Supporting Information

Optimization of 4-aminopiperidines as inhibitors of influenza A viral entry that are synergistic with oseltamivir.

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Table of contents:

CYP450 Inhibition Assay

Inhibitory activity of the compounds for CYP450 enzymes was evaluated using the Vivid® CYP3A4 Green and CYP2C9 Green Screening Kits by ThermoFisher Scientific according to manufacturer's protocol. Briefly, test compounds/DMSO were prepared at 250 µM in 1X Vivid CYP450 Reaction Buffer and were serially diluted two- or three-fold for 9-11 times with the Reaction Buffer to achieve 2.5X of the desired final concentrations. A 40ul volume of the 2.5X compound solutions was transferred into the wells of the 96-well black plates. A 50 µl volume of the Master Pre-Mix including P450 BACULOSOMES® Plus Reagent and Vivid® Regeneration System in the Reaction Buffer was subsequently added to each well and mixed on a rocking shaker for 10 minutes at room temperature (RT). After 10 minutes of incubation, 10 µl of a mixture of Vivid® Substrate and Vivid® NADP+ was added to each well to start the CYP450 reaction. Plates were placed on a rocking shaker for 25 minutes at RT and fluorescent signals were then immediately measured using a fluorescent microplate reader. Final concentrations of 100 µM to 0.39 µM or 100 µM to 0.0017 µM of test compounds with 1% DMSO were achieved for calculation of IC_{50} values. Final concentrations of 10 µM ketoconazole and 30 µM sulfaphenazole were used as positive control inhibitors for CYP3A4 and CYP2C9, respectively. 1% DMSO was used as a negative control.

Evaluation of compound 16 against oseltamivir-resistant strain of influenza A virus (H1N1) with H274Y mutation.

Virus titer reduction assay. Monolayers of MDCK cells grown in 24-well plates were infected with a Tamiflu-resistant strain of influenza A virus (H1N1) at an MOI of 0.01. After incubation at 37 °C for 1 h, the medium was replaced by fresh Opti-MEM (2 µg/mL TPCK-trypsin)

containing DMSO or increasing concentrations of compound **16** or oseltamivir carboxylate. The plates were incubated for 24 hours at 37 °C and supernatants were harvested for virus titration.

