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Research Article

Discovery of GST-HG171, a Potent and Selective Oral 3CL Protease Inhibitor for the Treatment of COVID-19

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Abstract

The coronavirus 3C-Like (3CL) protease, aka Main protease (Mpro), has become a clinically validated therapeutic target for developing new COVID-19 therapeutics with the recent success of Paxlovid (nirmatrelvir/ritonavir) in treating high-risk COVID-19 patients in clinic. However, there is still a huge unmet medical need for effective drugs for treating COVID-19 patients with standard-risk as well as those who experience rebounds after Paxlovid treatment. Here we report the discovery of a novel 3CL protease inhibitor, GST-HG171 that is more potent and effective than nirmatrelvir in pre-clinical studies both *in vitro* and *in vivo*. GST-HG171 has broad-spectrum activity against different variants of SARS-CoV-2, including Beta, Delta, Omicron B.1.1.529, Omicron BA.4, BA.5 variants with 5-10 fold higher potency than nirmatrelvir when tested head-to-head in cytopathic effect assay. *In vivo*, GST-HG171 demonstrated higher efficacy than nirmatrelvir in reducing the viral load of lung tissue in mice infected with SARS-CoV-2 virus. Furthermore, GST-HG171 has demonstrated a more preferable lung tissue distribution in rats than nirmatrelvir, with a 4-5 fold higher lung/plasma exposure ratio. Finally GST-HG171 has an excellent safety profile in both pre-clinical studies and Ph1 clinical study in healthy human volunteers. In a Single Ascending Dose (SAD) Ph1 clinical trial, GST-HG171 demonstrated a dose-proportional increase in Cmax and exposure up to 600 mg. At 600 mg, GST-HG171 has an exposure of 23166 h*ng/mL, which is 4.2-fold higher than that reported for nirmatrelvir at a similar dose (5465 h*ng/mL at 500 mg). In sum, GST-HG171 is a novel, safe and more potent 3CL protease inhibitor than nirmatrelvir, and has the potential to become a superior broad-spectrum and effective COVID-19 therapeutic. Currently, GST-HG171 is under investigation in a large Ph3 trial in mild and moderate COVID-19 patients in China.

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Introduction

The COVID-19 pandemic, caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has resulted in more than 769 million infections and 6.9 million deaths worldwide as of August 2023 according to the World Health Organization (WHO). Effective and safe vaccines were believed to be, and may still be, the best option for preventing viral infection and its progression to more severe disease, hospitalization, and death. However, the effectiveness of COVID-19 vaccines and many neutralizing monoclonal antibodies is being compromised by the continuous emergence of immune-escape SARS-CoV-2 variants [1,2]. To combat this immune escape, primarily caused by mutations in viral surface proteins, the development of broad-spectrum and effective small molecule drugs targeting the intrinsic and more conserved viral replication cycle, has

become crucial in the fight against the outbreak. Small molecule drugs also have additional advantages over traditional biologics, including the ability to be taken orally for convenience, stability at room temperature, and lower production costs.

3CL protease is the Main protease (Mpro) found in coronaviruses, and has become a popular anti-viral therapeutic target for small molecule drug development against SARS-CoV-2 in recent years. 3CL protease is responsible for processing two large viral polyproteins at 11 distinct sites to generate non-structure proteins that are essential for viral genome replication and transcription, including RNA-dependent RNA polymerase (RdRp), helicase, among others [3,4]. The structure and substrate specificity of 3CL protease is highly conserved among beta-coronaviruses such as SARS-CoV-2, SARS-CoV, and MERS-CoV (Middle East respiratory syndrome coronavirus), making it an ideal target for developing broad-spectrum coronavirus inhibitors [5-7]. Furthermore, no human homolog of 3CL protease exists, indicating that selective 3CL protease inhibitors will only target the virus, not the human host, and should have a good safety profile in clinic.

Furthermore, 3CL protease is a clinically validated therapeutic target for developing small molecule inhibitors for COVID-19 treatment. Several 3CL protease inhibitors including Paxlovid and Ensitrelvir were conditionally approved for treating COVID-19 patients in recent years [8,9]. Paxlovid was shown in a Ph3 trial in mild and moderate COVID-19 patients with high risk to reduce the rate of progression to severe disease, hospitalization, or death by 89% [8], and has since become the most prescribed anti-SARS-CoV-2 medicine worldwide. More 3CL protease inhibitors are currently at various stages of development.

There is still huge unmet medical need for developing safe and more effective COVID-19 therapeutics. Here we report the discovery of a novel 3CL protease inhibitor, GST-HG171, which is more potent and effective than Paxlovid in pre-clinical studies *in vitro* and *in vivo*. GST-HG171 has excellent safety profile in both pre-clinical studies and a Ph1 clinical study in healthy volunteers. Moreover, GST-HG171 has demonstrated favorable PK characteristics in a SAD study of Ph1 trial, with up to 4-fold higher exposure in human plasma than Paxlovid at similar dose range. Taken together, GST-HG171 has the potential to become a superior broad-spectrum and effective COVID-19 therapeutic, and is currently under investigation in a large Ph3 trial in mild and moderate COVID-19 patients in China.

