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ORIGINAL ARTICLE

Discovery of SARS-CoV-2 PLpro inhibitors and RIPK1 inhibitors with synergistic antiviral efficacy in a mouse COVID-19 model



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Abstract SARS-CoV-2 continues to propagate globally, posing non-negligible risks of severe COVID-19. Although several clinical antivirals and immunosuppressants offer crucial protection, there is a persistent need for additional therapeutic options to counter emerging viral variants and drug resistances. New strategies focusing on host targets, or simultaneously suppressing viral replication and inflammation,

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RIPK1;
Infective diseases;
Cytokine storms;
Small molecule inhibitors;
Combination therapy

particularly require rigorous validation. Compared to established antiviral targets, PLpro presents an alternative actionable vulnerability in SARS-CoV-2 infection. Meanwhile, RIPK1 was pinpointed to enhance both viral replication and the resulting cytokine storm in host cells. However, inhibitors targeting PLpro or RIPK1 require further optimization for preclinical studies, and their combined efficacy *in vivo* has yet to be explored. Here, we report the discoveries of potent and selective PLpro inhibitors and RIPK1 inhibitors through high-throughput approaches. Our lead compounds, SHY1643 and QY1892, demonstrated synergistic and robust effects in reducing the viral loads and cytokine release syndromes in SARS-CoV-2-infected mice. These findings establish a proof-of-concept combination therapy strategy for treating severe COVID-19, and provide promising leads for the clinical drug development.

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1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has persisted for four years, resulting in millions of deaths and immeasurable economic calamity. Driven by emerging variants and waning immunity from prior infections and vaccinations, SARS-CoV-2 continues to propagate globally as influenza viruses and other milder coronaviruses, albeit with reduced but still significant risks of causing severe, potentially fatal diseases and long-term sequelae¹. Currently, several antiviral drugs targeting the RNA-dependent polymerase (RdRp) or the main protease (Mpro) of SARS-CoV-2 have received clinical approval². While these drugs have shown greater efficacy in treating mild to moderate COVID-19, their controversial effectiveness, potential mutagenic risks, and inevitable challenges posed by resistant variants remain concerns^{3,4}. On the other hand, anti-inflammatory agents, such as corticosteroids and Janus kinase (JAK) inhibitors, have been used alongside antivirals or alone to mitigate cytokine storm, the primary driver of mortality in severe COVID-19^{2,5}. However, these immunosuppressants have fallen short of meeting clinical needs due to variable patient responses and adverse effects^{6,7}. In light of these limitations, the development and validation of additional drugs and alternative therapeutic strategies are imperative to address potential future outbreaks of coronaviruses.

In addition to RdRp and Mpro, the papain-like protease (PLpro) represents another essential and highly conserved enzyme among multiple pathogenic coronaviruses. PLpro plays a critical role in processing viral nonstructural proteins (Nsp1-3), which are indispensable for SARS-CoV-2 replication. Furthermore, PLpro can cleave K48-linked ubiquitin (Ub) chains and interferon-stimulated gene 15 (ISG15) modifications in host cells, thereby suppressing the host immune response (Fig. 1A)⁸. Building on the advancements in targeting SARS-CoV PLpro^{9,10}, inhibitors against SARS-CoV-2 PLpro are being developed through high-throughput screening (HTS) or structure-guided rational design. However, lead optimization efforts based on HTS hits have been less productive compared to those derived from known SARS-CoV PLpro inhibitors, such as GRL-0617 and Cmpd-3k^{11,12}. Through structure-guided optimization of the Cmpd-3k series, we successfully developed a PLpro inhibitor, Cmpd-19, which demonstrated significantly stronger potency than previously reported SARS-CoV PLpro inhibitors¹². Alongside our efforts, substantial progress has been made in the preclinical optimization of PLpro inhibitors¹³⁻¹⁶. Recently, PLpro inhibitors derived from GRL-0617 and rational design have shown explicit antiviral

efficacy in SARS-CoV-2-infected mouse models, albeit at relatively high dosages (Fig. 1A)¹⁷⁻²¹. However, the therapeutic potential of PLpro inhibitors to synergize with other treatments remains to be explored.

Meanwhile, many immunomodulators originally developed for autoimmune diseases had been clinically or preclinically investigated for their potential to treat severe COVID-19, either in combination with antivirals or as standalone therapies^{22,23}. Among them, kinase inhibitors (KIs) targeting receptor-interacting serine/threonine-protein kinase 1 (RIPK1), such as DNL758, and SIRI-365, had advanced into phase I clinical trials and shown preliminary clinical improvements in severe COVID-19 patients (Fig. 1B)^{24,25}. RIPK1 is a master regulator in the activation of inflammatory response and programmed cell death mediated by tumor necrosis factor receptor 1 (TNFR1) or other cytokine receptors. Inhibition of its kinase activity has demonstrated efficacy in alleviating pathological conditions across a wide range of inflammatory and degenerative disease models²⁶. Notably, compared to many immunosuppressants, RIPK1 KIs exhibit minimum risks of compromising the innate immune system and may even enhance immune reconstruction²⁷. Beyond their anti-inflammatory effects, we discovered that RIPK1 KIs can also inhibit of SARS-CoV-2 viral propagation by disrupting the Nsp12-RIPK1-viral receptors cascades, further solidifying RIPK1 as a promising target for treating severe COVID-19^{28,29}. However, current RIPK1 KIs are not without limitations, such as mediocre potency, inactivity against rodent RIPK1, off-target effects on the immune system, or poor brain penetration, which could be addressed through development of new pharmacophores²⁶. Additionally, their therapeutic potential in combination with antivirals has yet to be thoroughly explored.

Here, we report the development of SHY1643, a potent and selective SARS-CoV-2 PLpro inhibitor, derived from Cmpd-19 through a high-throughput derivation approach followed by structure-guided lead optimization. Additionally, we describe the discovery of QY1892, a potent and selective RIPK1 inhibitor, identified *via* phenotypic HTS and intensive structure-activity relationship (SAR) studies. Both inhibitors demonstrated significant antiviral activity against SARS-CoV-2 in cellular/organoid models and *in vivo*, with SHY1643 also exhibiting effectiveness against other pathological coronaviruses. Crucially, combination therapy with SHY1643 and QY1892 synergistically and effectively reduced both viral loads and inflammatory cytokine levels in a severe COVID-19 mouse model. These findings establish dual targeting of viral PLpro and host RIPK1 as a viable and effective therapeutic strategy for severe COVID-19, while also providing

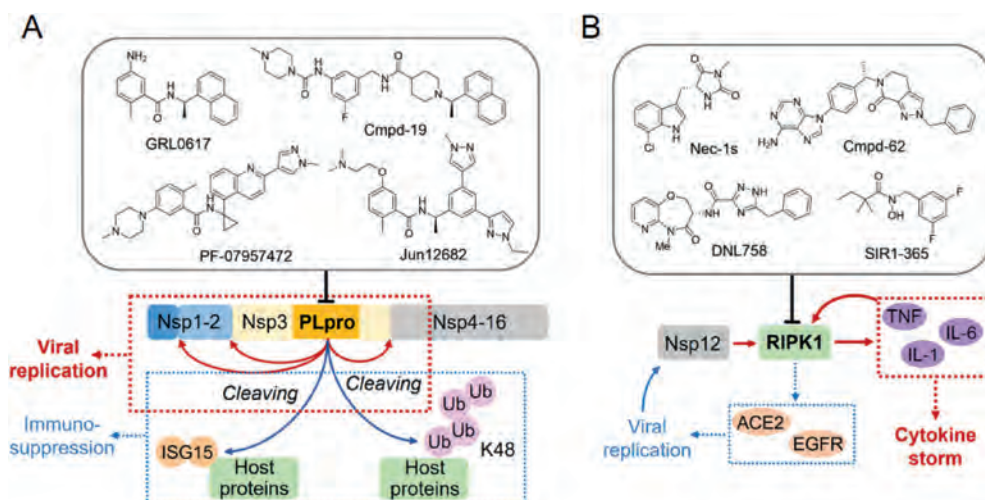


Figure 1 Diagrams depicting the pathological roles and representative inhibitors of SARS-CoV-2 PLpro (A) and RIPK1 (B) in COVID-19.

promising preclinical leads for the development of anti-infective and anti-inflammatory drugs.

2. Results and discussion

To further optimize our first-generation SARS-CoV-2 PLpro inhibitor, Cmpd-19¹², we adopted a high-throughput derivation and screening strategy leveraging SuFEx (sulfur(VI) fluoride exchange)-catalyzed azidation and click-chemistry techniques (Fig. 2A)³⁰. This approach enabled the effective conversion of diverse primary amines into azido building blocks in 96-well plates, which were then reacted with alkynes (**I–IV**) derived from Cmpd-19 scaffold *via* copper-catalyzed azide-alkyne cycloaddition (CuAAC) *in situ*. This process generated thousands of triazole analogs of Cmpd-19, which were subsequently and conveniently screened for inhibitory activity against the PLpro enzyme upon dilution. Among the four alkynes, alkyne-I, structurally most similar to Cmpd-19, was used to generate 4608 analogs, while the others each yielded 1140 analogs (Fig. 2B).

All the resulting triazole analogs were screened for their inhibitory potency against PLpro using our previously developed HTS assay, which utilizes recombinant SARS-CoV-2 PLpro protein and a peptidomimetic fluorogenic activity probe, with Cmpd-19 serving as the positive control¹². The screening results revealed that many analogs were more potent than Cmpd-19, with nearly 20 showing at least a 1.5-fold increase in potency (Fig. 2C). Notably, the top hits were all derivatives based on Alyne-I, whose scaffold is the most structurally conserved relative to Cmpd-19. Following the identification of these hits, the top four hits were cherry-picked (Fig. 2D), resynthesized and purified, and subjected to further characterization and optimization.

The four top hits were subsequently titrated for their IC₅₀s against SAR-CoV-2 PLpro. The findings revealed that **1**, **2**, and **4** exhibited comparable activity, whereas **3** demonstrated markedly reduced potency (Fig. 3A). Notably, the substituents (R) of **1** and **4** are enantiomeric, both featuring an aliphatic carboxylic group, which may contribute to suboptimal permeability and absorption. However, the removal of these carboxylic groups resulted in decreased activity (**5**, **6**). Consequently, our focus shifted to **2**, which bears an 8-methyl-[1,2,4]triazolo[4,3-*a*]pyridine-3-yl substituent, as it presented a more promising profile for optimization.

To guide the optimization of **2**, we employed molecular docking to predict its binding mode, utilizing the co-crystal structure of SAR-CoV-2 PLpro in complex with an analog of Cmpd-19, Cmpd-12 (PDB ID: 7E35)¹². The co-crystal structure reveals that two chains of PLpro form an asymmetric dimer, creating a continuous hydrophobic pocket between them. Within this pocket, two molecules of Cmpd-12 interact with both chains of PLpro, functioning as molecular glues (Fig. 3B). Cmpd-19 is predicted to bind similarly to Cmpd-12, although its 4-methylpiperazinyl-1-carboxamide group must orient away from the binding pocket's center due to its larger size compared to the acetamide group of Cmpd-12 (Fig. 3C). With an even bulkier and more hydrophobic substituent, **2** adopts a U-shape conformation to fit within the binding pocket, occupying the space independently other than as a paired interaction (Fig. 3B and C).

The SAR study revealed that replacing the naphthyl group of **2** with substituted phenyl groups resulted in varying degrees of potency decrease (**7–9**), discouraging further exploration of modifications to this moiety. Introducing a *meta*-fluorine to the phenyl group of **2** improved the docking score (**10**), but this modification did not translate into enhanced activity in experiments. In contrast, replacing the methyl at the 8-position of the triazolopyridine group with chlorine or fluorine yielded significant improvements in both docking scores and inhibitory activities, leading to the identification of **11** and SHY1643, both with IC₅₀ values in the two-digit nanomolar range (Fig. 3D). Notably, SHY1643 demonstrated consistent potency against the DUB activity of recombinant SARS-CoV-2 Nsp3^{179–1329} protein, which comprises the PLpro domain and seven adjacent domains and represents a more physiologically relevant target during viral infection, with an IC₅₀ value of 28 nmol/L (Supporting Information Fig. S1). Similarly, Cmpd-19 and a recently reported PLpro inhibitor, Jun12682¹⁷, also exhibited comparable IC₅₀ values against both PLpro and Nsp3^{179–1329} enzymatic activities.

