Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Bioorganic & Medicinal Chemistry Letters 19 (2009) 2722–2727

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

New developments for the design, synthesis and biological evaluation of potent SARS-CoV 3CL^{pro} inhibitors

Thomas Regnier [a,†], Diganta Sarma [a,†], Koushi Hidaka [a], Usman Bacha [b], Ernesto Freire [b], Yoshio Hayashi [a,c,*], Yoshiaki Kiso [a,*]

[a] Department of Medicinal Chemistry, Center for Frontier Research in Medicinal Science, 21st Century COE Program, Kyoto Pharmaceutical University, Kyoto 607-8412, Japan
[b] Department of Biology, Johns Hopkins University, Baltimore, MD, USA
[c] Department of Medicinal Chemistry, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan

ARTICLE INFO

Article history:
Received 24 February 2009
Revised 24 March 2009
Accepted 25 March 2009
Available online 28 March 2009

Keywords:
SARS-CoV
Protease inhibitors
Drug design
Synthesis

ABSTRACT

A series of trifluoromethyl, benzothiazolyl or thiazolyl ketone-containing peptidic compounds as SARS-CoV 3CL protease inhibitors were developed and their potency was evaluated by in vitro protease inhibitory assays. Three candidates had encouraging results for the development of new anti-SARS compounds.

In November 2002, it was reported an emergence of severe acute respiratory syndrome (SARS) as a highly contagious and fatal respiratory disease infecting more than 8000 individuals of which 9.6% patients died within a few months.[1] Due to highly efficient international cooperation, two groups rapidly reported that a novel coronavirus (CoV) was the causative agent of SARS.[2,3] CoV encodes a chymotrypsin-like protease (3CL^{pro}) that plays a pivotal role in the replication of the virus.[4] 3CL^{pro}, a cysteine protease, is functionally analogous to the main picornavirus protease 3C^{pro} with a catalytic dyad (Cys-145 and His-41) in the active site. Cys acts as a nucleophile, whereas His functions as a general base.[5,6] In order to find compounds that can inhibit SARS-CoV, numerous 3CL^{pro} inhibitors have been described, including C_2-symmetric diols,[7] bifunctional aryl boronic acids,[8] keto-glutamine analogs,[9] isatin derivatives,[10] α,β-unsaturated esters,[11] anilide,[12] benzotriazole[13] as well as glutamic acid and glutamine peptides possessing a trifluoromethyl ketone group as reported by us and our collaborators since 2006[14] and recently by another group.[15] However, no effective therapy has been developed so far and it is still a matter of necessity to discover new potent structures in case the disease re-emerges.

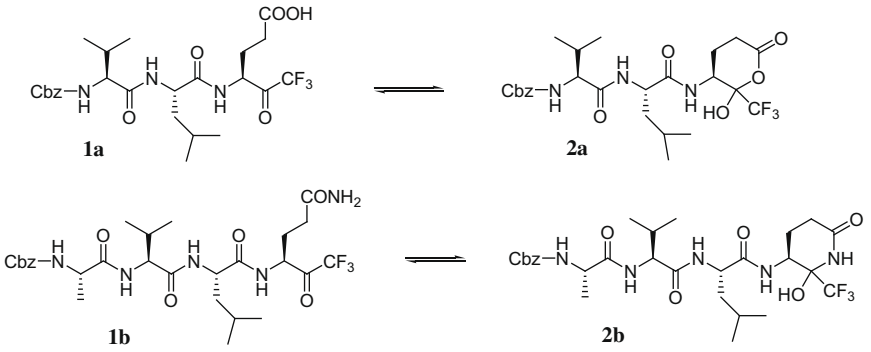
In our previous report, two compounds (Scheme 1, **1a,b**) were found to be moderate SARS-CoV 3CL^{pro} inhibitors (K_i = 116 and 134 μM, respectively).[14a] As mentioned by Cai and co-workers in 2006, the moderate activity can be the result of the formation of a typical cyclic structure (Scheme 1, compounds **2a,b**) that is not expected to interact effectively with the active site of SARS-CoV 3CL^{pro}.[16]

Herein, we report our results on improving the inhibitory activity of these compounds, by focusing on two strategies. First, keeping the trifluoromethylketone moiety in place, we investigated chemical modifications on the side chain of Glu or Gln residue at the P1 position, in order to block the formation of the cyclic structure (Scheme 1) and modulate the hydrogen bonding ability of this P1 position toward the active site, as well as modifying the amino acid residues at the P2 and P3 positions. Second, we investigated a replacement of the chemical warhead of the inhibitor, that is, the trifluoromethyl unit, by other moieties such as electron-withdrawing thiazolyl and benzothiazolyl groups. We believe that this modification would be valuable for enhancing the reactivity of the covalent-adduct formation to the active site cysteine residue in SARS-CoV 3CL^{pro}.

