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Crystal structure of SARS-CoV 3C-like protease with baicalein

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ABSTRACT

The 3C-like protease (Mpro, 3CLpro) plays a key role in the replication process in coronaviruses (CoVs). The Mpro is an essential enzyme mediates CoVs replication and is a promising target for development of antiviral drugs. Until now, baicalein has been shown the specific activity for SARS-CoV Mpro in vitro experiments. In this study, we resolved the SARS-CoV Mpro with baicalein by X-ray diffraction at 2.25 Å (PDB code 7XAX), which provided a structural basis for the research and development of baicalein as an anti-CoVs drug.

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1. Introduction

CoVs are the largest RNA viruses, which have a positive-sense, single-stranded RNA genome [1]. The severe acute respiratory syndrome (SARS) is caused by a novel species of CoVs (SARS-CoV) [2]. The symptoms of SARS-CoV infection are the lower respiratory tract disease including fever, lymphopenia, malaise and mildly elevated serum hepatic enzymes etc. [3,4]. At present, no anti-viral drugs have been found to be beneficial for SARS. The world is facing with a pandemic caused by SARS-CoV-2 now, which is a strain of CoVs spreading rapidly across the globe. But SARS-CoV-2 resulted in less widespread morbidity and mortality compared to SARS-CoV [5]. Although vaccination campaigns are underway globally, the efficacy is reduced because of the variants of concern (VOCs) [6]. Potential risk exists for SARS-CoV-2 VOCs to develop and gain some mutations similar to life threaten SARS happened in 2003. There is a need to fully understand the SARS-CoV and even the whole sub-type of coronavirus.

Mpro is an attractive drug target among CoVs due to its essential role in processing the polyproteins which were translated from the viral RNA [7]. Studies show Mpro is an essential target for inhibition by interaction with Cys145 of its catalytic site [8–10]. The substrate-binding site and active site of the SARS-CoV-2 Mpro crystal structure in the apo state was more flexible than the ligand-binding mode [11,12]. Various complexes of the Mpro structure of SARS-CoV-2 with natural products and novel inhibitors have emerged. The elucidation of the mechanism of shikonin against CoVs laid the foundation for more natural products and traditional Chinese medicines as a source for antivirus drug candidates [13–15].

Flavonoids, found in various plants, are a class of polyphenolic compounds which have a structural unit of 2-phenylchromone [16]. Some flavonoid compounds have antiviral activity against CoVs by inhibiting the activity of Mpro. Studies showed herbacetin, gallo-catechin gallate and rhoifolin can block the enzymatic activity of SARS-CoV Mpro due to S1, S2 and S3 sites [17,18]. Baicalein is an ingredient of Shuanghuanglian, mainly derived from the root of Scutellaria baicalensis. Baicalein shown superior binding effect to Mpro. Previous data showed baicalein was identified as potential noncovalent inhibitors for SARS-CoV-2 Mpro of IC50 values at 0.94 μM [19,20]. In this paper, we resolved the crystal structure of SARS-CoV Mpro-baicalein at 2.25 Å, analyzed and compared with the structure of SARS-CoV-2 Mpro-baicalein. It provides a structural basis and theoretical basis for the drug research and development of treating CoVs in the near future.

2. Materials and methods

2.1. Expression and purification of human SARS-CoV

The codon-optimized cDNAs for the SARS-CoV was synthesized fused with 6_His at the N terminus. Synthesized gene was
subcloned into the pET-28a vector. The expression and purification of protease was performed by a standard method previously described [21].

2.2. X-ray crystallography

Details of the crystallization, data collection, structure solution, and refinement are provided in Table 1. Briefly, all crystallization trials were conducted using a sitting-drop vapor diffusion method at 20 °C. Baicalein was soaked with the crystal of SARS-CoV-2 Mpro with baicalein at 2.20 Å (PDB code 6M2N) (Fig. 1B). The structure of baicalein has been shown in (Fig. 1C). The Mpro of SARS-CoV and SARS-CoV-2 had 96% similarity and 95% amino acid homology [24,25]. A comparison of the sequences shows that twelve residues are different between the Mpro of SARS-CoV and SARS-CoV-2 (Fig. 1D).