Results

The Discovery Of GST-HG171

In an effort to identify potent and selective SARS-CoV-2 3CL protease inhibitors, we carried out multiple rounds of CADD-guided P1 through P3 ligand fragment design and docking score prediction. Our efforts started with docking nirmatrelvir (PF-07321332) into the co-crystal structure of GC376 with the SARS-CoV-2 Mpro (PDB: 6WTT) [10]. Later, we confirmed our docking model is highly consistent with the published nirmatrelvir (PF-07321332) co-crystal structure (PDB: 7VH8, (Figure 1A) [11].

With this docking model in hand, we docked series of P1, P2 and P3 modifications, and then carefully selected optimal fragments based on right binding poses and good docking scores. On the basis of this docking effort, we decided to selectively synthesize and investigate the peptide-mimetic compounds by rational design on the key positions interacting with the active domain of the viral protease. Extensive SAR exploration (to be published elsewhere) yielded GST-HG171 (Patent WO2022218442A1). In (Figure 1B), we compare the docked GST-HG171 and nirmatrelvir (PF-07321332) co-crystal structure: the unchanged ligand fragments were highly consistent in terms of binding to the protein. The nitrile group formed a covalent bond with the catalytic residue C145. The 2-pyrrolidinone fragment made hydrogen bond interactions with E166 and H163. A water-mediated hydrogen bond was found between Q189 and the amide between P1 and P2, with this amide making another hydrogen bond with H163. Meanwhile, the modified fragment of GST-HG171, which changed from the [3,4] bicyclic-pyrrolidine

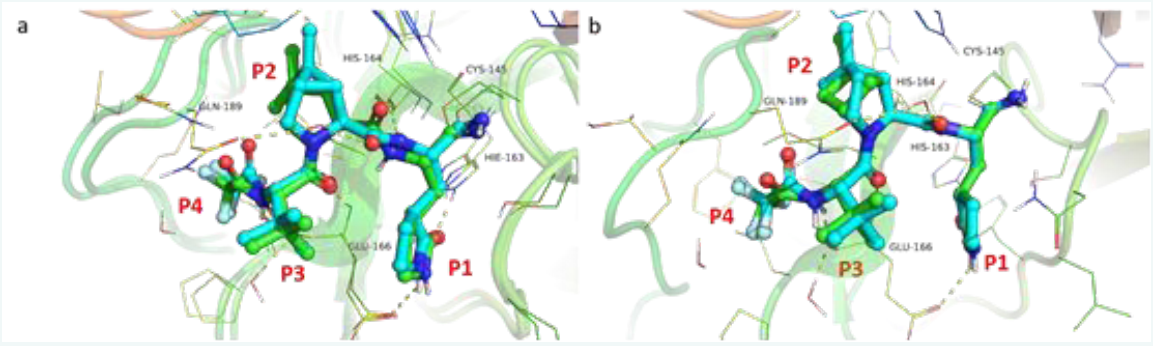

Figure 1 Binding model of GST-HG171 and Nirmatrelvir (PF-0732332).

a. Docked PF-0732332 (Green) vs co-crystal nirmatrelvir (PF-0732332) (Cyan): the γ-lactam ring in P1 pocket hold the same set of H-bonds to the neighboring residues as well as other key interactions.

b. Docked GST-HG171 (Green) overlay with nirmatrelvir (PF-0732332) (Cyan) co-crystal structure: unchanged fragments were highly aligned to each other in P1, P2, and P3 pockets and the modified piece of GST-HG171 in P2 pocket occupied more extensive space vs the space occupied by nirmatrelvir (PF-0732332). Yellow dashes are the H-bond network.

in the P2 pocket in nirmatrelvir (PF-07321332) to [3,5] bicyclic-pyrrolidine, filled up more of the hydrophobic space in the P2 pocket and resulted in better binding and higher potency. This led to the discovery of GST-HG171, which demonstrated superior *in vitro* and *in vivo* activities as well as favorable PK properties over nirmatrelvir (PF-07321332) in pre-clinical studies, and was thus selected as the Pre-Clinical Candidate (PCC) for further development.

