Harringtonine has the effects of double blocking SARS-CoV-2 membrane fusion

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Abstract

Fusion with host cell membrane is the main mechanism of infection of SARS-CoV-2. Here, we propose a new strategy to double block SARS-CoV-2 membrane fusion by using Harringtonine (HT), a small-molecule antagonist. By using cell membrane chromatography (CMC), we found that HT specifically targeted the SARS-CoV-2 S protein and host cell TMPRSS2, and then confirmed that HT can inhibit pseudotyped virus membrane fusion. Furthermore, HT successfully blocked SARS-CoV-2 infection, especially in the delta and Omicron mutant. Since HT is a small-molecule antagonist, it is minimally affected by the continuous variation of SARS-CoV-2. Our findings show that HT is a potential small-molecule antagonist with a new mechanism of action against SARS-CoV-2 infection, and thus HT mainly targets the S protein, and thus, greatly reduces the damage of the S protein's autotoxicity to the organ system, has promising advantages in the clinical treatment of COVID-19.

Results and discussion

Since the outbreak of SARS-CoV-2 infection in December 2019, it has still wreaked havoc worldwide (**Fig. 1a, b**). Currently, many types of vaccines have been widely used for the prevention of SARS-CoV-2 infection[1], while only two anti-COVID-19 drugs have been approved in the world[2,3]. These drugs target the replication process after SARS-CoV-2 enters host cells[4,5]. Given the continuous variation of SARS-CoV-2 into mutants such as Delta and Omicron mutants, effective therapeutic drugs that are capable to treat mutant infections are an urgent demand in the clinic.

