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Supplemental Information

Pim1 Impacts Enterovirus A71 Replication and Represents a Potential Target in Antiviral Therapy

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Supplemental Figures

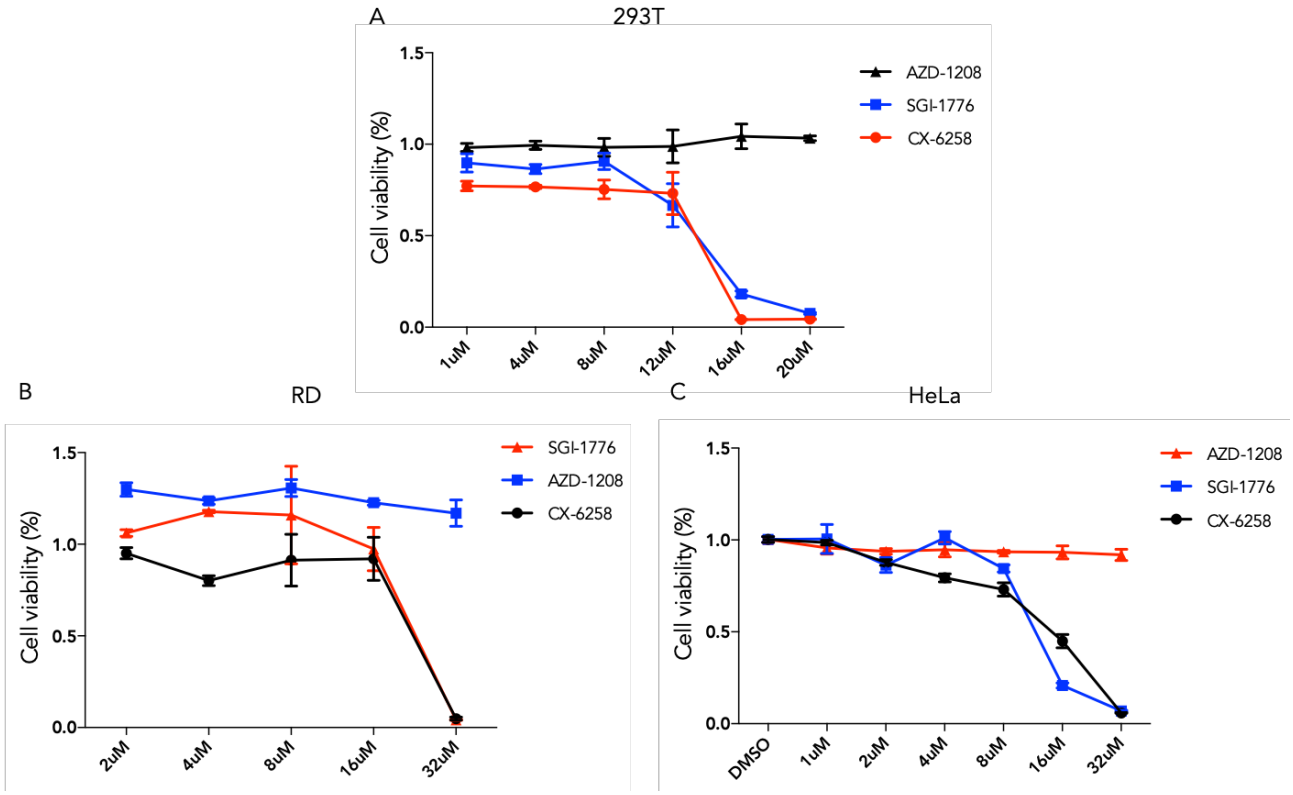

Figure S1. Cell viability with inhibitor treatment in different cells (related to Figure 5)

(A) We tested all Pim1 inhibitors (SGI-1776, CX-6258 and AZD-1208) for their effect on cell proliferation in 293T cell lines. Cells were incubated with the indicated concentrations for 48h. The cell viabilities were determined by MTT assay. (B). A similar experiment to that seen in panel A was performed on RD cells. (C) A similar experiment to that seen in panel A was performed on HeLa cells. Results are represented as mean ± SD from three independent experiments.

Transparent Methods

1. Viruses and cells

RD cells (ATCC number CCL-136), HeLa cells and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 μg/ml streptomycin. EV-A71 (SHZH98 strain, GenBank accession number AF302996) was obtained from Shenzhen Center for Disease Control and Prevention, Shenzhen, China. The virus was propagated as previously described (Lu et al., 2011; Yi et al., 2011).