In vitro Antiviral Activity of GST-HG171 against SARS-Cov-2

GST-HG171 is a highly potent and selective inhibitor of SARS-CoV-2 3CL protease with an IC_{50} of 3.0 nM in biochemical assay with recombinant viral 3CL protease (Table 1). GST-HG171 has no activity against other common mammalian proteases such as cathepsin B/D/L, chymotrypsin C and thrombin (IC_{50} > 100 μM) (**Table S1**). The cellular antiviral activity was evaluated using Cytopathic Effect (CPE) assay in Vero E6 cells in the presence of a P-glycoprotein inhibitor (CP-100356). GST-HG171 exhibited potent and broad-spectrum anti-viral activities against SARS-CoV-2 wild type strain, as well as various SARS-CoV-2 variants including Delta, Omicron B.1.1.529, Omicron BA.4 and BA.5, with EC_{50} values of 79, 49, 48, 49 and 70 nM, respectively in the cellular assays. As a reference compound, nirmatrelvir (PF-07321332) was included in these assays. GST-HG-171 demonstrated 2.5- to 10-fold higher inhibitory potency than nirmatrelvir (PF-07321332) in these head-to-head comparison assays (Table 2). No cytotoxicity was observed up to 100 μM of GST-HG171 in Vero E6 cells. The anti-viral activity of GST-HG171 was specific to the coronavirus family since it has no or very low activities against herpes simplex virus 1, human rhinovirus (1b), respiratory syncytial virus (A long) and influenza virus (H1N1) (EC_{50} > 100 μM) (**Table S2**). We also evaluated the activity of GST-HG171 against 12 emergent mutations of SARS-CoV-2 3CL protease. GST-HG171 was active against most of these mutations in the enzymatic assays (IC_{50} < 40 nM, Table 1), with more potent activity in several mutants that were reported to be resistant to nirmatrelvir (PF-07321332) (S144A, E166A and H172Y) (Paxlovid, Prescribing Information). Together, these results demonstrate that GST-HG171 is a potent and selective 3CL protease inhibitor *in vitro*.

Table 1: Enzymatic activity of GST-HG171 and Nirmatrelvir (PF-07321332) against SARS-COV-2 wild type and mutant 3CL proteins.

SARS-CoV-2 wildtype and mutant 3CL proteins	IC_{50} (nM)	
	GST-HG171	Nirmatrelvir (PF-07321332)
WT	3	7.6
G15S	2.3	6.8
Y54A	15	69
K90R	2	5.4
T135I	4.9	14
F140A	17	72
S144A	10	59
H164N	6.6	21
E166A	20	266
H172Y	37	245
Q189K	5.7	14
D248E	4.3	9.2
A260V	4.7	10

Pharmacokinetic Characteristics and Tissue Distribution of GST-HG171 in Animals

We next evaluated the pharmacokinetic properties of GST-HG171 in rat and dog *in vivo*. GST-HG171 has comparable PK profiles as nirmatrelvir (PF-07321332) in animal studies. The key pharmacokinetic parameters of GST-HG171 in rat and dog were summarized in (**Table S3,S4**). GST-HG171 displayed moderate plasma clearance with an elimination half-life of 1-2 hours after intravenous dosing. Following oral administration in dogs, GST-HG171 at 5 mg/kg had an AUC value of 11300 h*ng/ml, a half-life of 1.47 hr and oral bioavailability of 86.6% (**Table S4**). In rats, GST-HG171 at 30 mg/kg had an AUC of 5850 h*ng/ml, a half-life of 1.02 h and oral bioavailability of 67.3% (**Table S3**).

Using drug substrates in Human Liver Microsomes (HLM) and recombinant CYP isoforms studies, the CYP3A4 was identified as the major contributor for the metabolism of GST-HG171 (> 80% contribution, **Table S5**). Ritonavir (RTV) is known to be used as a pharmacokinetic enhancer for drugs subjected to CYP3A4-mediated metabolism [12,13]. The Cmax and AUC of GST-HG171 was increased 6-fold by RTV co-administration in a rat PK study (data not shown).

We also determined the tissue distribution in rats after co-administration of GST-HG171 with RTV. The concentration and AUC of GST-HG171 in lung tissue were significantly higher than the level in plasma with a lung/plasma ratio of 2.62 (Table 3). As a comparison, nirmatrelvir (PF-07321332) with RTV was included in the same study. GST-HG171 demonstrated about 5-fold higher lung/plasma ratio in exposure than nirmatrelvir (PF-07321332) at the same dose (30 mg/kg). These results indicate that GST-HG171 has a preferable lung tissue distribution over plasma compared to nirmatrelvir (PF-07321332), which may be an advantage in treating respiratory viral infection such as COVID-19.

In vivo Efficacy of GST-HG171 in Mouse Model of SARS-Cov-2 Infections

To evaluate antiviral activity *in vivo*, we assessed the efficacy of GST-HG171 in the transgenic H11-k18-hACE2 mice, which preferably express human ACE2 receptor in the lung of the mice, making the mice sensitive to human SARS-CoV-2 infection as reported in literature [14]. H11-k18-hACE2 mice at age of 8-9 weeks were challenged intra-nasally with $1x10^5$ TCID50 of wildtype hSARS-CoV-2 virus (2019-nCoV). Vehicle control, GST-HG171 at 150 mg/kg and 450 mg/kg and nirmatrelvir (PF-07321332) at 150 mg/kg and 450 mg/kg were administrated to the mice by oral gavage (PO) two hours after viral challenge. The drugs were given twice a day for 5 days, and the lung tissues of the

Table 2: *In vitro* antiviral activity of GST-HG171 and Nirmatrelvir (PF-07321332) against SARS-CoV-2 variants.