In addition, the size exclusion chromatography (SEC) analysis results indicated that incubation with SHY1643 could induce dimerization of Nsp3 protein, consistent with the predicted binding mode of this inhibitor series (Supporting Information Fig. S2). Additionally, SHY1643 exhibited markedly improved metabolic stability compared to **2** in mouse microsomes (Supporting Information Fig. S3). To assess its selectivity, SHY1643 was tested

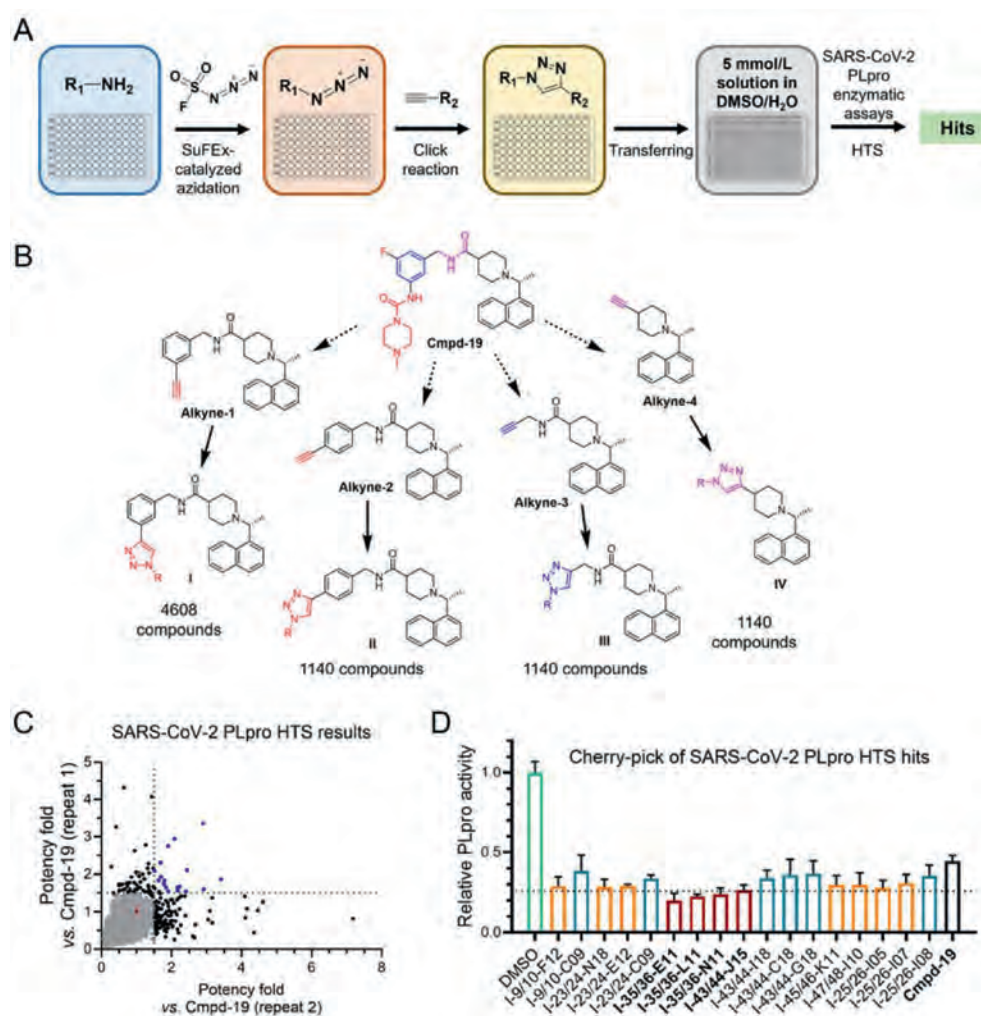


Figure 2 High throughput derivation and screening of SARS-CoV-2 PLpro inhibitors. (A) Diagrams summarizing the preparation and screening of modular click chemistry libraries. (B) The derivation strategies based on Cmpd-19. (C) HTS results based on SARS-CoV-2 PLpro enzymatic activities. Each dot represents a compound with scores from repeats in two independent plates, and the compounds were screened at 5 μ mol/L assuming the starting alkynes were completely converted. (D) Bar graph showing confirmation run results of cherry-picked hits (blue dots in panel C) at 2 μ mol/L. Data represent means $\pm$ SD ($n = 3$).

against a panel of other DUBs, including USP7, USP8, USP14, UCHL1, OTULIN, and OTUB1. The results confirmed that SHY1643 had no significant inhibitory effects on these DUBs, even at a concentration of 10 μ mol/L (Fig. 3E). Collectively, these findings underscore SHY1643 as a potent and highly selective inhibitor of SARS-CoV-2 PLpro.

To develop novel RIPK1 kinase inhibitors, we conducted a HTS campaign using a chemical library comprising approximately 30,000 compounds. These compounds were pre-selected for their potential to penetrate the central nervous system (CNS), based on favorable physicochemical properties such as molecular weight, number of flexible bonds, polar surface area, and calculated partition coefficient. The screening was performed using a phenotypical HTS assay designed to detect inhibition of TNF α -induced, RIPK1-dependent necroptosis in *FADD* deficient (*FADD*^{-/-}) Jurkat T lymphocytes. This approach successfully identified multiple hits capable of effectively rescuing the cells from necroptosis (Fig. 4A). Among the top four hits selected for further verification, the second hit (**12**) stood out as a particularly

promising candidate, exhibiting a sub-micromolar IC₅₀ value. This level of potency is highly encouraging for a screen hit and highlights the potential of **12** for further optimization (Fig. 4B).

We then systematically investigated the SAR of each subunit of **12** (Fig. 4C). Noting that the *N*-acyl-2-aryl pyrrolidine moiety of **12** bears a structural resemblance GSK'963, a reported RIPK1 inhibitor³¹, we replaced the 3-methyl-1,2,4-oxadiazole group of **12** with a phenyl group, which resulted in a notable increase in potency. On the other hand, introducing small hydrophobic substitutions, such as fluorine and methoxy, at the 2-position of the 4-chlorophenyl group of **12** also enhanced activity. By combining both modifications, we obtained **13**, which demonstrated significantly improved potency in both human *FADD*^{-/-} Jurkat cells and mouse L929 fibroblasts, achieving two-digit nanomolar IC₅₀ values under TNF α -only stimulation. Consistently, **13** exhibited superior activity with sub-micromolar IC₅₀ values compared to **12** or Nec-1s under harsher necroptotic conditions induced by TNF α combined with SM164, a cIAP inhibitor that suppresses the ubiquitination and inactivation of RIPK1 by cIAP³² (Fig. 4C and D).

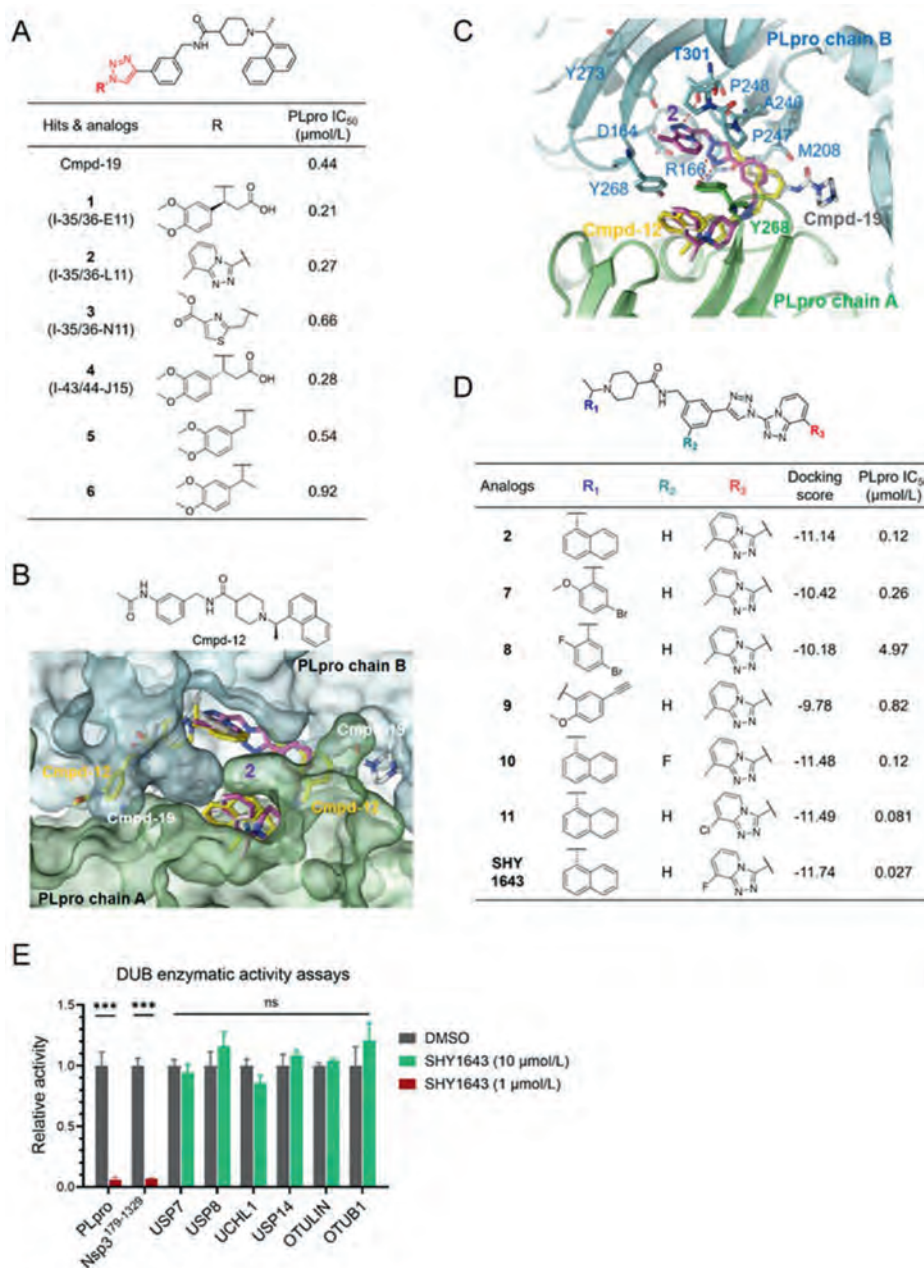


Figure 3 Lead optimization of the SARS-CoV-2 PLpro inhibitor, SHY1643. (A, D) The SAR studies based on HTS hits and **2**. Data represent means of three replicates. (B, C) Predicted binding mode of Cmpd-19 and **2** based on the coordinates of SARS-CoV-2 PLpro co-crystallized with Cmpd-12. The PLpro protein structures are shown as ribbons and surfaces, key residues and inhibitors are shown as sticks, and potential H-bonds are highlighted with dashes. (E) Bar graph showing selectivity profiling results of SHY1643 based on DUB enzymatic assays. Data represent means $\pm$ SD ($n = 3$).

However, modifications to the linker between the pyrrolidine group and the chlorophenyl group resulted in varying degrees of potency decrease, prompting us to focus further optimization efforts on both phenyl groups of **13**. We discovered that introducing small hydrophobic groups, such as methyl (**14**) and chlorine, at the *para*-position of the phenyl group on the pyrrolidine ring further enhances the potency of **13**. Additionally, the *S*-isomer (**14**) was confirmed to be more potent than the *R*-isomer, consistent with the trend observed for GSK'963. Inspired by recent advances in deuterated drug development³³, and aiming to improve its

metabolic stability and hydrophobic interactions with RIPK1, we replaced the 4-methylphenyl group of **14** with a deuterated analog, yielding **15** with favorable potency in mouse cells. Meanwhile, substituting the 4-chloro-2-fluorophenyl group of **14** with an indolin-2-one-6-yl group reduced the IC₅₀ values to single-digit nanomolar levels (**16**). Further improvement in both activities and aqueous solubility was achieved by introducing an isosteric 1*H*-indazole-6-yl group. Finally, combining both the indazole group and the deuterated methylphenyl group led to discovery of QY1892, an extraordinarily potent analog with IC₅₀ values

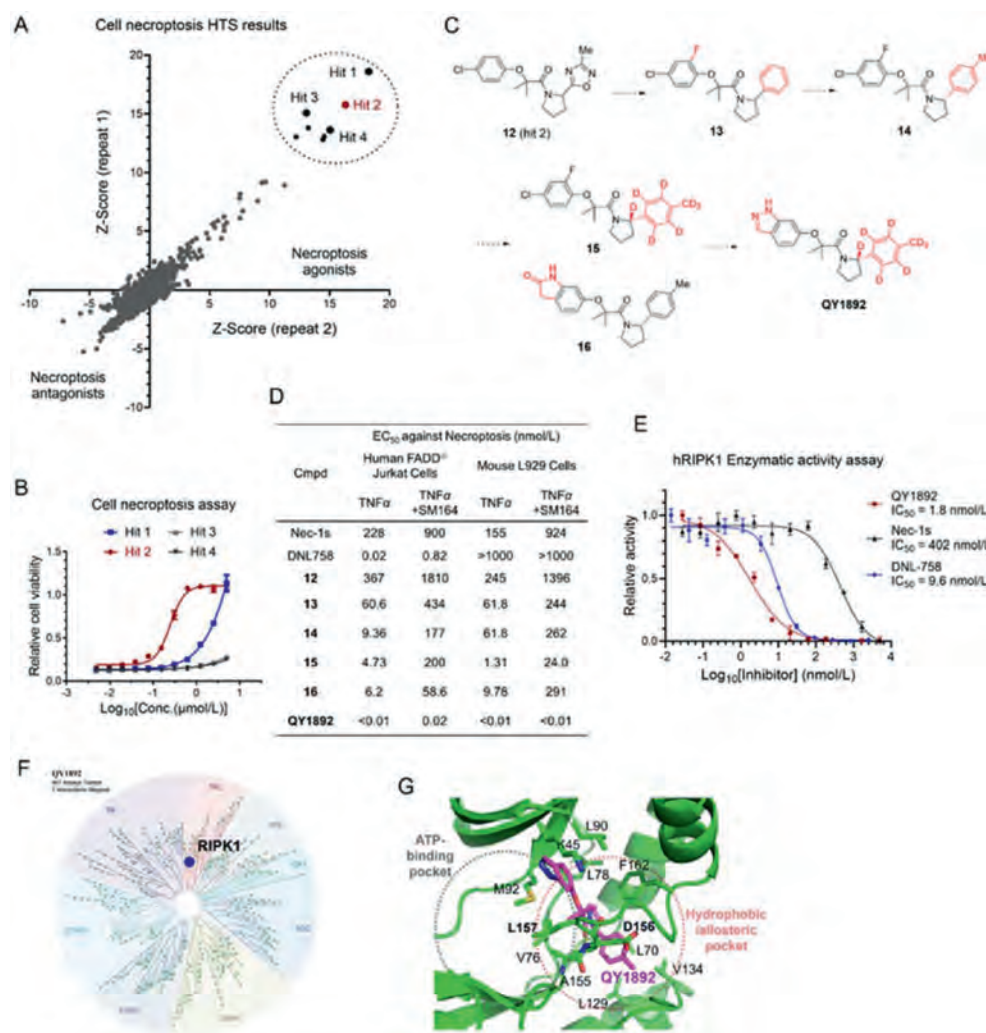


Figure 4 Development of the RIPK1 inhibitor, QY1892. (A) HTS results based on FADD^{-/-} cell necroptosis. Each dot represents a compound with scores from repeats in two independent plates, and the compounds were screened at 5 μg/mL concentrations. (B) Protection curves for selected hits against FADD^{-/-} cell necroptosis. Data represent means ± SD (*n* = 3). (C, D) SAR study of the hit 2 and lead optimization of QY1892. Data represent means of three replicates. (E) Inhibition curves and IC₅₀ values for QY1892 and reported inhibitors against human RIPK1 based on an enzymatic assay. Data represent means ± SD (*n* = 3). (F) Kinase selectivity profiles for QY1892 at 1 μmol/L against a diverse panel of more than 300 wild-type human kinases. (G) Predicted binding mode of QY1892 based on the coordinates of RIPK1 co-crystallized with Nec-1s. The RIPK1 protein structure is shown as green ribbons, key residues and QY1892 are shown as sticks, and potential H-bonds are highlighted with dashes.

reaching picomolar levels in both human and mouse cells. In stark contrast, the clinical RIPK1 inhibitor DNL758 showed almost no activity against mouse RIPK1 (Fig. 4C and D).

