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Discovery of novel bicyclic[3.3.0]proline peptidyl α-ketoamides as potent 3CL-protease inhibitors for SARS-CoV-2

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ABSTRACT

The outbreak of SARS-CoV-2 has caused global crisis on health and economics. The multiple drug-drug interaction risk associated with ritonavir warrants specialized assessment before using Paxlovid. Here we report a multiple-round SAR study to provide a novel bicyclic[3.3.0]proline peptidyl α-ketoamide compound **4a**, which is endowed with excellent antiviral activities and pharmacokinetic properties. Also, *in vivo* HCoV-OC43 neonatal mice model demonstrated compound **4a** has good *in vivo* efficacy. Based on these properties, compound **4a** worth further SAR optimization with the goal to develop compounds with better pharmacokinetic properties and finally to realize single agent efficacy in human.

Although three years have passed since the first patient reported, COVID-19 is still causing a global health emergency.[1,2] Billions of cases and millions of fatalities have been reported worldwide, indicating the urgent need of antiviral drugs.[3] Remdesivir,[4] which interferes with the function of RNA-dependent RNA polymerase, was recommended by World Health Organization (WHO) in 2020. However, it needs to be administered intravenously, which limits its widespread use during the pandemic. Oral SARS-CoV-2-specific therapeutics are urgently needed to prevent more severe disease, hospitalization and death.

3-chymotrypsin-like cysteine protease enzyme ($3CL^{pro}$/M^{pro}) plays an essential role in coronavirus replication. It can cleave the polyproteins at 11 different sites to yield shorter, non-structural proteins vital to viral replication.[5,6] Paxlovid, targeting 3C-like protease,[7] was granted Emergency Use Authorization in December 2021 as therapy of nonhospitalized patients (adults and children 12 years or older). Paxlovid consists of two components, nirmatrelvir (**1**) and ritonavir.[8,9] Nirmatrelvir is a $3CL^{pro}$ inhibitor and has low nanomolar inhibition potency against the COVID-19. Ritonavir is a protease inhibitor and potent inhibitor of enzyme (CYP3A4) responsible for the metabolism of nirmatrelvir, as a result, it enables higher peak level and more prolonged half-life of nirmatrelvir (Fig. 1).

However, combination with ritonavir requires cautious use with other medications because interfering with CYP3A4 enzyme would increase drug concentration in serum of other medicines, which may result in unexpected side effects. Paxlovid should also not be given to patients on pharmacological agents that act as CYP inducers. These can lead to drastically reduced levels of nirmatrelvir, resulting in worse therapeutic effect.

With the goal of overcoming those issues associated with the combination use of PK booster ritonavir while retaining great antiviral activity, herein we would like to report on the discovery of a novel series of α-ketoamide based potent $3CL^{pro}$ inhibitors that may be used as single anti-Covid agent without the need to be used along with ritonavir.[10]

At the beginning of our study, we first studied the binding mode of nirmatrelvir by docking it to a published co-crystal structure of GC-376 with the SARS-CoV-2 M^{pro} (PDB: 6WTT).[11] This established docking model was found aligned well with later published nirmatrelvir co-crystal structure (PDB: 7VH8).[12] The γ-lactam ring in nirmatrelvir forms hydrogen-bond interactions with Glu166 and His163. The nitrile warhead forms a reversible covalent thioimidate adduct with the Cys145 (Fig. 2). The dimethyl cyclopropanyl group at *P*2 and the *tert*-butyl group at *P*3 show hydrophobic interactions with the binding pockets,

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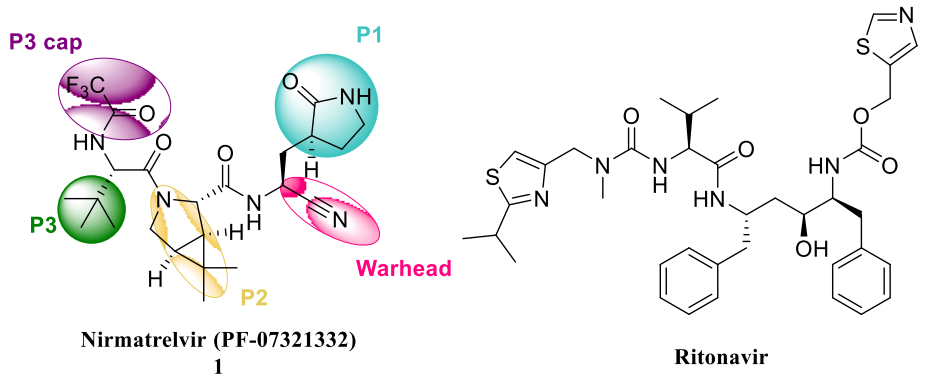


Fig. 1. Chemical structure of nirmatrelvir and ritonavir.