Coronavirus	EC_{50} (μM)	
	GST-HG171	PF-07321332
SARS-CoV-2 WT	0.079	0.522
SARS-CoV-2 Delta	0.049	0.492
SARS-CoV-2 Omicron B.1.1.529	0.048	0.418
SARS-CoV-2 Omicron BA.4	0.049	0.12
SARS-CoV-2 Omicron BA.5	0.07	0.351

CC_{50} values of GST-HG171 and nirmatrelvir (PF-07321332) in Vero E6 cells.

Cell line	CC_{50} (μM)	
	GST-HG171	Nirmatrelvir (PF-07321332)
Vero E6	> 100	> 100

Table 3: Plasma and lung concentrations of GST-HG171 and nirmatrelvir (PF-07321332) co-administered with RTV in rats.

Compound	GST-HG171			Nirmatrelvir (PF-07321332)		
Dose	30 mg/kg + 10 mg/kg RTV			30 mg/kg + 10 mg/kg RTV		
Formulation	3 mg/mL in 10% Solutol : 30% PEG 400 : 2 % Tween 80 : 58% Water, Nearly clear solution			3 mg/mL in saline, clear solution		
Time (h)	Plasma concentration (nM)	Lung concentration (nmol/kg)	Lung/plasma ratio	Plasma concentration (nM)	Lung concentration (nmol/kg)	Lung/ plasma ratio
0.25	2860	15800	5.5	15127	10013	0.67
1	5490	18450	3.24	8955	5218	0.59
6	4875	7850	1.61	1277	623	0.49
$AUC_{0\text{-last}}$ (h*nmol/L or h*nmol/kg)	29371	76840	2.62	30431	17579	0.58

mice were collected after the final drug treatment, and measured for RNA copy numbers of SARS-CoV-2 ORF1ab, N, and S viral genes by quantitative RT-PCR. In the vehicle-treated group, log 10.8, log 10, log 10.3 copies of ORF1ab, N,S genes were detected in the lung tissues, respectively. After GST-HG171 treatment at 150 mg/kg for 5 days, the viral copies dropped to log 7.6, 6.7, and 7.6 for ORF1ab, N, S genes, respectively (a drop of 3.2, 3.4, and 2.7 log viral copies for each gene) (Table 4). At the same dose (150 mg/kg) of nirmatrelvir (PF-07321332), the viral copies were reduced to log 9.3, 8.7, and 8.9 for the three genes, respectively (a drop of 1.5, 1.3, and 1.4 log) (Table 4). GST-HG171 reduced the viral copies of the three genes for 1.7, 2.1, and 1.3 log more than Paxlovid at the same dose in this study. Similarly, GST-HG171 had a higher viral copy reduction than nirmatrelvir (PF-07321332) at 450 mg/kg although the effect seemed to be plateaued at the higher dose. Thus, GST-HG171 displayed significant higher viral load reduction than nirmatrelvir (PF-07321332) at both dose levels (Figure 2A,2B) (Table 4).

The body weight of the mice in vehicle group experienced a big drop at Day 5 post-infection, while the treatment of both compounds alleviated the body weight drop significantly. The effect of GST-HG171 on body weight recovery seems to be dose-proportional. Again, GST-HG171 at both doses shown a trend of better protection from the body weight drop than nirmatrelvir (PF-07321332) (Figure 2C). Collectively, the results demonstrate that GST-HG171 inhibits the viral replication and protects mice from body weight reduction caused by SARS-CoV-2 infection. GST-HG171 was more efficacious than nirmatrelvir (PF-07321332) in this animal model.

Pharmacokinetic Characteristics of GST-HG171 in Human Ph1 Study

As a pre-clinical candidate, GST-HG171 was evaluated in a package of pre-clinical safety and toxicity studies in mouse, rats, and dogs required for the Investigational New Drug (IND) application. GST-HG171 demonstrated excellent safety profile in the animal studies, and the NOAEL for GLP 14- day repeat-dose toxicity study in rats (dosed at 50, 200, 600 mg/kg/day) was 600 mg/kg/day, and the NOAEL for GLP 14-day repeat-dose toxicity study in dogs (dosed at 30, 100, 300 mg/kg/day) was 300 mg/kg/day (to be published elsewhere). GST-HG171 received the authorization for entering clinical development in human from Chinese National Medical Products Administration (NMPA) in September, 2022.

The safety, tolerability, and pharmacokinetics of GST-HG171 were evaluated in a randomized, double-blind, placebo-controlled Phase 1 studies in Chinese healthy subjects (www.ClinicalTrials.gov identifier: NCT05668897). GST-HG171 demonstrated excellent safety and tolerability, and favorable pharmacokinetics in this study. In the Single-Ascending Dose (SAD) study, a total

Table 4: Reduction of viral load in lung tissues of mice infected with SARS-CoV-2.