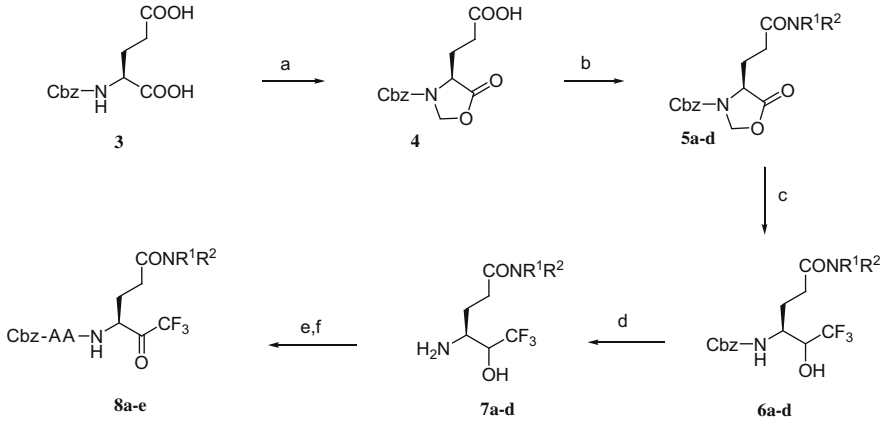
From a synthetic point of view, the preparation of the target compounds was envisioned following the synthetic routes illustrated in Schemes 2–4. Compounds **8a–e** were prepared from Cbz-L-Glu-OH (**3**) that was converted to the corresponding oxazolidinone acid **4** under the conditions described by Moore et al.[17] Amides **5a–d** were next prepared by coupling compound **4** with four kinds of amines using a standard HOBt–EDC·HCl coupling method for peptides, resulting in excellent yields. Compounds **5a–d** were then converted in a one-pot reaction to the correspond-

* Corresponding authors. Tel.: +81 75 595 4635; fax: +81 75 591 9900 (Y.K.).
E-mail address: kiso@mb.kyoto-phu.ac.jp (Y. Kiso).
† Equal contribution.

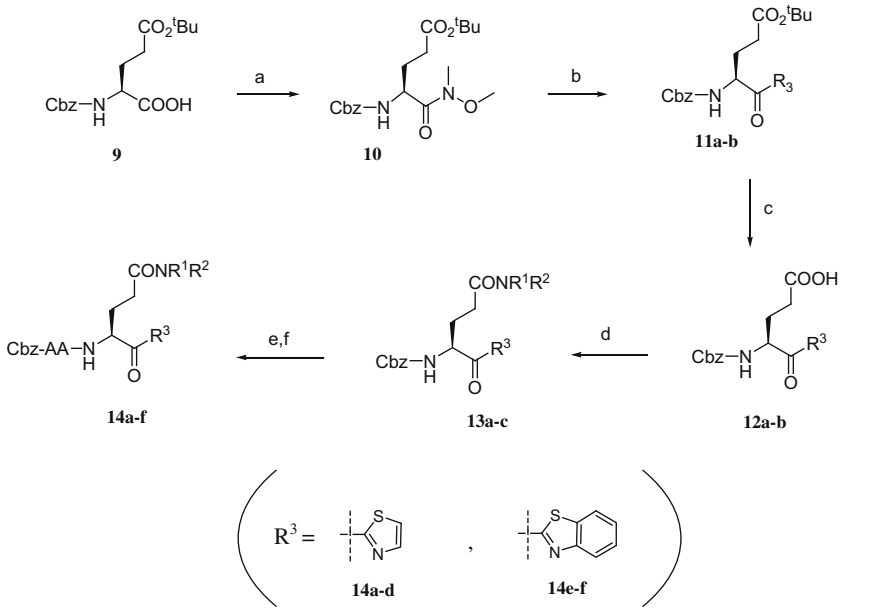
0960-894X/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2009.03.118



Scheme 1. Previously reported trifluoromethyl ketone-containing peptides and their corresponding cyclic non-active counterparts.