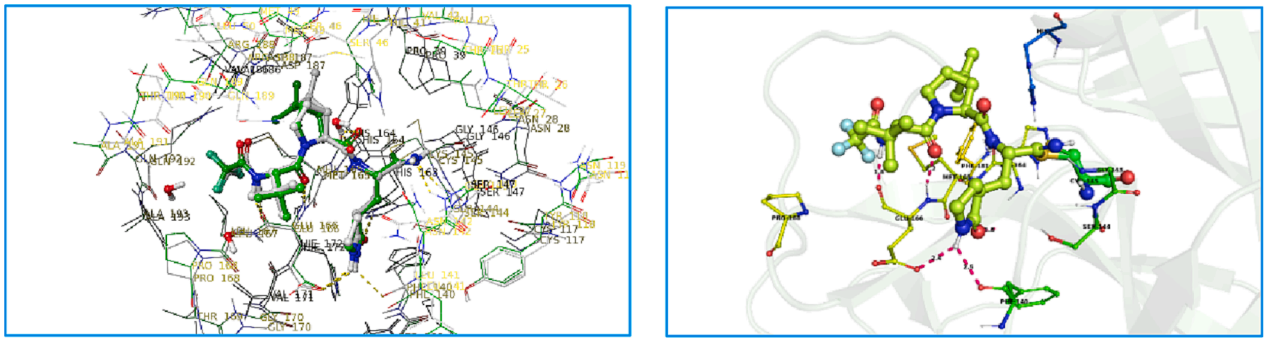
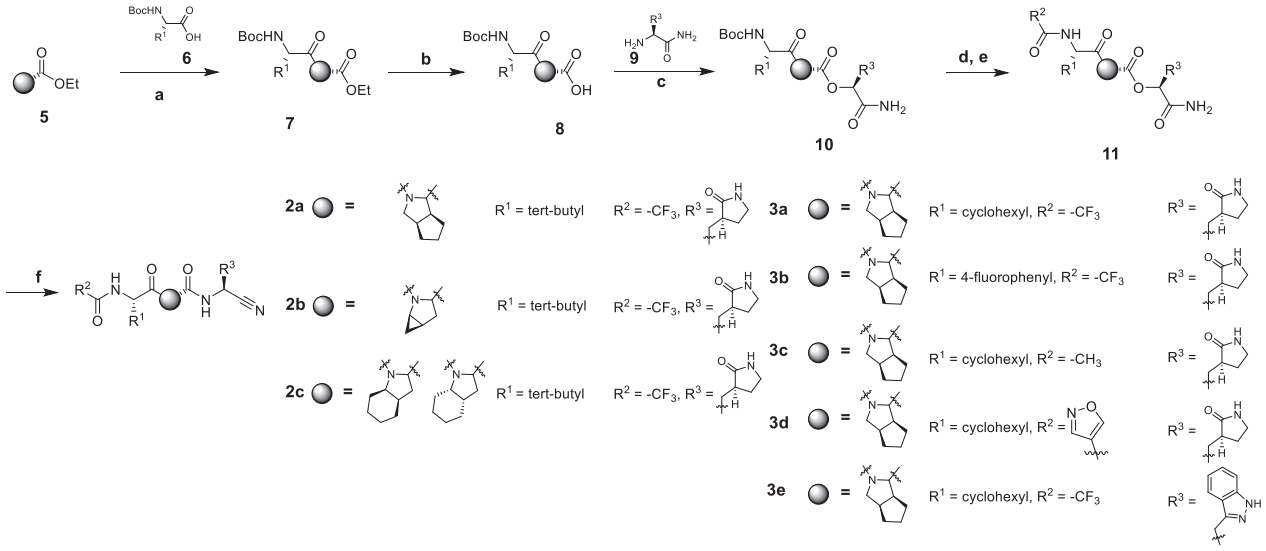


Fig. 2. a) Predicted binding mode of nirmatrelvir and SARS-CoV-2 M^{pro} (PDB: 6WTT). Pink dashes are potential hydrogen bonds. b) Nirmatrelvir's co-crystal structure with SARS-CoV-2 M^{pro}[12].