		Quantity-ORF1ab	Quantity-N	Quantity-S
Log viral load drop *vs* vehicle control	150 mpk Nirmatrelvir (PF-07321332) *vs* vehicle	1.46	1.3	1.36
	450 mpk Nirmatrelvir (PF-07321332) *vs* vehicle	2.71	2.52	2.37
	150 mpk GST-HG171 *vs* vehicle	3.15	3.38	2.69
	450 mpk GST-HG171 *vs* vehicle	3.08	2.85	2.67

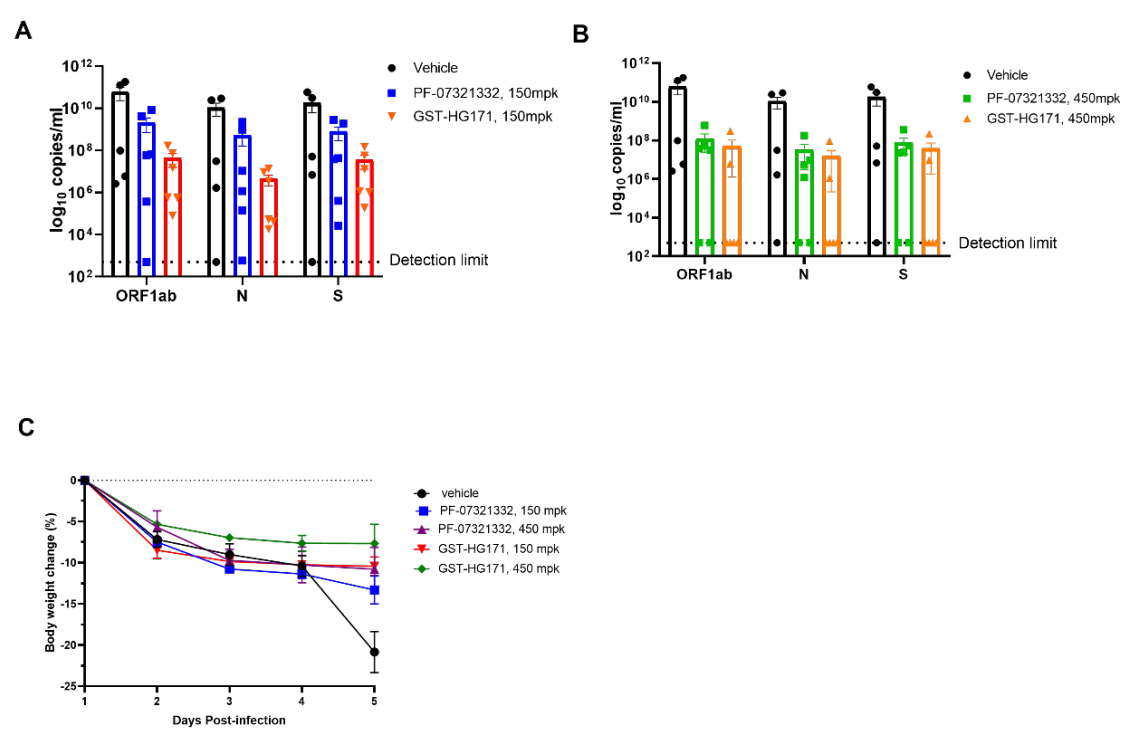

Figure 2 *In vivo* efficacy of GST-HG171 and Nirmatrelvir (PF-07321332) in SARS-CoV-2 mouse model.

K18-hACE2 transgenic mice infected with 1×105 TCID50 of wildtype SARS-CoV-2 were orally administered with vehicle control, GST-HG171 (150 and 450 mg/kg) and Nirmatrelvir (PF-07321332) (150 and 450 mg/kg) for 5 days (n = 6).

A,B. viral RNA levels (ORF1ab, N and S genes) in lung tissues were determined at 5 days post-infection.

C.Body weight was monitored daily. Data are expressed as mean ± SEM.

of 32 eligible subjects were randomized to receive a single-dose administration of GST-HG171 at 150, 300, 600, 900 mg, or placebo. GST-HG171 was safe and well tolerated in all dose cohorts. No severe AEs or SAEs were reported. All treatment-emergent AEs were mild in severity. The Cmax and AUC of GST-HG171 increased in an approximately dose proportional manner across the dose range from 150 to 600 mg, and approaching saturation in Cmax and exposure at 900 mg (to be published elsewhere). The AUC_{inf} of GST-HG171 was 23166 h*ng/ml for 600 mg groups, whereas the reported AUC_{inf} of nirmatrelvir (PF-07321332) at a similar dose (500 mg) was 5465 h*ng/ml (Figure 3A) [13], demonstrating that GST-HG171 has about 4-fold higher exposure in human plasma than nirmatrelvir at the similar dose. At higher doses (600 or 900 mg), the free plasma concentrations of GST-HG171 stayed above the EC_{50} values (Table 2) of the compound in the CPE assays against COVID-19 for over 12 hours, but significantly lower than the NOAEL Cmax concentration observed in 14-day toxicity study in dogs (85103 ng/ml), indicating an appropriate efficacy and safety window for GST-HG171. Since GST-HG171 is mainly metabolized by CYP3A4 similar as nirmatrelvir, we also evaluated the PK characteristics of GST-HG171 in combination with ritonavir (RTV), a known PK enhancer. At 150 mg dose of GST-HG171, the AUC in human healthy subjects was increased for over 6-fold (35992 h*ng/ml) in the presence of RTV (100 mg), compared to the AUC derived without RTV (5768 h*ng/ml), which was similar to the published data for nirmatrelvir (Figure 3B) [13]. The detailed results from the Phase 1 study will be reported separately.

