**Supporting information:**

Title: Allosteric regulation of phosphatidylinositol 4-kinase III beta by an anti-picornavirus compound MDL-860

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The number of pages: 10
The number of figures: 6

**Figure S1. Effects of MDL-860 on the localization of OSBP and the amount of unesterified cholesterol in the cells.**

(**A**) Effect of MDL-860 on the localization of OSBP-EGFP in the non-infected cells. OSBP-EGFP-HEK293 cells were treated with 20 μM of T-00127-HEV2, itraconazole, or MDL-860 at 37°C for 20 min.
(**B**) Effect of MDL-860 on the amount of UC of the cells. RD cells were treated with 20 μM of T-00127-HEV1, T-00127-HEV2, or MDL-860 at 37°C for 6 h or 24 h. The amount of UC in the cells (%) is shown, where the amount of UC in the absence of compounds was taken as 100%. $n = 3$. *, $P < 0.05$.

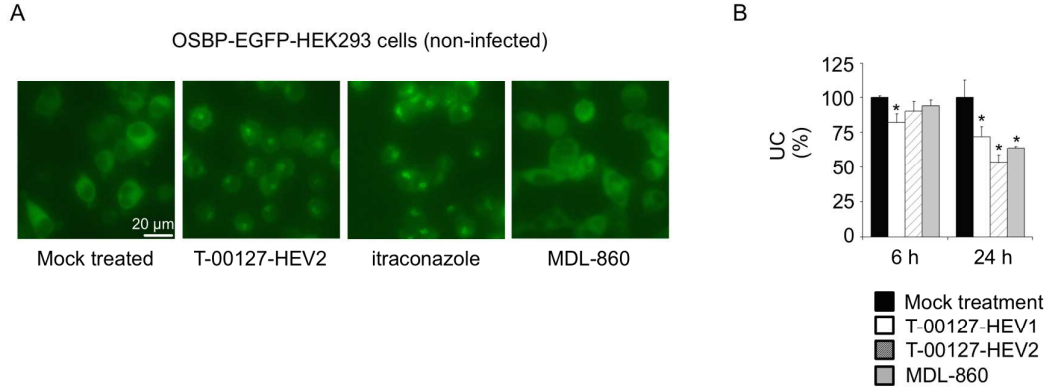


**Figure S2. Specificity of the anti-viral activity of MDL-860.**

AG1478 (PI4KA inhibitor) and T-00127-HEV1 (PI4KB inhibitor) were used as positive control for inhibition of PV and EMCV replicon [1], respectively. Replication of each replicon in the absence of compounds was taken as 100%. Effective concentration of each compound was indicated by line. $n$ =3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

$EC_{50}$ and $EC_{90}$ values of T-00127-HEV1 were as below:
$EC_{50}$ (T-00127-HEV1): 1.4 μM (PV replicon) vs. 7.2 μM (EMCV replicon)
$EC_{90}$ (T-00127-HEV1): 4.3 μM (PV replicon) vs. 30 μM (EMCV replicon))