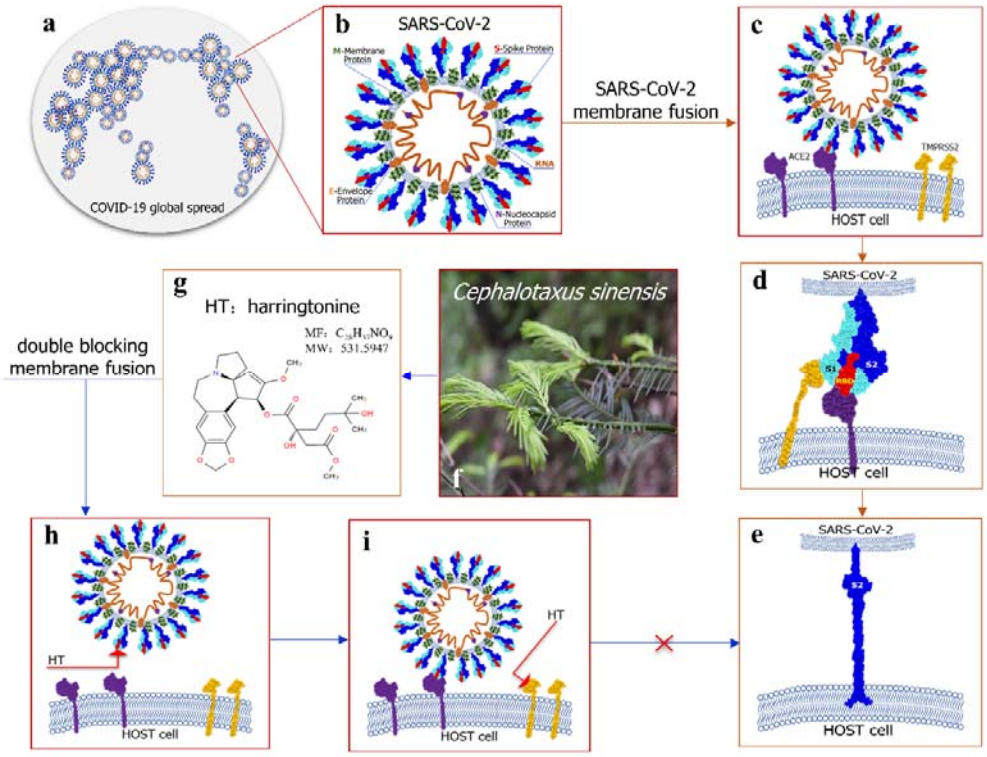

Fig. 1 A new strategy of double blocking SARS-CoV-2 membrane fusion using harringtonine (HT). a, The COVID-19 pandemic is spreading globally. **b,** SARS-CoV-2 is mainly composed of the spike (S), membrane (M), and envelope (E) proteins. There is a viral RNA chain of the nucleocapsid (N) protein inside SARS-CoV-2. **c-e,** SARS-CoV-2 membrane fusion process includes the formation of the S-ACE2 complex, which is hydrolysed by TMPRSS2 and S2 fusion peptide anchored to the host membrane. **f-g,** HT was isolated from *Cephalotaxus sinensis*. **h-i,** Our aim is to find an antagonist such as HT that blocks SARS-CoV-2 membrane fusion by simultaneously targeting S protein and the host TMPRSS2.

Natural medicinal plants are an important source of small molecule new drugs[6]. The *Cephalotaxus sinensis* is a unique medicinal plant from Qinling Mountains of China[7], and the average content of HT is 0.0045% (**Fig 1. f-g**). In this study, we propose a new strategy of treatment to be directed against SARS-CoV-2 membrane fusion through the S protein (**Fig.1c-e**). HT, a small molecule antagonist, targets the S protein to inhibit its binding to the ACE2 receptor in host cells; simultaneously, it targets the host cell TMPRSS2 against the enzymatic hydrolysis of S protein (**Fig1. h-i**). Our experiments showed that HT is an effective antagonist and acts by double blocking SARS-CoV-2 membrane fusion.

1. HT Targeted the S protein of SARS-CoV-2, and to TMPRSS2 in host cells

Cell membrane chromatography (CMC) is a biomimetic analysis technology used to study interactions between drugs and their receptors[8]. In this study, the S/CMC and TMPRSS2/CMC models were established to test the affinities between HT and S protein as well as TMPRSS2[9]. The reversibility of HT on the S protein and TMPRSS2 was determined. In addition, the binding modes of HT were simulated by computer molecular docking technology.

The K_D values of HT were 1.36 ± 0.10 μM (S protein) and 15 ± 3.34 μM (TMPRSS2) [10]. The A_S values were 1.71 (S protein) and 5.27(TMPRSS2) (**Fig. 2a-d**). CMD showed that HT can form hydrogen bonds with the GLY696, SER494, TYR449, and ARG403 residues in the RBD domain of the S protein (PDB ID: 6M0J), and these sites were key binding sites between the S protein and ACE2. HT can also bind the SER441, GLY462, and GLY464 residues in the domain of TMPRSS2 (PDB ID: 7MEQ) (**Fig. 2e-f**). These findings show that HT has a strong affinity for

SARS-CoV-2 S protein and TMPRSS2, and also shows some irreversibility, which is very beneficial for blocking SARS-CoV-2 membrane fusion.