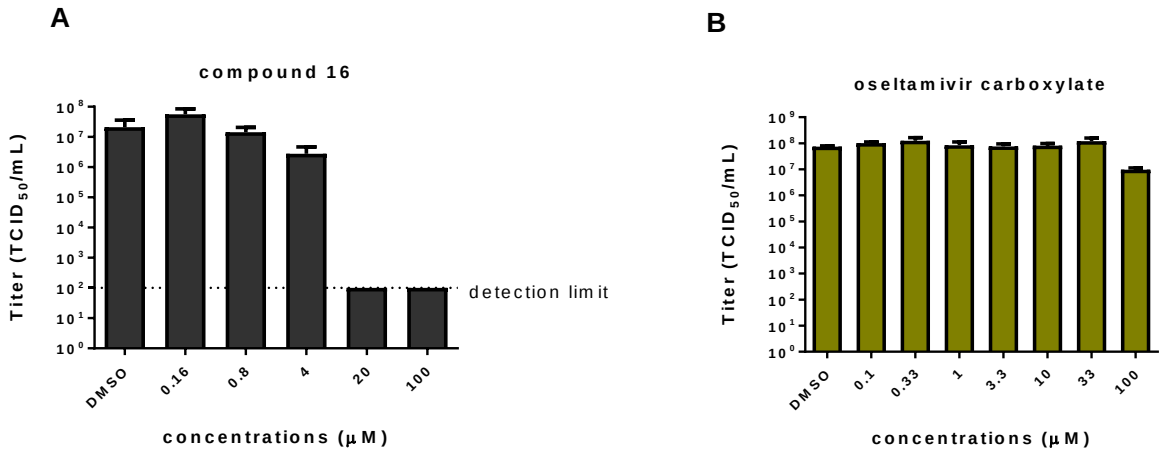
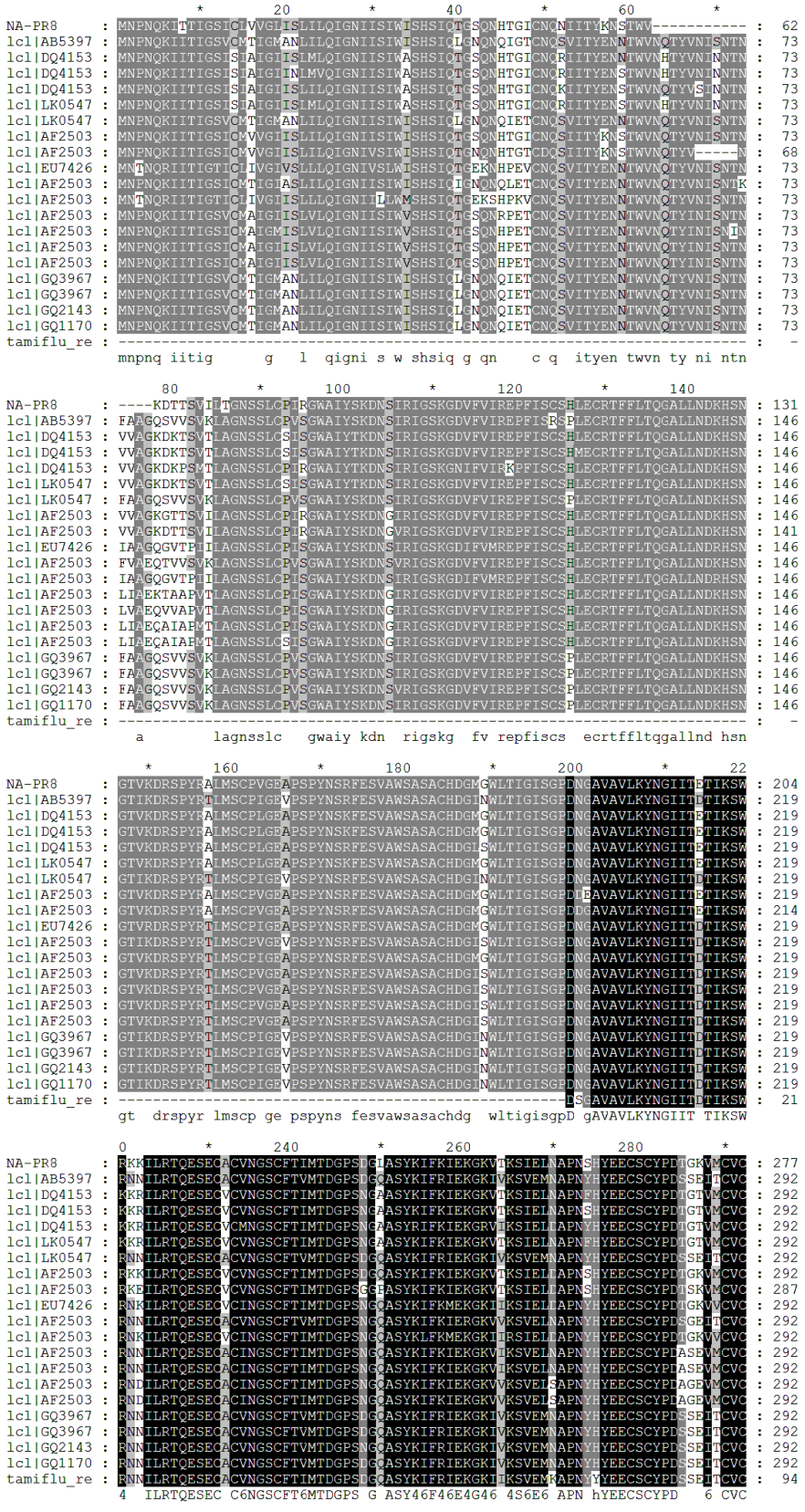


Figure S1. Virus titer reduction assay with influenza A virus (H1N1) with H274Y. Compound **16** (A) almost completely inhibited virus replication at a concentration of 20 µM. Oseltamivir carboxylate (B), as a control, did not show any inhibitory effect at 33 µM.

Characterization of the oseltamivir-resistant strain of influenza A virus (H1N1). The segment NA of oseltamivir-resistant influenza A virus (H1N1) was sequenced. As a result, the H274Y mutation, which was known to contribute to oseltamivir resistance, was found in the NA of the oseltamivir-resistant influenza A virus (H1N1) (Figure 2S).