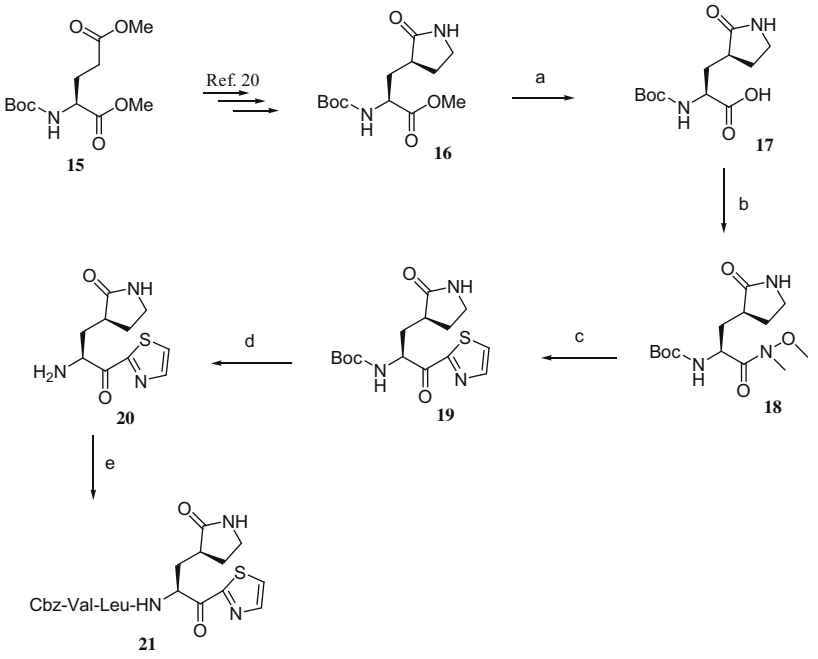


Scheme 2. Reagents and conditions: (a) paraformaldehyde, *p*-TsOH·H_2O, toluene, reflux, 2 h, 98%; (b) HNR^1R^2, HOBt, EDC·HCl, DMF, 0 °C–rt, overnight, 80–98%; (c) CsF, $CF_3Si(CH_3)_3$, THF, sonication, rt, 3 h then MeOH, rt, 30 min then $NaBH_4$, rt, overnight, 48–61%; (d) H_2, Pd/C (10%), MeOH, rt, overnight, 100%; (e) Cbz-AA-OH, HOBt, EDC·HCl, DMF, 0 °C–rt, overnight; (f) Dess–Martin periodinane, CH_2Cl_2, rt, 16 h, EtOAc then filtration through Celite followed by HPLC purification.



Scheme 3. Reagents and conditions: (a) *N*,*O*-dimethylhydroxylamine hydrochloride, EDC·HCl, HOBt, TEA, DMF, rt, 12 h, 90%; (b) thiazole or benzothiazole, *n*-BuLi, −78 °C, 2.5 h, 70%; (c) formic acid, rt, 12 h, 100%; (d) HNR^1R^2, EDC·HCl, HOBt, DMF, rt, 12 h, 90%; (e) triflic acid, DCM, rt, 5 min, 100% (f) Cbz-AA-OH, HOBt, EDC·HCl, DMF, rt, 12 h followed by HPLC purification.

ing trifluoromethylalcohols **6a–d**, whose Cbz group was de-protected after silica gel column chromatography, and the amino function in the resultant compounds **7a–d** was coupled to the appropriate peptide fragments.[14] The peptide fragments were synthesized according to known procedures.[14,18] Finally, the resulting peptides were directly engaged in the last oxidation step affording



Scheme 4. Reagents and conditions: (a) LiOH, THF/H_2O, 92%; (b) HN(OCH_3)CH_3, EDC·HCl, HOBt, DMF, rt, 12 h, 90%; (c) thiazole, *n*-BuLi, −78 °C, 2.5 h, 70%; (d) TFA/H_2O, 4 h, >99%; (e) Cbz-Val-Leu-OH, EDC·HCl, HOBt, DMF, rt, 12 h followed by HPLC purification.

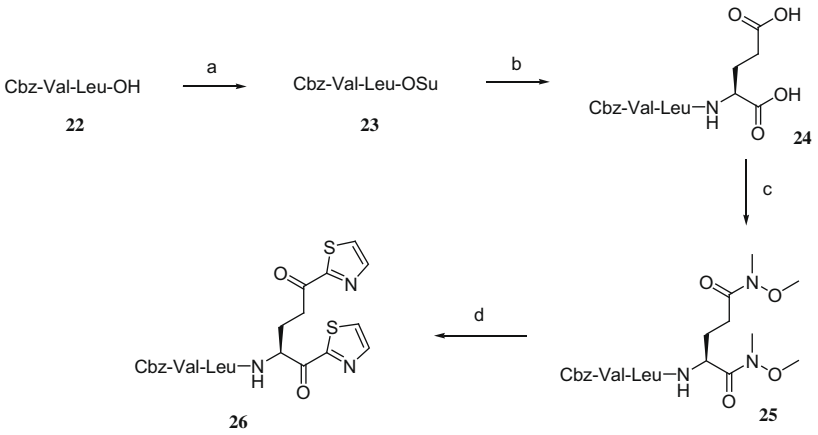
pure target compounds **8a–e** with moderate overall yields after RP-HPLC purification by a CH_3CN:(0.1% TFA/H_2O) system.