Consistent with its robust anti-necroptotic activity in cellular assays, QY1892 demonstrated extraordinarily potent inhibition of human RIPK1 kinase activity *in vitro*, with an IC₅₀ value of 1.8 nmol/L. This represents 200- and 5-fold improvement over the IC₅₀ values of Nec-1s and DNL758, respectively (Fig. 4E). To evaluate its selectivity, QY1892 was profiled against a diverse panel of over 300 wild-type human kinases^{34,35}. Remarkably, QY1892 exhibited exclusive selectivity for RIPK1 at a concentration of 1 μmol/L, with no significant off-target activity observed across the kinome (Fig. 4F, Supporting Information Table S1). Molecular docking studies, based on the co-crystal structure of RIPK1 in complex with Nec-1s (PDB ID: 4ITH)³⁶, provided insights into the binding mode of QY1892. Specifically, the

deuterated methylphenyl group of QY1892 occupies the unique hydrophobic pocket of RIPK1 near the DLG-motif, a feature shared by most allosteric RIPK1 inhibitors (Fig. 4G). The linker of QY1892 adopts a specific conformation that enables the formation of an H-bond with the amide-NH of RIPK1 Asp156. This interaction allows the linker and the indazole group to fit snugly into the tight space surrounded by RIPK1 residues, engaging in additional stabilizing interactions through van der Waals forces. Overall, QY1892 is a potent and selective RIPK1 kinase inhibitor.

The optimized PLpro inhibitor, SHY1643, was evaluated for its antiviral activity in human cells. Using the expression levels of the SARS-CoV-2 nucleocapsid (N) protein as a metric, SHY1643 demonstrated significantly greater effectiveness in suppressing SARS-CoV-2 replication compared to Cmpd-19 in human angiotensin-converting enzyme 2 (ACE2)-expressing HeLa cells (hACE2-HeLa), with a reduced EC₅₀ value of 0.38 *versus*

0.88 $\mu\text{mol/L}$ for Cmpd-19 based on viral titer quantification (Fig. 5A and B). Notably, SHY1643 also exhibited robust antiviral activity against other pathogenic coronaviruses, including OC43 and 229E³⁷, consistently outperforming Cmpd-19 in human Huh7 hepatocytes (Fig. 5C and D). In parallel, SHY1643 was assessed for cytotoxicity in HeLa cells and the human lung epithelial cells (BEAS-2B). The results revealed that SHY1643 is considerably less toxic than Cmpd-19, with CC_{50} values exceeding 50 $\mu\text{mol/L}$ in both cells (Fig. 5E).

On the other hand, given that SARS-CoV-2 viral propagation may depend on RIPK1 activation in immunocompetent microenvironments, we evaluated our optimized RIPK1 inhibitor, QY1892, in a viral infection model using mouse airway organoids, which comprise both epithelial and immune cells²⁸. In the organoid cultures, treatments with QY1892 at 4 $\mu\text{mol/L}$ effectively suppressed the viral replication, whereas Nec-1s showed only weak inhibition even at 10 $\mu\text{mol/L}$ (Fig. 6A). Furthermore, QY1892 significantly reduced the acutely elevated phosphorylation levels of RIPK1 Ser166 following viral infection, as well as the expression levels of ACE2, a key viral entry receptor whose expression is putatively RIPK1-dependent in the host cells²⁸. In

contrast, the PLpro inhibitor, SHY1643, did not affect these two host markers (Fig. 6B). In contrast, neither QY1892 nor DNL758 exhibited detectable effects against SARS-CoV-2 in ACE2-HeLa cells even at 10 $\mu\text{mol/L}$, further confirming that QY1892 suppressed viral replication through indirect antiviral mechanisms (Supporting Information Fig. S4A).

Remarkably, co-treatment with QY1892 and SHY1643, each at 1 $\mu\text{mol/L}$, achieved comparable suppression of viral replication to QY1892 or SHY1643 alone at 4 $\mu\text{mol/L}$, indicating synergistic effects between the two inhibitors (Fig. 6A–C). In addition, while QY1892 was significantly more effective than SHY1643 in reducing cytokine secretion from the organoids upon viral infection, combining both inhibitors at half concentrations further enhanced the inhibitory effects (Fig. 6D). Consistently, co-treatment with QY1892 and SHY1643 in human bronchial epithelial cells (NHBE) also demonstrated synergistic inhibition of SARS-CoV-2 replication (Fig. S4B). Similar synergy was observed with reported PLpro inhibitor Jun12682 and RIPK1 inhibitor DNL758. Importantly, co-treatment with QY1892 at 10 $\mu\text{mol/L}$ hardly increased SHY1643's cytotoxicity in either NHBE or BEAS-2B cells (Fig. S4C). Cumulatively, these results

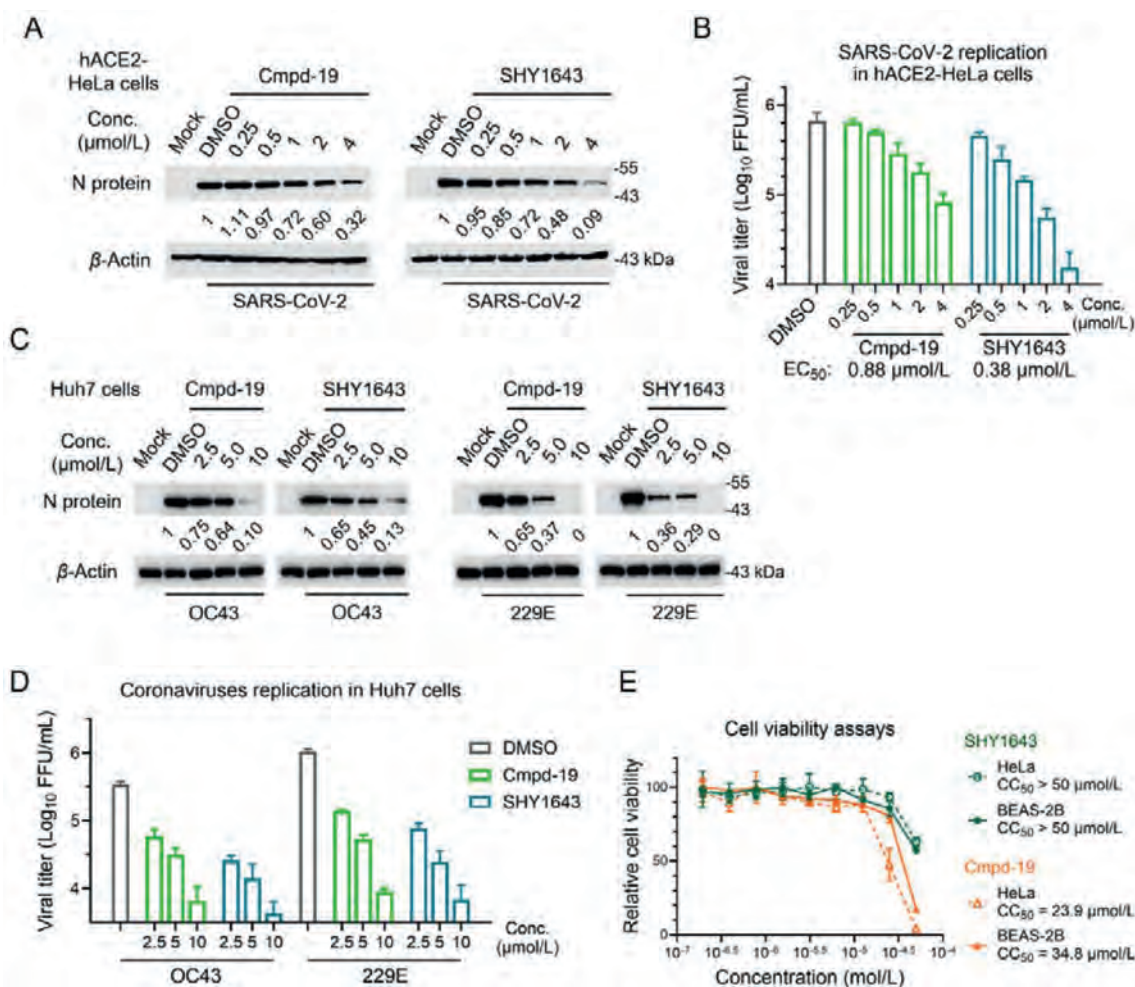


Figure 5 Antiviral activities of SHY1643 and QY1892 in cell infection models. (A–D) hACE2-HeLa cells (A, B) were infected with SARS-CoV-2 (0.1 MOI) for 2 h, and Huh7 (C, D) cells were infected with OC43 (0.1 MOI) or 229E (0.01 MOI) for 2 h, then washed out with PBS and cultured with normal medium and indicated compounds for an additional 48 h, then lysed and followed by Western blot analysis. The viral loads were quantified based on the N protein band grayscale (A, C) and Focus-Forming units (FFU) counting of the plaque-forming viruses (B, D). (E) Cytotoxicity test results for SHY1643 and Cmpd-19 in HeLa and BEAS-2B cells. Data represent means $\pm$ SD ($n = 3$).

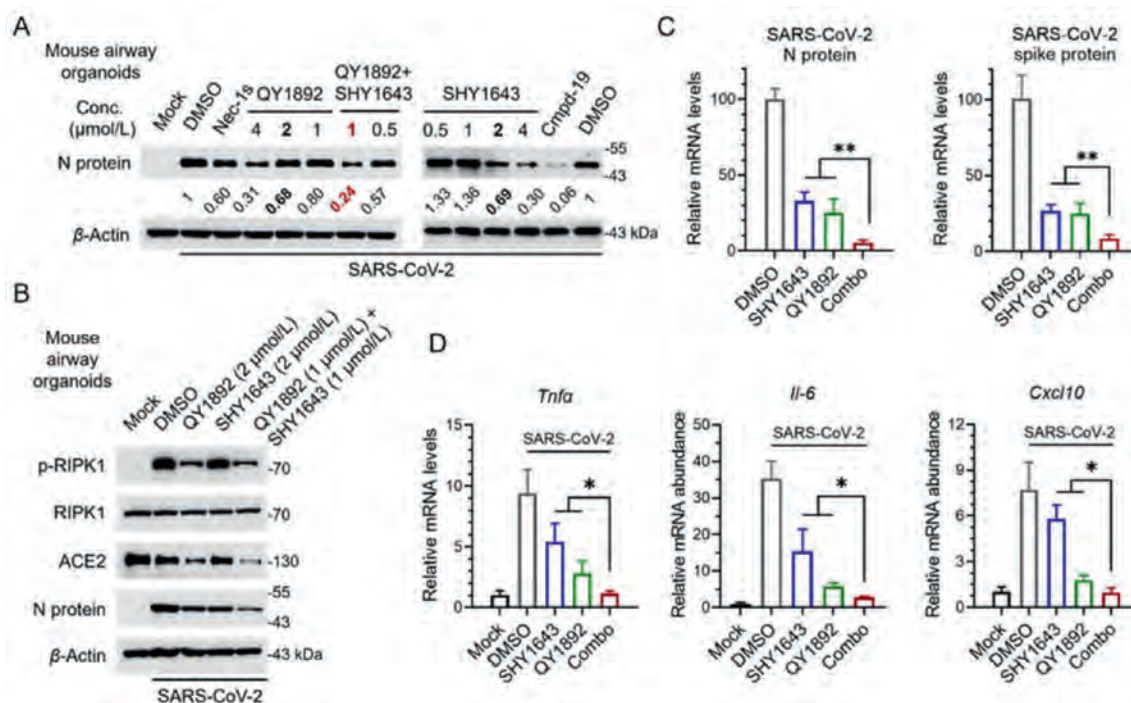


Figure 6 Antiviral activities of SHY1643 and QY1892 in organoid infection models. Mouse lung organoids were infected with SARS-CoV-2 (0.1 MOI) for 2 h, then washed out with PBS and cultured with normal medium and indicated compounds for an additional 48 h, then lysed and followed by Western blot analysis (A, B) and RT-PCR (C, D). The viral loads were quantified based on the viral protein band grayscale (A) and mRNA expression levels (C), and the cytokine secretion levels were quantified based on their mRNA expression levels (D). Data represent means $\pm$ SD ($n = 3$); * $P < 0.05$, ** $P < 0.01$, unpaired Student's t -test.

demonstrated that SHY1643 and QY1892 can individually and synergistically inhibit SARS-CoV-2 propagation in cellular models. Moreover, QY1892, either alone or in combination with SHY1643, effectively mitigated the inflammatory responses associated with viral infection.

To evaluate their potential for *in vivo* application, we conducted pharmacokinetic (PK) studies of SHY1643 and QY1892 in mice following intravenous (iv) and intraperitoneal (ip) administration. Despite QY1892 having a relatively short half-life, both inhibitors exhibited overall favorable PK profiles following a single ip injection at 10 mg/kg (mpk). They achieved micromolar maximum concentrations (C_{max}), decent area under the curve (AUC) values, and good bioavailability (Fig. 7A). In addition, QY1892 exhibited significant brain distribution post-administration (Supporting Information Fig. S5), suggesting its potential effectiveness against RIPK1 in CNS.

Next, we assessed the *in vivo* antiviral efficacy of SHY1643 and QY1892, both as single agents and in combination. We utilized an acute infectious disease model based on K18-hACE2 transgenic mice, which express high levels of hACE2 and develop cytokine storms and severe weight loss acutely upon nasal infection with SARS-CoV-2, leading to 100 percent mortality within less than one week (Fig. 7B). In our study, based on PK analysis and preliminary dose screening, infected mice received twice-daily (BID) intraperitoneal treatment for five days with either vehicle, SHY1643 (10 mpk), QY1892 (5 mpk), or a combination of both inhibitors at half dose. The mice were then sacrificed on Day 6 for analysis of viral load and pathological changes.

Based on the *in vivo* study results, the vehicle-treated group experienced severe and continuous weight loss following

infection, with mortality beginning on Day 6 due to drastic decreases in bodyweight (Fig. 7C). In contrast, treatment with SHY1643 at 10 mpk or QY1892 at 5 mpk significantly slowed the weight loss in infected mice. Remarkably, co-treatment with SHY1643 at 5 mpk and QY1892 at 2.5 mpk for four days not only halted weight loss but also reversed it, demonstrating the anticipated synergistic efficacy. Notably, by day 6, all mice receiving the co-treatment were as active as healthy controls and exhibited no signs of illness.