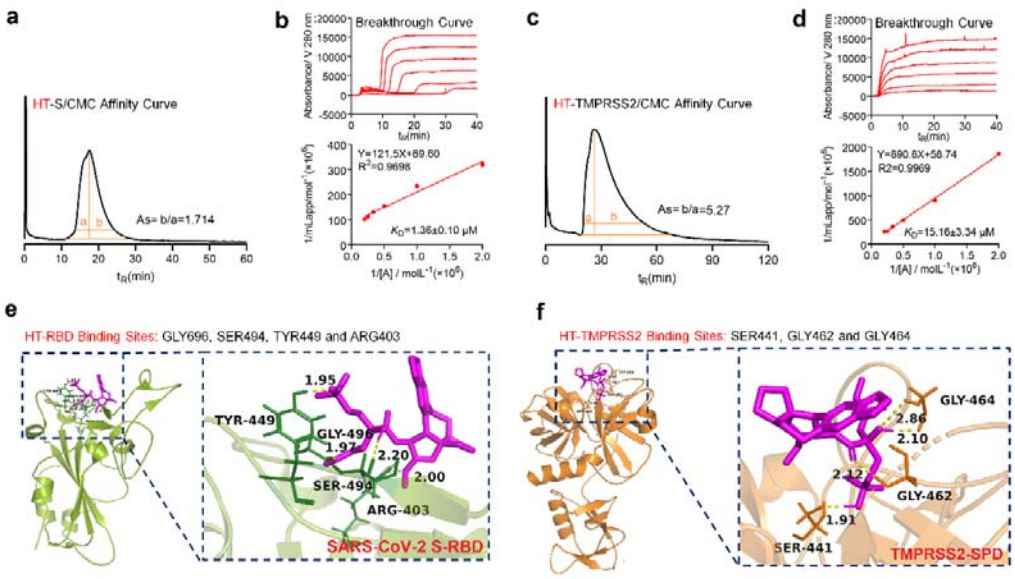

Fig. 2 HT specifically targets the S protein of SARS-CoV-2 and host cell TMPRSS2. a-b, Affinity and breakthrough curves of HT were obtained using the S/CMC-HPLC method and **c-d,** the TMPRSS2/CMC-HPLC method. **e-f,** Computer molecular docking (CMD) was used to identify the hydrogen bonds between HT and the receptor-binding domain (RBD) of S protein, and those between HT and the serine protease domain (SPD) of TMPRSS2. Graphs display mean±S.E.M. One-way ANOVA (and nonparametric or mixed) (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* 0)

2. HT inhibits pseudotyped virus membrane fusion

The interaction between ACE2 and the S protein plays a key role in SARS-CoV-2 infection[11]. We used NanoBiT® to evaluate the effect of HT on the interaction between ACE2 and The S protein **(Fig. 3a)**. As shown in Fig 3b, HT can dose-dependently inhibit cell-to-cell membrane fusion induced by the binding of ACE2 and S proteins (S495) **(Fig. 3b-c, and e)**. In addition, HT also effectively inhibited ACE2^{h} cell membrane fusion induced by Delta virus mutated S protein-L452R, T478K, and P681R sites (S605) **(Fig. 3b, d, and f)**. It was evident from the micrographs that HT dose-dependently inhibited S495 and S605-induced syncytial formation.

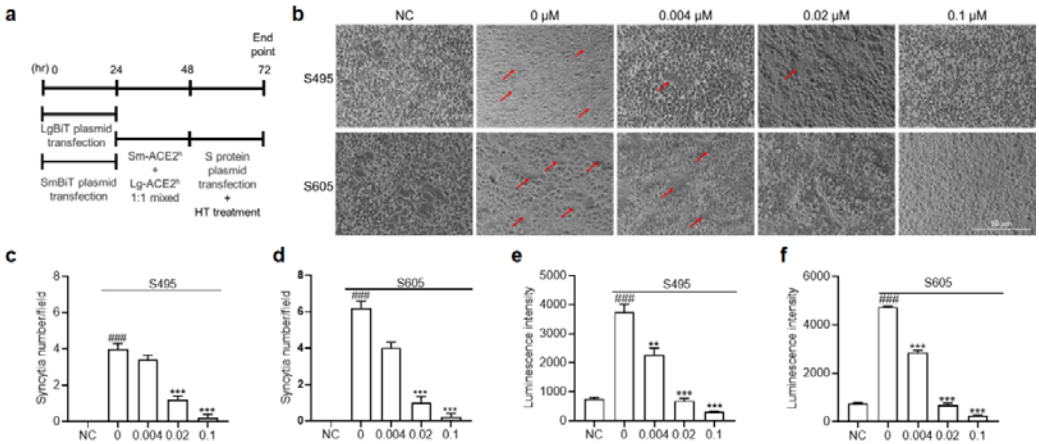

Fig. 3 HT simultaneously membrane fusion through S protein. a, Protocol of ACE2h cells membrane fusion assay; ACE2h cells were transfected with LgBit plasmid and SmBit plasmid respectively for 24h.Then, 1:1 mixed and incubated for the night. After that, mixed cells were transfected with S protein plasmid for 24h and the cells were collected and used to evaluate Luminescence intensity. **b,** HT inhibited S495 and S605 induced ACE2h cells membrane fusion. **c-d,** Quantitation of numbers of syncytia in cells as described in (b). Values are syncytia number per microscope field. **e-f,** Luciferase were measured to indicate the effect of HT to S495 and S605-induced syncytial formation. Graphs display mean ± S.E.M. One-way ANOVA (and nonparametric or mixed) (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* 0)

