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HIGHLIGHT

Jun12682, a potent SARS-CoV-2 papain-like protease inhibitor with exceptional antiviral efficacy in mice

KEY WORDS

SARS-CoV-2;
Papain-like protease;
Broad-spectrum;
Anti-drug resistance;
Drug candidate

Recently, a collaborative research study published in *Science*, led by Jun Wang, Xufang Deng, Eddy Arnold, and Francesc Xavier Ruiz[1], identified a series of potent small molecule inhibitors that specifically target SARS-CoV-2 papain-like protease (PL^{pro}). The study demonstrated nanomolar PL^{pro} inhibitory potency with K_i values ranging from 13.2 to 88.2 nmol/L. By employing a structure-based drug design strategy, the researchers discovered an exceptionally promising compound, named Jun12682, that effectively targets both the newly discovered ubiquitin Val70 ($Val70^{Ub}$)-binding site and the known blocking loop (BL2) groove near the S4 subsite of PL^{pro}. Furthermore, studies on the mechanism of action revealed that Jun12682 inhibits the deubiquitinating and deISGylating activities of PL^{pro}, which are crucial for antagonizing the host's innate immune response upon viral infection. Structural biology studies confirmed the "two-pronged" binding mode of Jun12682, aligning perfectly with their drug design rationale. Importantly, Jun12682 exhibited potent antiviral activity against SARS-CoV-2 and its variants, including nirmatrelvir-resistant mutants, in Caco-2 cells (EC_{50}: 0.44−2.02 μmol/L). It is noteworthy that its oral administration significantly improved survival rates and alleviated both lung virus loads and histopathological lesions in a lethal SARS-CoV-2 mouse model. In conclusion, these findings support the therapeutic potential of Jun12682 as an up-and-coming oral drug candidate targeting SARS-CoV-2 PL^{pro} for clinical applications.

Although the public health emergency related to the COVID-19 pandemic officially ended last year, SARS-CoV-2 circulation continues to be a serious public health concern, with ongoing transmission posing a substantial threat to human social life and the economy. Vaccines and antivirals developed to date have contributed to overcoming the challenges of the pandemic over the last four years. However, these interventions have encountered new difficulties due to the emergence of SARS-CoV-2 variants or mutants, which exhibit limited sensitivity to direct-acting antivirals, including therapeutic neutralizing antibodies, and possess immune evasion capabilities. This means they can bypass immunity acquired from vaccination or prior infection, leading to subsequent infection waves[2]. As a result, scientists are exploring novel therapeutic strategies to address the future emergence of antiviral-resistant coronavirus variants. Currently, three small-molecule antiviral drugs (remdesivir, molnupiravir, and nirmatrelvir) have received FDA approval. However, each of these drugs has its limitations, such as insufficient antiviral efficacy, potential toxicity, and accessibility issues. For instance, remdesivir requires intravenous administration, which significantly restricts patient compliance, and its clinical efficacy remains controversial. Molnupiravir is contraindicated for pregnant women due to risks of mutagenicity and genotoxicity. Nirmatrelvir must be administered with ritonavir, a CYP3A4 inhibitor, to improve its pharmacokinetic (PK) properties[3]. Furthermore, clinical use and laboratory studies have shown that remdesivir and nirmatrelvir can lead to drug-resistant mutations in their target proteins of SARS-CoV-2 through multiple evolutionary pathways[4]. This situation underscores the necessity of developing new therapeutic targets or mechanisms to robustly counter SARS-CoV-2 resistance in antiviral treatment.

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PL^{pro} is a cysteine protease that specifically cleaves viral nonstructural polyproteins (Nsps) between Nsp1 and Nsp2, Nsp2 and Nsp3, and Nsp3 and Nsp4, establishing it as an attractive therapeutic target. It also plays a pivotal role in the SARS-CoV-2 life cycle by counteracting the host's innate immune system, particularly by regulating interferon and NF-κB pathways through the cleavage of ubiquitin and interferon-stimulated gene 15 (ISG15) from host proteins[5]. Consequently, antagonizing PL^{pro} with antiviral agents could offer dual benefits: direct antiviral activity and restoration of the host immune response. This dual functionality represents a distinctive advantage not observed with other inhibitors of the main protease (M^{pro}) or RNA-dependent RNA polymerase (RdRp). However, the discovery of small molecule inhibitors for PL^{pro} is complicated by the enzymatic dynamics and structural flexibility of two representative target pockets, the catalytic domain, and the BL2 groove domain. The catalytic domain typically adopts a closed conformation that only opens upon substrate approach[6], posing challenges for condition-wise availability for effective small molecule binding. Consequently, only a limited number of covalent inhibitors have been reported, which exhibit relatively low enzymatic inhibitory activity. Additionally, the high flexibility of the BL2 domain results in weak binding when interacting with small molecules[7]. To date, no small molecule drugs targeting PL^{pro} have been launched on an international scale. Compared to drugs targeting M^{pro} and RdRp, developing PL^{pro} inhibitors represents a new frontier filled with both substantial challenges and opportunities.