Histopathological analysis further supported these findings, revealing a significant reduction in segmental consolidation in the lungs of drug-treated mice, with co-treatment showing the most pronounced improvement (Fig. 7D). To quantify the antiviral effects, we measured SARS-CoV-2 viral loads in lung samples using RT-PCR analysis and immunohistochemical (IHC) staining. Both SHY1643 and QY1892 treatment effectively reduced viral loads in the lungs, with co-treatment synergistically reducing viral loads by at least two orders of magnitude (Fig. 7D and E). In the end, we assessed the expression levels of key inflammatory cytokines, including *Tnfa*, *Il-1b*, *Il-6*, *Ifn- γ* , *Cxcl9*, and *Cxcl13*, in lung samples from infected mice (Fig. 7F). The results demonstrated significant inhibition of cytokine secretion in the lungs of drug-treated mice, with co-treatment reducing cytokine levels by over 80%. Altogether, these findings highlight the synergistic efficacy of combining SHY1643 and QY1892 in mitigating severe COVID-19, confirming that dual inhibition of PLpro and RIPK1 not only suppresses viral replication but also alleviates the associated inflammatory response *in vivo*.

In contrast to the typically low-yield outcomes of traditional HTS efforts, derivations based on reported optimized SARS-CoV

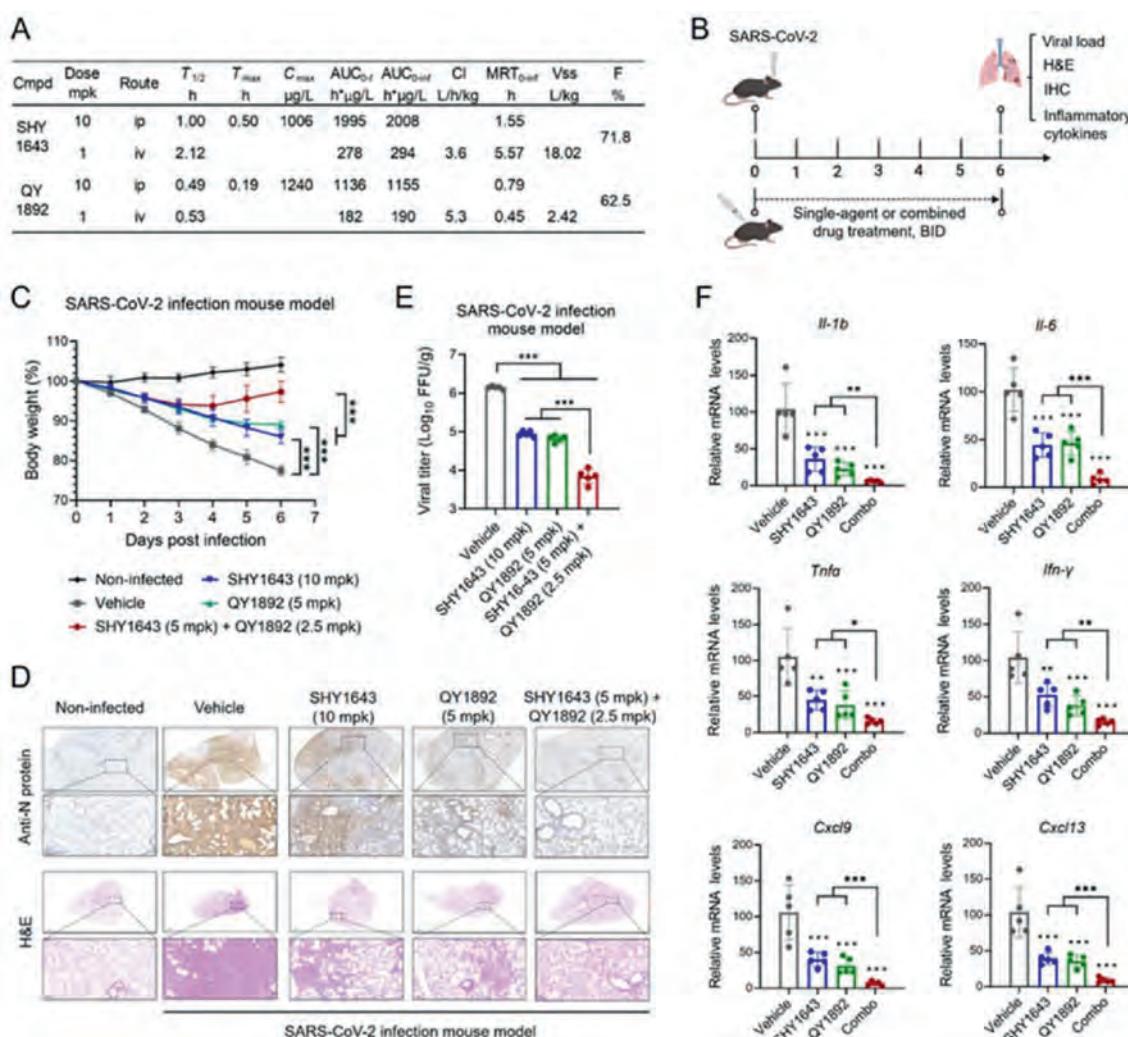


Figure 7 *In vivo* studies with SHY1643 and QY1892. (A) PK parameters of SHY1643 and QY1892 in mice. (B) Schematic diagram of the efficacy study model based on K18-hACE2 transgenic mice. (C) Quantification of the bodyweight for each group of mice infected by SARS-CoV-2 and treated with vehicle or indicated drugs ($n = 5$). (D) Representative immunohistochemistry (N protein) and hematoxylin and eosin (H&E) staining images from mouse lung histopathology 6 days after infection and treatments. (E, F) Quantification of the viral loads (E) and cytokine levels (F) from mouse lung samples 6 days after infection and treatments ($n = 5$). Data represent means $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, paired Student's *t*-test.

inhibitor scaffolds have proven to be a highly efficient strategy for developing potent and selective SARS-CoV-2 PLpro inhibitors^{12,17-19}. In the present study aimed at optimizing SARS-CoV-2 PLpro inhibitors, we employed a robust high-throughput derivation approach leveraging SuFEx-catalyzed diazotization and modular click chemistry libraries³⁰. This innovative methodology enabled the parallel and rapid generation of thousands of derivatives of Cmpd-19, facilitating *in situ* screening without the need for cumbersome purification and preparation steps, and ensuring the efficient identification of **2** and other promising hits. This approach could not only significantly accelerate the discovery of PLpro inhibitors, but also provide a scalable and versatile platform for lead optimization efforts targeting other pathogens.

We attempted to determine the crystal structure of PLpro in complex with compound **2** or its analogs but were unsuccessful. However, molecular docking studies predicted a unique molecular-glue-like binding mode for **2** and analogs. In this model, **2** simultaneously and asymmetrically interacts with both

chains of PLpro, providing a robust foundation for the rational optimization of **2** as a potent PLpro inhibitor. Through further structure-guided lead optimization, we successfully developed SHY1643, a potent, selective, and bioavailable SARS-CoV-2 PLpro inhibitor suitable for *in vivo* studies. While recently reported bioavailable PLpro inhibitors have predominantly been derived from the GRL0617 scaffold, SHY1643 represents an alternative and highly promising scaffold for the development of next-generation PLpro inhibitors, and offers new opportunities for exploring diverse therapeutic strategies against SARS-CoV-2 and other coronaviruses.

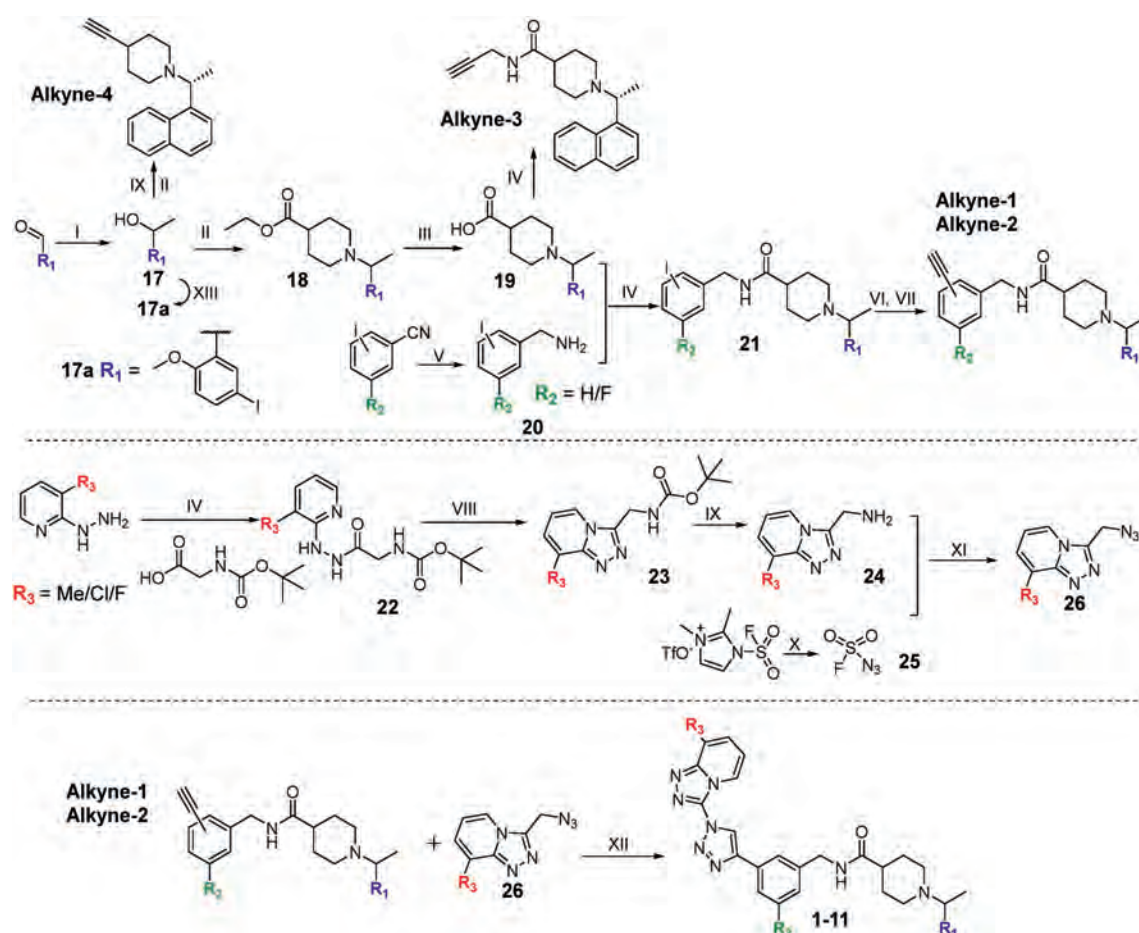
Despite sequence divergence among coronavirus PLpro subunits, SHY1643 demonstrated similar effectiveness against OC43 and 229E, suggesting structural similarities in their PLpro binding pockets. Consistently, a recently reported PLpro inhibitor exhibited pan-activity against NL63 PLpro, although it showed much weaker activity against 229E PLpro³⁸. We anticipate minimal resistance arising from the naturally occurring mutations in

SARS-CoV-2 PLpro, as these mutations are located outside the inhibitor binding pocket¹⁸. However, given that Mpro inhibitors are already facing challenges from resistant mutations due to their widespread use^{3,39}, it would be a matter of time before resistance emerges against PLpro inhibitors once they are deployed clinically. Therefore, there is an urgent need to develop therapeutic agents that target host mediators of viral replication while minimizing disruption to normal physiological processes.

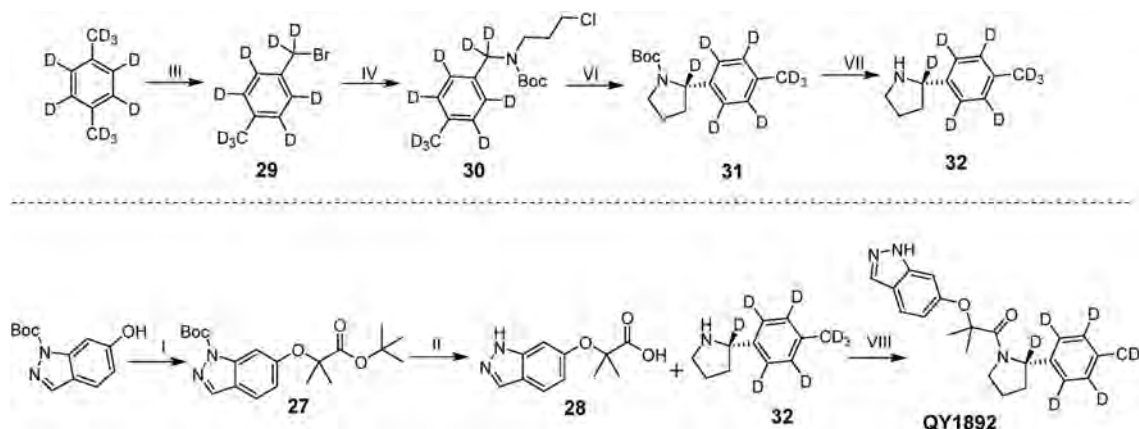
While RIPK1 KIs have demonstrated preliminary improvements as immunomodulators for treating severe COVID-19 in clinic trials^{24,25}, research studies have revealed their unexpected yet potent effects against SARS-CoV-2 viral replication^{28,29}. These findings highlight their significant therapeutic potential in combating evolving coronaviruses. However, existing RIPK1 KIs face limitations, such as lack of applicability for rodent infection models, poor brain-penetration, or off-target immunoactivating effects. In contrast, QY1892 stands out with its exclusive selectivity and extraordinary potency against both human and rodent RIPK1, allowing for comprehensive preclinical validation of RIPK1 in animal models. Additionally, its capability to penetrate

CNS positions it as a promising candidate for ameliorating neuroinflammation associated with COVID-19⁴⁰⁻⁴².