Scheme 1. Synthesis of bicyclic[3.3.0]proline peptidyl cyano compounds.[a] Reagents and conditions: a) HATU, DIEA, **6**, DMF; b) $LiOH{\cdot}H_2O$, THF/H_2O; c) EDCI, HOBt, DIEA, **9**; d) TFA, DCM; f) TFAA, pyridine, THF; For compound **3c** d) TFAA, pyridine, THF; e) TFA, DCM; f) Acetyl chloride, TEA, DMAP; For compound **3d**: d) TFA, DCM; e) EDCI, HOBt, DIEA, 1, 2-oxazole-3-carboxylic acid , 2-butanone; f) TFAA, pyridine, THF [a] The two isomers of compound 2c were not isolated.

which are suitable for more structure–activity relationship studies.

We first started to explore with different greasy fragments to fill up the hydrophobic *P*2 pocket. Compounds **2a**, **2b**, **2c** were selected as our initial SAR exploration targets. The syntheses of **2a-c** are shown in Scheme 1. SARS-CoV-2 M^{pro} enzymatic potencies, HCoV OC43 CPE assay and SARS-CoV-2 IFA test were examined and compared with nirmatrelvir. HCoV OC43 CPE assay was selected because its 3CL protease has high homology with SARS-CoV-2 M^{pro}.[13]

As shown in Table 1, among *P*2 modified compounds, compound **2a** was found to be the most potent one with M^{pro} enzymatic IC_{50} value of 43 nM, HCoV OC43 CPE EC_{50} of 136 nM and SARS-CoV-2 IFA EC_{50} value of 64 nM. In contrary, compounds **2b** and **2c** were both found to be inactive.

Compound **2a** was further evaluated through *in vitro* ADME and *in vivo* PK tests. As shown in Table 2, for ADME, both nirmatrelvir and **2a** displayed significant species different stability profiles and seemed quite

Table 1
Antiviral activities of *P*1, *P*2, *P*3, *P*3 cap modification compounds and nirmatrelvir.

Assays	Compounds Nirmatrelvir(1)	2a	2b	2c[a]	3a	3b	3c	3d	3e
M^{pro} enzyme IC_{50} (nM)	14	43	356	>1000	47	165	551	135	>1000
HCoV OC43 CPE EC_{50} (nM)	70	136	5806	>10000	112	677	2778	573	4887
SARS-CoV-2 IFA EC_{50} (nM)	27	64	–	–	43	–	–	–	–

[a] The two isomers were tested together and showed no antiviral activities.

Table 2
ADME and rodents PK properties of compounds **2a**, **3a** and nirmatrelvir.

	Assay Type	nirmatrelvir		2a		3a	
In vitro stability[a]	Plasma stability (Remaining% at 2 h)	91, 84, 74		102, 100, 106		100, 92, 108	
	Microsome stability ($CL_{int(liver)}$, mL/min/kg)	329, <17.3, 29		262, <17.3, 13		215, <17.3, 19	
	Hepatocytes stability ($CL_{int(liver)}$, mL/min/kg)	169, <29.9, <17.8		435, 32, <17.8		455, 51, <17.8	
Dose route	PK parameter[b]	mouse	rat	mouse	rat	mouse	rat
iv	CL (mL min^{-1} kg^{-1})	30.2	42.0	79.3	75.4	92.2	62.7
	V_{ss} (L/kg)	0.456	1.24	0.640	1.25	1.01	1.34
	$T_{1/2}$ (h)	0.714	0.770	0.111	0.239	0.188	0.378
po	C_{max} (μM)	2.72	2.14	0.79	2.98	1.16	3.47
	AUC (μM × h)	3.58	2.38	0.553	2.31	0.478	2.92
	F (%)	32.7	29.9	13.3	52	14.2	57.6

[a] *In vitro* stability data is listed in the sequence of mouse, rat, human.
[b] Mice were administered at 3 mg/kg for iv group and 10 mg/kg for po group. Rats were administered at 2 mg/kg for iv group, and 10 mg/kg for po group. The vehicle was saline for all administration groups.

