High-throughput screening and evaluation of repurposed drugs targeting the SARS-CoV-2 main protease

Signal Transduction and Targeted Therapy (2021) 6:356

Dear editor,

To date, a number of clinically approved drugs have been evaluated for potential to treat coronavirus disease 2019 (COVID-19), such as lopinavir/ritonavir, hydroxychloroquine, cobicistat, and darunavir. Some of these drugs have been proven to be effective in vitro; however, clinical trials showed that none of these compounds led to a significant improvement in symptoms or length of hospitalization. Thus, it is essential and more reliable to start from a defined target to identify candidate drugs.

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) main protease M\textsuperscript{pro}, also known as 3CL protease, is one of the best-characterized drug targets among coronaviruses. In the current study, structure and sequence alignment based on two different structures of SARS-CoV-2 M\textsuperscript{pro} (PDB IDs: 6lu7 and 6m2q) showed an obvious change between Ser46/CA and Leu167/CA, which indicated that the substrate-binding pocket of M\textsuperscript{pro} exhibits a certain extent of flexibility (Supplementary Fig. S1). Therefore, we developed a multiple cross-docking strategy with these two structures, to perform a computer-based high-throughput virtual screening of possible inhibitors from a drug database and our in-house automatic processing scripts using AutoDock Vina software (Supplementary Fig. S2). A total of 108 molecules with a docking score of \(-8.0\) kcal/mol were found against both of these structures. Then, 37 molecules with molecular weights between 330 and 700 g/mol with respect to the pocket volume and pKa values > 12 were selected. After that, we focused on 11 antiviral, antibacterial, and target-oriented antitumor drugs (Supplementary Table 1). Moreover, several previously reported drugs were used for reference, such as GC376, lopinavir, nelfinavir, and darunavir.

Full-length SARS-CoV-2 M\textsuperscript{pro} was expressed and purified based on its coding sequence (GI: 1897214688) and the affinity of each of the screened candidates for M\textsuperscript{pro} was detected by the surface plasmon resonance (SPR) technique. The response units were measured with a Biacore instrument using a gradient concentration of the small molecules that interacted with M\textsuperscript{pro}; then, the receptor-ligand binding affinity was measured and reported as the equilibrium dissociation constant (K\textsubscript{D}) based on curve fitting under steady-state analysis. Six drugs exhibited excellent binding affinity for M\textsuperscript{pro}, including entrectinib, indinavir, cloxacillin, dolutegravir, saquinavir, and enasidenib, with K\textsubscript{D} values of 55 μM or below (Fig. 1a).

Next, mutagenesis studies were performed to confirm the specificity of the interaction between the screened molecules and SARS-CoV-2 M\textsuperscript{pro}. First, an in-depth analysis of the substrate-binding pocket of M\textsuperscript{pro} identified seven residues that may interact with these molecules, including His41, Asn142, Cys145, His164, Met165, Asp187, and Gln189. Among them, His41, Cys145, His164, and Asp187 have been reported as potential catalytic residues.\textsuperscript{2} Then, a mutant of M\textsuperscript{pro} was designed in which all seven of these residues were replaced with Ala. Computer-aided simulation indicated that the main structure of this mutant remained stable, except for the substrate-binding pocket (Supplementary Fig. S3b).

In contrast to previous methods,\textsuperscript{3} in the inhibitory assay in this study, after testing a gradient of concentrations of M\textsuperscript{pro} (1.5, 0.5 and 0.25 μM), we performed the enzymatic inhibitory experiment with a higher concentration of M\textsuperscript{pro} (1.5 μM) and a longer reaction time to improve the signal-basal state rate and decrease the background noise. Thus, the obtained inhibitory rates were relative values, generating more reliable comparative data for us to determine the different inhibitory performances of these drugs on the activity of SARS-CoV-2 M\textsuperscript{pro}. The results showed that not all of the high-affinity drugs had significant inhibitory efficacy; thus,
functional experiments are critical for the final validation. For instance, we detected the nelfinavir had a high affinity for Mpro ($K_D = 2.36 \mu M$) and its EC50 value (the drug concentration that inhibits half of the maximal efficacy) was reported to be 1.13 µM in the cellular experiment, but it finally failed to block the enzymatic activity of Mpro in this functional assay. Then we measured the IC50 (the drug concentration that inhibits half of the enzymatic activity) of entrectinib identified in this study. An enzymatic inhibitory experiment with 200 nM Mpro was performed by using a concentration gradient of entrectinib and the reference compounds. Based on the fitting curves of inhibition (Supplementary Fig. 4), the IC50 value of entrectinib was 10.6 µM, which was
compared to that of GC376 (13.8 µM) but significantly lower than that of nelfinavir (41.5 µM). We noticed that the IC50 values were inconsistent in different reports, so it is very important to set an internal reference in each experiment, such as GC376 in our study. It testified that the comparative data are more reliable than a single value to evaluate the activity of compounds.

Finally, the antiviral activity of entrectinib was examined in Vero E6 cells infected with SARS-CoV-2 at the biosafety level 3 laboratory of the Kunming High-level Biosafety Primate Research Center, Yunnan, China. Infected cells were treated with different concentrations of drugs at safe doses (Supplementary Fig. S5) for 48 h and viral RNA copies were detected by a quantitative reverse transcriptase PCR assay using SARS-CoV-2-specific primers. The EC50 values of entrectinib and GC376 were determined to be 198 and 74 nM, respectively, as calculated by the fit curve (Fig. 1d). This result demonstrated that entrectinib exhibited promising anti-SARS-CoV-2 activity at the cellular level.

Taken together, based on a virtual drug screening workflow followed by experimental affinity and inhibitory efficacy evaluations, we identified several drugs that not only have a definite docking conformation and binding affinity to SARS-CoV-2 Mpro but also exhibit a stronger ability to suppress its activity than previously reported drugs, such as lopinavir, darunavir, and nelfinavir. This study provides more solid evidence at the molecular level to interpret the differences and mysteries between previous cellular experiments and clinical trials for antiviral treatment of COVID-19. Our results suggest that some clinically approved drugs, such as entrectinib, may serve as promising candidates to treat SARS-CoV-2 infection.

DATA AVAILABILITY
The data used and analyzed in this study are available in the main text and the Supplementary Materials. Any other raw data that support the findings of this study are available from the corresponding author upon reasonable request.

ACKNOWLEDGEMENTS
This work was supported by grants from the Emergency Project of West China Hospital (number HX-2019-nCoV-025) and the Program from Chongqing Education Commission (number KYJ202010).

AUTHOR CONTRIBUTIONS
Q.M.Z., G.G. and R.L. conceived the study and participated in the overall design, supervision, and coordination of the study. Y.L., J.Y.Z. and Z.L.D. designed and performed most experiments. Y.L. performed the virtual screening and SLP. participated in the data analysis. N.W., X.C.S. and K.Y.L. did the molecular cloning, gene expression and enzymatic assays. Y.J.Z., L.F., X.C.S. and Y.J.Y. carried out the experiments of molecular interaction. Z.L.D. and R.L. performed the viral and cellular experiments. Y.L. and J.Y.Z. wrote the manuscript. Q.M.Z., Y.S., H.Z. and G.G. critically reviewed the manuscript and contributed to the discussion.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41392-021-00763-5.