2. Real-Time Polymerase Chain Reaction

RT-qPCR assays was used a total 1 μg RNA extracted from RD, HeLa or 293T cells. RT-qPCR assay was used ImProm-II™ Reverse Transcription System (Promega, USA). Real-time PCR was carried out in the ABI 7500 Real-Time PCR system with Power SYBR Green Master Mix (Applied Biosystems, USA), using the following program: 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 15s and 60°C for 1 min. Sets of primers for these genes are available upon request. All samples were run in triplicate and the experiment was repeated three times. The messenger RNA (mRNA) level of each target gene was normalized to the mRNA copies of GAPDH in the same sample and results were expressed as a percentage of the negative control (set as 1).

3. RNA interference

RNA interference was carried out using siRNA purchased from Genepharma (ShangHai, China). Two separate siRNAs corresponding to the Pim1 mRNA (siPim1-1-Sense: AACCUUCGAAGAAAUCCAGAACCAU, siPim1-1-Antisense: AUGGUUCUGGAUUUCUUCGAAGGUU; siPim1-2-Sense: GUAUGAUAUGGUGUGUGGAGAUAUUC, siPim1-2-Antisense: GAAUAUCUCCACACACCAUAUCAUAC) were used at 40nM to inhibit endogenous Pim1 expression. For the rescue assay (Fig. 3G&3H), siRNA was designed by targeting Pim1 3'-UTR (sense: 5′-ACAUUUACAACUCAUUCCA-3′, antisense: 5′-UGGAAUGAGUUGUAAAUGU-3′) (Park et al., 2015). Scramble siRNA was used as the control purchased from GenePharma (Shanghai, China). Transfection of siRNA was performed according to the manufacturer's instructions. In brief, cells at 50% confluence were transfected with 40 nM siRNA using Lipofectamine 3000.

4. Plasmids construction

Human Pim1 (Accession NM_001243186) was amplified using Platinum Taq DNA Polymerase high fidelity (Invitrogen, USA). The PCR product was cloned into pcDNA4/HisMax B (Invitrogen, USA)

vector between BamH I and Xba I sites. The EV-A71 report plasmid pRIRESF was constructed as follows: Renilla Luciferase gene (RLuc) was inserted into pcDNA4/HisMax B between BamH I and EcoR V sites (Wang M et al., 2016); EV-A71 IRES was amplified from EV-A71 virus strain (SHZH98). Also, Firefly Luciferase gene (FLuc) was amplified by using a primer which has an overlapping sequence with the C-terminal of EV-A71 IRES. Finally, the IRES-FLuc constructs were then amplified by using overlap PCR and inserted downstream of the Renilla Luciferase gene by using EcoRV and XbarI. The control plasmid pRF was constructed in similar way except it contained EV-A71 IRES upstream of the FLuc gene (Fig. 4A). The FLuc gene was amplified and then inserted downstream of the Renilla Luciferase gene by using EcoRV and XbarI. All primers used in plasmid construction are available upon request.

5. Western blotting

Cells were lysed in Nonidet-P40 (NP-40) buffer (150 mM sodium chloride, 1.0% NP-40, 50 mM Tris, pH 8.0, 1×Roche protease inhibitor cocktail) with occasional vortex. The cell lysates were then centrifuged to remove debris at 14,000 rpm for 20 min at 4°C. The concentration of proteins in the lysates was determined by the Bradford assay (Bio-Rad). Equal amounts of total protein for each sample were loaded and separated by 8%-12% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked with 5% bovine serum albumin (BSA) in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 h and incubated with the following specific antibodies: Pim1 (2907, CST), EV71 VP1 (MAB1255-M05, Abnova), eIF4G (sc-373892, Santa Cruz), AUF1 (ab50692, Abcam), Sam68 (ab26803, Abcam), hnRNPK (ab18195, Abcam), FUBP1 (sc-271241, Santa Cruz). Beta-actin (sc-517582, Santa Cruz) or GAPDH (sc-47724, Santa Cruz) served as the loading control. Target proteins were detected with corresponding secondary antibodies (Santa Cruz Biotechnology, USA) and visualized with a C600 western blot imaging system from Azure Biosystems. Each immunoblot assay was carried out at least three times.