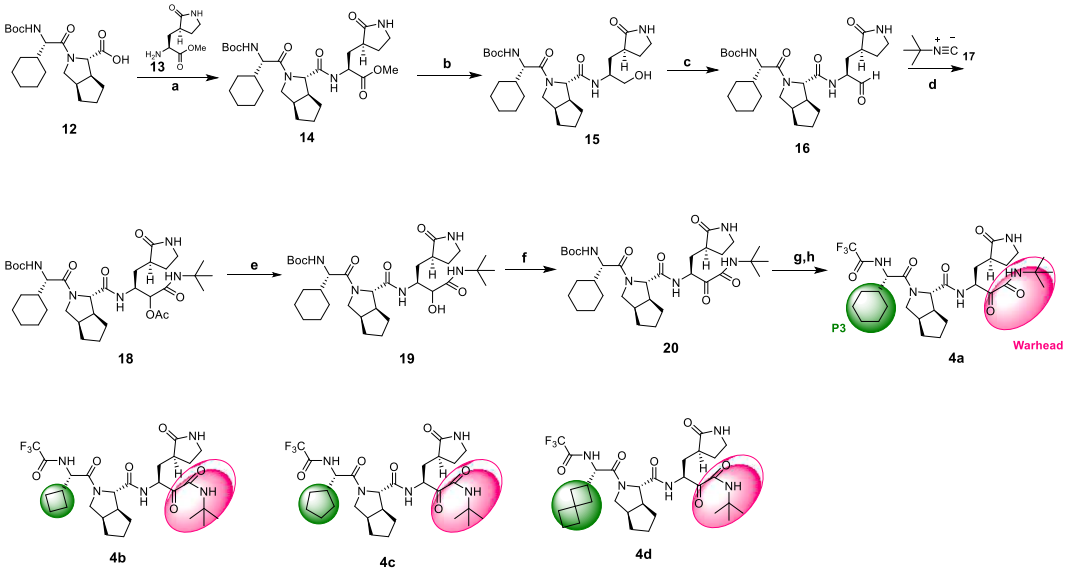
Table 3
Antiviral potency of compound **4a** in different assays.

Compound	results Nirmatrelvir(1)	4a	4b	4c	4d
M^{pro} enzyme IC_{50} (nM)	14	34	104	50	77
HCoV OC43 CPE EC_{50} (nM)	70	146	–	421	444
SARS-CoV-2 IFA EC_{50} (nM)	27	53	–	–	–

unstable in mice. Compound **2a** showed comparable liver microsome and hepatocytes stability with nirmatrelvir, along with slightly better plasma stability. For rat PK results, which should be more consistent with human according to the *in vitro* ADME data, compound **2a** showed similar oral exposure albeit with better bioavailability. However, the $T_{1/2}$ value determined for **2a** was still relatively short (Table 3).

To continue effort towards discovery of potent 3CL inhibitors endowed with better pharmacokinetic properties, we began another round of SAR optimization on *P*3 and *P*3 cap. We kept octahydrocyclopenta[c] pyrrole moiety in compound **2a** and designed a series of compounds and ranked them according to docking results. Through this exercise, compounds **3a** and **3b** were first selected for synthesis and evaluation.

To our satisfaction, compound **3a** exhibited comparable antiviral potencies to that displayed by compound **2a** (Table 1). On another hand, compound **3b** was found to be 4-fold weaker in enzymatic potency relative to **2a**. Several modifications in *P*3 cap (**3c** and **3d**) with high docking scores were then tested. However, they both showed 3–10 folds



Scheme 2. Synthesis of compounds **4a-4d**. Reagents and conditions: a) T_3P, DIEA, **13**, DCM, 67%; b) $LiBH_4$, THF, 94%; c) DMP, DCM, 94%; d) HOAc, **17**, DCM, 84%; e) K_2CO_3, MeOH/H_2O, 100%; f) DMP, DCM, 96%; g) HCl/EA, THF; h) TFAA, pyridine, 53% for 2 steps.