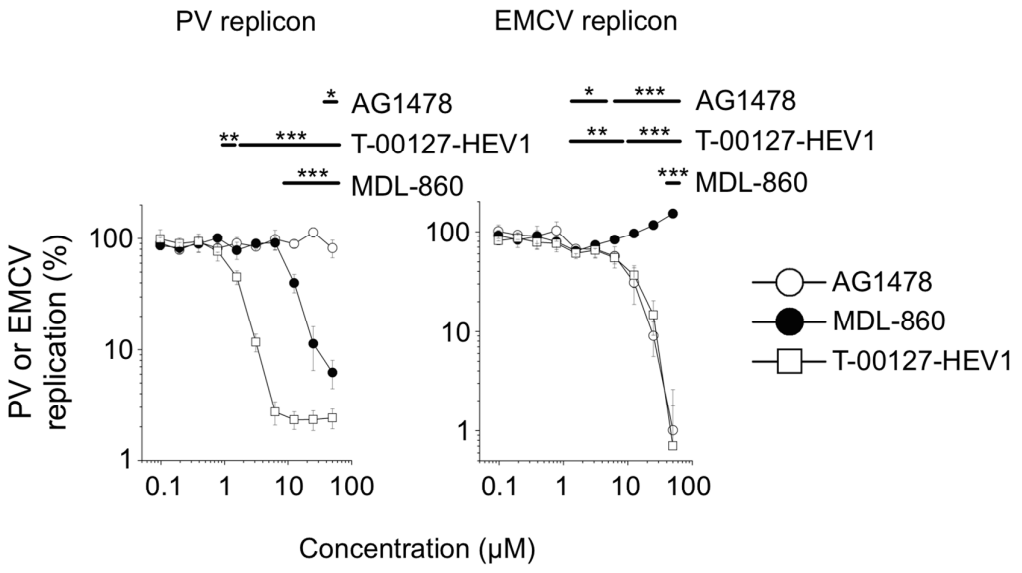


**Figure S3. Effect of MDL-860 on the localization of PI4KB in the cells.**

(**A**) Effect of MDL-860 on the localization of PI4KB and EGFP-RAB11A in non-infected cells [2]. The EGFP-RAB11-RD cells were treated with MDL-860(20 μM) or T-00127-HEV1(PI4KB inhibitor, 10 μM) at 37 C for 4 h (Top), or 1, 2, or 3 h (Bottom), and then subjected to indirect immunofluorescence**.** Green, EGFP-RAB11A; magenta, PI4KB, blue, nucleus.
(**B**) Effect of MDL-860 on the localization of PI4KB and PI4P in non-infected cells. RD cells were treated with MDL-860(20 μM) or T-00127-HEV1(20 μM) at 37 C for 24 h, and then subjected to indirect immunofluorescence**.** Green, PI4KB; magenta, PI4P, blue, nucleus.

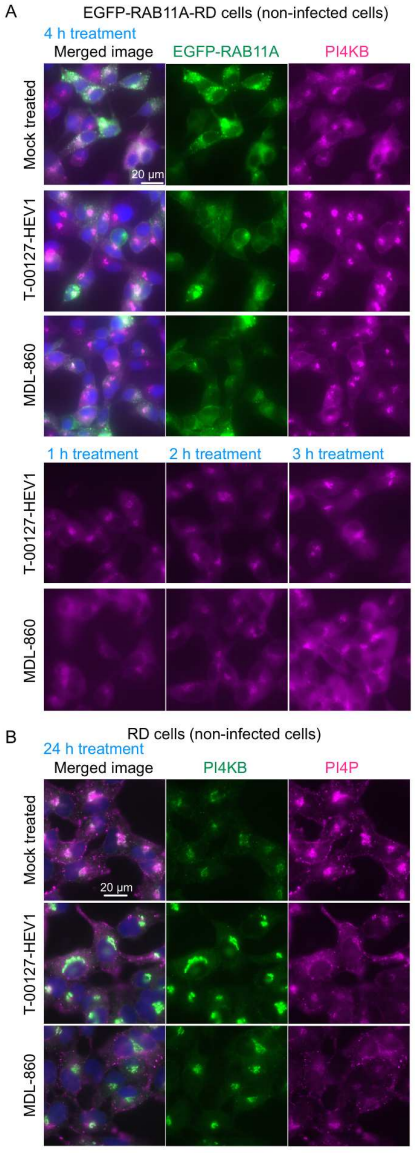


**Figure S4. Quantitation of PI4P in $PV1_{pv}$-infected cells.**

Quantitation of the net amount of produced PI4P in the $PV1_{pv}$-infected cells measured by flow cytometry. The net amount of produced PI4P in $PV1_{pv}$-infected cells with mock treatment was taken as 100%. $n = 3$. *, $P < 0.05$; **, $P < 0.01$,***, $P < 0.001$ (vs. 3 h treatment).