To advance the development of PL^{pro} inhibitors, Wang et al.[1] designed the hybrid covalent inhibitor Jun11313, evolving from the previously reported inhibitors, XR8-24 and Cp7 (Fig. 1). Innovation included the replacement of the naphthalene ring in Cp7 with 3-phenylthiophene, enhancing the molecule's covalent binding affinity for PL^{pro}. Jun11313 demonstrated a prominent inhibitory effect on PL^{pro} (IC_{50} = 0.12 μmol/L), outperforming XR8-24 (IC_{50} = 0.56 μmol/L) and being comparable to Cp7 (IC_{50} = 0.094 μmol/L), making it a promising starting point for further structural optimization. Crystallographic analysis revealed the fumarate ester of Jun11313 forms a covalent bond with Cys111. Detailed structural insights suggested unique interactions between Jun11313 and the PL^{pro} protein, notably the thiophene group making distinct van der Waals contacts with Pro247 and Pro248, as well as engaging in CH−π and S−π interactions with Met208. This study highlighted the importance of a hydrophobic pocket that is occupied by the thiophene moiety, as it is accomplished similarly with the Val70^{Ub} motif (a narrow area around Val70 of ubiquitin), providing a novel strategy for designing future PL^{pro} inhibitors. Their studies using an alignment approach additionally showed that the thienyl group of Jun11313 interacts uniquely with the Asn151-Leu152 site on the PL^{pro}-bound ISG15. Building on these findings, a library of 85 biaryl phenyl-substituted benzamide compounds was synthesized and screened, leading to the identification of Jun12682. This compound (K_i = 37.7 nmol/L, EC_{50} = 1.1 μmol/L) exhibited approximately 20-fold higher potency than GRL0617 (a known PL^{pro} inhibitor, EC_{50} = 22.4 μmol/L) in FlipGFP PL^{pro} assay and SARS-CoV-2 antiviral experiments in Vero cells. It is noteworthy that Jun12682 consistently displayed inhibitory activity against Omicron and Delta variants, as well as against three recombinant nirmatrelvir-resistant viruses (rNsp5-S144M, rNsp5-L50F/E166V, and rNsp5-L50F/E166A/L167F), indicating its potential as a broad-spectrum antiviral agent.

The FRET-based assay of Jun12682 targeting PL^{pro} utilizing Ub-AMC (Ub-7-amino-4-methylcoumarin) and ISG15-AMC substrates proved its efficacy in hindering both deubiquitination and deISGylation, with K_i values of 63.5 and 38.5 nmol/L, respectively. To assess the specificity of Jun12682 and its possible off-target effects, it was tested against the structurally related human enzymes USP7 and USP14. Remarkably, even at high concentrations up to 40 μmol/L, Jun12682 showed no inhibitory activity on the hydrolytic functions of these cellular enzymes. The findings highlight the selectivity of Jun12682 and substantiate targeting PL^{pro} as an effective dual strategy for SARS-CoV-2 treatment. The data provided a solid foundation for anticipating that this approach would suppress viral protein maturation and

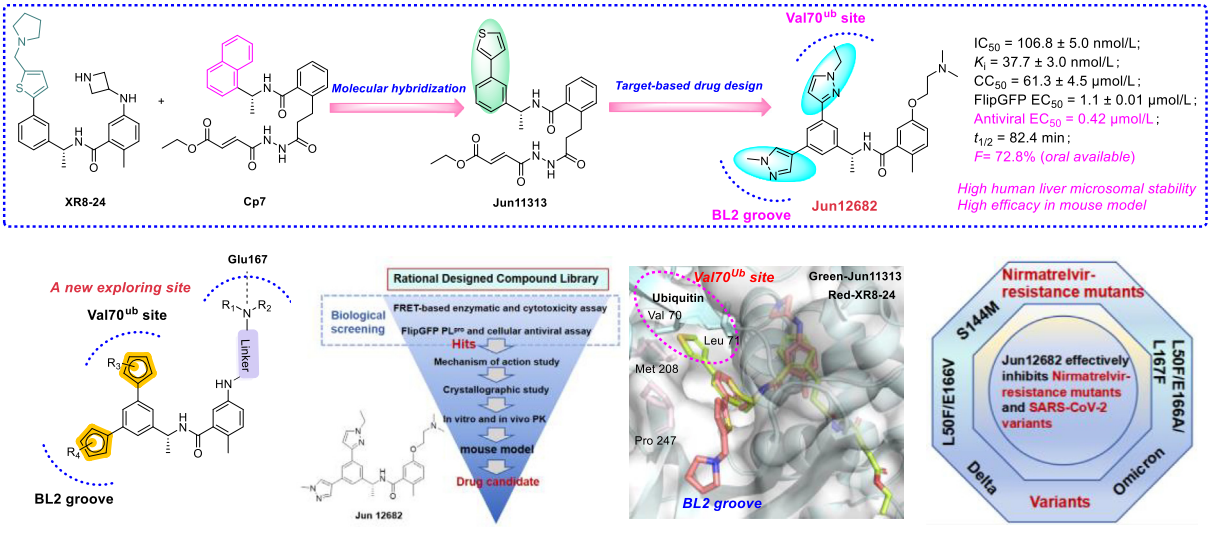

Figure 1 Graphical representation of the discovery of the SARS-CoV-2 PL^{pro}-targeting drug candidate Jun12682.

modulate the host's innate immune response, while leaving human endogenous deubiquitination mechanisms unaffected.