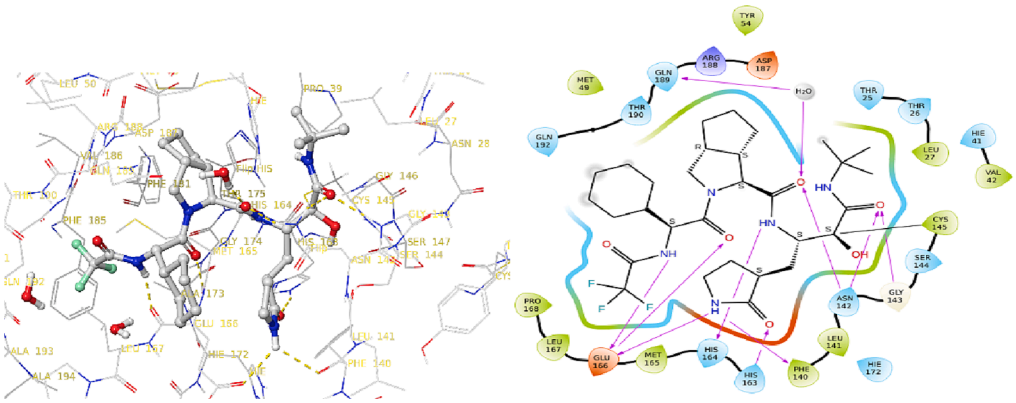


Fig. 3. Predicted binding mode of compound **4a**; a potential reversible covalent Cys145 adduct is formed with the α-ketoamides in compound **4a**.