By targeting the pro-inflammatory and virus–host interaction mechanisms downstream of RIPK1, QY1892 simultaneously inhibited both SARS-CoV-2 replication and inflammatory cytokines secretion in the immunocompetent airway organoids. This effect was further synergistically enhanced when combined with SHY1643. Notably, QY1892 as a single agent demonstrated antiviral efficacy comparable to SHY1643 in a severe COVID-19 mouse model, underscoring the critical role of RIPK1-mediated immune microenvironments in SARS-CoV-2 propagation *in vivo*. More importantly, co-treatment with both inhibitors at lower doses resulted in significant synergy, robustly reducing viral loads and cytokine secretion to levels near the limit of detection. This combined approach ameliorated weight loss, lung damage, and CRS in infected mice. The current study employed a commonly used animal protocol, initiating treatment before the onset of severe COVID-19 symptoms^{17,18}. However, the exploration of combined treatments involving clinical antivirals with immunomodulators remains in its early stages. Building on our encouraging results, it



Scheme 1 Reaction and condition: I) Methylmagnesium bromide (3 mol/L in THF); N₂, 0 °C to room temperature; II) step1: Ms₂O, DIEA, DCM, 40 min, 0 °C; step2: Ethyl isonipicotate/4-ethynylpiperidine, DCM, 16 h, room temperature; III) 1 mol/L NaOH, MeOH, 16 h, room temperature; IV) HATU, DIEA, DCM, 16 h, room temperature; V) H₂, Ni-Raney, MeOH (7 mol/L NH₃), 16 h, room temperature; VI) Trimethylsilylacetylene, Et₃N, CuI, (PPh₃)₂PdCl₂, THF, 50 °C, 1 h; VII) MeOH (7 mol/L NH₃), room temperature; VIII) Lawesson's reagent, DME, N₂, 80 °C; IX) TFA/4 mol/L HCl in dioxane, DCM, room temperature; X) NaN₃, MTBE, MeCN, H₂O, room temperature, 10 min; XI) KHCO₃, DMF, room temperature, 1 h; XII) sodium ascorbate, CuSO₄·5H₂O, H₂O, *t*-BuOH, DCM, 60 °C, 3 h; XIII) CuI, NaI, *trans*-(1*R*,2*R*) *N,N'*-dimethylcyclohexane-1,2-diamine, 1,4-dioxane, N₂, 110 °C.



Scheme 2 Reagents and conditions: I) *tert*-butyl 2-bromo-2-methylpropanoate, K_2CO_3 , MeCN, reflux; II) TFA, CH_2Cl_2 ; III) Br_2 , CCl_4 , 40 °C, under N_2 ; IV) *tert*-butyl (3-chloropropyl)carbamate, NaH, THF, 60 °C; V) *S*-BuLi, (–)-sparteine, xylene, –78 °C under N_2 ; VI) TFA, CH_2Cl_2 ; VII) HATU, DIEA, CH_2Cl_2 .

is imperative to further investigate how such co-treatment strategies perform when initiated at more advanced stages of the disease.

3. Conclusions

In conclusion, the development and combined validation of SHY1643 and QY1892 provide proof-of-concept regimens for treating severe COVID-19. Synergistically targeting viral PLpro and host RIPK1 hold immense promise for improving patient outcomes and overcoming drug resistance in COVID-19. This dual-targeting strategy not only addresses viral replication but also mitigates the hyperinflammatory response, offering a comprehensive therapeutic approach against coronavirus diseases and potentially other viral infections.

4. Experimental

4.1. Chemistry

The synthesis of alkyne1–4 and compounds 1–11 is outlined in Scheme 1. Starting from diverse commercially available aldehyde precursors, the corresponding alcohols (**17**) were synthesized *via* Grignard reaction with methylmagnesium bromide, while some certain alcohol derivatives were directly obtained from commercial sources. Treatment of alcohol intermediates (**17**) with methanesulfonic anhydride (Ms_2O) provided the corresponding mesylate esters (leaving groups), which were then subjected to nucleophilic substitution with appropriate piperidine derivatives to yield alkyne-4 and compounds **18**. Hydrolysis of **18** to remove the ethyl group, followed by condensation with the corresponding amines, afforded alkyne-3 and intermediates **21**. Subsequent coupling of **21** with trimethylsilylacetylene and deprotection of trimethylsilyl group under basic conditions yielded alkyne-1 and alkyne-2. These alkynes were then subjected to a Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC, “click” reaction) with the corresponding azide derivatives (**26**) to furnish the final compounds 1–11. The synthesis of azide derivatives (**26**) commenced with commercially available 2-hydrazinopyridine analogs. Condensation of these analogs provided intermediate **22**, which was subsequently cyclized using Lawesson’s reagent. After deprotection, the resulting intermediate was treated with a custom-synthesized azidation reagent (**25**) to deliver the target azide compounds (**26**).

The synthesis of QY1892 is outlined in Scheme 2. Other final compounds were prepared using similar procedures. QY1892 was synthesized through the condensation of intermediates **32** and **28**. The preparation of **32** commenced with commercially available perdeuterated *p*-xylene, which was brominated to afford **29**. Subsequent nucleophilic substitution of **29** with *tert*-butyl (3-chloropropyl)carbamate, followed by cyclization under *sec*-butyllithium conditions and deprotection, yielded **32**. On the other hand, **28** was prepared from commercially available 6-hydroxy-1*H*-imidazole-1-carboxylic acid *tert*-butyl ester. This starting material underwent nucleophilic substitution with *tert*-butyl 2-bromo-2-methylpropanoate, followed by deprotection to provide **28**.

4.2. Cell lines and reagents

Cell lines were cultured as follows: hACE2-HeLa, Huh7, L929, and HepG2 cells were cultured in DMEM supplemented with 10% (*v/v*) fetal bovine serum (FBS); Jurkat and BEAS-2B cells were cultured in RPMI-1640 with 10% FBS; NHBE cells were cultured in HBE135-E6E7 cell complete medium (Pricella, CM-0732); All the cells were maintained at 37 °C and 5% CO_2 . All culture media and FBS were GIBCO™ and purchased from ThermoFisher. Common reagents were purchased from Sigma-Aldrich unless otherwise specified. The following antibodies were used in this study: TNF α (Novoprotein), mTNF α (Cell Sciences), RIPK1 (Cell Signaling Technology (CST), 3493), phospho-RIPK1 (CST, 53286), ACE2 (CST, 92485), β -actin (TransGen, I10813), and SARS-CoV-2 N protein (Sino Biological). Chemical probes were purchased from Selleck or prepared according to the literature.

4.3. Mouse airway organoid culture

Mouse airway tissues were cut into pieces of approximately 0.5 cm^3 in size and washed 2 to 3 times with PBS containing antibiotics, then resuspended with an appropriate amount of primary tissue solution digest (Mogeng Biotech, MB-0818L06S-100) for 30–60 min at 37 °C, 100 rpm (Allsheng, MSC-100, Hangzhou, China) in a thermostatic shaking incubator, before being added FBS (1%–5%) to terminate the digestion. The tissue suspension was then filtered through a 100 μm cell filter and centrifuged for 3 min (300 $\times g$, 4 °C), and the precipitate was mixed with extracellular matrix (Mogeng Biotech, 082755/

082701/082703) on ice (10,000 cells: 25 μ L extracellular matrix). The mixture was injected into the 96-well plates and incubated (37 °C, 5% CO₂) for 20 min until the extracellular matrix solidified, then slowly added complete medium (Mogeng Biotech, MA-0817H005S). The P0 organoids should be cultured for 4 days or so until obvious cell clusters can be observed.

4.4. Modular click chemistry library building and screening

The modular click chemistry libraries were prepared following previously reported procedures³⁰. From the azide library plates, 96 azide solutions (10 μ L from each well, containing 0.5 μ mol/L of an azide) in each microplate were transferred to the corresponding empty 96-well microplate. The terminal alkyne compound solution (50 mmol/L in DMSO, 10 μ L, 0.5 μ mol/L), Sodium ascorbate aqueous solution (250 mmol/L, 10 μ L, 2.5 μ mol/L), CuSO₄/THPTA aqueous solution (2.5 mmol/L, 10 μ L, 0.025 μ mol/L for both CuBr and THPTA) and DMSO (10 μ L) were added sequentially to each well of these newly loaded plates. These plates were sealed and swirled at 800 rpm (Eppendorf, Centrifuge 5810R, Hamburg, Germany) and 40 °C for 12 h to afford the corresponding triazole derivative in each well. After the reaction, DMSO (50 μ L) was added to dilute the triazole derivative solution in each well to the concentration of 5 mmol/L. The resulting triazole derivative library was used for the functional screening, which followed previously reported procedures except that Cmpd-19 was used as the positive PLpro inhibitor control¹². Compounds that showed at least 1.5-fold stronger potency than Cmpd-19 were cherry-picked for further analysis.

4.5. In vitro profiling assays

SARS-CoV-2 PLpro and NSP3^{179–1329} recombinant protein was expressed and purified following previously reported procedures^{12,43}. For the PLpro, NSP3, or DUB enzymatic activity assay, the final concentrations were 0.2 μ mol/L for PLpro and NSP3^{179–1329}, or optimized concentration for DUBs, and 0.5 μ mol/L for Ub-Rho, respectively.

The human RIPK1 kinase activities were tested using a commercially available assay kit from Promega. IC₅₀ values for all inhibitors were determined in 384-well format at room temperature. The kinase selectivity assays were done by DiscoverX (KINOMEScan™), and the scores were reported as a percent of DMSO control, with the lower score usually indicating a higher probability of being a hit^{34,35}. The assays for microsomal stability were performed according to the literature⁴⁴.

4.6. Molecular docking studies

The docking studies of Plpro inhibitors and RIPK1 inhibitors were performed based on the coordinates of SARS-CoV-2 Plpro co-crystallized with Cmpd-12 (PDB 7E35)²⁸ or the coordinates of RIPK1 co-crystallized with Nec-1s (PDB 4ITH)³⁶, using Schrödinger Glide software⁴⁵. The best ligand poses were chosen based on the docking score, and scores of -10 or lower usually represent very good binding. All the structural figures were prepared in the program PyMol (Schrödinger).

4.7. Cell viability assays

General cell survival was measured by the ATP luminescence assay CellTiterGlo (Promega). The percentage of viability was

normalized to readouts of vehicle-treated cells of each genotype. The HTS of RIPK1 inhibitors was performed using the small molecule libraries at 5 μ g/mL on FADD-deficient Jurkat cells treated with TNF α (20 ng/mL) in 384-well plates in two independent confirmation runs. Hits with z-scores above 12 were selected and subjected to 10-point twofold dilution series concentration-response assays starting from a maximum concentration of 5 μ mol/L.

4.8. Viral infection and evaluation of antiviral activities

SARS-CoV2 (Beta strain), HCoV-OC43 and HCoV-229E were propagated in Vero-E6, BHK21, and Huh7 cells, respectively. The viral titer was determined by a Focus-Forming assay (FFA). All the infection experiments related to SARS-CoV-2 were performed in a Biosafety Level-3 (BSL-3) laboratory. To evaluate the antiviral efficacy of these inhibitors, hACE2-HeLa, Huh7, or NHBE cells, or mouse airway organoids were cultured in 24-well plates (1×10^5 cells/well) overnight, and then infected with the indicated virus for 2 h. The supernatant was removed and the cells or organoids were washed 3 times with PBS, then cultured with fresh medium plus inhibitors at indicated doses. At 48 h post-infection, cells were collected with an SDS sample buffer for immunoblotting or RT-PCR analysis.

4.9. Immunoblotting

The whole-cell lysates from cells or organoids treated with inhibitors were collected with 1% SDS lysis buffer (1% SDS, 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.4)), and then boiled at 95 °C for 10 min. Total protein concentration was analyzed by BCA kit (ThermoFisher) and aligned with lysis buffer. Lysates were mixed with an equal volume of $2 \times$ loading buffer (100 mmol/L Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, bromophenol blue, and 5% β -mercaptoethanol) and boiled at 95 °C for 10 min, then separated in 10% polyacrylamide gel electrophoresis. Protein was transferred onto 0.2 μ mol/L nitrocellulose membrane and blotted with specific antibodies as indicated.

4.10. In vivo studies

For PK studies, inhibitors were dissolved in dimethylacetamide (10% v/v) and Solutol HS 15 (10% w/v) in normal saline, then injected into male ICR mice *via* tail vein (iv, 1 mpk) or intraperitoneally (ip, 10 mpk). Blood samples were collected at 5, 15, 30, 60, 120, 240, 360, 480, 720, and 1440 min (iv) and at 15, 30, 60, 120, 240, 360, 480, 720, and 1440 min (ip). The animal was restrained manually at the designated time points, and approximately 30–50 μ L of blood sample was collected *via* the retro-orbital plexus into tubes with EDTA2K. Plasma samples were separated by centrifugation of whole blood, then diluted in acetonitrile with internal standard. Brain samples are diluted in phosphate-buffered saline and acetonitrile (1:8) with internal standard, and then lysed on TissueLyser at 30 Hz for 10 min. The above samples were centrifuged at 4 °C with a speed of 14,000 rpm (Eppendorf, Centrifuge 5424R, Hamburg, Germany) for 10 min, then analyzed using a 1290 Infinity II (Agilent) coupled to a Triple Quad™ 3500 (SCIEX).

K18-hACE2 transgenic mice were used to study the efficacy of SARS-CoV-2 infection. The mice were anesthetized and administered with 100 FFU of the Beta SARS-CoV-2 strain through intranasal inoculation. The inhibitors were administered using the

same formulation as in the PK studies. Their body weights were monitored daily, and their lungs were collected for the assessment of viral load, mRNA abundance, immunohistochemistry, and H&E staining. The animal experimental procedures used in the study were approved by the Institutional Animal Care and Use Committee of Shenzhen Third People's Hospital (No. 2023-015).

4.11. General chemical experimental information

Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. Reactions were run in round bottom flasks or glass vials and stirred with Teflon-coated magnetic stir bars. Solvent evaporation was performed on rotary evaporator (Büchi) under reduced pressure. Reactions were monitored by thin layer chromatography or LC–MS (Agilent 6120). Acquity H-Class LC–MS with QDa mass spectrometer using water +0.05% formic acid (solvent A) and acetonitrile + 0.05% formic acid (solvent B). Preparative HPLC was performed on a Waters SunFire C18 column (19 mm × 100 mm, 5 µm) using a gradient of 10%–95% acetonitrile in water containing 0.05% trifluoroacetic acid (TFA), or on a Daicel CHIRALPAK®IC polysaccharide chiral column (4.6 mm × 250 mm, 5 µm) using 50% hexane in ethanol, over 10 or 20 min (15 or 25 min run time) at a flow rate of 15 or 0.5 mL/min, respectively. All final compounds were >95% pure by LC–MS analysis. ¹H/¹³C NMR spectra were recorded at ambient temperature on Bruker DMX 400 (400 MHz for ¹H NMR and 101 MHz for ¹³C NMR) instruments in the specified deuterated solvents. Observed proton absorptions are reported as δ units of parts per million (ppm) relative to tetramethylsilane (δ 0.0). Multiplicities are reported: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets) and m (multiplet). Coupling constants are reported as a *J* value in Hertz (Hz).