Derivatives **14a–d** with a thiazole-ketone and **14e,f** with a benzothiazole-ketone structure at the P1 residue were prepared as shown in Scheme 3. Cbz-Glu(tBu)-OH **9** was converted to Weinreb amide **10** and successively coupled to thiazole or benzothiazole in the presence of *n*-BuLi as a base to afford ketones **11a,b**.[19] After deprotection of the *tert*-butyl group by HCOOH, the resultant carboxyl group of compounds **12a,b** was coupled to the amines to obtain compounds **13a–c**, followed by coupling of the peptide fragments based on a similar approach depicted in Scheme 2. Compounds **14a–f** were obtained with moderate yields after HPLC purification.

Compound **21**, which has a pyrrolidone structure at the P1 side chain, was synthesized by a different approach as shown in Scheme 4. Lactam ester **16** was prepared from commercially available *N*-Boc-L-(+)-glutamic acid dimethyl ester **15** by following a previously published procedure.[20] Lactam ester **16** was then hydrolyzed to obtain the lactam acid **17**, followed by the synthetic steps depicted in Scheme 3 to get desired compound **21**.

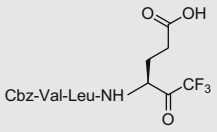
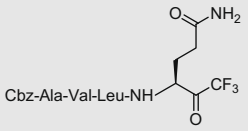
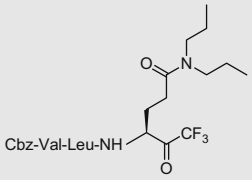
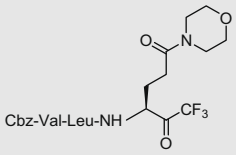
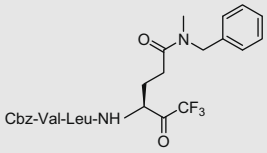
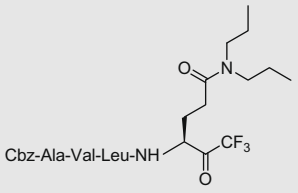
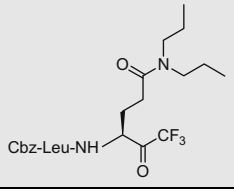
Dithiazolyl compound **26** was synthesized starting from Cbz-Val-Leu-OH **22** (Scheme 5) that was first converted to succinimide ester **23** followed by treatment with L-glutamic acid to obtain compound **24**. Dicarboxylate compound **24** was then converted to the corresponding α,γ-double-Weinreb amide (**25**), followed by a treatment with 2 equiv of thiazole in the presence of *n*-BuLi to afford the desired N-Cbz-protected dithiazolylketone compound (**26**).

The inhibitory activities (K_i) of the first series of compounds against SARS-CoV 3CL^{pro} are shown in Table 1. The inhibition assay was performed using a procedure mentioned earlier.[14a] In our previous report, compounds **1a,b** (Scheme 1) were found to be moderate SARS-CoV 3CL^{pro} inhibitors with K_i values of 116 and 135 μM, respectively (Table 1, entries 1 and 2). These compounds were used as leads for structural optimization in the development of potent SARS-CoV 3CL^{pro} inhibitors.



Scheme 5. Reagents and conditions: (a) *N*-hydroxysuccinimide, DCC, THF, 0 °C–rt, 12 h, 93%; (b) L-glutamic acid, NaOH solution (aq), dioxane, 4 h, 0 °C–rt, 90%; (c) HN(OCH_3)CH_3, EDC·HCl, HOBt, DMF, rt, 12 h, 90%; (d) thiazole, *n*-BuLi, −78 °C, 2.5 h followed by HPLC purification.

Table 1
Structure and activity of the synthesized CF_3-inhibitors

Entry	Compound	Structure[a]	K_i[b] (μM)
1	**1a**		116 ± 14
2	**1b**		135 ± 32
3	**8a**		363 ± 128
4	**8b**		21.0 ± 4.3
5	**8c**		34.1 ± 4.1
6	**8d**		297 ± 49
7	**8e**		584 ± 167

[a] Cbz, benzyloxycarbonyl.
[b] K_i, inhibition constant against SARS-CoV $3CL^{pro}$.

Compound **8a** was our first attempt at modifying the P1 residue by converting the γ-carboxylic acid of compound **1a** to a *N,N*-diethylamide. However, compound **8a** exhibited lower inhibitory activity than lead compound **1a**. A plausible reason for the observed low activity could be attributed to a disruption of hydrogen bonding interactions at the P1 position with the active site of the enzyme, or an unfavorable steric hindrance to a covalent bond formation at the CF_3-ketone moiety with the active site cysteine residue in the enzyme. On the other hand, replacements of P1 *N,N*-diethylamide in compound **8a** with *N*-morpholine- and *N,N*-methylbenzyl-amides increased the inhibitory activity (Table 1, entries 4 and 5). Indeed, the activity of compounds **8b** and **8c** with K_i values of 21.0 and 34.1 μM, respectively, was fivefold higher affinity than reference compound **1a**. Next, to develop smaller sized inhibitors, we prepared CF_3-ketone compounds **8d** and **8e** containing the same P1 structure as compound **8a** but different peptide chain lengths, because peptide chain length plays a significant role in the inhibition of enzymes by forming crucial hydrogen bonding interactions. However, the observed change in activity was negligible with either an increase or decrease of one residue in the peptide chain length (compare entries 3, 6 and 7).