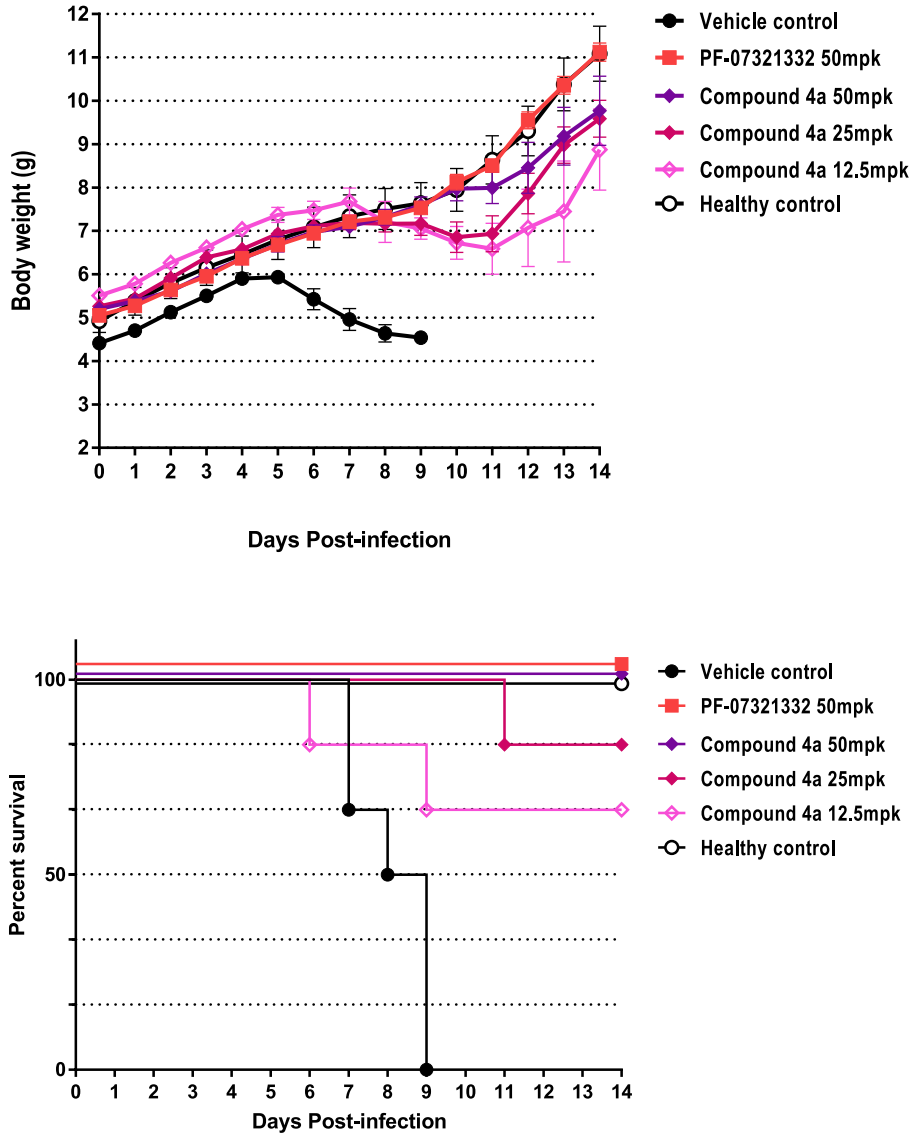


Fig. 4. Survival Percentage- dosing relationship curve and body weight change-{Citation}dosing relationship Curve.

loss in enzymatic and cell potencies, suggesting the trifluoromethyl group in nirmatrelvir may picked up extra hydrogen-halogen bonds other than pure hydrophobic interactions at *P*3 cap position (Table 1). 2-isoxazole group in compound **3d** maintained some of the hydrogen bonds while trimethyl group in compound **3c** did not have this interaction. This may explain the difference of antiviral potencies with **3c** and **3d**.

We then evaluated DMPK profiles of **3a** and found that it had

Table 4
ADME and rodents PK properties of compounds **4a** and **4c.**

Assay Type		4a		4c
In vitro stability[a]	Plasma stability (Remaining% at 2 h)	106, 96, 102		-
	Microsome stability ($CL_{int(liver)}$, mL/min/kg)	212, 25, 33		-
	Hepatocytes stability ($CL_{int(liver)}$, mL/min/kg)	1023, 42, <17.8		-
Dose route	PK parameter [b]	mouse	rat	mouse
iv	CL (mL min^{-1} kg^{-1})	2.50	2.55	5.06
	V_{ss} (L/kg)	1.27	0.929	0.911
	$T_{1/2}$ (h)	6.24	4.36	3.27
po	C_{max} (μM)	4.81	3.79	4.80
	AUC (μM × h)	33.0	37.4	16.8
	F (%)	32	37.4	30.8

[a] *In vitro* stability data is listed in the sequence of mouse, rat, human.
[b] Mice were administered at iv 3 mg/kg and po 10 mg/kg. Rats were administered at iv 2 mg/kg and po 10 mg/kg. Vehicle was 40% PEG400 + 60 % saline for all groups. -: not tested.

comparable PK properties as compound **2a** (Table 2). Once again, significant species difference and relatively inferior PK profiles of **3a** existed.

As for *P*1 SAR, we decided to employ indazole moiety. Docking model showed indazole could maintained the same set of hydrogen bonds as γ-lactam within *P*1 pocket. However the phenol ring clashed slightly to the *P*3 moiety within the molecule which led to a lower binding score. Triggered by indazole's anti-viral properties showed in other programs, compound **3e** was still synthesized and tested. Disappointedly, this compound did not possess any activity in bioassays evaluate.

After initial examination of SAR on *P*1, *P*2, *P*3 and *P*3 cap moieties, we obtained several novel compounds (e.g., **2a** & **3a**, all containing nitrile group as warhead) displaying comparable potencies as nirmatrelvir. However, none of these compounds showed improvement in pharmacokinetic properties both in terms of oral exposure level (AUC) and $T_{1/2}$ value. Therefore, we turned our attention to warhead alteration (nitrile group as employed by PF-series). Towards that end, aldehyde and α, β-unsaturated ester are two commonly used warheads in antiviral medicines, but both aldehyde and α, β-unsaturated ester may cause unexpected toxicity and instability problems. After careful literature search, we were delighted to find the α-ketoamide bearing HCV protease inhibitor Telaprevir reported by Eli Lilly group.[14–17] α-Ketoamides have been shown to possess better pharmacokinetic properties such as improved membrane permeability and enhanced stability toward plasma esterases.[18,19] It has also been used in exploring broad-spectrum inhibitors of coronavirus and enterovirus replication.[20] Inspired by these findings, we decided to introduce α-ketoamide as the warhead for our anti-Covid-19 3CL inhibitor program.[21]

To explore the warhead pocket of α-ketoamide bearing Covid-19 3CL protease inhibitor series, we docked various types of *P*1′ groups and found a greasy pocket that could accommodate appropriate substituent group to gain extra binding. Therefore, we decided to install *tert*-butyl group on the warhead moiety as seen in compound **4a** (see Scheme 2). The docking results of **4a** with viral enzyme are outlined in Fig. 3. The α-carbonyl group of α-ketoamide could form a reversible hemi-thioketal with Cys145, which is similar to the role of cyano group in nirmatrelvir. Moreover, the amide group of α-ketoamide may form additional hydrogen bonds with Asn142 and Gly143. The hydrogen bonds and hydrophobic interactions on other sites are also maintained (Fig. 4).