We used a pseudotyped virus, it can be used to evaluate the antiviral effects of drugs only in a P2 Lab[12,13]**(Fig. 4a)**. The pseudovirus has a significant infection advantage for $ACE2^h$ cells[14]. $ACE2^h/TMPRSS2^h$ cells were successfully established by stably overexpressing TMPRSS2 in $ACE2^h$ cells[15]. The pseudo-type entry rates in $ACE2^h/TMPRSS2^h$ cells were greatly improved compared with those in $ACE2^h$ cells when pseudotyped viruses were used to infect different cells. The $ACE2^h/TMPRSS2^h$ cell model works well because the Camostat (TMPRSS2 antagonist) considered to be an effective antagonist of TMPRSS2 and can effectively inhibit pseudovirus infection (**Fig. 4b**).

The TMPRSS2 enzyme on host cells plays a key role in pseudovirus virus membrane fusion. HT dose-dependently inhibited pseudovirus infection in $ACE2^h$ and $ACE2^h/TMPRSS2^h$ cells, with IC_{50} values of 1.091 ± 0.157 μM and 0.580 ± 0.15 μM, respectively. The latter is obviously stronger than the former (**Fig. 4c-f**). The results show that HT is an effective antagonist against SARS-CoV-2 infection, and

TMPRSS2 has a key synergistic effect.

