The Main Protease of SARS-CoV-2 and its inhibitors

Subjects: Biochemistry & Molecular Biology

Contributor: Shaza S. Issa, Sofia V. Sokornova, Roman R. Zhidkin, Tatiana V. Matveeva

Mpro, also known as 3-chymotrypsin-like protease (3CLpro), is a 33.8 kDa, three-domain cysteine protease, essential for proteolytic maturation and viral replication of coronaviruses. This enzyme is an excellent target for a potential drug, as it is essential for viral replication and has no closely related homologues in humans, making its inhibitors unlikely to be toxic.

Keywords: SARS-CoV-2 ; COVID-19 ; main protease ; phytochemicals ; potential inhibitor

1. Introduction

In late December 2019, a viral pneumonia outbreak emerged in Wuhan, China caused by a new strain of coronavirus that was identified as SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) [1][2][3][4]. Soon after, the outbreak was declared a public health emergency of international concern by the WHO, and later in March it was declared a global pandemic, named COVID-19 (coronavirus disease 2019) [5][6]. The virus spread vastly all around the world, causing, to date, more than 500 million confirmed cases and millions of deaths in one of the worst global health crises in modern history [7]. Although many vaccines have been approved worldwide, so far there is still no treatment for COVID-19, and only supportive and preventive measures are being applied to reduce the disease's complications [8][9][10]. Moreover, in trying to adapt to changing environments, the virus has developed a number of mutations that could strongly affect its transmissibility and infectivity. These mutations are also prone to increasing and spreading worldwide, due to natural selection [11][12][13][14].

2. SARS-CoV-2 Structure and the Main Protease of SARS-CoV-2 as a Potential Protein Target

Similar to other viruses in the Coronaviridae family, SARS-CoV-2 has a single-stranded, positive-sense RNA (+ssRNA) genome of approximately 29 kb [15][16]. The viral RNA is composed of more than six open reading frames (ORFs), the first one of which (ORF1) serves as a template for producing two polyproteins essential for viral replication and transcription: pp1a and pp1ab [17][18]. These two polyproteins undergo extensive processing by the viral main protease (Mpro) and another protease known as papain-like protease (PLP), producing 16 nonstructural proteins (NSPs) [3][19]. The other ORFs encode at least four main structural proteins: the spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins [17][20][21].

Mpro, also known as 3-chymotrypsin-like protease (3CLpro), is a 33.8 kDa, three-domain cysteine protease, essential for proteolytic maturation and viral replication [9][18][22][23]. Mpro was found to be conserved among coronaviruses (CoVs), along with some common features of its substrates in different CoVs [18][24]. In addition to its vital role in the SARS-CoV-2 life cycle, the absence of any closely related human homologous Mpro makes it an ideal protein target for potential antiviral drugs, as its inhibitors are unlikely to be toxic in humans [25]. Furthermore, vaccines, as researchers have learned from previous viruses, can represent a selection pressure resulting in the evolution of novel resistant viral mutants, which again highlights SARS-CoV-2 Mpro as a good drug target, as it is less subject to such selection pressure caused by vaccines targeting the viral spike protein [26][27].

3. Standard Approaches That Could Be Applied in the Search for Mpro Potential Inhibitors

3.1. Virtual In Silico Screening: (The Data-Driven Approach)

Enabled by the development of bioinformatics tools along with eased access to protein databases, virtual screening has proven to be a fundamental tool in drug design and drug repurposing research [28][29]. In the virtual screening approach, automated molecular docking tools are usually used to predict the best possible variant for binding one molecule to another, considering the best orientation with the best binding affinity [30]. These tools enable the screening of large

numbers of candidates against a specific studied target, at a very low cost [31]. Virtual screening is a data-driven approach that can be either target-based, where a library of candidate ligands is docked against the target and analyzed, or ligand-based, where a similarity search or a machine learning strategy can be applied [32][33][34][35].