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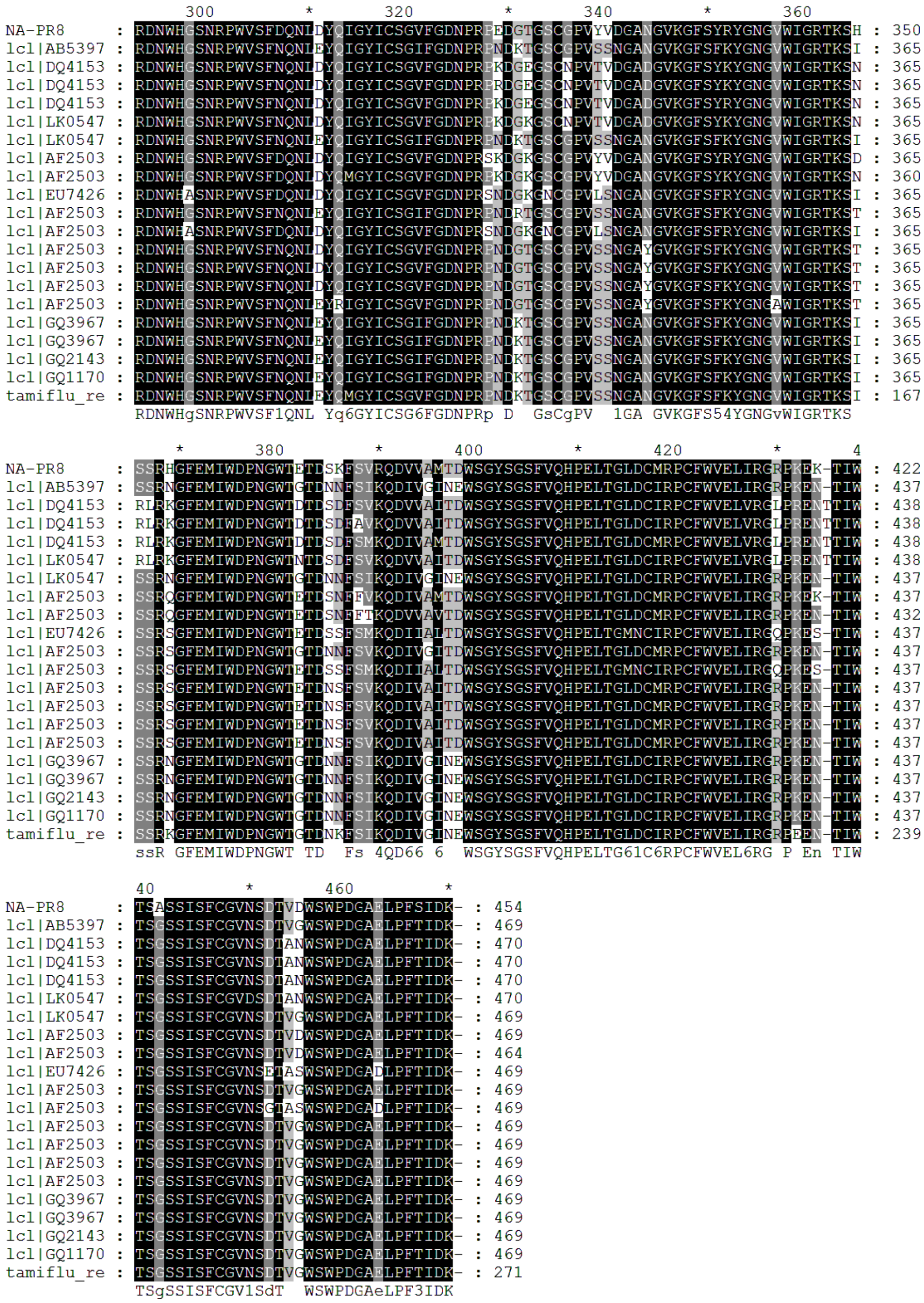

Figure S2. Sequence analysis of the segment NA of oseltamivir resistant influenza A virus (H1N1).