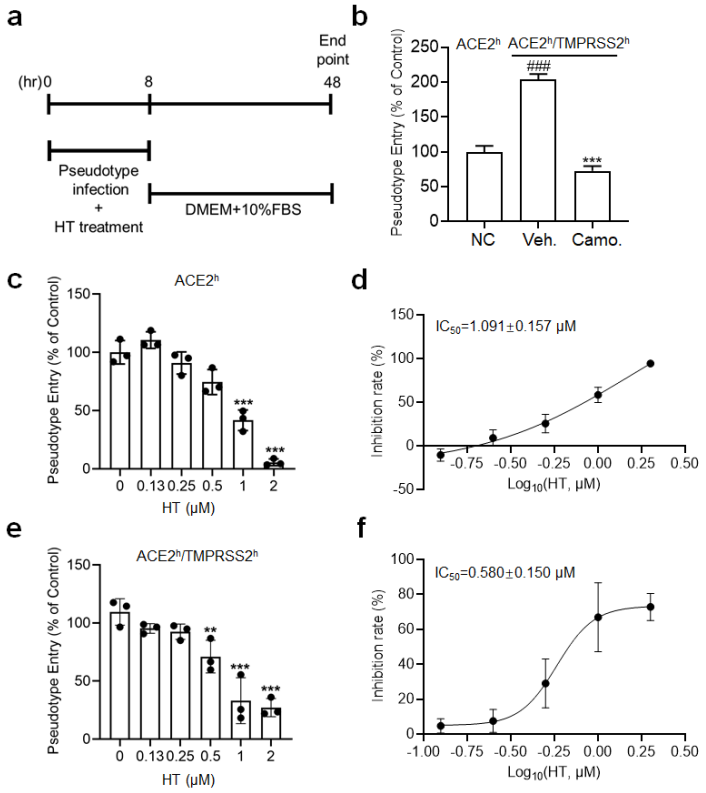

Fig.4 Effect of HT on the entrance of SARS-CoV-2 spike pseudotyped virus into ACE2^h and ACE2^h/TMPRSS2^h cells. a, Protocol of pseudotyped virus entry assay; ACE2^h cells were pretreated with pseudotyped SARS-CoV-2 virus combined with different doses of HT for 8 h. Then, the cells were transferred to fresh cell culture medium and incubated for 48 h; the cells were collected and used to evaluate pseudotype entry rates. **b,** TMPRSS2 was transfected into ACE2^h cells (ACE2^h/TMPRSS2^h cell), which were then infected with pseudotype combined with vehicle (Veh.) or camostat (Cam.) The entrance of pseudotype virus into ACE2^h/TMPRSS2^h cells was inhibited by camostat.**c-d,** HT inhibited pseudotyped virus entry into ACE2^h cells**. e-f,** HT inhibited pseudotyped virus entry into ACE2^h/TMPRSS2^h cells dose-dependently. Graphs display mean±S.E.M. One-way ANOVA (and nonparametric or mixed) (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* 0)

3. HT as an antagonist for anti-SARS-CoV-2 infection

The anti-SARS-CoV-2 effects of HT in vitro were investigated in SARS-CoV-2ΔN trVLP with N protein deficient[16], SARS-CoV-2-WIV04 original strain, and SARS-CoV-2-Delta 630 mutant strain.

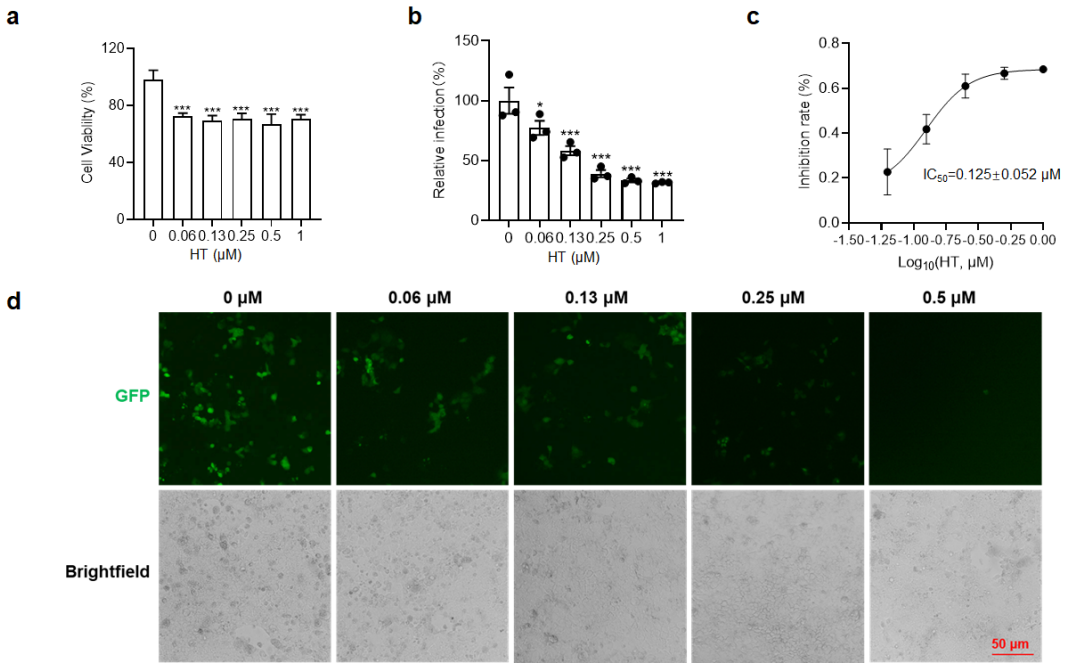

Fig. 5 HT successfully blocks SARS-CoV-2/ΔN infecting Caco 2-N cells. a, Viability of Caco2-N cells treated with 1 μM HT for 72 h. **b-c,** HT in the range of 0.06~1 μM dose-dependently decreased SARS-CoV-2ΔN trVLP infection in Caco-2-N cells. **d,** Representative image of HT inhibiting SARS-CoV-2/ΔN infecting Caco 2-N cells. Graphs display mean ± S.E.M. One-way ANOVA (and nonparametric or mixed) (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* 0)

The survival rate of Caco-2-N cells was more than 70% under 1 μM HT treatment (**Fig. 5a**). HT dose-dependently decreased SARS-CoV-2ΔN trVLP infection in Caco-2-N cells, with an IC_{50} value of 0.125 ± 0.052 μM (**Fig. 5b-d**).