Since the beginning of the COVID-19 pandemic, a large number of studies around the world have used this approach to search for potential inhibitors of SARS-CoV-2 [22][36][37][38]. Joshi R.S. et al. used this approach in their study conducted in 2020 to scan over 7000 compounds from different origins against SARS-CoV-2 Mpro [39]. Another study conducted by Tallei E.T. et al. in 2020 used this approach to evaluate the potency of plant-derived bioactive compounds against Mpro, resulting in the identification of pectolinarin, hesperidin, nabiximols, rhoifolin, and epigallocatechin gallate as potential antiviral phytochemicals [40]. Research by Tahir Ul Qamar et al. also resulted in the identification of 5,7,3′,4′-Tetrahydroxy-2′-(3,3-dimethylallyl) isoflavone, amaranthin, licoleafol, calceolarioside B, and methyl rosmarinate as potential inhibitors of the target, using this approach [41]. Khaerunnisa S. et al. extended the list with kaempferol, quercetin, luteolin-7-glucoside, demetoxycurcumine, naringenin, apigenin-7-glucoside, oleuropein, catechin, curcumin, zingerol, gingerol, and allicin [42]. Essential oils have also shown their effectiveness against SARS-CoV-2 Mpro in silico [43][44][45]. Therefore, using this approach, multiple natural compounds have been identified as strong binders of SARS-CoV-2 Mpro, and some of them were also identified as multi-target inhibitors that could be applied in COVID-19 management approaches [36][37][38][39][40][41][42][43][44][45].

3.2. The Classical Approach of High-Throughput Screening (HTS)

High-throughput screening (HTS) is a method for automated testing of thousands to millions of compounds for their biological activity against specific targets on model systems [46]. The development of robotics, laboratory equipment, laboratory methods, and software for the control of sample preparation, incubation, results detection, and data processing has allowed the HTS approach to be used to quickly search for lead compounds. Therefore, it is possible to quickly and inexpensively test large libraries of chemical compounds for their biochemical activity [47].

In practice, HTS is implemented in the form of a large number of miniature in vitro assays to identify molecules that can modulate the activity of a biological target. These reactions are run in 96-well, 386-well, or 1536-well plates [48]. Most often, the results of such biochemical analyses are obtained using various fluorescence detection methods [49], for example, direct measurement of fluorescence, fluorescence polarization, fluorescence resonance energy transfer (FRET), fluorescence quenching energy transfer (QFRET), or time-resolved fluorescence [46].

Since the early stage of COVID-19 pandemic, a large number of HTS assays have been developed worldwide to screen huge libraries of either previously approved drugs or potential inhibitors against SARS-CoV-2 [50][51][52][53]. Using this approach in their study to screen a library of 10,755 potential inhibitory compounds against SARS-CoV-2 Mpro, along with drugs previously approved for other viruses, Zhu W. et al. identified 23 potential inhibitors with different half-maximal inhibitory concentration values (IC_{50}), the efficacy of 7 of which was confirmed in a later cytopathic effect assay [54]. Given the high safety level required of laboratories studying and manipulating the live SARS-CoV-2 virus (BSL-3 laboratories), Zhang, Q.Y. et al. proposed a new HTS assay to enable potential antiviral testing in a BSL-2 research facility, where they constructed a reporter replicon of the virus using *Renilla* luciferase (Rluc) reporter gene and validated it later using hit natural compounds [52]. Froggatt H. M. et al. also developed a fluorescence-based HTS assay using a protein derived from green fluorescent protein (GFP) to serve as a target for SARS-CoV-2 Mpro, and hence a reporter for the enzyme's inhibition and activation, enabling rapid screening of libraries and identification of lead compounds [55]. A further improvement of HTS can be achieved by combining it with the previous in silico approach to yield an ultra-high-throughput virtual screening approach, where huge libraries can be tested against multiple viral targets efficiently and rapidly [56]. Gorgulla C. et al. conducted a study to search for SARS-CoV-2 inhibitors using this large-scale HTS screening approach and were able to screen over one billion candidate molecules against 40 different target sites on 17 potential targets, both in the virus and the host [56]. Although the results were obtained from computational data and have not all been tested with experimental analyses yet, this filtration of candidates could narrow down research targets for later more detailed and efficient analyses.

3.3. Antiviral Activity Cell-Based Assays

Cell-based assays offer an advantage over virtual or biochemical screening assays, as they provide a whole physiological environment, reflecting the complexity of a living system rather than focusing on a specific isolated target and thereby enabling a more accurate evaluation of the biological activity and potential toxicity of screened compounds [57][58][59]. Due to practical considerations, it is important to develop and test drug compounds that exhibit inhibitory activity at various stages of the virus life cycle. Therefore, test systems have been developed to evaluate the effectiveness of inhibitors of entry, uncoating, replication, assembly (in which viral proteases are active), and maturation of viruses [60]. However, the

whole variety of such systems can be reduced to two main mechanisms for their implementation: cytopathic and reporter mechanisms [61]. In the first case, the activity of antiviral agents is assessed by reducing the formation of plaques due to the accumulation of coloring or luminescent agents in living cells [62][63]. In the second, viruses and cells with report inserts are used, and the activity of inhibitors helps in reducing the expression of the reporter protein [64].