The synthesis of the α-ketoamide **4a** is outlined in Scheme 2. Starting from the acid precursor **12** described in Scheme 2, compound **16** was obtained through condensation and oxidation state adjustment. Reaction with *tert*-butyl isocyanide **17** and then de-acylation provided the keto amide precursor **19**. Oxidation of compound **19** with DMP, followed by deprotection and trifluoroacetylation provided the desired compound **4a**. After finished the synthesis of compound **4a**, more modifications on *P*3 were done. Compounds **4b**, **4c** and **4d** were then synthesized according to the route displayed.

Consistent with the docking model prediction, α-ketoamide inhibitor **4a**, **4b**, **4c**, **4d** had good enzymatic potencies with IC_{50} values ranging from 30 to 100 nM. Compound **4a** also exhibits 2-fold weaker yet still impressive potencies in different cell and viral assays compared with nirmatrelvir. Encouraged by the promising antiviral potency demonstrated by compound **4a** and **4c**, we continued to evaluate ADME/PK profiles on these compounds (see Table 4).

In the subsequent ADME and PK evaluations, compound **4a** also displayed excellent plasma stability, liver microsome and hepatocytes stability data indicated **4a** is very stable in human plasma. For PK results, compound **4a** has longer $T_{1/2}$, lower clearance, higher plasma exposure and moderate oral bioavailability in both mice and rat. Predicted human clearance is also very low, which is about 2.9 ml/min/kg using simple allometric scaling method. Compound **4c** also exhibited significant improvement in pharmacokinetic parameters compared with nirmatrelvir, but it is slightly inferior to **4a**. Overall, α-ketoamide compounds exhibit excellent PK properties with much lower clearance and longer half-life than the nitrile-series, compounds **2a** and **3a**.

Owing to its impressive anti-COVID-19 activities and desirable DMPK profile, compound **4a** was further evaluated in *in vivo* efficacy study. HCoV-OC43 neonatal mice model was used as a surrogate model for COVID-19 infection. This model was chosen because of the following considerations: 1) high viral sequence homology between HCoV-OC43 and SARS-CoV-2 M^{pro}, and 2) laboratory execution convenience (BSL-2 vs. BSL-3).

Compound **4a** was administered via intraperitoneal injection at three doses. The results showed that none of the mice (0/6) in vehicle group survived at Day 9. All mice (6/6) in healthy group, nirmatrelvir and high dosing group of compound **4a** survived at day 14. Five out of six mice survived in medium dosing group while four out of six mice survived in low dosing group, indicating a dose dependent *in vivo* efficacy being observed with compound **4a**. The body weight change curve also showed this dose-dependent tendency.

In conclusion, we have discovered a series of bicyclic[3.3.0]proline peptidyl α-ketoamide compounds through multiple rounds of SAR optimization. The most promising one, compound **4a**, displayed impressive potencies and much better PK profile which has higher oral exposure and longer $T_{1/2}$. Based on these properties, compound **4a** is warranted further SAR optimization with the goal to achieve single agent efficacy in human (without the need to co-administered with Ritonavir). **RAY1216**[21], successor of compound **4a**, finally realized with this goal and have recently been approved for NDA in China. The details of **RAY1216** SAR investigation will be disclosed in the subsequent manuscript in the near future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2023.129324.

