**LETTER**

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PIKfyve inhibitors against SARS-CoV-2 and its variants including Omicron

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**Dear Editor,**

COVID-19 pandemic, caused by SARS-CoV-2 infection, is raging around the world and results in millions of deaths since the end of 2019. Although various therapies including vaccines and neutralizing antibodies have been developed to defend against the horrible pandemic, current strategies are inevitably at risk of failure due to high mutagenicity of the viral genome. In fact, the most worrying situation is that the monoclonal antibodies of existing vaccines against the rapidly spreading Omicron variant are ineffective.1 Facing the great threat posed by COVID-19, there is an urgent need to develop small molecule antiviral drugs. At present, only a few drugs are authorized to treat COVID-19 in emergency medicine clinics. To identify and evaluate molecular target for COVID-19 becomes a top priority for worldwide health professionals.

It has been reported that PIKfyve might be a potential antiviral target.2 PIKfyve is a phosphoinositide 5-kinase that synthesizes PtdIns5P and PtdIns(3,5)biphosphate, which in turn regulates endomembrane homeostasis. Apilimod, an established PIKfyve inhibitor, shows a certain effect in blocking the entry of SARS-CoV-2 into host cells.2 Although apilimod has entered a clinical trial against the COVID-19 (NCT04446377), the results have not been published yet and might not be satisfactory because of its unexpected low plasma concentration and poor bioavailability shown in previous failed clinical trials in patients with Crohn’s disease and rheumatoid arthritis.3 Coincidentally, our previously internal research on cancer methuosis inducers found a series of 2,4-disubstituted-5H-pyrrolo[3,2-d]pyrimidine derivatives as PIKfyve inhibitors which has distinct scaffold compared with apilimod (Fig. 1a). Among them, XMU-MP-7 (cmpd 38), displayed high affinity for PIKfyve with average Kd of 6.4 nM (Supplementary Fig. S1) and moderate pharmacokinetic property (Supplementary Table. S1). Molecular docking study revealed the binding mode of XMU-MP-7 with PIKfyve (Supplementary Fig. S2). In this study, we aim to evaluate the antiviral activity of XMU-MP-7 against SARS-CoV-2 and its various variants, especially the highly contagious Delta and the heaviest mutated Omicron, in comparison with apilimod and other FDA-approved small molecule drugs for COVID-19 treatment.

Accumulating huge vacuoles in cell cytoplasm is the characteristic of PIKfyve inhibition. As expected, XMU-MP-7 dramatically induced cyttoplasmic vacuoles in vero E6 cells as apilimod did. Cmpd 24, an XMU-MP-7 analog without PIKfyve binding affinity (Kd > 30,000 nM), could not induce visible vacuoles in the same condition (Fig. 1b). We then determined their antiviral activity against SARS-CoV-2 wild-type isolate XOMP8T, which was highly homologous to the SARS-CoV-2 isolate Wuhan-Hu-1. Vero E6 cells were pretreated with individual drugs for 2 h before infection, and virus released in supernatants was detected via quantitative real-time PCR (qRT-PCR) at 48 h post infection (p.i.) (Fig. 1c). To our surprise, XMU-MP-7 showed the half-maximal effective concentration (EC50) of 9.3 nM which was far better than approved antiviral agents, as adenosine analogue Remdesivir showed an EC50 of 1642 nM and lysosomal acidification inhibitor Chloroquine had an EC50 of about 500 nM. PF-07321332, the key component of the newly approved oral drug PAXLOVID, exhibited an EC50 of 487 nM, which is comparable to that of other studies.4 Given the extremely low cytotoxicity of XMU-MP-7 (CC50 > 150 μM), its selectivity index (SI, CC50/EC50) was even higher than apilimod. In contrast, cmpd 24, showed a fairly weak antiviral activity (EC50 = 12277 nM; SI = 4.21) (Fig. 1c). Taken together, PIKfyve inhibitor XMU-MP-7 exhibits potent antiviral ability against wildtype SARS-CoV-2 in vitro.