Since work with a live virus is accompanied by significant organizational restrictions, approaches have been developed for evaluating the effectiveness of antiviral agents that model various stages of the life cycle of viruses in cells of HeLa [65], *Escherichia coli* [66], and *Saccharomyces cerevisiae* [61]. Moreover, cell-based assays are nowadays increasingly integrated into HTS assays to accomplish rapid screens in a relevant physiological environment [58].

Several recent studies have used antiviral activity cell-based assays, either after or combined with the previously described approaches, to investigate previously approved drugs and herbal medicines for their potential inhibition potency against SARS-CoV-2 [9][67][68][69][70]. Applying this methodology followed by a further in vivo validation, Jan J.T. et al. screened a 3000-candidate library of both pharmaceuticals and herbal medicines to test their effectiveness against SARS-CoV-2 Mpro and RNA polymerase and proposed multiple herbal extracts as potential herbal inhibitors against the targeted viral enzymes [68]. Another recent study conducted by Qiao J. et al. also applied this approach to investigate 32 different inhibitors against SARS-CoV-2 Mpro, 6 of which were found to have a high inhibition potency and were used to select candidates for further in vivo investigation [71].

The phased use of these three approaches makes it possible to identify those that exhibit the targeted therapeutic activity from the variety of known plant secondary metabolites. Among these compounds, there may be those that have not previously exhibited such properties. Furthermore, compounds for which hypothetical activity is found can be quickly tested for their effectiveness on cell-free and then on cellular systems. Such a screening strategy has shown to be effective in the search for inhibitors of SARS-CoV-2 Mpro, which indicates its potential in the search for drugs against new pathogens [9].

References

1. Lee, P.I.; Hsueh, P.R. Emerging threats from zoonotic coronaviruses-from SARS and MERS to 2019-nCoV. J. Microbiol. Immunol. Infect. 2020, 53, 365–367.
2. Li, Q.; Guan, X.; Wu, P.; Wang, X.; Zhou, L.; Tong, Y.; Feng, Z. Early Transmission Dynamics in Wuhan, China, of Novel Coronavirus-Infected Pneumonia. N. Engl. J. Med. 2020, 382, 1199–1207.
3. Wu, F.; Zhao, S.; Yu, B.; Chen, Y.; Wang, W.; Song, Z.; Hu, Y.; Tao, Z.; Tian, J.; Pei, Y.; et al. A new coronavirus associated with human respiratory disease in China. Nature 2020, 579, 265–269.
4. Zhu, N.; Zhang, D.; Wang, W.; Li, X.; Yang, B.; Song, J.; Zhao, X.; Huang, B.; Shi, W.; Lu, R.; et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. N. Engl. J. Med. 2020, 382, 727–733.
5. Rodríguez-Morales, A.J.; MacGregor, K.; Kanagarajah, S.; Patel, D.; Schlagenhauf, P. Going global—Travel and the 2019 novel coronavirus. Travel Med. Infect. Dis. 2020, 33, 101578.
6. Silveira, M.M.; Moreira, G.M.S.G.; Mendonça, M. DNA vaccines against COVID-19: Perspectives and challenges. Life Sci. 2020, 267, 118919.
7. WHO. COVID-19 Dashboard. 2022. Available online: https://covid19.who.int/ (accessed on 5 May 2022).
8. Chen, P.; Shi, X.; He, W.; Zhong, G.; Tang, Y.; Wang, H.; Zhang, P. mRNA vaccine-a desirable therapeutic strategy for surmounting COVID-19 pandemic. Hum. Vaccines Immunother. 2022, 18, 2040330.
9. Jin, Z.; Du, X.; Xu, Y.; Deng, Y.; Liu, M.; Zhao, Y.; Yang, H. Structure of Mpro from SARS-CoV-2 and discovery of its inhibitors. Nature 2020, 582, 289–293.
10. Yamamoto, K.Z.; Yasuo, N.; Sekijima, M. Screening for Inhibitors of Main Protease in SARS-CoV-2: In Silico and In Vitro Approach Avoiding Peptidyl Secondary Amides. J. Chem. Inf. Model. 2022, 62, 350–358.
11. Hossain, M.J.; Rabaan, A.A.; Mutair, A.A.; Alhumaid, S.; Emran, T.B.; Saikumar, G.; Dhama, K. Strategies to tackle SARS-CoV-2 Mu, a newly classified variant of interest likely to resist currently available COVID-19 vaccines. Hum. Vaccin. Immunother. 2022, 18, 2027197.
12. Konings, F.; Perkins, M.D.; Kuhn, J.H.; Pallen, M.J.; Alm, E.J.; Archer, B.N.; Van Kerkhove, M.D. SARS-CoV-2 Variants of Interest and Concern naming scheme conducive for global discourse. Nat. Microbiol. 2021, 6, 821–823.