Table 1

| Statistics for data processing and model refinement of SARS-CoV Mpro with baicalein. |
|-----------------------------------------------|
| PDB code | 7XAX |
| Synchrotron | SSRF |
| Beam line | BL02U1 |
| Wavelength (Å) | 0.97919 |
| Space group | P1 |
| a, b, c (Å) | 55.51, 60.55, 68.28 |
| α, β, γ (°) | 90.95, 120.71, 108.65 |
| Total reflections | 129,291 |
| Unique reflections | 37,931 |
| Resolution (Å) | 2.25 (2.21-2.25) |
| Mean I/σ (I) | 11.0/1.8 |
| Completeness (%) | 97.7 (96.2) |
| Redundancy | 3.4 (3.5) |
| Resolution (Å) | 66.31-2.25 |
| Rwork/Rfree (%) | 22.92/27.74 |
| Atoms | 4442 |
| Mean temperature factor (Å2) | 46.1 |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 0.973 |
| Ramachandran plot (%) | 97.11 |
| Preferred | 97.11 |
| Allowed | 2.89 |
| outliers | 0 |

The binding modes of SARS-CoV Mpro-baicalein were compared with structure of SARS-CoV-2 Mpro-baicalein. In order to identify the key residues binding to baicalein, we obtained the crystal structure of SARS-CoV Mpro with baicalein at 2.25 Å (PDB code 7XAX) (Fig. 1A). SARS-CoV-2 Mpro with baicalein at 2.20 Å (PDB code 6M2N) (Fig. 1B). The structure of baicalein has been shown in (Fig. 1C). The Mpro of SARS-CoV and SARS-CoV-2 had 96% similarity and 95% amino acid homology [24,25]. A comparison of the sequences shows that twelve residues are different between the Mpro of SARS-CoV and SARS-CoV-2 (Fig. 1D).

3. Results

3.1. Structures of SARS-CoV Mpro-baicalein

The protomer is composed of three domains. Domain I and domain II have an antiparallel β-barrel structure. Domain III contains three α-helices arranged into a largely antiparallel globular cluster, and it is connected to domain II by a long loop region [13](Fig. 2A). From the structure of SARS-CoV Mpro, the S2 and S2′ subsites are critical for substrate binding to the SARS-CoV Mpro [26-28]. SARS-CoV Mpro with baicalein had a Cys145-His41 catalytic dyad in the S2 subsite, which located in a cleft between domain I and domain II [19,29-31](Fig. 2B). SARS-CoV Mpro with baicalein is shown in (Fig. 2C). Three phenolic hydroxyl groups of baicalein form hydrogen bonds with the main chains of Cys145/Ser144/Gly143 as well as the side chains of Asn142/Asn141. The free benzene ring inserted into S2 subsite by making hydrophobic interactions with multiple residues Gln189/Arg188/Glu166/Met165/His164/Asp48/His41. With the aid of an array of direct hydrogen bonds with Cys145/Ser144/Gly143, baicalein served to stabilize the tetrahedral transition state of the proteolytic reaction (Fig. 2D).

3.2. Crystal structure of SARS-CoV Mpro with baicalein

The protomer is composed of three domains. Domain I and domain II have an antiparallel β-barrel structure. Domain III contains five α-helices arranged into a largely antiparallel globular cluster, and it is connected to domain II by a long loop region [13](Fig. 2A). From the structure of SARS-CoV Mpro, the S2 and S2′ subsites are critical for substrate binding to the SARS-CoV Mpro [26-28]. SARS-CoV Mpro with baicalein had a Cys145-His41 catalytic dyad in the S2 subsite, which located in a cleft between domain I and domain II [19,29-31](Fig. 2B). SARS-CoV Mpro with baicalein is shown in (Fig. 2C). Three phenolic hydroxyl groups of baicalein form hydrogen bonds with the main chains of Cys145/Ser144/Gly143 as well as the side chains of Asn142/Asn141. The free benzene ring inserted into S2 subsite by making hydrophobic interactions with multiple residues Gln189/Arg188/Glu166/Met165/His164/Asp48/His41. With the aid of an array of direct hydrogen bonds with Cys145/Ser144/Gly143, baicalein served to stabilize the tetrahedral transition state of the proteolytic reaction (Fig. 2D).
3.3. Comparison of structure of SARS-CoV M\textsuperscript{pro} and SARS-CoV-2 M\textsuperscript{pro} with baicalein