Next, the researchers determined the co-crystal structure of PL^{pro} with eight inhibitors, including Jun12682. The results revealed that Jun12682 and its analogues bind simultaneously to both the hydrophobic pocket of the BL2 groove domain and the $Val70^{Ub}$ site, thereby solidifying the initial rational design. An *in vitro* PK study demonstrated that Jun12682 exhibited favorable metabolic stability, with a half-life of 131.9 min, intrinsic clearance of 10.5 μL/min/mg, and adequate solubility, exceeding 5 mg/mL in an aqueous solution. Notably, the compound did not exhibit any inhibitory effects on the five major drug-metabolizing cytochrome P450 (CYP450) enzymes, including CYP1A2, 2C9, 2C19, 2D6 and 3A-M ($IC_{50} > 50.0$ μmol/L), suggesting a low likelihood of drug−drug interactions, unlike Paxlovid. In line with the *in vitro* data, *in vivo* PK studies in C57BL/6J mice confirmed its excellent oral bioavailability (72.8%), with a peak plasma concentration of 4537 ng/mL and a half-life of 2.0 h. Considering the comprehensive data on mouse microsomal stability and PK profiles, the authors shifted their focus to studying the *in vivo* efficacy using a mouse lethal model. Oral administration of Jun12682 showed evident improvements in survival rate and reduced body weight loss in BALB/c mice infected with mouse-adapted SARS-CoV-2, offering complete survival at a dose of 250 mg/kg, administered twice a day. It also reduced the levels of infectious viral titer and the viral nucleocapsid (N) gene in the lungs, accompanied by decreases in the expression of various inflammatory factors such as IFN-β, IL-1β, IL-6, and CXCL10. Decisively, histopathological and immunohistochemical analyses visualized that Jun12682 effectively alleviated lung inflammation and reduced viral replication in the target tissue.

From the perspective of antiviral agent development targeting PL^{pro}, the advantage lies in its ability to inhibit viral replication and restore the attenuated innate immune response. With comprehensive knowledge of the biological functions of PL^{pro}, its tertiary structure at the molecular level, and pharmacokinetic indications, the study successfully designed highly efficient PL^{pro} inhibitors by extending the antiviral target site from the conventional BL2 loop to the newly discovered $Val70^{Ub}$-binding pocket. Eventually, Jun12682 shows remarkable antiviral activity against various mutant strains of SARS-CoV-2 and demonstrates exceptional efficacy *in vivo*, positioning it as a strong drug candidate for combating SARS-CoV-2 and providing insights for the continued development of other PL^{pro} inhibitors.

Despite the long journey ahead in developing PL^{pro} inhibitors into marketable drugs, we consider the work of Wang et al.[1] to be a valuable breakthrough. The interaction between the human immune system and PL^{pro} involves a complex process that remains to be fully elucidated. For instance, SARS-CoV-2 PL^{pro} suppresses the interferon response through deISGylation of IRF3, as reported by Shin et al.[5], and also through the deubiquitination of the STING protein, as noted by Cao et al.[8]. Exploring the interconnected inflammatory pathways is likely to offer groundbreaking insights for the therapeutic management of COVID-19 or long-term COVID. This includes exploring the synergistic impact of STING or IRF3 agonists and PL^{pro} inhibitors for treating different clinical spectrums of SARS-CoV-2 infections. To meet clinical demands, assessing the efficacy of combination therapies with RdRp or M^{pro} inhibitors might be required. Targeting non-catalytic sites with allosteric inhibitors of the PL^{pro} presents a novel approach for developing broad-spectrum antiviral drugs against coronaviruses. Finally, leveraging state-of-the-art technologies and innovative strategies like DNA-encoded library (DEL) screening, protein degradation technologies (proteolysis-targeting chimera, PROTAC; hydrophobic tag, HyT; molecular glue), and miniaturized synthesis technologies could expedite the discovery of novel inhibitors or degraders that targeting PL^{pro} with unique mechanisms to potentiate their antiviral effectiveness.

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Author contributions

Mianling Yang: Writing − review & editing, Writing − original draft. Meehyein Kim: Writing − review & editing. Peng Zhan: Writing − review & editing, Funding acquisition.

Conflicts of interest

The authors declare no competing financial interest.

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