13. Sharun, K.; Tiwari, R.; Dhama, K.; Emran, T.B.; Rabaan, A.A.; Al Mutair, A. Emerging SARS-CoV-2 variants: Impact on vaccine efficacy and neutralizing antibodies. Hum. Vaccin. Immunother. 2021, 17, 3491–3494.

14. Van Egeren, D.; Novokhodko, A.; Stoddard, M.; Tran, U.; Zetter, B.; Rogers, M.; Chakravarty, A. Risk of rapid evolution ary escape from biomedical interventions targeting SARS-CoV-2 spike protein. PLoS ONE 2021, 16, e0250780.

15. Brant, A.C.; Tian, W.; Majerciak, V.; Yang, W.; Zheng, Z.M. SARS-CoV-2: From its discovery to genome structure, trans cription, and replication. Cell Biosci. 2021, 11, 136.

16. Zhou, P.; Yang, X.-L.; Wang, X.-G.; Hu, B.; Zhang, L.; Zhang, W.; Si, H.-R.; Zhu, Y.; Li, B.; Huang, C.-L.; et al. A pneum onia outbreak associated with a new coronavirus of probable bat origin. Nature 2020, 579, 270–273.

17. Chen, Y.; Liu, Q.; Guo, D. Emerging coronaviruses: Genome structure, replication, and pathogenesis. J. Med. Virol. 202 0, 92, 418–423.

18. Dai, W.; Zhang, B.; Jiang, X.M.; Su, H.; Li, J.; Zhao, Y.; Liu, H. Structure-based design of antiviral drug candidates targe ting the SARS-CoV-2 main protease. Science 2020, 368, 1331–1335.

19. Ramajayam, R.; Tan, K.-P.; Liang, P.-H. Recent development of 3C and 3CL protease inhibitors for anti-coronavirus an d anti-picornavirus drug discovery. Biochem. Soc. Trans. 2011, 39, 1371–1375.

20. Cao, Y.; Yang, R.; Lee, I.; Zhang, W.; Sun, J.; Wang, W.; Meng, X. Characterization of the SARS-CoV-2 E Protein: Seq uence, Structure, Viroporin, and Inhibitors. Protein Sci. 2021, 30, 1114–1130.

21. Kim, D.; Lee, J.Y.; Yang, J.S.; Kim, J.W.; Kim, V.N.; Chang, H. The Architecture of SARS-CoV-2 Transcriptome. Cell 20 20, 181, 914–921.e10.

22. Reiner, Ž.; Hatamipour, M.; Banach, M.; Pirro, M.; Al-Rasadi, K.; Jamialahmadi, T.; Radenkovic, D.; Montecucco, F.; Sa hebkar, A. Statins and the COVID-19 main protease: In silico evidence on direct interaction. Arch. Med. Sci. 2020, 16, 4 90–496.

23. Saravanan, K.M.; Zhang, H.; Senthil, R.; Vijayakumar, K.K.; Sounderrajan, V.; Wei, Y.; Shakila, H. Structural basis for th e inhibition of SARS-CoV2 main protease by Indian medicinal plant-derived antiviral compounds. J. Biomol. Struct. Dy n. 2022, 40, 1970–1978.

24. Yang, H.; Xie, W.; Xue, X.; Yang, K.; Ma, J.; Liang, W.; Rao, Z. Design of wide-spectrum inhibitors targeting coronavirus main proteases. PLoS Biol. 2005, 3, e324.

