Sunitinib reduces the infection of SARS-CoV, MERS-CoV and SARS-CoV-2 partially by inhibiting AP2M1 phosphorylation

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Dear Editor,

In the past 17 years, coronaviruses including SARS-CoV, MERS-CoV and SARS-CoV-2 have crossed the species barrier and resulted in remarkable epidemics in human for three times. Each disease caused by them, especially COVID-19 that is caused by SARS-CoV-2¹, led to tremendous life threatening and economic loss. There is no effective treatment against them currently, and the development of druggable target is urgently needed. Considering the frequent invasion into human by various coronaviruses, broad spectrum drugs against coronaviruses are particularly important.

HIV backbone-based pseudotyped virus carries a luciferase reporter gene, which is a safe and convenient tool to study the entry of highly virulent pathogens such as SARS-CoV and MERS-CoV. Using this tool, we have previously identified ACE2 as the receptor of SARS-CoV², and analyzed the immunoreactivity of the sera from MERS-CoV-infected animals³. In the current study, we used SARS-CoV pseudotyped virus (HIV/SARS-CoV pseudovirus) to screen a siRNA library, and identified AP2M1 as a crucial host factor for SARS-CoV infection. Based on the discovery, we further demonstrated that sunitinib, a kinase inhibitor involving in the regulation of AP2M1, not only inhibited the entry of HIV/SARS-CoV pseudovirus, but also functioned on SARS-CoV-2 and MERS-CoV, thus held great potential as an anti-coronavirus drug.

The siRNA library used for screening is an intracellular membrane traffic siRNA library targeting 144 host molecules, and the primary screening results suggested that AP2M1 may play an important role in SARS-CoV infection. AP2M1 encodes the μ2 subunit of AP2 complex, which is an adapter protein complex for clathrin. AP2M1, clathrin as well as some other factors constitute a clathrin-dependent endocytic pathway by which cells absorb metabolites, hormones, proteins—as well as some viruses—by the inward budding of the plasma membrane. To validate the function of AP2M1 in coronavirus entry, we used two siRNAs to knock down AP2M1 expression (Fig. 1a), and then analyzed the impact on SARS-CoV pseudovirus infection. Neither of the two siRNAs showed cytotoxicity in transfected cells, as revealed by CCK8 assay (Supplementary Fig. S1). In cells transfected with these two siRNAs, the infectivity of HIV/SARS-CoV was significantly reduced to a similar level as HIV/VSV, which was used as a control in the experiment (Fig. 1b). Next, we examined the effect of clorpromazine (CPZ), the inhibitor of clathrin-mediated endocytosis, on pseudotyped virus infection. CPZ effectively inhibited the infection of HIV/SARS-CoV in a dose-dependent manner, but had no effect on the infection of HIV/AMLV which entered cells in a clathrin-independent way (Fig. 1c), showing that SARS-CoV infection depended on clathrin-mediated endocytosis.

As SARS-CoV and SARS-CoV-2 both utilize ACE2 as the receptor for viral entry¹²⁴, we asked whether AP2M1 functions for SARS-CoV-2. SARS-CoV-2 pseudovirus system was similarly established and used to test the effect of siAP2M1 in viral entry. The result showed that knock down of AP2M1 inhibited the infectivity of HIV/SARS-CoV and HIV/SARS-CoV-2

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Fig. 1 (See legend on next page.)
but not HIV/AMLV (Fig. 1d), suggesting potential interaction between ACE2 and AP2M1.

There are at least 20 clathrin adaptor proteins, and each adaptor is generally considered to be involved in a different trafficking step. Specifically, AP2 functions in assisting proteins in plasma membrane to be incorporated into endocytic clathrin-coated vesicles. By recognizing YXXΦ (Φ is a hydrophobic residue—L/I/M/V/F) or dileucine motifs, AP2M1 links the cytoplasmic tail of a transmembrane protein to clathrin5. To interrogate the hypothesis that AP2M1 may interact with ACE2 and mediated clathrin-mediated endocytosis, we analyzed the sequence of ACE2 and found that a YASI sequence, fulfilling the requirement of the YXXΦ element, was presented in the middle of the cytoplasmic region of ACE2. Further analysis showed that this motif is conserved among mammalian species including aquatic mammals and bat, which is believed to be the natural host of SARS-CoV among mammalian species including aquatic mammals. Further analysis showed that this motif is conserved in the middle of the cytoplastic region of ACE2.