Alkyne1–4, compounds **1**–**11**, and SHY1643 were synthesized according to the procedures described below.

4.11.1. General procedure A

To a solution of benzaldehyde (9.3 mmol) in anhydrous THF (47 mL) was added 3 mol/L methylmagnesium bromide solution in ether (4.7 mL, 14 mmol) at 0 °C under nitrogen atmosphere, and the mixture was stirred at room temperature for 1.5 h. After the reaction was complete, it was quenched with a saturated ammonium chloride solution, extracted with ethyl acetate, washed with saturated sodium bicarbonate solution and sodium chloride solution, and dried with anhydrous sodium sulfate. Then it was concentrated under reduced pressure to furnish compound **17** in 99% yield.

4.11.2. General procedure B

17 (8 mmol) was dissolved in anhydrous DCM (30 mL) and the solution was cooled to 0 °C under N₂ atmosphere, before DIEA (40 mmol) and Ms₂O (12 mmol) in DCM (10 mL) were added dropwise. After stirred for 40 min at 0 °C, piperidine analog (24 mmol) was added, and the mixture was stirred overnight at room temperature. The mixture was extracted with ethyl acetate. The organic layer was evaporated *in vacuo* and the residue was purified by silica gel column chromatography with appropriate solvent to afford compound **18** in 28%–46% yield.

4.11.3. General procedure C

18 (0.54 mmol) was dissolved in methanol (8 mL), followed by the addition of 1 mol/L sodium hydroxide (2 mmol) in one

portion. The resulting mixture was stirred for 16 h at room temperature, then concentrated *in vacuo*, and dissolved in small amount of water. The solution was treated with AcOH dropwise until the solution was acidic. The residue was purified by reversed phase column chromatography to furnish compound **19** in 78%–98% yield.

4.11.4. General procedure D

A solution of **19** (0.18 mmol) and HATU (0.21 mmol) in dry DCM (1 mL) were added a solution of DIEA (0.88 mmol) and amine (0.21 mmol) in dry DCM at 0 °C under argon atmosphere. It was allowed to stir overnight at room temperature. The reaction mixture was quenched with water and extracted with DCM. The organic layers were dried over Na₂SO₄ and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to furnish compound **21**, **22** and alkyne-3 in 30%–93% yield.

4.11.5. General procedure E

To a solution of **19** (1.68 mmol) in saturated ammonia in MeOH, Raney-Ni (10%) was added, and the mixture was stirred under H₂ atmosphere at room temperature for 16 h. The reaction was filtered through acelite pad, and the filtrate was concentrated under reduced pressure. The residue was purified by silica gel column chromatography to furnish compound **20** in 95% yield.

4.11.6. General procedure F

To a solution of trimethylsilylacetylene (7.1 mmol), CuI (0.094 mmol), Et₃N (14 mmol) in THF (12 mL) add **21** (4.7 mmol) and bis(triphenylphosphine)palladium(II) chloride (0.094 mmol) under N₂ atmosphere. The reaction was stirred at 50 °C for 1 h. Then the mixture was purified by flash silica gel column chromatography with appropriate solvent to afford compound trimethylsilylacetylene coupling products in 71%–96% yield.

4.11.7. General procedure G

The trimethylsilylacetylene coupling products (6.4 mmol) were dissolved in a 7 mol/L ammonia methanol solution (24 mL). It was stirred overnight at room temperature, and then purified by flash silica gel column chromatography with appropriate solvent to afford compound alkyne-1 and alkyne-2 in 87%–95% yield.

4.11.8. General procedure H

To a solution of **22** (0.178 mmol) in anhydride DME (0.6 mL) add Lawesson's reagent (0.089 mmol) at N₂ atmosphere. The reaction was stirred at 90 °C for 5 h. The residue was purified by flash silica gel column chromatography with appropriate solvent to furnish compound **23** in 50% yield.

4.11.9. General procedure I

To a solution of **23** or *tert*-butyl 4-ethynylpiperidine-1-carboxylate (24 mmol) in DCM (64 mL) add TFA (6.4 mL) or 4 mol/L HCl dioxane (120 mmol). The reaction was stirred overnight at room temperature, and then purified by flash silica gel column chromatography with appropriate solvent to afford compound **24** and 4-ethynylpiperidine in 99% yield.

4.11.10. General procedure J

To a solution of aqueous NaN₃ solution (0.50 mol/L, 40 mL, containing 1.3 g, 20 mmol NaN₃) add MTBE (40 mL). Trifluoromethanesulfonate 1-(fluorosulfonyl)-2,3-dimethyl-1H-

imidazole-3-ium (7.9 g, 24 mmol) was dissolved in MeCN (2 mL), and the solution was added rapidly to the stirred Na₃/H₂O/MTBE mixture in ice-water bath. This was followed by a rinse of the vial used for preparing the solution with additional MeCN (2 mL), which was also added to the reaction mixture. The reaction mixture was stirred vigorously in ice-water bath for 10 min, then the mixture was poured into a glass separating funnel. The mixture was rested in the funnel at room temperature for 30 min for phase separation. The organic phase was separated from the aqueous phase, and rested for at least 12 h. The orange-red residual aqueous phase developed during the 12-h resting period, and was removed to afford the colorless organic phase (**25**) in MTBE without further purification.

4.11.11. General procedure K

To a solution of **24** (1 mmol) in DMF (2.5 mL) was added **25** (2.5 mL, 1 mmol) and 3 mol/L K₂HCO₂ aqueous (1.33 mL, 4 mmol). The reaction was stirred at room temperature for 1 h. The mixture was extracted with ethyl acetate. The organic layer was evaporated *in vacuo* and the residue was purified by silica gel column chromatography with appropriate solvent to afford compound **26** in 90%–99% yield.

4.11.12. General procedure L

To a solution of alkyne-1 (0.1 mmol) in water/*tert*-butanol/DCM (3/5.4/2.8 mL) mixture add **26** (0.1 mmol) and VcNa (0.22 mmol). The mixture was stirred at 60 °C for 3 h in microwave. The residue was purified by flash silica gel column chromatography with appropriate solvent to furnish compound **1–11** and SHY1643.

4.11.13. General procedure M

To a solution of **17** (0.54 mmol) in dioxane (1 mL) was added CuI (5 mg, 0.03 mmol), NaI (162 mg, 1.08 mmol) and (1*R*,2*R*)-*N,N'*-dimethyl-1,2-cyclohexanediamine (8 mL, 0.054 mmol) under N₂ atmosphere. The reaction was stirred overnight at 110 °C. The residue was purified by flash silica gel column chromatography with appropriate solvent to furnish compound **17a** in 99% yield.

4.11.14. (R)-N-(3-Ethynylbenzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (alkyne-1)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.47 (t, *J* = 5.9 Hz, 1H), 8.39 (d, *J* = 8.5 Hz, 1H), 8.05 (d, *J* = 8.2 Hz, 2H), 7.89 (d, *J* = 7.3 Hz, 1H), 7.65 (m, 3H), 7.32 (d, *J* = 8.8 Hz, 3H), 7.25 (d, *J* = 6.7 Hz, 1H), 5.47–5.37 (m, 1H), 4.25 (d, *J* = 5.9 Hz, 2H), 4.17 (s, 1H), 3.94 (m, 1H), 3.10 (m, 2H), 2.89 (m, 1H), 2.42 (m, 1H), 2.04 (m, 1H), 1.98–1.89 (m, 1H), 1.82 (m, 1H), 1.75 (d, *J* = 6.5 Hz, 4H). MS-ESI: *m/z* calculated for C₂₇H₂₈N₂O. Exact Mass: 396.22, found 397.2 [M+H]⁺.

4.11.15. (R)-N-(4-Ethynylbenzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (alkyne-2)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.46 (d, *J* = 8.0 Hz, 1H), 8.29 (t, *J* = 6.0 Hz, 1H), 7.91 (d, *J* = 7.2 Hz, 1H), 7.80 (d, *J* = 8.1 Hz, 1H), 7.58–7.36 (m, 6H), 7.20 (d, *J* = 7.9 Hz, 2H), 4.24 (d, *J* = 5.9 Hz, 2H), 4.14 (d, *J* = 10.1 Hz, 2H), 3.07 (d, *J* = 10.9 Hz, 1H), 2.79 (d, *J* = 11.1 Hz, 1H), 2.14 (t, *J* = 11.6 Hz, 1H), 2.05–1.96 (m, 2H), 1.71 (m, 1H), 1.56 (m, 3H), 1.40 (d, *J* = 6.6 Hz, 3H). MS-ESI: *m/z* calculated for C₂₇H₂₈N₂O. Exact Mass: 396.22, found 397.2 [M+H]⁺.

4.11.16. (R)-1-(1-(Naphthalen-1-yl)ethyl)-N-(prop-2-yn-1-yl)piperidine-4-carboxamide (alkyne-3)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.39 (d, *J* = 7.6 Hz, 2H), 8.05 (d, *J* = 8.3 Hz, 2H), 7.89 (d, *J* = 7.3 Hz, 1H), 7.65 (m, 3H), 5.41 (s, 1H), 3.92 (m, 1H), 3.84 (d, *J* = 5.6 Hz, 2H), 3.07 (m, 3H), 2.86 (m, 1H), 2.36 (s, 1H), 1.93 (m, 3H), 1.74 (d, *J* = 6.4 Hz, 3H), 1.67 (s, 1H). MS-ESI: *m/z* calculated for C₂₁H₂₄N₂O. Exact Mass: 320.19, found 321.1 [M+H]⁺.

4.11.17. (R)-4-Ethynyl-1-(1-(naphthalen-1-yl)ethyl)piperidine (alkyne-4)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.47–8.40 (m, 1H), 7.94–7.87 (m, 1H), 7.79 (d, *J* = 8.1 Hz, 1H), 7.56–7.45 (m, 4H), 4.16 (q, *J* = 6.7 Hz, 1H), 2.87 (d, *J* = 2.3 Hz, 1H), 2.81 (s, 1H), 2.62 (m, 1H), 2.34 (s, 1H), 2.22 (s, 1H), 2.13 (m, 1H), 1.80–1.74 (m, 1H), 1.69 (m, 1H), 1.50–1.42 (m, 2H), 1.39 (d, *J* = 6.8 Hz, 3H). MS-ESI: *m/z* calculated for C₁₉H₂₁N. Exact Mass: 263.17, found 264.1 [M+H]⁺.

4.11.18. (S)-3-(3,4-Dimethoxyphenyl)-3-(4-(3-((1-(R)-1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamido)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl)propanoic acid (**1**)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.73 (d, *J* = 8.5 Hz, 1H), 8.50 (t, *J* = 5.9 Hz, 1H), 8.40 (d, *J* = 8.5 Hz, 1H), 8.09–8.01 (m, 2H), 7.90 (d, *J* = 7.3 Hz, 1H), 7.72 (s, 1H), 7.66 (m, 4H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.17 (d, *J* = 7.6 Hz, 1H), 7.10 (s, 1H), 6.91 (d, *J* = 2.3 Hz, 2H), 6.05 (d, *J* = 9.8 Hz, 1H), 5.42 (s, 1H), 4.29 (t, *J* = 4.6 Hz, 2H), 3.94 (m, 1H), 3.74 (s, 3H), 3.72 (s, 3H), 3.59 (m, 1H), 3.28 (m, 1H), 3.10 (m, 2H), 2.87 (m, 1H), 2.42 (m, 2H), 2.00 (m, 2H), 1.82 (m, 1H), 1.75 (d, *J* = 6.9 Hz, 3H), 1.71 (s, 1H). MS-ESI: *m/z* calculated for C₃₈H₄₁N₅O₅. Exact Mass: 647.31, found 648.2 [M+H]⁺.

4.11.19. (R)-N-(3-(1-(8-Methyl-[1,2,4]triazolo[4,3-*a*]pyridin-3-yl)methyl)-1*H*-1,2,3-triazol-4-yl)benzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (**2**)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.69 (s, 1H), 8.50 (t, *J* = 5.9 Hz, 1H), 8.45 (d, *J* = 6.9 Hz, 1H), 8.40 (d, *J* = 8.5 Hz, 1H), 8.08–8.02 (m, 2H), 7.89 (d, *J* = 7.4 Hz, 1H), 7.74 (s, 1H), 7.70–7.65 (m, 3H), 7.62 (t, *J* = 7.3 Hz, 1H), 7.37 (t, *J* = 7.7 Hz, 1H), 7.26 (d, *J* = 6.9 Hz, 1H), 7.18 (d, *J* = 7.6 Hz, 1H), 7.03 (t, *J* = 6.9 Hz, 1H), 6.32 (d, *J* = 2.5 Hz, 2H), 5.42 (s, 1H), 4.29 (d, *J* = 5.7 Hz, 2H), 3.94 (m, 1H), 3.10 (m, 2H), 2.89 (m, 1H), 2.55 (s, 3H), 2.42 (m, 1H), 2.04 (m, 1H), 1.95 (m, 1H), 1.82 (m, 1H), 1.74 (d, *J* = 6.6 Hz, 3H), 1.71 (d, *J* = 5.8 Hz, 1H). MS-ESI: *m/z* calculated for C₃₅H₃₆N₈O. Exact Mass: 584.30, found 585.2 [M+H]⁺, 293.1 [(M+2H)/2]⁺.