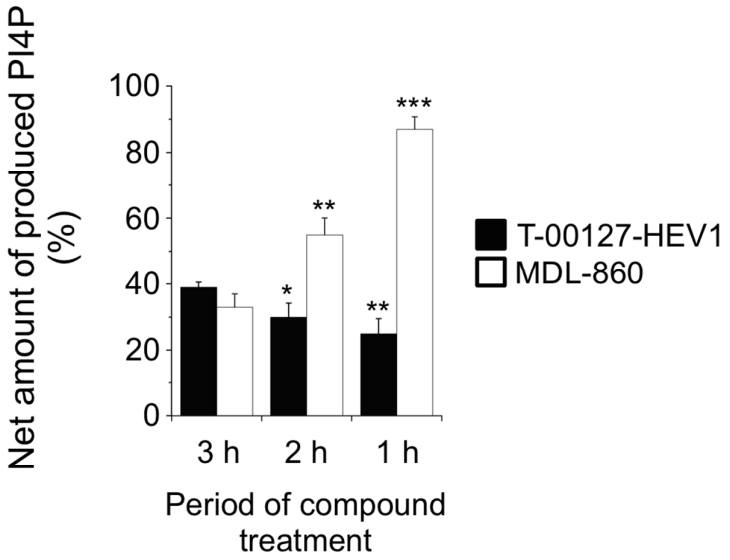


**Figure S5. Anti-PV activity of sulforaphane.**

RD cells were pre-treated with indicated concentration of sulforaphane for 0 or 24 h at 37°C before $PV1_{pv}$ infection. $PV1_{pv}$ infection in the absence of anti-PV compounds at 7 h p.i. was taken as 100 %.

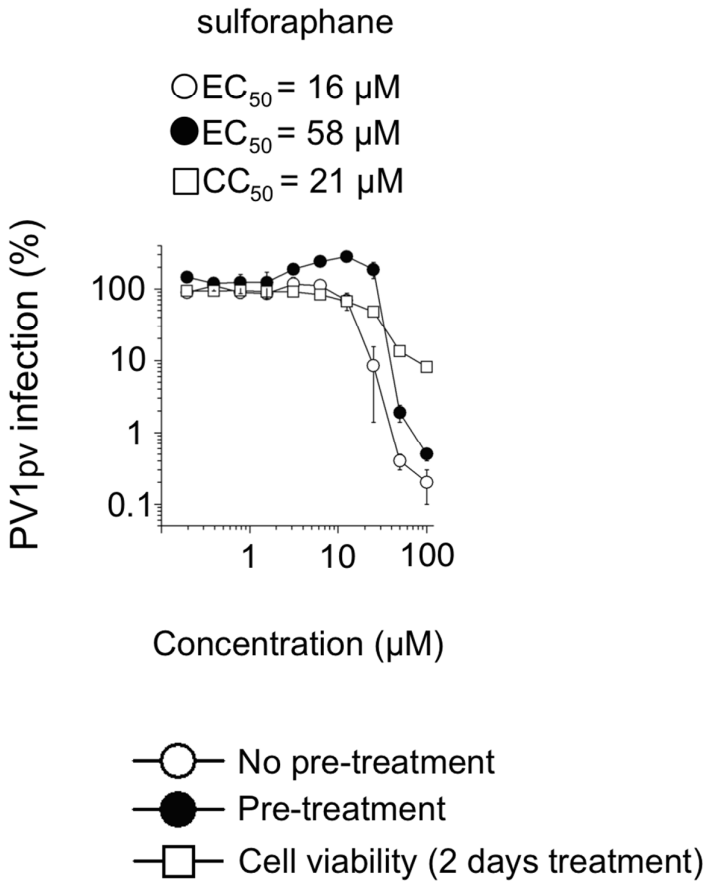
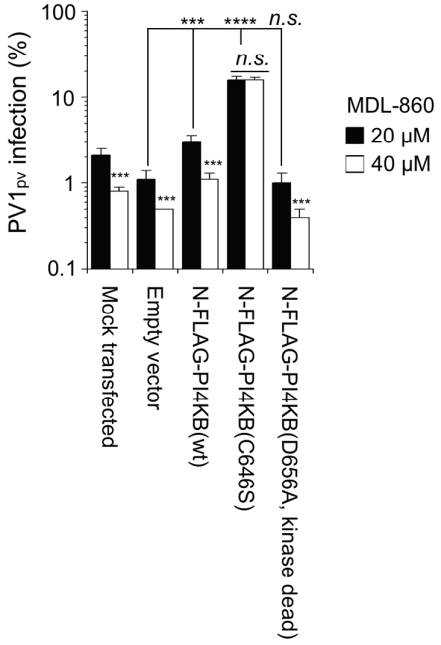


