REPLY TO BEHNAM AND KLEIN:

Potential role of the His-tag in C-terminal His-tagged SARS-CoV-2 main protease

Zhe Li^a, Runduo Liu^a, Chang-Guo Zhan^{b,c,1}, Xin Wang^{d,e,1}, and Hai-Bin Luo^{a,f,1}

Behnam and Klein (1) carried out *in vitro* assays on two severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease (M^{pro}) inhibitors, including atazanavir reported by us (2), using an M^{pro} protein with C-terminal His-tag under various assay conditions, and find that both the Michaelis–Menten constant (K_m) and inhibitory activity (IC_{50}) are sensitive to the assay conditions. It is interesting to examine whether the assay conditions significantly affect the activities of an enzyme and its inhibitors. On the other hand, Behnam and Klein (1) do not account for possible effects of the C-terminal His-tag on the K_m and IC_{50} , despite the fact that the tag-free M^{pro} (without any N- or C-terminal tag) was used in our reported assays (2), whereas the C-terminal His-tagged M^{pro} was used in their assays (1).

As discussed recently (3), both the N and C termini of M^{pro} are close to the active-site cavity, according to available X-ray crystal structures. Thus, a His-tag on the N or C terminus could interfere in M^{pro} binding with a ligand (substrate or inhibitor). Depicted in Fig. 1 is a modeled structure of the C-terminal His-tagged M^{pro} protein. The modeling started from an available X-ray crystal structure (Protein Data Bank ID code 6M2N) (4). Notably, the His-tag was outside of the M^{pro} active site in the initial structure (Fig. 1A) built by using the Prime module (5, 6) of the Schrodinger 2013 software. After 50-ns molecular dynamics (MD) simulations, the His-tag

moved to the M^{pro} active site (Fig. 1B), suggesting that the His-tag added to the C terminus may compete with the substrate/inhibitor for binding. For this reason, the C-terminal His-tag could lower the binding affinity of a given ligand (substrate or inhibitor) with M^{pro} . In fact, it has been reported that $K_m = 1.41 \mu M$ for the tag-free M^{pro} (3) and $K_m = 28.2 \mu M$ for the C-terminal His-tagged M^{pro} (7), suggesting that the C-terminal His-tag may lower the binding affinity with the substrate by ~20-fold. The remarkable difference explains why the IC_{50} values determined by Behnam and Klein (1) and Ma and Wang (8) using C-terminal His-tagged M^{pro} are significantly larger than the corresponding IC_{50} values determined by us using the tag-free M^{pro} (2). It is also possible that the interaction between the His-tag and the active site is affected by the assay conditions. Hence, the results described by Behnam and Klein (1) are not surprising based on the structural information about the His-tagged M^{pro} protein discussed above.

Acknowledgments

We cordially acknowledge the National Key R&D Program of China (Grant 2017YFB0202600), National Natural Science Foundation of China (Grants 81903542, 81522041, and 21877134), Science Foundation of Guangdong Province (Grant 2020A111128007), NSF (Grant CHE-1111761), Taishan Scholars Program (Grant tsqn201909170), and Innovative Leader of Qingdao Program (Grant 19-3-2-26-zhc).

^aGuangdong Provincial Key Laboratory of New Drug Design and Evaluation, School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, People's Republic of China; ^bMolecular Modeling and Biopharmaceutical Center, College of Pharmacy, University of Kentucky, Lexington, KY 40536; ^cDepartment of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, KY 40536; ^dCenter for Innovative Marine Drug Screening & Evaluation, School of Medicine and Pharmacy, Ocean University of China, Qingdao 266100, China; ^eMarine Biomedical Research Institute of Qingdao, Qingdao 266100, China; and ^fKey Laboratory of Tropical Biological Resources of Ministry of Education, School of Life and Pharmaceutical Sciences, Hainan University, Haikou 570228, China

Author contributions: C.-G.Z. designed research; Z.L., R.L., and X.W. performed research; Z.L., R.L., C.-G.Z., X.W., and H.-B.L. analyzed data; and C.-G.Z. and H.-B.L. wrote the paper.

The authors declare no competing interest.

This open access article is distributed under [Creative Commons Attribution License 4.0 \(CC BY\)](https://creativecommons.org/licenses/by/4.0/).

¹To whom correspondence may be addressed. Email: zhan@uky.edu, wx8399@ouc.edu.cn, or luohb77@mail.sysu.edu.cn.

Published September 2, 2021.

Fig. 1. Structure of the M^{Pro} protein with His-tag (LEHHHHHH) added to the C terminus. (A) Initial structure of the C-terminal His-tagged M^{Pro} protein with a ligand bound in the active site. (B) The last snapshot of the MD-simulated C-terminal His-tagged M^{Pro} protein structure (in which the ligand in the active site was removed before the MD simulation) in a TIP3P water box. The system, after energy minimization, was first heated to 300 K in a 50-ps MD simulation (with NVT) followed by 50-ps MD simulation [with NPT, i.e., the constant amount of substance (N), pressure (P), and temperature (T)] for equilibration. Finally, a 50-ns MD simulation (with NPT) was performed without any restraints for the system.

- 1 M. Behnam, C. Klein, Inhibitor potency and assay conditions: A case study on SARS-CoV-2 main protease. *Proc. Natl. Acad. Sci. U.S.A.*, 10.1073/pnas.2106095118 (2021).
- 2 Z. Li *et al.*, Identify potent SARS-CoV-2 main protease inhibitors via accelerated free energy perturbation-based virtual screening of existing drugs. *Proc. Natl. Acad. Sci. U.S.A.* **117**, 27381–27387 (2020).
- 3 Z. Li *et al.*, Reply to Ma and Wang: Reliability of various in vitro activity assays on SARS-CoV-2 main protease inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2024937118 (2021).
- 4 H. X. Su *et al.*, Anti-SARS-CoV-2 activities in vitro of Shuanghuanglian preparations and bioactive ingredients. *Acta Pharmacol. Sin.* **41**, 1167–1177 (2020).
- 5 M. P. Jacobson *et al.*, A hierarchical approach to all-atom protein loop prediction. *Proteins* **55**, 351–367 (2004).
- 6 M. P. Jacobson, R. A. Friesner, Z. Xiang, B. Honig, On the role of the crystal environment in determining protein side-chain conformations. *J. Mol. Biol.* **320**, 597–608 (2002).
- 7 C. Ma *et al.*, Boceprevir, GC-376, and calpain inhibitors II, XII inhibit SARS-CoV-2 viral replication by targeting the viral main protease. *Cell Res.* **30**, 678–692 (2020).
- 8 C. Ma, J. Wang, Dipyrindamole, chloroquine, montelukast sodium, candesartan, oxytetracycline, and atazanavir are not SARS-CoV-2 main protease inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **118**, e2024420118 (2021).