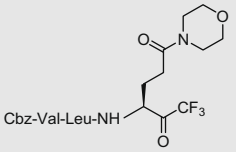
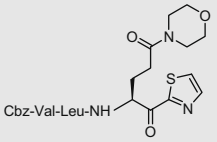
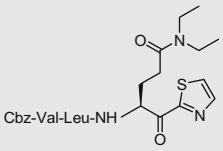
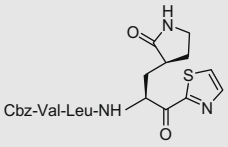
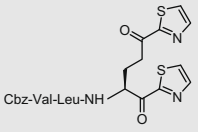
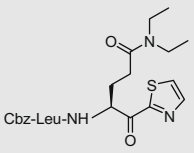
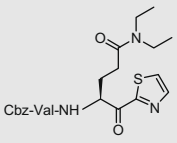
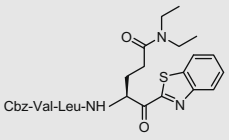
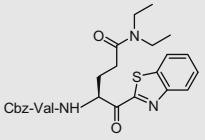
In the commonly adopted mechanism of inhibition of SARS-CoV $3CL^{pro}$, a covalent bond is formed between the carbonyl group located at the warhead of the inhibitor and the active site cysteine residue in the enzyme. Modifying the electronegativity at this warhead carbonyl moiety could accelerate the covalent bond formation. Hence, as a second series of investigations, the trifluoromethylketone moiety was replaced with an electron withdrawing thiazolyl or benzothiazolyl counterpart.

As shown in Table 2, when compared to trifluoromethylketone containing compound **8b**, corresponding compound **14a** with thiazolylketone exhibited drastically decreased inhibition (K_i = 478 μM; entry 2). However, a thiazolylketone derivative **14b** with *N,N*-diethylamide at the P1 side chain slightly increased inhibitory activity (K_i = 112 μM; entry 3) over the CF_3-ketone derivative **8a** with a more bulky *N,N*-diisopropylamide structure. Moreover, when the pyrrolidine structure was adapted to the P1 side chain, thiazolylketone derivative **21** (entry 4) drastically increased inhibitory activity with a K_i value of 2.2 μM. Slightly smaller and rigid cyclic amide structure seemed to be well accommodated by the S1 site. The introduction of a thiazolylketone to the P1 side chain resulted in a compound (entry 5) that exhibited decreased but relatively strong activity (K_i = 45.2 μM) in comparison to pyrrolidine derivative **21**. Inhibitory activity was again decreased by reducing the peptide chain length of compound **14b** (compare entries 3, 6 and 7). A little improvement in inhibitory activity was observed when the warhead thiazole was replaced by a benzothiazole (compare entries 3, 7–9). Of the compounds synthesized, inhibitor **21** possessing P1-pyrrolidone and P1′-thiazole was found to be the most potent SARS-CoV $3CL^{pro}$ inhibitor.

Our studies suggest that all the synthesized inhibitors are tight-binding substrate mimetics that target the active site of SARS-$3CL^{pro}$.[22] Similar to the trifluoromethyl ketone compounds, the thiazole and benzothiazole ketone compounds did not show an increase in inhibition after they were equilibrated with the protease for 10 min. Thus, the inhibition of the compounds to the protease was not time dependent indicating a reversible tight-binding interaction with the protease. Moreover, to better understand the potent activity of compound **21** with the enzyme, computational molecular studies were performed (Fig. 1).

A binding model of **21** with SARS-CoV $3CL^{pro}$ was simulated using a modeling package (MOE *2007.09*, Chemical Computing Group, Inc., Montreal, Canada). The initial conformation of **21** was built by changing a structurally similar ligand (Fig. 1) obtained from X-ray crystal data (PDB ID, 1WOF, K_i = 10.7 μM obtained from Ref. 21). Several energy minimization processes with an MMFF94x force field were additionally performed in a water soak environment around the inhibitor, followed by a molecular dynamics simulation. During the simulation, the moieties from P_1 to P_3 interacted in a similar manner as the original ligand except for the thiazolyl ketone and benzyloxycarbonyl moieties (Fig. 1a). The oxoanion of the ketone generated from an attack of Cys145, interacted with the amide backbone of Gly143, Ser144 and Cys145 (Fig. 1b). The nitrogen in the thiazole was also in contact with His41 through hydrogen bonding interactions. These interactions indicate an acceptance of the unique ketone. On the other hand, the benzyloxycarbonyl group barely made hydrophobic