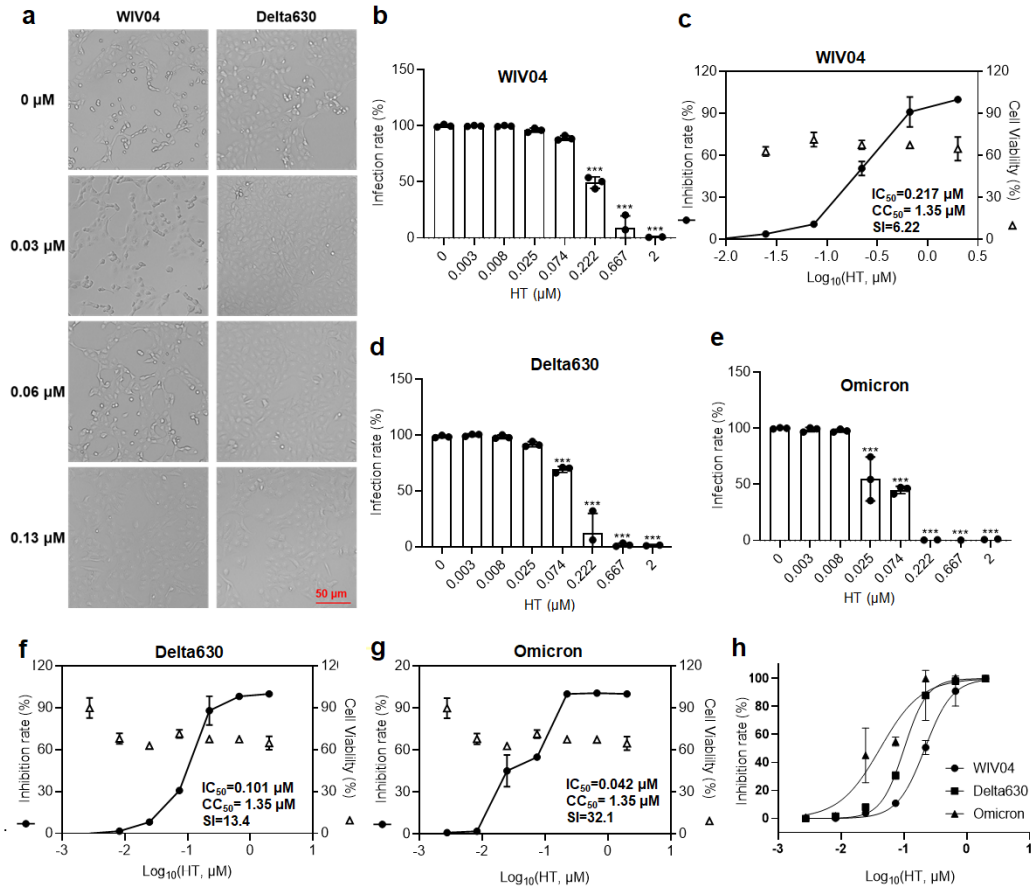

Fig. 6 HT successfully blocks SARS-CoV-2 infection. a, Representative image of HT inhibiting SARS-CoV-2-WIV04 and SARS-CoV-2-Delta 630 virus-induced cytopathic effects (CPE). **b-c,** Real-time RT-PCR showed that HT in the range of 0.03~2 μM dose-dependently inhibited SARS-CoV-2-WIV04 virus infection in Vero E6 cells. **d-e,** Real-time RT-PCR showed that HT significantly inhibited SARS-CoV-2-Delta 630 and Omicron virus infection in Vero-E6 cells, with a lower inhibiting dose. **f-g,** The IC_{50}, CC_{50} and SI of HT on the two variant strains of SARS-CoV-2. **h,** HT inhibits SARS-CoV-2 and its variant strains from infecting Vero E6 cells. Graphs display mean ± S.E.M. One-way ANOVA (and nonparametric or mixed) (**P*<0.05, ***P*<0.01, ****P*<0.001 *vs.* 0)