**Figure S6. Rescue of $PV1_{pv}$ infection in the presence of MDL-860 by ectopic expression of PI4KB.**

$PV1_{pv}$ infection in RD cells expressing N-FLAG-PI4KB (wt, C646S, or D656A [kinase dead]) in the presence of MDL-860 (20 or 40 μM). $PV1_{pv}$ infection in the absence of MDL-860 was taken as 100 %. $n = 3$. ***, $P < 0.001$; ****, $P < 0.0001$.



**Methods for Supporting information**

***Measurement of 50% cytotoxic concentration ($CC_{50}$) and 50% effective concentration ($EC_{50}$).***

For the measurement of $CC_{50}$, RD cells ($7 \times 10^3$ cells per well in 20 μL medium) were cultured at 37 °C in 384-well plates (catalog no. 781080; Greiner Bio-One), followed by addition of 20 μL MDL-860 solution (final concentrations of 0.19 to 100 μM). The cells were incubated at 37 °C for 2 days and then the cell viability was measured by using a Cell Titer-Glo Luminescent Cell Viability Assay kit (Promega) using a 2030 ARVO X luminometer (PerkinElmer).

For the measurement of $EC_{50}$, RD cells ($7 \times 10^3$ cells per well in 20 μL medium) in 384-well plates (catalog no. 781080; Greiner Bio-One) were inoculated with 10 μL of $PV1_{pv}$ (800 infectious units [IU]) and 10 μL of compound solution (the final concentrations of 0.15 to 80 μM for MDL-860, or indicated final concentrations for other compounds). The cells were incubated at 37 °C for 7 h. Luciferase activity in the infected cells was measured at 7 h postinfection (p.i.) with the Steady-Glo luciferase assay system (Promega) using a 2030 ARVO X luminometer (PerkinElmer). The $EC_{50}$ values of the compounds were obtained by nonlinear regression analysis of the dose-response curves.

***Immunofluorescence microscopy***.

Cells were fixed with 3% paraformaldehyde for 10 min at room temperature, and then permeabilized with 20 μM digitonin in HBS (21 mM HEPES buffer [pH 7.4], 1.8 mM disodium hydrogenphosphate, 137 mM NaCl, 4.8 mM KCl). The cells were stained by indirect immunofluorescence with primary antibodies against viral protein 2B or rabbit anti-PI4KB antibody (Millipore), secondary antibodies conjugated with Alexa Fluor 594 dyes (Molecular Probes), Hoechst 33342 (Molecular Probes) for counterstaining of nuclei. Samples were observed with a fluorescence microscope (BZ-9000, Keyence).

***Quantification of cholesterol.***

RD cells ($1.75 \times 10^5$ cells) were treated with 20 μM of T-00127-HEV1, T-00127-HEV2, or MDL-860 at 37°C for 6 h or 24 h. The cells were washed by 0.8 ml of HBS 3 times, and then cholesterol were extracted in 100 μL of extraction buffer (chloroform : isopropanol : NP-40 = 7 : 11: 0.1 volumes). The extracts were air dried, and then the amount of UC were quantified by using a Cholesteol/Choleteryl Ester Quantification Colorimetric/Fluorometric Kit (BioVision Inc.), according to the manufacturer's instructions.