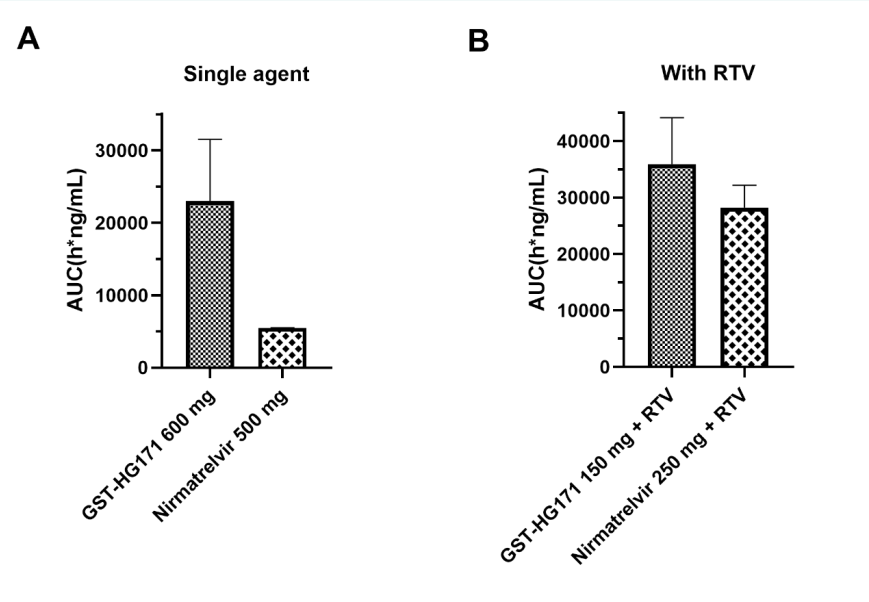

Figure 3 The plasma exposure of GST-HG171 and Nirmatrelvir in healthy adult subjects.

A. The mean AUC (area under curve) of GST-HG171 600 mg and Nirmatrelvir 500 mg administered as a single agent.

B. The mean AUC of GST-HG171 150 mg/Ritonavir 100 mg and Nirmatrelvir 250 mg/ Ritonavir 100 mg. The AUC values for Nirmatrelvir are from its phase 1 SAD study [13]. Data are shown as mean ± SD.

Discussion

The recent clinical success of Paxlovid (nirmatrelvir/ ritonavir) in preventing high-risk COVID-19 patients from progressing to more severe disease and death has validated 3CL protease as an anti-SARS-CoV-2 therapeutic target [8]. However, the effectiveness of Paxlovid for treating standard-risk COVID-19 patients is still controversial [15]. Furthermore, 5-10% of Paxlovid treated patients experienced a rebound of the virus within 30 days after the treatment [16]. In addition, Paxlovid needs to be co-administrated with a metabolic booster, Ritonavir (RTV), which has complicated its use in elderly patients, who often have chronic illnesses and need to take other medications simultaneously. More potent and effective 3CL inhibitors are needed to improve treatment effectiveness and to support a repertory of therapeutic choice. Here we have identified a potent and selective oral 3CL protease inhibitor, GST-HG171 that is more effective than nirmatrelvir (PF-07321332) in both *in vitro* and *in vivo* pre-clinical studies in inhibiting SARS-CoV-2 virus and its variants. Moreover, it has superior pharmacokinetic properties, such as preferable lung tissue distribution in animals. Finally, GST-HG171 demonstrated excellent safety and tolerability, as well as favorable pharmacokinetics as a single agent in a Ph1 trial in healthy human subjects. In the SAD study, GST-HG171 achieved over 4-fold higher exposure than nirmatrelvir in human plasma at a similar dose.