SARS-CoV M\textsuperscript{pro} and SARS-CoV-2 M\textsuperscript{pro} have the same binding mode with baicalein, both have the binding sites include the Cys145-His41 catalytic dyad (Fig. 3A and D). Baicalein in the active site of SARS-CoV M\textsuperscript{pro} and SARS-CoV-2 M\textsuperscript{pro} (Fig. 3B and E). The phenolic hydroxyl group of baicalein in SARS-CoV M\textsuperscript{pro} forms hydrogen bonds with main chains and side chains has been shown (Fig. 3D). The SARS-CoV-2 M\textsuperscript{pro} with baicalein complexes are hydrogen-bonded to the Ser144/Gly143/Leu141 and side chains via the water molecule, where the only carbonyl group established a hydrogen bond with the main chain of Glu166 [32–34]. The free benzene ring also inserted into the S2 subsite by hydrophobic interactions with His41 residue (Fig. 3F). The electrostatic potential surface surrounding the active pocket in SARS-CoVs with baicalein are also shown in Fig. 3. It was revealed that SARS-CoV-apo (Fig. 3G) is different from that of SARS-CoV M\textsuperscript{pro}-baicalein(Fig. 3H). SARS-CoV-2-apo (Fig. 3I) is different from SARS-CoV-2 M\textsuperscript{pro}-baicalein (Fig. 3J).

4. Discussion

Recently, as the cases of SASR-CoV-2 infections, the effective drugs and vaccines has already been found [35]. But there are currently no antivirus drugs approved for the prevention or treatment of highly virulent SARS-CoV infection. M\textsuperscript{pro} plays a key role involved in the replication and transcription of CoVs among the few available targets for anti-CoVs drugs, which has become an essential and relatively mature drug target in anti-CoVs drug research. M\textsuperscript{pro} inhibitors mainly exhibit reversible binding with the amino acid residues in S1, S2, and S4 pockets. The inhibitors contain unsymmetrical aromatic disulphides showing inhibitory activity including the flavonoids compounds [36].

Flavonoid compounds displayed good inhibition toward M\textsuperscript{pro} [37]. Correspondingly, baicalein which belongs to flavonoid compounds has been shown the specific activity for SARS-CoV M\textsuperscript{pro} in vitro experiments. SARS-CoV M\textsuperscript{pro} has a Cys145-His41 catalytic dyad in the cleft between domains I and II, can recognize the eleven cleavage sites of nsp4-16 specifically and exhibit self-hydrolytic cleavage activity [38]. Here, we resolved the crystal structure of...
SARS-CoV Mpro with baicalein that can bind to the substrate pocket between domain I and domain II. Three phenolic hydroxyl groups of baicalein make hydrogen bonds with the main chains of Cys145/Ser144/Gly143 as well as the side chains of Asn142/Asn141, providing a structural basis and theoretical basis for baicalein to inhibit the replication of SARS-CoV.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2022.04.086.