25. Rocha, R.E.; Chaves, E.J.; Fischer, P.H.; Costa, L.S.; Grillo, I.B.; da Cruz, L.E.; de Lima, L.H. A higher flexibility at the SARS-CoV-2 main protease active site compared to SARS-CoV and its potentialities for new inhibitor virtual screening targeting multi-conformers. J. Biomol. Struct. Dyn. 2021, 10, 1–21.

26. Luo, R.; Delaunay-Moisan, A.; Timmis, K.; Danchin, A. SARS-CoV-2 biology and variants: Anticipation of viral evolution and what needs to be done. Environ. Microbiol. 2021, 23, 2339–2363.

27. Mótyán, J.A.; Mahdi, M.; Hoffka, G.; Tőzsér, J. Potential Resistance of SARS-CoV-2 Main Protease (Mpro) against Prot ease Inhibitors: Lessons Learned from HIV-1 Protease. Int. J. Mol. Sci. 2022, 23, 3507.

28. Mohan, A.; Rendine, N.; Mohammed MK, S.; Jeeva, A.; Ji, H.F.; Talluri, V.R. Structure-based virtual screening, in silico docking, ADME properties prediction and molecular dynamics studies for the identification of potential inhibitors against SARS-CoV-2 M(pro). Mol. Divers. 2021, 26, 1645–1661.

29. Morris, G.M.; Lim-Wilby, M. Molecular docking. Methods Mol. Biol. 2008, 443, 365–382.

30. Sari, S.; Avci, A.; Aslan, E.K. In silico Repurposing of Drugs for pan-HDAC and pan-SIRT Inhibitors: Consensus Structu re-based Virtual Screening and Pharmacophore Modeling Investigations. Turk. J. Pharm. Sci. 2021, 18, 730–737.

31. Pinzi, L.; Rastelli, G. Molecular Docking: Shifting Paradigms in Drug Discovery. Int. J. Mol. Sci. 2019, 20, 4331.

32. Bonanno, E.; Ebejer, J.-P. Applying Machine Learning to Ultrafast Shape Recognition in Ligand-Based Virtual Screenin g. Front. Pharmacol. 2020, 10, 1675.

33. Muegge, I.; Mukherjee, P. An overview of molecular fingerprint similarity search in virtual screening. Expert Opin. Drug Discov. 2015, 11, 137–148.

34. Pinto, G.P.; Hendrikse, N.M.; Stourac, J.; Damborsky, J.; Bednar, D. Virtual screening of potential anticancer drugs bas ed on microbial products. In Seminars in Cancer Biology; Academic Press: Cambridge, MA, USA, 2021.

35. Pinto, G.P.; Vavra, O.; Filipovic, J.; Stourac, J.; Bednar, D.; Damborsky, J. Fast Screening of Inhibitor Binding/Unbinding Using Novel Software Tool CaverDock. Front. Chem. 2019, 7, 709.

36. Islam, M.T.; Sarkar, C.; El-Kersh, D.M.; Jamaddar, S.; Uddin, S.J.; Shilpi, J.A.; Mubarak, M.S. Natural products and thei r derivatives against coronavirus: A review of the non-clinical and pre-clinical data. Phytother. Res. 2020, 34, 2471–249 2.

37. Olubiyi, O.O.; Olagunju, M.; Keutmann, M.; Loschwitz, J.; Strodel, B. High Throughput Virtual Screening to Discover Inhibitors of the Main Protease of the Coronavirus SARS-CoV-2. Molecules 2020, 25, 3193.

38. Zhang, D.-H.; Wu, K.-L.; Zhang, X.; Deng, S.-Q.; Peng, B. In silico screening of Chinese herbal medicines with the potential to directly inhibit 2019 novel coronavirus. J. Integr. Med. 2020, 18, 152–158.

39. Joshi, R.S.; Jagdale, S.S.; Bansode, S.B.; Shankar, S.S.; Tellis, M.B.; Pandya, V.K.; Kulkarni, M.J. Discovery of potential multi-target-directed ligands by targeting host-specific SARS-CoV-2 structurally conserved main protease. J. Biomol. Struct. Dyn. 2021, 39, 3099–3114.

