SUPPLEMENTAL METHODS, TABLES AND FIGURES

Peptide-mediated interference of PB2-eIF4G1 interaction inhibits influenza A viruses replication *in vitro* and *in vivo*

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Supplemental Methods

Cells, plasmids and peptides

The human lung carcinoma A549 cells, Madin-Darby canine kidney MDCK cells, and human embryonic kidney 293T cells were maintained in DMEM medium supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 50 units/ml penicillin and 50 μg/ml streptomycin (P/S). Mouse lung adenoma LA-4 cells were cultured in the DMEM/F12 medium (Invitrogen) supplemented with 20% FBS and 50 units P/S. The pCMV3-His-PB2 plasmid was purchased from Sino Biological Company (Beijing, China). Peptide were synthesized from Cellmano Biotech Limited (Hefei, China) with > 95% purity.

Immunoprecipitation assay

HEK293T cells were seeded in 6-well plate and transfected with pCMV3-His-PB2 plasmid by Lipofectamine 3000 transfection reagent (Invitrogen), followed by addition of individual peptide to the cell cultures 6 h post-transfection and maintained. Twenty-four hours later, cytosolic extracts were prepared using the lysis buffer containing 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.5). After centrifugation, the extracts were analyzed by co-immunoprecipitation kit (Pierce), in which specific antibodies against eIF4G1 antibody (PA5-17140, Thermo Fisher) were applied for the capture of PB2-eIF4G1 immunocomplexes, followed by the detection of PB2 and eIF4G1 presence with PB2-specific (GTX125926, GeneTex) and eIF4G1-specific (ab2609, Abcam) antibodies, respectively

Multi-cycle virus growth assay

Cells seeded in 96-well plates were infected with indicated influenza virus strain at multiplicity of infection (MOI) of 0.1. One hour after virus inoculation, the inoculum was

removed and replaced by fresh medium containing 0.1 μg/ml TPCK trypsin and serial-diluted peptides (200, 100, 50, and 25 μM). The cell-free supernatants were collected at 24 h post-infection (p.i.) and applied to virus titration by either plaque assay [1] or RT-qPCR.[2]

Regimen of mouse study

Three subtypes of influenza viruses (i.e. H1N1, H5N1 and H7N9) were tested, respectively. In each subtype a total of 42 mice (14 mice/group) were evaluated. After anesthesia, mice were intranasally (i.n.) inoculated with 20 μl of virus suspension, i.e. five lethal dose 50% (LD_{50}) of influenza viruses. The therapeutic treatment initiated 6 h post-virus-challenge by intranasal route. One group of mice was i.n. inoculated with 20 μl of 25 mg/ml Mouse-eIF4G1-B-Tat (25 mg/kg). The second group of mice, as a positive control, was i.n. administered with the similar molar amount of zanamivir (2.5 mg/kg). The third group was i.n. inoculated with PBS as an untreated control. Two doses per day of Mouse-eIF4G1-B-Tat, zanamivir or PBS were i.n. administered for 2.5 days (total 5 doses/mouse). Animal survival and sick signals were monitored for 14 days or until the mouse death. A body weight loss of 30% was set as the humane endpoint.[3] Four mice in each group were euthanized randomly on day 5 post-challenge, mouse lungs were collected for virus titration and histopathologic analyses as described previously.[4]

Protein expression and purification

PB2 cap-binding domain ($PB2_{cap}$, residues 318–483) and 627-NLS domain (residues 538-760) were expressed in prokaryotic expression system, while the PB1- binding domain (residues 1- 37) was synthesized as a biotinylated peptide. Briefly, reverse-transcribed cDNA of A/Vietnam/1194/2004(H5N1) influenza virus (GenBank accession no. AY651718) was used as a template for cloning. $PB2_{cap}$ was purified with his-tag affinity chromatography according to the

protocol described previously, [5] while 627-NLS domain was purified with GST-tag affinity chromatography following the reported conditions.[6] Blank vectors, i.e. pET32a (+) (his-tag) and pGEX-6P-1 (GST-tag), were expressed and purified with same procedures of $PB2_{cap}$ and 627-NLS domain as negative controls.

The enzyme-linked immunosorbent assay (ELISA)

A 96-well streptavidin-coated plate (Pierce) was coated with 1 μM of biotinylated Human B peptide according to the manufacture's protocol. After wash, individual PB2 domain, i.e. PB1-binding, $PB2_{cap}$, and 627-NLS domain, were added to the wells with serial-dilutions (20 to 0.625 μM). One hour after incubation, intensive wash were carried out, followed by the addition of appropriate antibodies, i.e. HRP-conjugated streptavidin (for PB1-binding domain), HRP-conjugated his-probe (for $PB2_{cap}$), HRP-conjugated GST-probe (for 627-NLS). The absorbance was read at 450 nm on an ELISA plate reader after addition of TMB substrates and 0.5M H_2SO_4. To validate the binding specificity, a competitive binding assay was carried out, in which unlabeled Human B peptide were serial-diluted (200 to 3.125 μM) and were added in the presence of 1 μM of $PB2_{cap}$ protein. Detection of the bound $PB2_{cap}$ were performed 1 h after incubation.

MTT assay

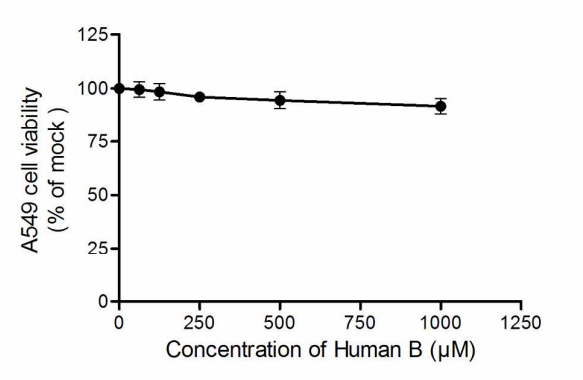
A 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay was performed to assess potential cytotoxicity of the peptide using MTT kit (Invitrogen). The experiment was carried out in a 96-well plate. At 37°C, A549 cells (4×10^4 cells/well) were cultured in fresh DMEM medium containing 0.5% FBS and serial-diluted Human B peptide (1000, 500, 250, 125, 62.5, and 0 μM) for 24 h. After that, 10 μl of freshly-prepared 5 mg/ml MTT

solution was added into each well and incubated for another 4 h. Next, 100 μl of 10% SDS with 10 mM HCl was added to each well and the mixture was incubated overnight. The final reading was obtained by an ELISA reader and represented by the value of OD570.

Supplemental Table 1. EC_{50}s of peptide Human B against different subtypes of influenza A viruses

Subtype	EC_{50} (μM)[a]
H1N1	33.5± 2.8
H3N2	72.5± 8.7
H5N1_VN	29.7± 2.4
H5N1_HK	53.5± 2.8
H7N7	37.9± 5.4
H7N9	56.2± 6.7
H9N2	29.8± 1.6

[a] EC_{50} was tested in A549 cells.



Supplemental Figure S1 Cytotoxicity evaluation of Human B peptide in A549 cells. The cytotoxicity of Human B in A549 cells was evaluated by an MTT assay. The highest concentration of Human B used was 1 mM, followed by two-fold-dilution. Experiments were carried out in triplicate and repeated twice. The mean value is shown with mean value ± SD

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