References

[1] Perlman Stanley, A. Dandekar Ajai, Immunopathogenesis of coronavirus infections: implications for SARS, Nat. Rev. Immunol. 5 (2005) 917–927. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7097326.
[2] Joseph S.M. Peiris, Kwok Y. Yuen, Albert D.M. E. Osterhaus, et al., The severe acute respiratory syndrome, N. Engl. J. Med. 349 (2003) 2431–2441. https://pubmed.ncbi.nlm.nih.gov/14681510.
[3] Satija Namita, K. Lal Sunil, The molecular biology of SARS coronavirus, Ann. N. Y. Acad. Sci. 1102 (2007) 26–38. https://pubmed.ncbi.nlm.nih.gov/17470909.
[4] J.S.M. Peiris, Y. Guan, K.Y. Yuen, Severe acute respiratory syndrome, Nat. Med. 10 (2004) S88–S97. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7096017.
[5] K.B. Anand, S. Karade, S. Sen, et al., SARS-CoV-2SARS-CoV-2: camazotz’s curse, Med. J. Armed Forces India 76 (2020) 136–141. https://pubmed.ncbi.nlm.nih.gov/32341622.
[6] Shapira Tirosh, Monreal I. Abrey, P. Dion Sébastien, et al., A TMPRESS inhibitor acts as a pan-SARS-CoV-2 prophylactic and therapeutic, Nature (2022). Mar 28, https://pubmed.ncbi.nlm.nih.gov/35344983.
[7] Linlin Zhang, Daizong Lin, Xinyuanyuan Sun, et al., Crystal structure of SARS-CoV-2 main protease provides a basis for design of improved α-ketoamide...
inhibitors, Science (2020) 368. https://pubmed.ncbi.nlm.nih.gov/32198291.

[8] Kidera Akinton, Moritsugu Keh, Eikemoto Toru, et al., Allosteric regulation of 3CL
protease of SARS-CoV-2 and SARS-CoV observed in the crystal structure
ensemble, Mol. Biol. 433 (2021), 167324. https://pubmed.ncbi.nlm.nih.gov/
34717972.

[9] J. Niesor Eric, Boivin Guy, Rheuma Eric, et al., Inhibition of the 3CL protease
and SARS-CoV-2 replication by dalfetrapib, ACS Omega 6 (2021)
16584–16591. https://pubmed.ncbi.nlm.nih.gov/34235330.

[10] Zhenming Jin, Yao Zhao, Yuan Sun et al Structural basis for the inhibition of
SARS-CoV-2 main protease by antineoplastic drug carmofur, Nat. Struct.
Mol. Biol. 27, 520–532. https://pubmed.ncbi.nlm.nih.gov/32383072.

[11] Zhenming Jin, Xiaoyu Du, Yechun Xu, et al., Structure of M(pro) from COVID-
19 virus and discovery of its inhibitors, Nature 582 (2020) 289–293. https://
pubmed.ncbi.nlm.nih.gov/3272481.

[12] Li Jian, Xuelan Zhou, Yan Zhang, et al., Crystal structure of SARS-CoV-2 main
protease in the apo state, Sci. China Life Sci. 64 (2021) 656–659. https://pubmed.ncbi.nlm.nih.gov/32880863.

[13] Riddhidiv Banerjee, Lalith Perera, L.M. Viranga Tillekeratne, Potential SARS-
CoV-2 main protease inhibitors, Drug Discov. Today (2021 Mar). https://
pubmed.ncbi.nlm.nih.gov/33309533.

[14] Li Juan, Xueling Zhou, Yan Zhang, et al., Crystal structure of SARS-CoV-2 main
protease in complex with the natural product inhibitor shikonin illuminates a
unique binding mode, Sci. Bull. 66 (2021) 661–663. https://pubmed.ncbi.nlm.nih.gov/
31362353.

[15] Yuting Zhang, Hongxia Gao, Xiaohui Hu, et al., Structure-Based discovery and
structural basis of a novel broad-spectrum natural product against the main
protease of coronavirus, J. Virol. 96 (2022), e0125321. https://pubmed.ncbi.nlm.nih.gov/34586857.

[16] Kangmei Wen, Xiaohuan Fang, Junli Yang, et al., Recent research on flavono-
oids and their biomedical applications, Curr. Med. Chem. 28 (2021)
1042–1066. https://pubmed.ncbi.nlm.nih.gov/32660393.

[17] Seri Jo, Suwon Kim, Dae Yong Kim, et al., Flavonoids with inhibitory activity
in vitro of Shuanghuanglian preparations and bioactive ingredients, Acta
Pharmacol. Sin. 41 (2020) 1167–1177. https://pubmed.ncbi.nlm.nih.gov/32717471.