Table 2
Structure and activity of the synthesized thiazolyl and benzothiazolyl inhibitors

Entry	Compound	Structure[a]	K_i[b] (μM)
1	**8b**		21.0 ± 4.3
2	**14a**		478 ± 193
3	**14b**		112 ± 34
4	**21**		2.20 ± 0.8
5	**26**		45.2 ± 7.8
6	**14c**		462 ± 145
7	**14d**		614 ± 139
8	**14e**		49.3 ± 10
9	**14f**		159 ± 25

[a] Cbz, benzyloxycarbonyl.
[b] K_i, inhibition constant against SARS-CoV 3CL^{pro}.

interactions, suggesting a possibility for further optimizations of the moiety.

In conclusion, we disclosed the inhibitory potency of compound **21**, containing a P1-pyrrolidone and warhead thiazolyl unit, as a SARS-CoV 3CL^{pro} inhibitor. The measured inhibitory activity coupled with possible structure modifications revealed by 3D docking give us new directions for a fast development of much more potent inhibitors. Further investigations on this new family of compounds are currently in progress in our laboratory.

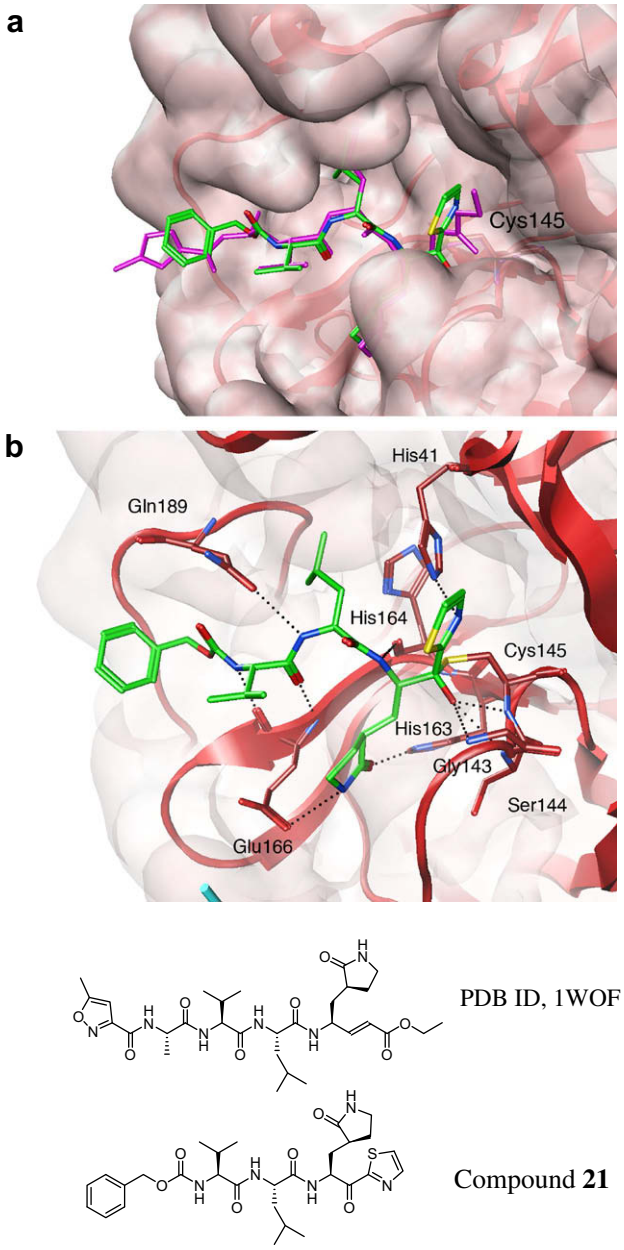


Figure 1. Molecular dynamics simulated pose of compound **21** (green stick) bound to SARS-CoV 3CL^{pro} (PDB 1WOF, with red and cyan ribbons with molecular surfaces). (a) Overlapped view with an original vinyl ester ligand (pink stick). (b) Contacted residues with hydrogen bonding interactions (dotted line).

Acknowledgements

This research was supported by The Frontier Research Program; The 21st Century COE Program from The Ministry of Education, Culture, Sports, Science and Technology, Japan; and Japan Society for the Promotion of Science's Post-Doctoral Fellowship for Foreign Researchers. E.F. also acknowledges support from a grant from the National Institutes of Health (GM 57144). The manuscript was thoroughly revised by Dr. Jeffrey-Tri Nguyen.