SARS-CoV-2-WIV04 has been one of the original strains since the outbreak of COVID-19. Vero-E6 cells are African green monkey kidney cell lines that highly express the ACE2 receptor[17]. In Vero E6, HT dose-dependently inhibited the cytopathic effect CPE caused by WIV04 virus (**Fig. 6a**). In Vero E6 cells, the CC_{50} and IC_{50} values of HT were 1.35 μM and 0.217 μM, respectively, and the selectivity index ($SI=CC_{50}/EC_{50}$) was 6.22 (**Fig. 6b-c**). Repeated epidemics are caused by the constant mutation of the SARS-CoV-2. The delta mutant strain was found in India as

early as October 2020[18,19] rapidly spreading and became a major concern in the containment of the pandemic[20,21]. On November 9, 2021, the first Omicron sequence available was from a specimen collected in Botswana. Ever since the identification of Omicron, the variant appears to rapidly spread. At the time of this writing, Omicron has become a major concern in the containment of the current pandemic[22,23]. The same evaluation tests for HT were performed on the Delta 630 and Omicron mutant. The results further confirmed that HT inhibit SARS-CoV-2-Delta 630 virus and Omicron infections at a lower inhibitory dose of 0.074 μM and 0.025 μM (**Fig. 6d-e**). The IC_{50} value of HT was as low as 0.101 μM and 0.042 μM, with a SI value of 13.4 and 32.1, which was almost five times that of the WIV04 original strain (**Fig. 6f-g**). With the increase of mutation sites on RBD, the antiviral effect of HT was significantly enhanced rather than decreased (**Fig. 6h**).