[18] Dafu Zhu, Haixia Su, Borkotoky Subhomoo, Dubei Vikash Kumar, Targeting two
unique binding mode, Sci. Bull. 66 (2021) 656–659. https://pubmed.ncbi.nlm.nih.gov/32880863.

[19] J. Feng, D. Li, J. Zhang et al. Biochemical and Biophysical Research Communications 611 (2022) 190–194.

[20] Dafu Zhu, Haixia Su, Changqiang Ke, et al., Ef
fects of main protease inhibitors, Science (2020) 368. https://pubmed.ncbi.nlm.nih.gov/32198291.

[21] Kanchan Anand, Ziebuhr John, Wadhwani Parvesh, et al., Coronavirus main
protease inhibitors, J. Proteome Res. 19 (2020) 4706–4717. https://pubmed.ncbi.nlm.nih.gov/32960661.

[22] W. Dai, B. Zhang, X.M. Jiang, et al., Structure-based design of antiviral drug
candidates targeting the SARS-CoV-2 main protease, Science 368 (2020)
1331–1335. https://pubmed.ncbi.nlm.nih.gov/32321856.

[23] A.S. Achutha, V.L. Pushpa, Suchitra Surendran, Theoretical insights into the
anti-SARS-CoV-2 activity of chloroquine and its analogs and in silico screening
of main protease inhibitors, J. Proteome Res. 19 (2020) 4706–4717. https://pubmed.ncbi.nlm.nih.gov/32960661.

[24]doi.org/10.1177/20416410221112076

[25] Mohanraj Dinesh, Whitelegg Alison, Trilogy of COVID-19: infection, vaccina-
tion, and immunosuppression, Int. Arch. Allergy Immunol. (2022) 1
934. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7409838.

[26] Yao Zhao, Yan Zhu, Xiang Liu, et al., Structural basis for replicase polyprotein
cleavage and substrate specificity of main protease from SARS-CoV-2, Proc.
Natl. Acad. Sci. U. S. A. 119 (2022), e211742119. https://pubmed.ncbi.nlm.nih.gov/35380892.

[27] Xiaoyu Xue, Haitao Yang, Wei Shen, et al., Production of authentic SARS-CoV
M(pro) with enhanced activity: application as a novel tag-cleavage endo-
pseudoesterase for protein overproduction, J. Mol. Biol. 366 (2007) 965–975.
https://pubmed.ncbi.nlm.nih.gov/17189929.

[28] Kidera Akinori, Moritsugu Kei, Ekimoto Toru, et al., Allosteric regulation of 3CL
protease of SARS-CoV-2 and SARS-CoV observed in the crystal structure
ensemble, Mol. Biol. 433 (2021), 167324. https://pubmed.ncbi.nlm.nih.gov/34717972.

[29] Seri Jo, Suwon Kim, Dong Hae Shin, Mi-Sun Kim, Inhibition of SARS-CoV 3CL
protease in the apo state, Sci. China Life Sci. 64 (2021) 656–659. https://pubmed.ncbi.nlm.nih.gov/32880863.

[30] Thi Thanh Hanh Nguyen, Hye-Jin Woo, Hee-Kyoung Kang, et al., Structural basis for replicate polyprotein
cleavage and substrate specificity of main protease from SARS-CoV-2, Proc.
Natl. Acad. Sci. U. S. A. 119 (2022), e211742119. https://pubmed.ncbi.nlm.nih.gov/35380892.

[31] Xuelan Zhou, Fanglin Zhong, Lin Cheng, et al., Structure of SARS-CoV-2 main
protease at different pH values, Acta Crystallogr. F Struct. Biol. Commun.
(2020 Oct). https://pubmed.ncbi.nlm.nih.gov/32350267.

[32] Haixia Su, Sheng Yao, Wen-feng Zhao, et al., Anti-SARS-CoV-2 activities
in vitro of Shuanghuanglian preparations and bioactive ingredients, Acta
Pharmacol. Sin. 41 (2020) 1167–1177. https://pubmed.ncbi.nlm.nih.gov/32717471.