References and notes

1. He, J.-F.; Peng, G.-W.; Min, J.; Yu, D.-W.; Liang, W.-L.; Zhang, S.-Y.; Xu, R.-H.; Zheng, H.-Y.; Wu, X.-W.; Xu, J.; Wang, Z.-H.; Fang, L.; Zhang, X.; Li, H.; Yan, X.-G.; Lu, J.-H.; Hu, Z.-H.; Huang, J.-C.; Wan, Z.-Y.; Hou, J.-L.; Lin, J.-Y.; Song, H.-D.; Wang, S.-Y.; Zhou, X.-J.; Zhang, G.-W.; Gu, B.-W.; Zheng, H.-J.; Zhang, X.-L.; He, M.; Zheng, K.; Wang, B.-F.; Fu, G.; Wang, X.-N.; Chen, S.-J.; Chen, Z.; Hao, P.; Tang, H.; Ren, S.-X.; Zhong, Y.; Guo, Z.-M.; Liu, Q.; Miao, Y.-G.; Kong, X.-Y.; He, W.-Z.; Li, Y.-X.; Wu, C.-I.; Zhao, G.-P.; Chiu, R. W. K.; Chim, S. S. C.; Tong, Y.-K.; Chan, P. K. S.; Tam, J. S.; Lo, Y. M. D. *Science* **2004**, *303*, 1666.
2. Ksiazek, T. G.; Erdman, D.; Goldsmith, C. S.; Zaki, S. R.; Peret, T.; Emery, S.; Tong, S.; Urbani, C.; Comer, J. A.; Lim, W.; Rollin, P. E.; Dowell, S. F.; Ling, A.-E.; Humphrey, C. D.; Shieh, W.-J.; Guarner, J.; Paddock, C. D.; Rota, P.; Fields, B.; DeRisi, J.; Yang, J.-Y.; Cox, N.; Hughes, J. M.; LeDuc, J. W.; Bellini, W. J.; Anderson, L. J. *N. Eng. J. Med.* **2003**, *348*, 1953.
3. Frosten, C.; Günther, S.; Preiser, W.; Van Der Werf, S.; Brodt, H.-R.; Backer, S.; Rabenau, H.; Panning, M.; Kolesnikova, L.; Fouchier, R. A. M.; Berger, A.; Burguiére, A.-M.; Cinatl, J.; Eickmann, M.; Escriou, N.; Grywna, K.; Kramme, S.; Manuguerra, J.-C.; Müller, S.; Rickerts, V.; Stürmer, M.; Vieth, S.; Klenk, H.-D.; Osterhaus, A. D. M. E.; Schmitz, H.; Doerr, H. W. N. *N. Eng. J. Med.* **2003**, *348*, 1967.
4. Rota, P. A.; Oberste, M. S.; Monroe, S. S.; Nix, W. A.; Campagnoli, R.; Icenogle, J. P.; Peñaranda, S.; Bankamp, B.; Maher, K.; Chen, M.; Tong, S.; Tamin, A.; Lowe, L.; Frace, M.; DeRisi, J. L.; Chen, Q.; Wang, D.; Erdman, D. D.; Peret, T. C. T.; Burns, C.; Ksiazek, T. G.; Rollin, P. E.; Sanchez, A.; Liffick, S.; Holloway, B.; Limor, J.; McCaustland, K.; Olsen-Rasmussen, M.; Fouchier, R.; Günther, S.; Osterhaus, A. D. M. E.; Drosten, C.; Pallansch, M. A.; Anderson, L. J.; Bellini, W. J. *Science* **2003**, *300*, 1394.
5. Anand, K.; Ziebuhr, J.; Wadhwani, P.; Mesters, J. R.; Hilgenfeld, R. *Science* **2003**, *300*, 1763.
6. Snijder, E. J.; Bredenbeek, P. J.; Dobbe, J. C.; Thiel, V.; Ziebuhr, J.; Poon, L. L. M.; Guan, Y.; Rozanov, M.; Spaan, W. J.; Gorbalenya, A. E. *J. Mol. Biol.* **2003**, *331*, 991.
7. (a) Wu, C.-Y.; Jan, J.-T.; Ma, S.-H.; Kuo, C.-J.; Juan, H.-F.; Cheng, E. Y.-S.; Hsu, H.-H.; Huang, H.-C.; Wu, D.; Brik, A.; Liang, F.-S.; Liu, R.-S.; Fang, J.-M.; Chen, S.-T.; Liang, P.-H.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 10012; (b) Shao, Y.-M.; Yang, W.-B.; Peng, H.-P.; Hsu, M.-F.; Tsai, K.-C.; Kuo, T.-H.; Wang, A. H.-J.; Liang, P.-H.; Lin, C.-H.; Yang, A.-S.; Wong, C.-H. *ChemBioChem* **2007**, *8*, 1654.
8. Bacha, U.; Barrila, J.; Velasquez-Campoy, A.; Leavitt, S. A.; Freire, E. *Biochemistry* **2004**, *43*, 4906.
9. Jain, R. P.; Petterson, H. I.; Zhang, J.; Aull, K. D.; Fortin, P. D.; Huitema, C.; Eltis, L. D.; Parrish, J. C.; James, M. N. G.; Wishart, D. S.; Vederas, J. C. *J. Med. Chem.* **2004**, *47*, 6113.
