jan Wang:** data curation, funding acquisition, investigation, and roles/writing - original draft, **Yeh Chen; Wen-Chi Su; Wan-Jou Shen, and Yen-Yi Liu:** investigation, **Wei-Chao Chang and Sheng-Teng Huang:** formal analysis, **Cheng-Wen Lin; Yu-Chuan Wang; Chia-Shin Yang; Mei-Hui Hou; Yu-Chi Chou; Yang-Chang Wu, and Shao-Chun Wang:** methodology.

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### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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