As an RNA virus, SARS-CoV-2 continues to evolve even without selection pressure, and thus has an inherently high mutation rate. Furthermore, due to the selection pressure of an increasing vaccination rate globally in recent years, many new SARS-CoV-2 variants and their sublineages have emerged in an attempt to escape human's immune response. For example, more than 500 omicron variants and sublineages have been identified worldwide so far (WHO), with probably more to come. Thus, it is critical to develop broad-spectrum anti-SARS-CoV-2 inhibitors that can overcome the mutations that commonly occur on the spike protein on the viral surface. Existing biologic therapeutics, including vaccines and neutralizing antibodies, cannot penetrate inside the virus due to the nature of biologics, and thus can only target surface proteins. However, small molecule drugs targeting the conserved internal viral replication cycle can overcome this shortfall of biologics and suppress the virus from replicating from the inside regardless of mutations on surface proteins. Accordingly, GST-HG171, like nirmatrelvir, demonstrates a broad-spectrum anti-coronaviruses activity, including emerging omicron variants. Furthermore, GST-HG171 demonstrated a 2.5 to10-fold higher potency than nirmatrelvir in inhibiting the SARS-CoV-2 original strain and several variants including several most recent ones (beta, delta, omicron B.1.1.529, BA.4, BA.5). We did notice that the cellular potency of nirmatrelvir in our studies was several fold lower than was reported in a similar cellular assay [17], which may have been caused by differences in assay conditions or cell systems used. However, our data was generated with the two compounds head-to-head in the same assay under identical conditions, and was repeated three times with comparable results each time.

In addition to mutations of the viral surface spike protein in these variants, many mutations in internal viral proteins, including 3CL protease, were also reported [18,19]. For instance, more than 100 naturally occurring mutations on 3CL protease

were located at the nirmatrelvir binding site, among which 20 mutants showed comparable enzyme activity to the natural substrate, but resistance to nirmatrelvir, although no clinical relevance of the resistance is known at the moment [20]. We evaluated 12 of the 20 known mutants of 3CL protease that were readily available to us in a biochemical assay using recombinant 3CL enzymes, and observed a distinct inhibition selectivity profile between GST-HG171 and nirmatrelvir. For example, nirmatrelvir was reported to have significantly reduced activity against the S144A mutant (Paxlovid, Prescribing Information), which directly interacts with nirmatrelvir in the active site of the enzyme. Our data shows GST-HG171 is, however, potent against the S144A mutant with an IC_{50} of 10 nM in an enzymatic assay, which is 6-fold more potent than nirmatrelvir in the same assay (Table 1). Nirmatrelvir was also reported to have significantly reduced activity against the H172Y mutant (Paxlovid, Prescribing Information), whereas GST-HG171 is potent (37 nM) in inhibiting the H172Y mutant in a biochemical assay with about 6-fold higher potency than nirmatrelvir in a head-to-head comparison assay. Similarly, GST-HG-171 has an IC_{50} of 20 nM against the E166A mutant, another hotspot for drug resistant mutation [20,21], whereas nirmatrelvir has an IC50 of 266 nM in the same assay. In sum, there are significantly more pronounced differences in the potency of the two compounds for the mutants than for wildtype 3CL enzyme, as GST-HG171 is only ~2.5-fold more potent than nirmatrelvir on wildtype 3CL enzyme (Table 1). The clinical significance of the improved potency of GST-HG171 over nirmatrelvir on these mutants vs wildtype enzyme is, however, unknown, and will need to be further validated.

Recent clinical experience with Paxlovid observed that 5-10% of COVID-19 patients treated with Paxlovid has a rebound of the virus within 30 days after drug administration [16]. The exact extent or rate of rebound is currently still under investigation. It is also not known whether the rebound is compound-specific or target-specific, but the rebound was recently also observed by RdRp inhibitors such as molnupiravir. The exact mechanism of this viral rebound is not clear, but it may be a result of resistance mutations of the target protein that occurred under the therapeutic pressure of the drugs. It is encouraging to see GST-HG171 is also potent (< 40 nM) against all 12 mutations we tested, and has a distinct inhibition profile with higher potency than Paxlovid against these mutant 3CL proteins. This indicates that GST-HG171 may have the potential to overcome the viral rebounds sometimes observed with Paxlovid. No conclusion, however, can be drawn until the efficacy of GST-HG171 on viral rebounds is evaluated in large clinical studies.

Materials and Methods

Docking Model

The docking process was carried out with Covalent Docking module from Schrödinger (Version 2022-3) [22] and co-crystal structure of GC-376 with the SARS-CoV-2 Mpro (PDB: 6WTT) [10] was used as the docking template. Cys 145 was specified as Reactive Residue, and GC-376 was used to define the docking box. Nucleophilic Addition to a Triple Bond was used as reactive type. Pose Prediction (Thorough) was used as docking mode and minimized the residues of the final pose within 3Å radius of the ligand. "Perform MM-GBSA scoring" was selected and default values were used for other settings. "Protein Preparation Workflow" in Maestro [23] was used to prepare the protein downloaded from PDB bank and OPLS4 force field was used for minimizations. All docked ligands were prepared with LigPrep [24] to generate the initial conformation and the optimal ionization state at pH 7.4.