References

1 Estimating excess mortality due to the COVID-19 pandemic: a systematic analysis of COVID-19-related mortality, 2020-21. Lancet Lond Engl. 2022;399(10334):1513-1536.
2 Estimating global, regional, and national daily and cumulative infections with SARS-CoV-2 through Nov 14, 2021: a statistical analysis. Lancet Lond Engl. 2022;399 (10344):2351-2380.
3 Couzin-Frankel J. Antiviral pills could change pandemic's course. *Science*. 2021;374: 799–800.
4 Siegel D, Hui HC, Doerffler E, et al. Discovery and Synthesis of a Phosphoramidate Prodrug of a Pyrrolo[2,1-f][triazin-4-amino] Adenine C-Nucleoside (GS-5734) for the Treatment of Ebola and Emerging Viruses. *J Med Chem*. 2017;60:1648–1661.
5 Pillaiyar T, Manickam M, Namasivayam V, Hayashi Y, Jung SH. An Overview of Severe Acute Respiratory Syndrome-Coronavirus (SARS-CoV) 3CL Protease Inhibitors: Peptidomimetics and Small Molecule Chemotherapy. *J Med Chem*. 2016; 59:6595–6628.
6 Jin Z, Du X, Xu Y, et al. Structure of M(pro) from SARS-CoV-2 and discovery of its inhibitors. *Nature*. 2020;582:289–293.
7 Owen DR, Allerton CMN, Anderson AS, et al. An oral SARS-CoV-2 M(pro) inhibitor clinical candidate for the treatment of COVID-19. *Science*. 2021;374:1586–1593.
8 Hammond J, Leister-Tebbe H, Gardner A, et al. Oral Nirmatrelvir for High-Risk, Nonhospitalized Adults with Covid-19. *New Engl J Med*. 2022;386:1397–1408.
9 Li P, Wang Y, Lavrijsen M, et al. SARS-CoV-2 Omicron variant is highly sensitive to molnupiravir, nirmatrelvir, and the combination. *Cell Res*. 2022;32:322–324.
10 Xiaoxin Chen, Xiaodong Huang, Qinhai Ma, et al. Inhibition mechanism and antiviral activity of an α-ketoamide based SARS-CoV-2 main protease inhibitor. bioRxiv. Published online January 1, 2023:2023.03.09.531862.
11 Ma C, Sacco MD, Hurst B, et al. Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. *Cell Res*. 2020;30:678–692.
12 Zhao Y, Fang C, Zhang Q, et al. Crystal structure of SARS-CoV-2 main protease in complex with protease inhibitor PF-07321332. *Protein Cell*. 2022;13:689–693.
13 Jang M, Park R, Park YI, et al. EGCG, a green tea polyphenol, inhibits human coronavirus replication in vitro. *Biochem Biophys Res Commun*. 2021;547:23–28.
14 Chen SH, Lamar J, Yip Y, et al. P1 and P1; Optimization of [3,4]-Bicycloproline P2 Incorporated Tetrapeptidyl α-Ketoamide Based HCV Protease Inhibitors. *Lett Drug Des Discov*. 2005;2:118–123.
15 Victor F, Lamar J, Snyder N, et al. P1 and P3 optimization of novel bicycloproline P2 bearing tetrapeptidyl alpha-ketoamide based HCV protease inhibitors. *Bioorg Med Chem Lett*. 2004;14:257–261.
16 Yip Y, Victor F, Lamar J, et al. Discovery of a novel bicycloproline P2 bearing peptidyl alpha-ketoamide LY514962 as HCV protease inhibitor. *Bioorg Med Chem Lett*. 2004;14:251–256.
17 Lamar J, Victor F, Snyder N, et al. Novel P4 truncated tripeptidyl α-ketoamides as HCV protease inhibitors. *Bioorg Med Chem Lett*. 2004;14:263–266.
18 Stöckigt J, Antonchick AP, Wu F, Waldmann H. The Pictet-Spengler reaction in nature and in organic chemistry. *Angew Chem (Int Ed English)*. 2011;50:8538–8564.
19 Harbeson SL, Abelleira SM, Akiyama A, et al. Stereospecific synthesis of peptidyl alpha-keto amides as inhibitors of calpain. *J Med Chem*. 1994;37:2918–2929.
20 Zhang L, Lin D, Kusov Y, et al. α-Ketoamides as Broad-Spectrum Inhibitors of Coronavirus and Enterovirus Replication: Structure-Based Design, Synthesis, and Activity Assessment. *J Med Chem*. 2020;63:4562–4578.
21 Chen X, Wang J, Huang J, et al. Ketoamide derivative and application thereof. 2023; (CN 202111057236 A).