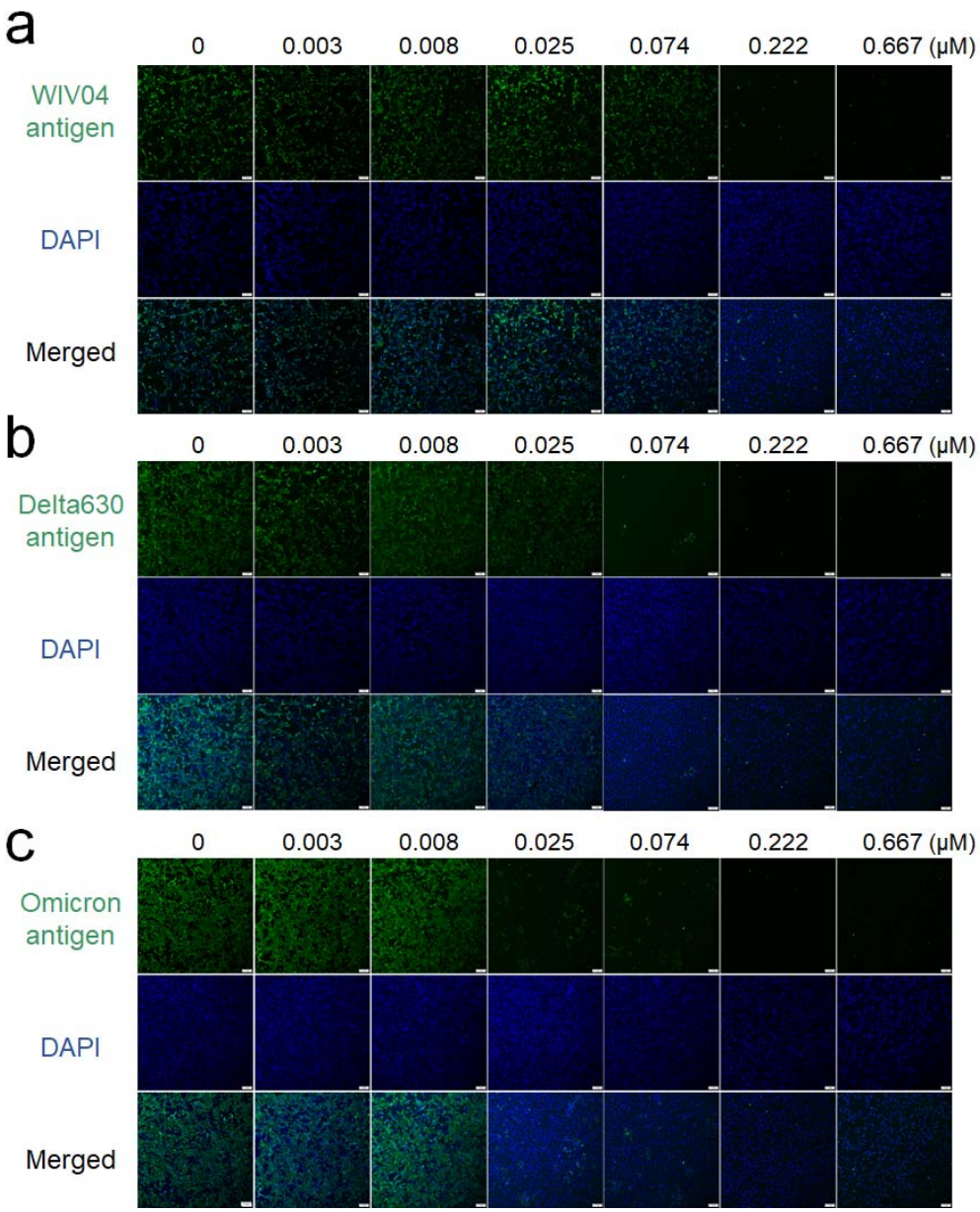

Fig.7 Representative immunofluorescence images of HT significantly inhibited SARS-CoV-2 infection. a, Immunofluorescence showed that HT significantly inhibited SARS-CoV-2-WIV04 virus infection in Vero-E6 cells. **b,** Immunofluorescence showed that HT significantly inhibited SARS-CoV-2-Delta 630 virus infection in Vero-E6 cells. **c**, Immunofluorescence showed that HT significantly inhibited SARS-CoV-2-Omicron virus infection in Vero-E6 cells, with a lower inhibiting dose. The scale of the figure is 1 mm.

In line with the viral immunofluorescence, the expression of S protein was dramatically decreased in those cells treated with 0.222 μM **(Fig.7a)**; for Delta630, 0.074 μM treatment could result in significantly less S protein expression compared to

the untreated infected cells **(Fig.7b)**; for Omicron, even 0.025 μM treatment could result in significantly less S protein expression compared to the untreated infected cells (Fig.7c). Thus, these findings show that HT is exceptionally valuable for anti-SARS-CoV-2 therapy. In summary, this study confirms that HT can inhibit SARS-CoV-2 membrane fusion by double blocking the S protein and TMPRSS2 pathway, thereby making it an effective antagonist for SARS-CoV-2 infection.

Conclusion

Collectively, HT has two potential advantages in the clinical treatment of COVID-19. First, HT mainly targets the S protein and inhibits its ability to bind to ACE2 protein on host cells. Compared with anti-SARS-CoV-2 replication drugs, HT greatly reduces the damage of S protein's autotoxicity to the organ system. Second, with continuing variation of SARS-CoV-2, the virus will "escape" the recognition of neutralizing antibodies and antibodies produced by inactivated vaccines. However, HT, as a small molecule antagonist, is minimally affected by SARS-CoV-2 variation. At present, the increased infectivity of Omicron reflects that it is stronger membrane fusion effect[24]. TH, as a small molecule antagonist for membrane fusion, is more targeted and can effectively block Omicron infection.

Conflicts of Interest

The authors declare no competing financial interest.

Acknowledgement

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