6. Viral RNA quantification

EV-A71 viral RNA was determined as previously described (Lu et al., 2011). Briefly, the total cellular RNA was isolated for intracellular viral RNA quantification. To calculate the extracellular virions, the culture media of infected cells was firstly harvested and briefly centrifuged to remove cell debris. Viral core particles were then precipitated with 10% polyethylene glycol 8000 containing 0.5 M NaCl at 4°C overnight. After centrifuging for 30 min at 16,000 g, viral particles were pelleted and treated with 100 μg/ml of RNase A (Sigma, USA). To isolate the intracellular virions, EV-A71 infected cells were lysed with lysis buffer (1% Triton 100 and 1 x Roche protease inhibitor cocktail in PBS). Then the cell lysates were used to isolate viral particles as described above. To set up the standard curve of infectious viruses, the viral titers were first determined by a CPE assay. Then the viral RNA was extracted from the infectious EV-A71 viruses. RNA was serial diluted at tenfold and used to reflect the calculated PFU from 10 to 1×10^7 live virions.

7. Virus titration.

Virus titration was performed as reported in our previously manuscripts (Lu et al., 2012; Wang et al., 2016). RD cells were seeded into 96-well plates for 24 h before infection, then cells were infected by 100 μl per well of serial 10-fold diluted supernatant in quintuplicate. The 50 % tissue culture-infected dose (TCID50) was calculated by the Reed-Muench method after 96 h of infection.

8. Luciferase assays

Luciferase assays were performed as previously described (Dan et al., 2019; Dong et al., 2018). 293T cells were plated in 24-wells one day before transfection. Over expression of the Pim1 plasmid or corresponding siRNA were transfected. Next, 24 hours later, cells were transfected with PRIF or PRF reporter plasmids. Two days after first-round transfection, cell extracts were prepared in passive buffer (Promega, USA) and assayed for Renilla luciferase (RLuc) and Firefly luciferase (FLuc)

activity in a Lumat LB9507 bioluminometer using a dual-luciferase reporter assay (Promega) according to the manufacturer's instructions.

9. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. After RD cells were grown in 96-well plates and treated with Pim1 inhibitors, 20 µl of MTT (5 mg/ml) was added to each well, and cells were further incubated for an additional 4 h at 37 °C. Next, the medium was carefully removed and subsequently 100 µl of Dimethyl Sulfoxide was added. The optical density was measured at a wavelength of 570 nm.

10. Isolation of nuclear and cytoplasmic protein

Briefly, cells (5 to 10 x 10^6 cells) were collected and washed by PBS three times. Then the cytoplasm proteins were extracted by cytoplasmic extract (CE) buffer (HEPES 10 mM, pH 7.9, KCl 10 mM, EDTA 0.1 mM, NP-40 0.3% (added just before use), protease inhibitors 1x (added just before use)). Subsequently, we used a nuclear extract (NE) buffer (HEPES 20 mM, pH 7.9, NaCl 0.4 M, EDTA 1 mM, Glycerol 25%, Protease Inhibitors 1x (added just before use)) to extract nuclear proteins.

11. Fluorescence microscopy

Fluorescence microscopy was performed on RD cells. RD cells grown on glass cover slips were pretreated with Pim1 inhibitor CX-6258. The culture media were removed, and cells were washed three times with PBS. The cells on the coverslip were fixed with 3.7% (wt/vol) formaldehyde at room temperature for 20 min. After being washed three times with PBS, cells on the coverslip were permeabilized with 0.5% Triton X-100 at room temperature for 5 min and washed again three times with PBS. For AUF1 immunostaining, the samples were blocked in solution (PBS, containing 5% bovine serum albumin [BSA]) for 60 min at room temperature and then incubated with anti-AUF1 (ab50692, San Francisco) for 1.5 h at room temperature and washed three times with PBS. The

samples were then reacted with rhodamine (tetramethyl rhodamine isothiocyanate [TRITC])-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc.) for 1 hour at room temperature. After being washed with PBS, the samples were treated with DAPI for 5 min at room temperature and washed again with PBS three times. Finally, coverslips with adhered cells were placed on a glass slide and sealed with transparent nail polish. Images were captured by confocal laser scanning microscopy (ZEISS LSM 880 Confocal Microscope) (Lin et al., 2014).

12. Statistical analysis

Results were expressed as mean ± standard deviation (SD). All statistical analyses were carried out with SPSS, version 16.0 software (SPSS Inc.). A two-tailed Student's T test was applied for two group comparisons. A p value <0.05 was considered statistically significant.

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