4.11.20. Methyl (R)-2-((4-(3-((1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamido)methyl)phenyl)-1*H*-1,2,3-triazol-1-yl)methyl)thiazole-4-carboxylate (**3**)

The title compound was obtained as described in the general procedure (white solid). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.70 (d, *J* = 4.8 Hz, 1H), 8.58 (d, *J* = 3.7 Hz, 1H), 8.53 (q, *J* = 5.6 Hz, 1H), 8.40 (d, *J* = 8.6 Hz, 1H), 8.09–8.01 (m, 2H), 7.89 (d, *J* = 7.2 Hz, 1H), 7.77 (s, 1H), 7.73–7.69 (m, 1H), 7.69–7.58 (m, 3H), 7.39 (t, *J* = 7.7 Hz, 1H), 7.20 (d, *J* = 7.6 Hz, 1H), 6.10 (s, 2H), 5.43 (d, *J* = 8.8 Hz, 1H), 4.35–4.28 (m, 2H), 3.95 (m, 1H),

3.82 (s, 3H), 3.11 (m, 2H), 2.88 (m, 1H), 2.43 (m, 1H), 2.08–1.94 (m, 2H), 1.83 (m, 1H), 1.75 (d, $J = 7.0$ Hz, 3H), 1.72 (s, 1H). MS-ESI: m/z calculated for $C_{33}H_{34}N_6O_3S$. Exact Mass: 594.24, found 595.1 $[M+H]^+$.

4.11.21. *(R)-3-(3,4-Dimethoxyphenyl)-3-(4-(3-((1-((R)-1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamido)methyl)phenyl)-1H-1,2,3-triazol-1-yl)propanoic acid (4)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.73 (d, $J = 8.7$ Hz, 1H), 8.50 (t, $J = 5.9$ Hz, 1H), 8.40 (d, $J = 8.5$ Hz, 1H), 8.05 (d, $J = 8.2$ Hz, 2H), 7.89 (d, $J = 7.2$ Hz, 1H), 7.72 (s, 1H), 7.71–7.60 (m, 4H), 7.37 (t, $J = 7.7$ Hz, 1H), 7.17 (d, $J = 7.6$ Hz, 1H), 7.10 (d, $J = 1.8$ Hz, 1H), 6.91 (d, $J = 2.6$ Hz, 2H), 6.05 (d, $J = 9.8$ Hz, 1H), 5.42 (s, 1H), 4.33–4.24 (m, 2H), 3.98–3.90 (m, 1H), 3.74 (s, 3H), 3.72 (s, 3H), 3.59 (d, $J = 7.0$ Hz, 1H), 3.28 (m, 1H), 3.10 (m, 2H), 2.88 (m, 1H), 2.42 (m, 1H), 2.05 (m, 1H), 1.95 (m, 1H), 1.82 (m, 1H), 1.74 (d, $J = 6.8$ Hz, 3H), 1.71 (s, 1H), 1.24 (s, 1H). MS-ESI: m/z calculated for $C_{38}H_{41}N_5O_5$. Exact Mass: 647.31, found 648.2 $[M+H]^+$.

4.11.22. *(R)-N-(3-(1-(3,4-Dimethoxybenzyl)-1H-1,2,3-triazol-4-yl)benzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (5)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.55 (s, 1H), 8.51 (q, $J = 6.0, 5.6$ Hz, 1H), 8.40 (d, $J = 8.7$ Hz, 1H), 8.05 (d, $J = 8.6$ Hz, 2H), 7.89 (d, $J = 7.3$ Hz, 1H), 7.75 (s, 1H), 7.66 (m, 4H), 7.36 (t, $J = 7.7$ Hz, 1H), 7.17 (d, $J = 7.6$ Hz, 1H), 7.04 (d, $J = 2.0$ Hz, 1H), 6.95 (d, $J = 8.2$ Hz, 1H), 6.90 (d, $J = 8.2$ Hz, 1H), 5.53 (s, 2H), 5.42 (t, $J = 6.8$ Hz, 1H), 4.32–4.27 (m, 2H), 3.94 (m, 2H), 3.74 (s, 3H), 3.73 (s, 3H), 3.10 (m, 2H), 2.88 (m, 1H), 2.46–2.39 (m, 1H), 2.04 (m, 1H), 1.98–1.90 (m, 1H), 1.82 (m, 1H), 1.75 (d, $J = 6.9$ Hz, 3H). MS-ESI: m/z calculated for $C_{36}H_{39}N_5O_3$. Exact Mass: 589.31, found 590.2 $[M+H]^+$.

4.11.23. *N-(3-(1-(1-(3,4-Dimethoxyphenyl)ethyl)-1H-1,2,3-triazol-4-yl)benzyl)-1-((R)-1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (6)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.66 (s, 1H), 8.51 (q, $J = 5.7$ Hz, 1H), 8.40 (d, $J = 8.6$ Hz, 1H), 8.08–8.02 (m, 2H), 7.89 (d, $J = 7.2$ Hz, 1H), 7.75 (s, 1H), 7.70–7.59 (m, 4H), 7.36 (t, $J = 7.7$ Hz, 1H), 7.16 (d, $J = 7.6$ Hz, 1H), 7.03 (d, $J = 2.1$ Hz, 1H), 6.95–6.86 (m, 2H), 5.89 (q, $J = 7.1$ Hz, 1H), 5.43 (d, $J = 7.3$ Hz, 1H), 4.33–4.26 (m, 2H), 3.94 (m, 2H), 3.74 (s, 3H), 3.73 (s, 3H), 3.10 (m, 2H), 2.93–2.84 (m, 1H), 2.42 (m, 1H), 2.02 (m, 2H), 1.91 (d, $J = 7.1$ Hz, 3H), 1.82 (m, 1H), 1.75 (d, $J = 6.8$ Hz, 3H). MS-ESI: m/z calculated for $C_{37}H_{41}N_5O_3$. Exact Mass: 603.32, found 604.30 $[M+H]^+$.

4.11.24. *1-(1-(5-Bromo-2-methoxyphenyl)ethyl)-N-(3-(1-((8-methyl-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)piperidine-4-carboxamide (7)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.71 (d, $J = 5.5$ Hz, 1H), 8.54–8.40 (m, 2H), 7.75 (t, $J = 1.9$ Hz, 1H), 7.70–7.65 (m, 2H), 7.62 (d, $J = 8.9$ Hz, 1H), 7.37 (t, $J = 7.8$ Hz, 1H), 7.26 (d, $J = 6.7$ Hz, 1H), 7.18 (d, $J = 7.7$ Hz, 1H), 7.12 (d, $J = 8.9$ Hz, 1H), 7.03 (t, $J = 6.8$ Hz, 1H), 6.33 (s, 2H), 4.71

(t, $J = 6.7$ Hz, 1H), 4.30 (d, $J = 5.7$ Hz, 2H), 3.85 (s, 2H), 3.74 (m, 2H), 3.21 (m, 1H), 2.92 (m, 1H), 2.73 (m, 1H), 2.55 (d, $J = 3.1$ Hz, 3H), 2.42 (m, 1H), 2.00–1.85 (m, 3H), 1.82–1.73 (m, 1H), 1.59 (d, $J = 7.0$ Hz, 3H). MS-ESI: m/z calculated for $C_{32}H_{35}BrN_8O_2$. Exact Mass: 642.21, found 643.17 $[M+H]^+$, 322.13 $[(M+2H)/2]^+$.

4.11.25. *1-(1-(5-Bromo-2-fluorophenyl)ethyl)-N-(3-(1-((8-methyl-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)piperidine-4-carboxamide (8)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.70 (d, $J = 3.4$ Hz, 1H), 8.48 (m, 2H), 7.87 (d, $J = 6.4$ Hz, 1H), 7.77–7.72 (m, 2H), 7.68 (d, $J = 7.7$ Hz, 1H), 7.37 (t, $J = 9.1$ Hz, 2H), 7.26 (d, $J = 6.7$ Hz, 1H), 7.18 (d, $J = 7.6$ Hz, 1H), 7.03 (t, $J = 6.8$ Hz, 1H), 6.33 (s, 2H), 4.73 (d, $J = 7.1$ Hz, 1H), 4.30 (d, $J = 5.8$ Hz, 2H), 3.71 (m, 1H), 3.32 (m, 1H), 2.96 (m, 1H), 2.80 (m, 1H), 2.55 (s, 3H), 2.43 (m, 1H), 1.96 (m, 3H), 1.80 (m, 1H), 1.66 (d, $J = 7.0$ Hz, 3H). MS-ESI: m/z calculated for $C_{31}H_{32}BrFN_8O$. Exact Mass: 630.19, found 631.12 $[M+H]^+$, 316.42 $[(M+2H)/2]^+$.

4.11.26. *1-(1-(5-Ethynyl-2-methoxyphenyl)ethyl)-N-(3-(1-((8-methyl-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)piperidine-4-carboxamide (9)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.70 (s, 1H), 8.53–8.31 (m, 2H), 7.74 (s, 1H), 7.68 (d, $J = 7.7$ Hz, 1H), 7.63 (d, $J = 2.1$ Hz, 1H), 7.56 (d, $J = 8.6$ Hz, 1H), 7.37 (t, $J = 7.7$ Hz, 1H), 7.26 (d, $J = 6.7$ Hz, 1H), 7.17 (m, 2H), 7.02 (t, $J = 6.9$ Hz, 1H), 6.33 (s, 2H), 4.72 (t, $J = 6.8$ Hz, 1H), 4.30 (d, $J = 5.8$ Hz, 2H), 4.15 (d, $J = 6.0$ Hz, 4H), 3.80 (s, 1H), 3.73 (m, 1H), 3.21 (m, 1H), 2.92 (m, 1H), 2.74 (m, 1H), 2.55 (s, 3H), 2.03–1.85 (m, 3H), 1.78 (m, 1H), 1.60 (d, $J = 7.1$ Hz, 3H). MS-ESI: m/z calculated for $C_{34}H_{36}N_8O_2$. Exact Mass: 588.30, found 589.26 $[M+H]^+$, 295.10 $[(M+2H)/2]^+$.

4.11.27. *(R)-N-(3-Fluoro-5-(1-((8-methyl-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (10)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.76 (d, $J = 5.5$ Hz, 1H), 8.55 (t, $J = 5.9$ Hz, 1H), 8.42 (m, 2H), 8.01 (m, 3H), 7.71–7.57 (m, 4H), 7.51 (d, $J = 9.3$ Hz, 1H), 7.25 (d, $J = 6.9$ Hz, 1H), 7.01 (q, $J = 9.5, 8.2$ Hz, 2H), 6.34 (s, 2H), 5.41 (s, 1H), 4.34–4.27 (m, 2H), 3.95 (m, 2H), 3.13 (m, 1H), 3.06 (m, 1H), 2.91–2.82 (m, 1H), 2.55 (s, 3H), 2.43 (s, 1H), 2.04 (s, 2H), 1.80 (s, 1H), 1.76 (d, $J = 6.7$ Hz, 3H). MS-ESI: m/z calculated for $C_{35}H_{35}FN_8O$. Exact Mass: 602.29, found 603.1 $[M+H]^+$, 302.1 $[(M+2H)/2]^+$.

4.11.28. *(R)-N-(3-(1-((8-Chloro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (11)*

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.69 (s, 1H), 8.62 (d, $J = 6.9$ Hz, 1H), 8.52 (q, $J = 6.0, 5.6$ Hz, 1H), 8.40 (d, $J = 8.5$ Hz, 1H), 8.05 (d, $J = 8.6$ Hz, 2H), 7.89 (d, $J = 7.3$ Hz, 1H), 7.74 (s, 1H), 7.70–7.64 (m, 4H), 7.61 (d, $J = 7.3$ Hz, 1H), 7.37 (t, $J = 7.7$ Hz, 1H), 7.18 (d, $J = 7.6$ Hz, 1H), 7.11 (q, $J = 7.2$ Hz, 1H), 6.35 (s, 2H), 5.42 (s, 1H), 4.29 (d, $J = 5.6$ Hz, 2H), 3.95 (m, 1H), 3.10 (m, 2H), 2.92–2.84 (m, 1H),

2.42 (m, 1H), 2.04 (m, 1H), 1.98–1.90 (m, 1H), 1.80 (s, 1H), 1.74 (d, $J = 6.6$ Hz, 4H). MS-ESI: m/z calculated for $C_{34}H_{33}ClN_8O$. Exact Mass: 604.25, found 605.19 $[M+H]^+$.

4.11.29. Ethyl (R)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxylate (18a)

The title compound was obtained as described in the general procedure B. 1H NMR (400 MHz, DMSO- d_6) δ 8.46–8.40 (m, 1H), 7.93–7.88 (m, 1H), 7.79 (d, $J = 8.1, 1.3$ Hz, 1H), 7.54–7.44 (m, 4H), 4.15 (q, $J = 6.7$ Hz, 1H), 4.06–4.01 (m, 2H), 2.96 (m, 1H), 2.76–2.68 (m, 1H), 2.25 (m, 1H), 2.08 (m, 2H), 1.84–1.77 (m, 1H), 1.74–1.67 (m, 1H), 1.55–1.45 (m, 2H), 1.39 (d, $J = 6.7$ Hz, 3H), 1.15 (t, $J = 7.1$ Hz, 3H). MS-ESI: m/z calculated for $C_{20}H_{25}NO_2$. Exact Mass: 311.19, found 312.1 $[M+H]^+$.

4.11.30. (R)-1-(1-(Naphthalen-1-yl)ethyl)piperidine-4-carboxylic acid (19a)

The title compound was obtained as described in the general procedure C. 1H NMR (400 MHz, DMSO- d_6) δ 8.38 (d, $J = 8.5$ Hz, 1H), 8.07–8.03 (m, 1H), 7.88 (m, 1H), 7.73–7.57 (m, 4H), 5.46–5.38 (m, 1H), 3.90 (m, 2H), 3.10 (s, 1H), 2.90 (m, 10.7 Hz, 1H), 2.44 (s, 1H), 2.13 (m, 1H), 1.94 (m, 1H), 1.83 (m, 1H), 1.75 (d, $J = 6.7$ Hz, 3H), 1.64 (m, 1H). MS-ESI: m/z calculated for $C_{18}H_{21}NO_2$. Exact Mass: 283.16, found 284.1 $[M+H]^+$.