3CL Protease Biochemical Assay

The C-6 His-tagged SARS-CoV-2 wild-type or mutant 3CL protease (Mpro) was cloned, expressed in *E.* coli and purified by WuXi AppTec. The enzyme activity assay was performed in the buffer containing 20 mM of Tris-HCl (pH 7.3), 100 mM of NaCl, 1 mM of EDTA, 5 mM of TCEP and 0.1% BSA. 25 µL of the 30 nM Mpro protein was mixed with serially diluted compounds and incubated at room temperature for 30 minutes. Then 5 µL of the 150 µM substrate (Dabcyl- KTSAVLQSGFRKM -Edans) was added to each well. The final concentrations of Mpro and substrate were 25 nM and 25 µM, respectively. For 100% inhibition control (HPE, hundred percent effect), no enzyme and compound was added. For no inhibition control (ZPE, zero percent effect), no compound was added. After 1h incubation at 30°C, the fluorescence signal was detected using a microplate reader SpectraMax M2e (Molecular Devices) at Ex/Em = 340nm/490nm. The inhibition activity was calculated using the formula below, and IC_{50} values were calculated using a four-parameter logstic fit in GraphPad Prism software.

Inhibition% = [(Sample- Average ZPE)/ (Average HPE-Average ZPE)]*100%

Cellular Antiviral Activity

The SARS-CoV-2 wild type, Delta, Omicron B.1.1.529, Omicron BA.4 and BA.5 variants were provided by the National Institute for Viral Disease Control and Prevention (NIVDC), Chinese Center for Disease Control and Prevention (China CDC). Vero E6 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS).

Antiviral activity against authentic SARS-CoV-2 was evaluated using the Cytopathic Effect (CPE) assay at the Biosafety Level-3 facility at the NIVDC. Tested compounds diluted with DMEM were mixed with 100 TCID50 SARS-CoV-2 various strains in a 96-well plate and incubated at 37°C for 2 h. Vero cells ($1x10^5$/ml) in the presence of 2 uM P-glycoprotein inhibitor CP-100356 were added into the mixture and incubated at 37°C and 5% CO_2 for 4 days. The CPE changes in the Vero cells were examined by the microscope. EC_{50} values were determined by plotting compound concentrations versus CPE inhibition and fitting data with linear regression. Cytotoxicity of test compounds were assessed under the same conditions, but without virus infection.

In vivo Studies

SARS-CoV-2 infection experiments were conducted in an animal biosafety level-3 facility in the National Institute for Viral Disease Control and Prevention (NIVDC), China CDC, and approved by the Institutional Animal Care and Use Committee

(IACUC) of NIVDC. K18-hACE2 transgenic mice aged 8-9 weeks (Gempharmatech Co., Ltd, n = 6 per group) were intranasally inoculated with SARS-CoV-2 wild type at a TCID50 of 1×10^5. Two hour after infection, the mice were orally administered with GST-HG171 (150 and 450 mg/kg, BID), or Nirmatrelvir (PF-07321332) (150 and 450mg/kg, BID), or vehicle control for 5 days. Mice were euthanized and their lungs were collected for viral load quantification by qPCR.

Rat Pharmacokinetic Study of GST-HG171 with RTV

GST-HG171 was dissolved in a formulation of 10% Solutol, 30%PEG400, 2% Tween80 and 58% deionized water. Male SD rats received co-administration of GST-HG171 at 30 mg/kg and RTV at 10 mg/kg (RTV was dosed two times at -12 h and 0 h), or of nirmatrelvir (PF-07321332) and RTV (RTV was dosed two times at -12 h and 0 h) at the same doses. Plasma samples and lung tissues were collected at 0.25, 1 and 6 h after dosing. Drug concentrations were determined by LC-MS/MS, and pharmacokinetic parameters were generated using Phoenix WinNonlin.

Human PK Study of GST-HG171 in Phase 1 Clinical Trial

The Single-Ascending Dose (SAD) study, which was a part of our muti-parts Phase 1 clinical trial (www.ClinicalTrials.gov identifier: NCT05668897), was conducted at the Phase I Clinical Research Center of the First Hospital of Jilin University, Jilin, China, from October 1, 2022 to December 2, 2022. The study protocols were approved by the Ethics Committee of the first hospital of Jilin University Hospital, and the informed consent forms were provided to all participants. Healthy subjects aged 18-50 years old, with a body mass index of 18-28 kg/m2 and a total body weight of ≥ 45 kg were recruited for this study. Key exclusion criteria included the evidence or history of clinically significant gastrointestinal, renal, hepatic, neurological, hematological, endocrine, cancer, pulmonary, immunological, psychiatric, cardiovascular or allergic diseases within 6 months before randomization, participants with a history of HIV, hepatitis B, or hepatitis C infection or a positive test for HIV at the screening. The SAD study was a randomized, double-blind and placebo-controlled clinical trial with 4 doses groups (150 mg, 300 mg, 600 mg, and 900 mg). A total of 32 participants were randomized to receive a single oral dose of GST-HG171 or placebo on a 3:1 ratio. Blood samples were collected at 0 h (pre-dose), 0.25 h, 0.5 h, 0.75 h, 1 h, 1.25 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, 24 h, 48 h, 72 h, 96 h post dosing. Pharmacokinetic parameters were calculated using Phoenix WinNolin software. The statistical analysis were performed using SAS V9.4.

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