40. Tallei, T.E.; Tumilaar, S.G.; Niode, N.J.; Kepel, B.J.; Idroes, R.; Effendi, Y.; Emran, T.B. Potential of Plant Bioactive Compounds as SARS-CoV-2 Main Protease (Mpro) and Spike (S) Glycoprotein Inhibitors: A Molecular Docking Study. Scientifica 2020, 2020, 6307457.

41. Ul Qamar, M.T.; Alqahtani, S.M.; Alamri, M.A.; Chen, L.L. Structural basis of SARS-CoV-2 3CL(pro) and anti-COVID-19 drug discovery from medicinal plants. J. Pharm. Anal. 2020, 10, 313–319.

42. Khaerunnisa, S.; Kurniawan, H.; Awaluddin, R.; Suhartati, S.; Soetjipto, S. Potential Inhibitor of COVID-19 Main Protease (Mpro) from Several Medicinal Plant Compounds by Molecular Docking Study. Preprints 2020, 2020, 2020030226.

43. da Silva JK, R.; Figueiredo PL, B.; Byler, K.G.; Setzer, W.N. Essential Oils as Antiviral Agents, Potential of Essential Oils to Treat SARS-CoV-2 Infection: An In-Silico Investigation. Int. J. Mol. Sci. 2020, 21, 3426.

44. Mohamed, M.E.; Tawfeek, N.; Elbaramawi, S.S.; Fikry, E. Agathis robusta Bark Essential Oil Effectiveness against COVID-19: Chemical Composition, In Silico and In Vitro Approaches. Plants 2022, 11, 663.

45. My, T.T.A.; Loan, H.T.P.; Hai, N.T.T.; Hieu, L.T.; Hoa, T.T.; Thuy, B.T.P.; Quang, D.T.; Triet, N.T.; Van Anh, T.T.; Dieu, N.T.X.; et al. Evaluation of the Inhibitory Activities of COVID-19 of Melaleuca cajuputi Oil Using Docking Simulation. ChemistrySelect 2020, 5, 6312–6320.

46. Attene-Ramos, M.S.; Austin, C.P.; Xia, M. High Throughput Screening. In Encyclopedia of Toxicology, 3rd ed.; Wexler, P., Ed.; Academic Press: Oxford, UK, 2014; pp. 916–917.

47. Ismail, F.M.; Nahar, L.; Sarker, S.D. High-Throughput Screening of Phytochemicals: Application of Computational Methods. Comput. Phytochem. 2018, 165–192.

48. Lundblad, R.L. Drug Design. In Encyclopedia of Cell Biology; Elsevier: Amsterdam, The Netherlands, 2016.

49. Hajare, A.A.; Salunkhe, S.S.; Mali, S.S.; Gorde, S.S.; Nadaf, S.J.; Pishawikar, S.A. Review on: High-throughput screening is an approach to drug discovery. Am. J. Pharm. Tech. Res. 2013, 4, 112–129.

50. Liu, J.; Li, K.; Cheng, L.; Shao, J.; Yang, S.; Zhang, W.; Zhou, G.; de Vries, A.A.; Yu, Z. A high-throughput drug screening strategy against coronaviruses. Int. J. Infect. Dis. 2020, 103, 300–304.

51. Xu, T.; Zheng, W.; Huang, R. High-throughput screening assays for SARS-CoV-2 drug development: Current status and future directions. Drug Discov. Today 2021, 26, 2439–2444.

52. Zhang, Q.Y.; Deng, C.L.; Liu, J.; Li, J.Q.; Zhang, H.Q.; Li, N.; Ye, H.Q. SARS-CoV-2 replicon for high-throughput antiviral screening. J. Gen. Virol. 2021, 102, 001583.

53. Zhao, Y.; Du, X.; Duan, Y.; Pan, X.; Sun, Y.; You, T.; Yang, H. High-throughput screening identifies established drugs as SARS-CoV-2 PLpro inhibitors. Protein Cell 2021, 12, 877–888.

54. Zhu, W.; Xu, M.; Chen, C.Z.; Guo, H.; Shen, M.; Hu, X.; Zheng, W. Identification of SARS-CoV-2 3CL Protease Inhibitors by a Quantitative High-Throughput Screening. ACS Pharmacol. Transl. Sci. 2020, 3, 1008–1016.

55. Froggatt, H.M.; Heaton, B.E.; Heaton, N.S. Development of a Fluorescence-Based, High-Throughput SARS-CoV-2 3CL(pro) Reporter Assay. J. Virol. 2020, 94, e01265-20.