We further evaluated the efficacy of the PIKfyve inhibitors against four SARS-CoV-2 variants of concern (VOCs), including Alpha, Beta, Delta and Omicron. Results showed similar drug sensitivity among SARS-CoV-2 wild-type and variants. XMU-MP-7 achieved complete viral inhibition at 200 nM, with the EC50 of 12.4 nM against Beta variant and below 6.9 nM against other three variants. Similarly, apilimod showed the EC50 below 6.9 nM to all tested variants. These data revealed that PIKfyve inhibitors exhibited much better inhibition with EC50 of 10–100 fold lower compared with Remdesivir and PF-07321332, respectively (Fig. 1d and Supplementary Fig. S3a). The remarkable antiviral activities of PIKfyve inhibitors were consistent with the data in our pseudovirions assay (Supplementary Fig. S3b and Table S2-S3). In addition, we treated Vero E6 cells for 48 h in cytopathic effect (CPE) assays (Supplementary Fig. S4a). Results showed that XMU-MP-7 and apilimod significantly rescued the cytopathic effects caused by SARS-CoV-2 and its variant Omicron (Supplementary Fig. S4b). Similarly, PIKfyve inhibitors also blocked the cytopathic effect induced by Omicron in A549, and their antiviral activities were better than that of Remdesivir and PF-07321332 (Supplementary Fig. S4c). Together, these results reveal that SARS-CoV-2 and its variants are much more sensitive to PIKfyve inhibitors.

PIKfyve plays a critical role in endocytosis that is often hijacked by virus for host cells entry.5 Here, we employed a genetically engineered sensor of fluorescent protein (Gamillus)-fused SARS-CoV-2 spike trimer (STG) to probe the dynamic virus entry and explore how PIKfyve inhibitors may affect this process.6 Three hours after incubation, the STG probes could be internalized and observed as green dots in cytoplasmic region. However, upon treatment with XMU-MP-7 or apilimod, STG probes were almost completely trapped on the enlarged cytoplasmic vacuoles and colocalized with internalized ACE2-mRuby to form yellow fluorescence similarly to that of the control group (Fig. 1e).

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It has been recently reported that PIKfyve inhibitor apilimod inhibits cathepsin class of lysosomal proteases, which is consistent with our observation that XMU-MP-7 impaired the maturation of active cathepsin B in time- and dose-dependent manners (Fig. 1f). Collectively, PIKfyve inhibitors terminate cell entry of SARS-CoV-2 by blocking viral/cell membrane fusion. Moreover, our time-of-addition experiment further indicated that PIKfyve inhibitors functioned at both entry and post-entry stages of the SARS-CoV-2 infection, strongly inhibiting viral replication (Fig. 1g) and the expression of viral nucleoprotein (Fig. 1h). By comparison, adenosine analogue remdesivir only exerted an inhibitory effect at post-entry stage, which was consistent with its
putative antiviral mechanism by hindering viral RNA replication. We noticed that both XMU-MP-7 and apilomid induced visible cytoplasmic vacuoles within a very short time (less than 20 min), which may contribute to their antiviral activity in post-entry stage. Even if a certain degree of virus got entry before drug treatment, the rapidly generated vacuoles and inactive cathepsins induced by PIKfyve inhibitors will seriously impair the new lifecycle of virus and consequently reduce infection. Furthermore, vacuolization could be maintained for up to 48 h or longer even when drug was withdrawn after only 4 h of treatment (Fig. 1i). The remaining vacuoles appeared to be sufficient to inhibit viral infection.

In summary, we demonstrate a novel PIKfyve inhibitor XMU-MP-7 effectively overrides SARS-CoV-2 and its variants including the most concerned Delta and Omicron in vitro. Moreover, XMU-MP-7 prevents SARS-CoV-2 from entering the cytoplasm and plays potent antiviral roles at both early and post-entry stages. The strong antiviral potency makes XMU-MP-7 as a good starting-point for developing antiviral agent against the current pandemic. Our findings support that pharmacological targeting PIKfyve to intervene the host cells’ endocytosis is an efficient way to block viral infection.

**DATA AVAILABILITY**

All data are available upon request from the corresponding author.

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**AUTHOR CONTRIBUTIONS**

J.S., J.Z., W.H. and Y.Z. performed the research (carrying out the experiments, data output and analysis) and contributed equally to the study. J.S. and J.Z. carried out the viral experiments in BSL-3 laboratory. W.H. and B.Z. designed and synthesized inhibitors. C.L. and L.J. carried out experiments in BSL-2 laboratory. Y.Z. was responsible for fluorescent visualization. T.C., Q.Y. and N.X. provided key reagents, materials and technical support. T.C., Q.Y., J.Z., L.L., X.D. interpreted the data. L.L., L.L. and X.D. designed and directed this project. J.S., L.L. and X.D. wrote the manuscript with comments and final approval from all authors.

**ADDITIONAL INFORMATION**

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