To analyze whether the YASI motif was essential for SARS-CoV entry, we replaced the coding sequence of ACE2 with YASI sequence of ACE2 and found that a YASI sequence, fully mediated clathrin-mediated endocytosis, we analyzed the expression levels of ACE2 protein in different species. Mutation of YXXΦ motif in mACE2 construct. Surface expression levels of ACE2 on ACE2-HeLa and mACE2-HeLa cells as determined by flow cytometry. Relative infectivity of HIV/SARS-CoV, HIV/SARS-CoV-2 and HIV/AMLV on ACE2-HeLa cells treated with siAP2M1 (siRNA-2) or NT siRNA. Sequence alignment of transmembrane domain and cytoplasmic tail of ACE2 protein in different species. The infection of pseudovirus was determined by measuring the luciferase activity, and expressed as relative infectivity compared with the control. C Relative infectivity of HIV/SARS-CoV and HIV/AMLV on ACE2-HeLa cells treated with different concentrations of CPZ. D Relative infectivity of HIV/SARS-CoV, HIV/SARS-CoV-2 and HIV/AMLV on ACE2-HeLa cells transfected with siAP2M1 (siRNA-2) or NT siRNA. E Sequence alignment of transmembrane domain and cytoplasmic tail of ACE2 protein in different species. F Mutation of YXXΦ motif in mACE2 construct. G Surface expression levels of ACE2 on ACE2-HeLa and mACE2-HeLa cells as determined by flow cytometry. H Relative infectivity of HIV/SARS-CoV, HIV/SARS-CoV-2 and HIV/AMLV on ACE2-HeLa and mACE2-HeLa cells. I Syncytia formation of HeLa cells expressing the S protein of SARS-CoV with ACE2-HeLa or mACE2-HeLa. J AP2M1 and phosphorylated AP2M1 levels in ACE2-HeLa cells treated with sunitinib, erlotinib or apatinib. K Cytoxicity of sunitinib on ACE2-HeLa cells. L–O Relative infectivity of HIV/AMLV (L), HIV/SARS-CoV (M), HIV/SARS-CoV-2 (N), or HIV/MERS-CoV (O) on target cells treated with sunitinib, erlotinib or apatinib. Data were derived from three independent experiments, shown as mean ± SD. **P < 0.01; *P < 0.05; ns not significant.
CoV-2 also uses ACE2 as receptor, the function of AP2M1 sequence in the cytoplasmic tail of ACE2, and mediates factor for coronaviral entry and can be targeted by kinase inhibiting in pathway, it may be helpful for COVID-19 therapy by As baricitinib can inhibit JAK1 and JAK2 signaling implied that AP2M1 was not associated with baricitinib. (Supplementary Fig. S2b). Nevertheless, this result only TMPRSS2 inhibitor 17. Second, the clathrin-dependent the importance of combination of sunitinib and drug for SARS-CoV-2. Since baricitinib has been pre-arthritis, has been suggested as a promising antiviral inhibitor and has been used for therapy of rheumatoid arthritis, has been suggested as a promising antiviral drug for SARS-CoV-2. Since baricitinib has been predicted to be an inhibitor for AAK1, it was also tried on three types of pseudovirus. Unexpectedly, although baricitinib has been shown to own high affinity to AAK1, it did not display obvious inhibition on the phosphorylation of AP2M1 unless at very high dose (10 μM) (Supplementary Fig. S2a), and consequently displayed no inhibitory effect on infection of each type of pseudovirus (Supplementary Fig. S2b). Nevertheless, this result only implied that AP2M1 was not associated with baricitinib. As baricitinib can inhibit JAK1 and JAK2 signaling pathway, it may be helpful for COVID-19 therapy by inhibiting inflammation.

Taken together, we identified AP2M1 as a crucial host factor for coronaviral entry and can be targeted by kinase inhibitors like sunitinib. AP2M1 interacts with YAS1 sequence in the cytoplasmic tail of ACE2, and mediates clathrin-dependent entry for SARS-CoV. While SARS-CoV-2 also uses ACE2 as receptor, the function of AP2M1 in SARS-CoV-2 entry may be similar to that in SARS-CoV entry. Based on the discovery of AP2M1, sunitinib was identified as a potential coronaviruses inhibitor with broad spectrum. Besides coronavirus, sunitinib also inhibits viral entry including HCV, rabies virus, dengue virus, and Ebola virus, suggesting that its application in anti-viral research should be paid more attention.

In spite of these advances, we have to keep two issues in mind. First, besides clathrin-dependent pathway, SARS-CoV, MERS-CoV and SARS-CoV-2 also utilizes TMPRSS2 to cleave its S protein and initiates a direct viral-cell membrane fusion to enter cells, suggesting the importance of combination of sunitinib and TMPRSS2 inhibitor. Second, the clathrin-dependent endocytosis is crucial for many cellular singling pathways, and its disruption in general might lead to severe cytoxicity and side effects. The therapy effects and side effects, therefore, should be carefully balanced before the application of these inhibitors.

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P.G.W. and Z.W. conceived and designed the project. D.J.T. screened the siRNA library. P.G.W. carried out experiments on SARS-CoV pseudovirus model. Z.W. performed the rest experiments. Z.H. and J.A. discussed the results and analyzed the data. Z.W. and P.G.W. wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.

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