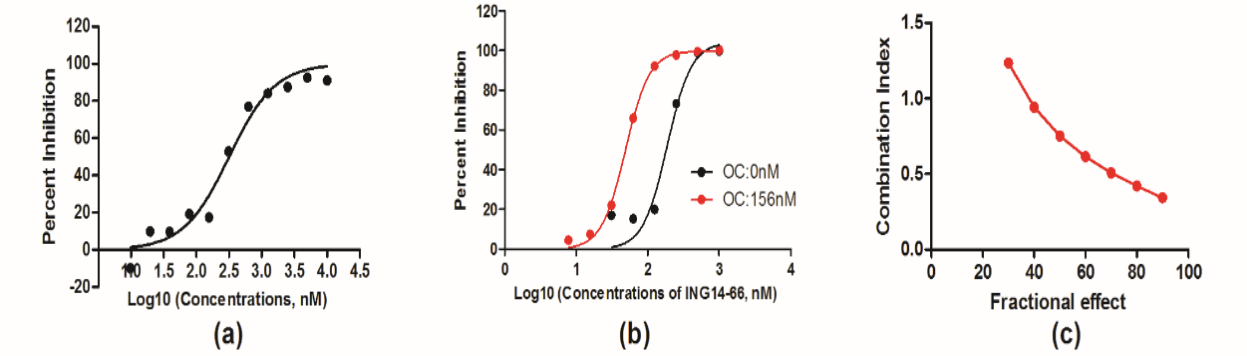

Figure S3. Oseltamivir carboxylate and compound **16** display synergistic combination index values for anti-influenza activity. (a) Dose dependent curves for oseltamivir carboxylate against IAV. (b) Dose dependent curves for **16** against IAV in the absence or presence of oseltamivir carboxylate (156 nM). (c) Combination index (CI) chart for the oseltamivir carboxylate and **16** combination was plotted with CI on the Y-axis and fractional effect on the X-axis.

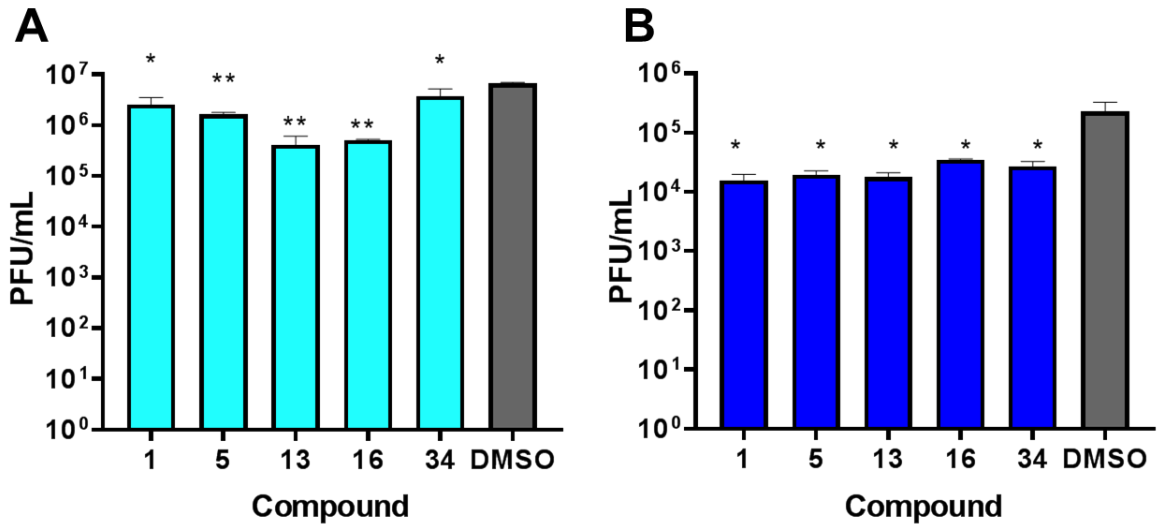

Figure S4. Infectious virus replication inhibition assay. H1N1 (A/Puerto Rico/8/1934) (A) or H5N1 (A/Vietnam/1203/2004) (B) viruses at the MOI of 0.01 were used to infect A549 cells in the presence or absence of selected 4-aminopiperidines at the 1 μM concentration. At 48 h post-infection, viral titers were determined by the standard plaque assay in MDCK cells. PFU/mL results represent the number of infective particles within the sample in the plaque assay. The assay was performed in triplicate; results are presented as mean ± SD. *$p < 0.05$ and **$p < 0.005$ versus DMSO control.