56. Gorgulla, C.; Das KM, P.; Leigh, K.E.; Cespugli, M.; Fischer, P.D.; Wang, Z.F.; Arthanari, H. A multi-pronged approach targeting SARS-CoV-2 proteins using ultra-large virtual screening. iScience 2021, 24, 102021.

57. Feng, Y.; Mitchison, T.J.; Bender, A.; Young, D.W.; Tallarico, J.A. Multi-parameter phenotypic profiling: Using cellular effects to characterize small-molecule compounds. Nat. Rev. Drug Discov. 2009, 8, 567–578.

58. Nierode, G.; Kwon, P.S.; Dordick, J.S.; Kwon, S.-J. Cell-Based Assay Design for High-Content Screening of Drug Candidates. J. Microbiol. Biotechnol. 2016, 26, 213–225.

59. Zaman, G.J. Cell-based screening. Comb. Chem. High Throughput Screen 2008, 11, 494.

60. Rumlová, M.; Ruml, T. In vitro methods for testing antiviral drugs. Biotechnol. Adv. 2017, 36, 557–576.

61. Green, N.; Ott, R.D.; Isaacs, R.J.; Fang, H. Cell-based assays to identify inhibitors of viral disease. Expert Opin. Drug Discov. 2008, 3, 671–676.

62. McLaren, C.; Ellis, M.; Hunter, G. A colorimetric assay for the measurement of the sensitivity of herpes simplex viruses t o antiviral agents. Antivir. Res. 1983, 3, 223–234.
63. Severson, W.E.; Shindo, N.; Sosa, M.; Fletcher, I.T.; White, E.L.; Ananthan, S.; Jonsson, C.B. Development and Validati on of a High-Throughput Screen for Inhibitors of SARS-CoV and Its Application in Screening of a 100,000-Compound L ibrary. SLAS Discov. Adv. Sci. Drug Discov. 2007, 12, 33–40.
64. Westby, M.; Nakayama, G.; Butler, S.; Blair, W. Cell-based and biochemical screening approaches for the discovery of novel HIV-1 inhibitors. Antivir. Res. 2005, 67, 121–140.
65. Lindsten, K.; Uhlíková, T.; Konvalinka, J.; Masucci, M.; Dantuma, N.P. Cell-Based Fluorescence Assay for Human Imm unodeficiency Virus Type 1 Protease Activity. Antimicrob. Agents Chemother. 2001, 45, 2616–2622.
66. Cheng, Y.-S.E.; Lo, K.-H.; Hsu, H.-H.; Shao, Y.-M.; Yang, W.-B.; Lin, C.-H.; Wong, C.-H. Screening for HIV protease inhi bitors by protection against activity-mediated cytotoxicity in Escherichia coli. J. Virol. Methods 2006, 137, 82–87.
67. Bertolin, A.P.; Weissmann, F.; Zeng, J.; Posse, V.; Milligan, J.C.; Canal, B.; Diffley, J.F. Identifying SARS-CoV-2 antiviral compounds by screening for small molecule inhibitors of nsp12/7/8 RNA-dependent RNA polymerase. Biochem. J. 202 1, 478, 2425–2443.
68. Jan, J.T.; Cheng TJ, R.; Juang, Y.P.; Ma, H.H.; Wu, Y.T.; Yang, W.B.; Wong, C.H. Identification of existing pharmaceutic als and herbal medicines as inhibitors of SARS-CoV-2 infection. Proc. Natl. Acad. Sci. USA 2021, 118, e2021579118.
69. Van Damme, E.; De Meyer, S.; Bojkova, D.; Ciesek, S.; Cinatl, J.; De Jonghe, S.; Van Loock, M. In vitro activity of itrac onazole against SARS-CoV-2. J. Med. Virol. 2021, 93, 4454–4460.
70. Zhao, J.; Guo, S.; Yi, D.; Li, Q.; Ma, L.; Zhang, Y.; Cen, S. A cell-based assay to discover inhibitors of SARS-CoV-2 RN A dependent RNA polymerase. Antiviral Res. 2021, 190, 105078.
71. Qiao, J.; Li, Y.S.; Zeng, R.; Liu, F.L.; Luo, R.H.; Huang, C.; Yang, S. SARS-CoV-2 M(pro) inhibitors with antiviral activity in a transgenic mouse model. Science 2021, 371, 1374–1378.