4.11.31. (R)-N-(3-Iodobenzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (21a)

The title compound was obtained as described in the general procedure D. 1H NMR (400 MHz, Chloroform- d) δ 8.46–8.40 (m, 1H), 7.86–7.81 (m, 1H), 7.73 (d, $J = 8.1$ Hz, 1H), 7.59–7.54 (m, 3H), 7.46 (dt, $J = 6.1, 2.5$ Hz, 2H), 7.42 (d, $J = 7.7$ Hz, 1H), 7.19 (d, $J = 7.6$ Hz, 1H), 7.02 (t, $J = 8.0$ Hz, 1H), 6.02 (t, $J = 5.8$ Hz, 1H), 4.34 (d, $J = 5.8$ Hz, 2H), 4.09 (q, $J = 6.7$ Hz, 1H), 3.25–3.17 (m, 1H), 2.90–2.83 (m, 1H), 2.05 (dddd, $J = 31.2, 17.1, 8.4, 4.7$ Hz, 4H), 1.79–1.66 (m, 3H), 1.45 (d, $J = 6.7$ Hz, 3H). MS-ESI: m/z calculated for $C_{25}H_{27}IN_2O$. Exact Mass: 498.12, found 499.0 $[M+H]^+$.

4.11.32. tert-Butyl (2-(2-(3-fluoropyridin-2-yl)hydrazineyl)-2-oxoethyl)carbamate (22a)

The title compound was obtained as described in the general procedure D. 1H NMR (400 MHz, DMSO- d_6) δ 9.69 (s, 1H), 8.58 (s, 1H), 7.86 (d, $J = 4.8$ Hz, 1H), 7.45 (dd, $J = 11.8, 7.9$ Hz, 1H), 6.99 (t, $J = 6.3$ Hz, 1H), 6.74 (dt, $J = 8.1, 4.1$ Hz, 1H), 2.70 (d, $J = 1.1$ Hz, 2H), 1.39 (s, 9H). MS-ESI: m/z calculated for $C_{12}H_{17}FN_4O_3$. Exact Mass: 284.13, found 284.94 $[M+H]^+$.

4.11.33. tert-Butyl ((8-fluoro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)carbamate (23a)

The title compound was obtained as described in the general procedure H. 1H NMR (400 MHz, DMSO- d_6) δ 8.35 (d, $J = 6.9$ Hz, 1H), 7.69 (d, $J = 6.8$ Hz, 1H), 7.59 (dt, $J = 13.0, 7.2$ Hz, 1H), 7.31 (m, 1H), 4.70 (d, $J = 5.9$ Hz, 2H), 1.38 (s, 9H). MS-ESI: m/z calculated for $C_{12}H_{15}FN_4O_2$. Exact Mass: 266.12, found 267.03 $[M+H]^+$.

4.11.34. (8-Fluoro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methanamine (24a)

The title compound was obtained as described in the general procedure I. 1H NMR (400 MHz, DMSO- d_6) δ 8.88 (s, 2H), 8.58 (d, $J = 6.8$ Hz, 1H), 7.62 (m, 1H), 7.41 (m, 1H), 4.68 (q,

$J = 5.7$ Hz, 2H). MS-ESI: m/z calculated for $C_7H_7FN_4$. Exact Mass: 166.07, found 166.89 $[M+H]^+$.

4.11.35. 3-(Azidomethyl)-8-fluoro-[1,2,4]triazolo[4,3-a]pyridine (26a)

The title compound was obtained as described in the general procedure K and directly used for next reaction without purification. MS-ESI: m/z calculated for $C_7H_5FN_6$. Exact Mass: 192.06, found 192.94 $[M+H]^+$.

4.11.36. (R)-N-(3-(1-((8-Fluoro-[1,2,4]triazolo[4,3-a]pyridin-3-yl)methyl)-1H-1,2,3-triazol-4-yl)benzyl)-1-(1-(naphthalen-1-yl)ethyl)piperidine-4-carboxamide (SHY1643)

The title compound was obtained as described in the general procedure L (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 8.70 (s, 1H), 8.51 (m, 2H), 8.40 (d, $J = 8.5$ Hz, 1H), 8.05 (d, $J = 8.4$ Hz, 2H), 7.89 (d, $J = 7.3$ Hz, 1H), 7.74 (s, 1H), 7.69–7.60 (m, 4H), 7.42–7.35 (m, 2H), 7.18 (d, $J = 7.7$ Hz, 1H), 7.11 (q, $J = 7.0$ Hz, 1H), 6.36 (s, 2H), 5.45–5.39 (m, 1H), 4.29 (d, $J = 4.6$ Hz, 2H), 3.95 (m, 1H), 3.10 (m, 2H), 2.87 (m, 1H), 2.42 (m, 1H), 2.04 (m, 1H), 1.95 (m, 1H), 1.80 (s, 1H), 1.74 (d, $J = 6.5$ Hz, 3H), 1.72 (d, $J = 5.3$ Hz, 1H). MS-ESI: m/z calculated for $C_{34}H_{33}FN_8O$. Exact Mass: 588.28, found 589.25 $[M+H]^+$, 295.09 $[(M+2H)/2]^+$.

QY1892 and analogs were synthesized according to the procedures described below.

4.11.37. General procedure N

To a solution of *tert*-butyl 6-hydroxy-1H-indazole-1-carboxylate (1.07 mmol) in MeCN (6 mL) was added K_2CO_3 (2.68 mmol). Then *tert*-butyl 2-bromo-2-methylpropanoate (1.6 mmol) was added dropwise to the reaction mixture at room temperature. The reaction was heated to reflux and stirred overnight. The mixture was filtered and evaporated *in vacuo* to remove organic solvent. The obtained residue was purified by silica gel column chromatography with appropriate solvent to afford compound **27** in 45% yield as light-yellow oil.

4.11.38. General procedure O

27 (0.48 mmol) was dissolved in DCM (2.5 mL), TFA (0.5 mL) was added to the solution and stirred at room temperature until reaction completed. The organic solvent was evaporated *in vacuo* to obtain compound **28** as yellow solid without further purification.

4.11.39. General procedure P

To a mixture of *p*-xylene- d_{10} (12.9 mmol) in CCl_4 (22 mL) were added bromine (12.9 mmol) dropwisely. Then the reaction was heated to 40 °C and stirred for another 2 h. The reaction was quenched by DCM (30 mL) and acetone (30 mL). The organic solvent was evaporated *in vacuo*, the residue was purified by silica gel column chromatography with appropriate solvent to afford compound **29** in 66% yield as light-yellow liquid.

4.11.40. General procedure Q

Sodium hydride (4.0 mmol) was added slowly to the solution of *tert*-butyl (3-chloropropyl)carbamate (4.0 mmol) in dry THF (12 mL) at 0 °C. The mixture kept stirring at 0 °C for 0.2 h before **29** was added. The reaction was warmed to room temperature slowly before heating to 60 °C then stirred at 60 °C overnight. The reaction was quenched by ice water and extracted with ethyl acetate. The organic layer was combined and washed with saturated

brine, dried over anhydrous sodium sulfate. Then the filtrate was removed under reduced pressure. The obtained residue was purified by silica gel column chromatography (EA:PE = 0%–20%) to afford compound **30** in 28% yield as light-yellow oil. MS-ESI: m/z calculated for $C_{16}H_{16}D_9ClNO_2$. Exact Mass: 306.21, found 251.1 $[M + H-56 (tert-butyl)]^+$.

4.11.41. General procedure R

To a solution of **30** dissolved in dry xylene (7 mL) were added (–)-sparteine (1.1 mmol) under N_2 atmosphere. After the mixture cooled to $-78^\circ C$, $s-BuLi$ (1.1 mmol) was added dropwise to the solution. The reaction kept stirring at $-78^\circ C$ for 5 h before quenched by ice water. Then extracted with ethyl acetate, the organic layer was combined and washed with saturated brine, dried over anhydrous sodium sulfate. Then the organic solvent was evaporated *in vacuo*. The obtained residue was purified by silica gel column chromatography (EA:PE = 0%–20%) to afford compound **31** in 22% yield as light-yellow oil. MS-ESI: m/z calculated for $C_{12}H_8D_8NO_2$. Exact Mass: 269.22, found 214.2 $[M + H-56 (tert-butyl)]^+$.

4.11.42. General procedure S

31 (0.15 mmol) was dissolved in DCM (3 mL). Trifluoroacetic acid (0.3 mL) was added to the solution and stirred at room temperature for 4 h. The organic solvent was removed under reduced pressure to furnish compound **32** as yellow oil without further purification. MS-ESI: m/z calculated for $C_{11}H_8D_8N$. Exact Mass: 169.17, found 170.2 $[M+H]^+$.

4.11.43. General procedure T

HATU (0.2 mmol) was added to the solution of **32** (0.16 mmol) and **18** (0.16 mmol) dissolved in dry DCM (3 mL). Then DIEA (0.8 mmol) was added to the reaction and stirred at room temperature for 3 h. DCM was removed under reduced pressure before the mixture was diluted and extracted with ethyl acetate. The organic layer was combined and washed with saturated brine, dried over anhydrous sodium sulfate. Then the organic solvent was removed under reduced pressure. The obtained residue was purified by silica gel (EA:PE = 0%–35%) and chiral (PE: EtOH = 50%) column chromatography to furnish compound QY1892 in 46% yield as colorless oil with 98.7% *ee*. MS-ESI: m/z calculated for $C_{22}H_{18}D_8N_3O_2$. Exact Mass: 371.24, found 372.3 $[M+H]^+$.

4.11.44. (S)-2-(4-Chlorophenoxy)-2-methyl-1-(2-(3-methyl-1,2,4-oxadiazol-5-yl)pyrrolidin-1-yl)propan-1-one (**12**)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 7.38–7.26 (m, 2H), 6.92–6.82 (m, 2H), 5.24 (dd, J = 8.4, 4.6 Hz, 1H), 3.89–3.62 (m, 2H), 2.34 (s, 3H), 2.25–2.15 (m, 1H), 1.96–1.86 (m, 2H), 1.82–1.74 (m, 1H), 1.50 (s, 3H), 1.46 (s, 3H). MS-ESI: m/z calculated for $C_{17}H_{21}ClN_3O_3$. Exact Mass: 349.12, found 350.1 $[M+H]^+$.

4.11.45. 2-(4-Chloro-2-fluorophenoxy)-2-methyl-1-(2-phenylpyrrolidin-1-yl)propan-1-one (**13**)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 7.53 (dd, J = 11.0, 2.6 Hz, 1H), 7.29 (t, J = 7.4 Hz, 2H), 7.24–7.08 (m, 4H), 6.79 (t, J = 9.0 Hz, 1H), 5.07 (dd, J = 8.1, 4.3 Hz, 1H), 3.97–3.66 (m, 2H), 2.19–2.07 (m, 1H), 2.05–1.70 (m, 3H), 1.58 (s,

3H), 1.48 (s, 3H). MS-ESI: m/z calculated for $C_{20}H_{22}ClFO_2$. Exact Mass: 361.12, found 361.9 $[M+H]^+$.

4.11.46. (S)-2-(4-Chloro-2-fluorophenoxy)-2-methyl-1-(2-(p-tolyl)pyrrolidin-1-yl)propan-1-one (**14**)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 7.52 (dd, J = 11.0, 2.6 Hz, 1H), 7.17–7.13 (m, 1H), 7.09 (d, J = 7.9 Hz, 2H), 6.98 (d, J = 8.0 Hz, 2H), 6.78 (t, J = 9.0 Hz, 1H), 5.03 (dd, J = 8.0, 4.4 Hz, 1H), 3.94–3.66 (m, 2H), 2.27 (s, 3H), 2.14–2.06 (m, 1H), 1.88–1.70 (m, 3H), 1.56 (s, 3H), 1.48 (s, 3H). MS-ESI: m/z calculated for $C_{21}H_{24}ClFO_2$. Exact Mass: 375.14, found 376.0 $[M+H]^+$.

4.11.47. (S)-2-(4-Chloro-2-fluorophenoxy)-2-methyl-1-(2-(4-(methyl- d_3)phenyl-2,3,5,6- d_4)pyrrolidin-1-yl-2- d)propan-1-one (**15**)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 7.52 (dd, J = 11.0, 2.6 Hz, 1H), 7.18–7.12 (m, 1H), 6.77 (t, J = 9.0 Hz, 1H), 3.91–3.68 (m, 2H), 2.14–2.05 (m, 1H), 1.92–1.68 (m, 3H), 1.55 (s, 3H), 1.48 (s, 3H). MS-ESI: m/z calculated for $C_{21}H_{16}D_8ClFO_2$. Exact Mass: 383.19, found 384.2 $[M+H]^+$.

4.11.48. (S)-5-((2-Methyl-1-oxo-1-(2-(p-tolyl)pyrrolidin-1-yl)propan-2-yl)oxy)indolin-2-one (**16**)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 10.24 (s, 1H), 7.08 (d, J = 7.5 Hz, 2H), 7.00 (d, J = 7.8 Hz, 2H), 6.69 (d, J = 9.6 Hz, 2H), 6.59 (dd, J = 8.4, 2.4 Hz, 1H), 5.04 (dd, J = 8.2, 4.2 Hz, 1H), 3.85–3.97 (m, 1H), 3.78–3.70 (m, 1H), 2.27 (s, 3H), 2.04–2.13 (m, 1H), 1.68–1.91 (m, 3H), 1.50–1.61 (m, 2H), 1.47 (s, 3H), 1.42 (s, 3H). MS-ESI: m/z calculated for $C_{23}H_{27}N_2O_3$. Exact Mass: 378.19, found 379.1 $[M+H]^+$.

4.11.49. (S)-2-((1H-Indazol-6-yl)oxy)-2-methyl-1-(2-(4-(methyl- d_3)phenyl-2,3,5,6- d_4)pyrrolidin-1-yl-2- d)propan-1-one (QY1892)

The title compound was obtained as described in the general procedure (white solid). 1H NMR (400 MHz, DMSO- d_6) δ 12.80 (s, 1H), 7.97 (d, J = 4.0 Hz, 1H), 7.66 (dd, J = 8.9, 4.1 Hz, 1H), 6.78 (t, J = 2.8 Hz, 1H), 6.71 (dt, J = 6.0, 3.1 Hz, 1H), 3.83 (d, J = 7.0 Hz, 1H), 3.73–3.56 (m, 1H), 2.02 (s, 1H), 1.74 (d, J = 7.6 Hz, 2H), 1.63 (d, J = 4.0 Hz, 3H), 1.54 (d, J = 4.0 Hz, 3H), 1.51–1.44 (m, 1H). MS-ESI: m/z calculated for $C_{22}H_{18}D_8N_3O_2$. Exact Mass: 371.24, found 372.1 $[M+H]^+$.

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Conflicts of interest

Li Tan, Ying Li, Ying Qin, and Dekang Li are inventors on patent applications relating to this work, owned by SIOC. All other authors declare they have no competing interests.

Appendix A. Supporting information

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