10. Chen, L.-R.; Wang, Y.-C.; Lin, Y.-W.; Chou, S.-Y.; Chen, S.-F.; Liu, L.-T.; Wu, Y.-T.; Kuo, C.-J.; Chen, T. S.-S.; Juang, S.-H. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3058.
11. (a) Ghosh, A. K.; Xi, K.; Ratia, K.; Santarsiero, B. D.; Fu, W.; Harcourt, B. H.; Rota, P. A.; Baker, S. C.; Johnson, M. E.; Mesecar, A. D. *J. Med. Chem.* **2005**, *48*, 6767; (b) Shie, J.-J.; Fang, J.-M.; Kuo, T.-H.; Kuo, C.-J.; Liang, P.-H.; Huang, H.-J.; Wu, Y.-T.; Jan, J.-T.; Cheng, E. Y.-S.; Wong, C.-H. *Bioorg. Med. Chem.* **2005**, *13*, 5240; (c) Ghosh, A. K.; Xi, K.; Grum-Tokars, V.; Xu, X.; Ratia, K.; Fu, W.; Houser, K. V.; Baker, S. C.; Johnson, M. E.; Mesecar, A. D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5876.
12. Shie, J.-J.; Fang, J.-M.; Kuo, T.-H.; Kuo, C.-J.; Liang, P.-H.; Huang, H.-J.; Yang, W.-B.; Lin, C.-H.; Chen, J.-L.; Wu, Y.-T.; Wong, C.-H. *J. Med. Chem.* **2005**, *48*, 4469.
13. Wu, C.-Y.; King, K.-Y.; Kuo, C.-J.; Fang, J.-M.; Wu, Y.-T.; Ho, M.-Y.; Liao, C.-L.; Shie, J.-J.; Liang, P.-H.; Wong, C.-H. *Chem. Biol.* **2006**, *13*, 4469.
14. (a) Sydnes, M. O.; Hayashi, Y.; Sharma, V. K.; Hamada, T.; Bacha, U.; Barrila, J.; Freire, E.; Kiso, Y. *Tetrahedron* **2006**, *62*, 8601; (b) Bacha, U.; Barrila, J.; Gabelli, B.; Kiso, Y.; Amzel, L. M.; Freire, E. *Chem. Biol. Drug Des.* **2008**, *72*, 34.
15. Shao, Y.-M.; Yang, W.-B.; Kuo, T.-H.; Tsai, K.-C.; Lin, C.-H.; Yang, A.-S.; Liang, P.-H.; Wong, C.-H. *Bioorg. Med. Chem.* **2008**, *16*, 4652.
16. Zhang, H.-Z.; Zhang, H.; Kemnitzer, W.; Tseng, B.; Cinatl, J., Jr.; Michaelis, M.; Doerr, H. W.; Cai, S. X. *J. Med. Chem.* **2006**, *49*, 1198.
17. Luesch, H.; Hoffman, D.; Hevel, J. M.; Becker, J. E.; Golakoti, T.; Moore, R. E. *J. Org. Chem.* **2003**, *68*, 83.
18. Kuo, C. J.; Chi, Y. H.; Hsu, J. T. A.; Liang, P. H.; Leavitt, S. A.; Freire, E. *Biochem. Biophys. Res. Commun.* **2004**, *318*, 862.
19. Maynard, G. D.; Cheng, H. C.; Kane, J. M.; Staeger, M. A. *Bioorg. Med. Chem. Lett.* **1993**, *4*, 753.
20. Tian, Q.; Nayyar, N. K.; Babu, S.; Chen, L.; Tao, J.; Lee, S.; Tibbetts, A.; Moran, T.; Liou, J.; Guo, M.; Kennedy, T. P. *Tetrahedron Lett.* **2001**, *42*, 6807.
21. Yang, H.; Xie, W.; Xue, X.; Yang, K.; Ma, J.; Liang, W.; Zhao, Q.; Zhou, Z.; Pei, D.; Ziebuhr, J.; Hilgenfeld, R.; Yuen, K. Y.; Wong, L.; Gao, G.; Chen, S.; Chen, Z.; Ma, D.; Bartlam, M.; Rao, Z. *PLoS Biol.* **2005**, *3*, 1742.
22. Briefly, the inhibitors were equilibrated with the enzyme for 10 min before the addition of the substrate to determine their activity respectively. The kinetic reaction was initiated by the addition of the substrate which has a sequence reminiscent of the N-terminal auto-cleavage site of the protease, flanked by the fluorescent groups, dabcyl and edans. The rate of substrate cleavage can be detected by an increase in fluorescence that can be monitored over time. The K_i or the inhibition constant is an indication of the compound as potency.