***Inhibitory effect of MDL-860 on EMCV(mengovirus) replicon.***

RNA transcripts of PV replicon or of EMCV(mengovirus) replicon were obtained by using a T7 RiboMAX Express Large-Scale RNA production system kit (Promega) with *Dra*I-linearized DNA of pPV-Fluc mc or with *Bam*HI-linearized DNA of pMwtLuz (a generous gift from Ann Palmenberg, Institute for Molecular Virology, University of Wisconsin-Madison), respectively, as the templates. Both replicons have firefly luciferase gene instead of the capsid-coding region. RNA transcripts were transfected

into the cells by using a Lipofectamine MessengerMAX reagent (Invitrogen). At 1 h p.t. of of the RNA transcripts, the compounds were added to the final concentrations indicated. Cells were harvested at 7 h p.t. (for PV replicon) or 7 h p.t. (for EMCV replicon) of the RNA transcripts, and then the luciferase activity in the cells was measured with Steady-Glo Luciferase Assay System (Promega) using 2030 ARVO X luminometer (PerkinElmer) according to the manufacturer's instructions.

***Microarray analysis.***

Total RNAs were extracted from RD cells that were treated with 20 μM of T-00127-HEV1, T-00127-HEV2, 25-HC, itraconazole, MDL-860, or mock-treated with DMSO (control) for 24 h. The total RNAs were labeled, and then were hybridized to SurePrint G3 Human 8x60K ver. 3.0 array (Agilent technologies), and then analyzed by using GeneSpring GX by normalization of 75 percentile shift (Hokkaido System Science Co., Ltd.). Identified cluster of the genes were subjected to the analyzed for the upstream target by using an Ingenuity Pathway Analysis software (QIAGEN). The accession numbers for the microarray data are GSE97542 (Series record), GSM2571857 (mock treatment), and GSM2571858 (MDL-860 treatment)

***Rescue of $PV1_{pv}$ infection in the presence of MDL-860 by ectopic expression of PI4KB.***

Expression vectors for N-FLAG-PI4KB (wt, C646S, or D656A [kinase dead]) were constructed with pTK-Gluc vector (NEW ENGLAND BioLabs, Inc.), by replacing the coding region of *Gaussia* luciferase with that of N-FLAG-PI4KB. RD cells ($2.2 \times 10^4$ cells) were transfected with the expression vectors in the absence (mock treatment) or the presence of MDL-860 (20 or 40 μM). The cells were infected with 80,000 IU of $PV1_{pv}$ at 18 h p.t. Luciferase activity in the infected cell was measured at 7 h p.i.

***Peptide mapping***.

N-FLAG tagged PI4KB purified from MDL-860-treated HEK293 cells (1.6 μg) were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining, and then the corresponding bands were collected from the gel. Gel-purified N-FLAG-PI4KB was washed with 25 mM ammonium bicarbonate and acetonitrile, and then reduced with 10 mM dithiothreitol at 60°C followed by alkylation with 50 mM iodoacetamide at room temperature. Reduced/alkylated N-FLAG-PI4KB were digested with trypsin, chymotrypsin, or with elastase at 37°C, for 4 h, 12 h, or 12 h, respectively, and were then quenched with formic acid. Digested N-FLAG-PI4KB were subjected to nano LC/MS/MS with a Waters NanoAcquity HPLC system (Waters) and Orbitrap Fusion Lumos (60,000FWHM resolution for MS and 15,000FWHM resolution for MS/MS) (Thermo Scientific). The data were analyzed by using a Byonic software (wildcard search for 120-170 Da modification) and a Scaffold software to identify modifications of the amino acid residues.

## Reference

1. Fata-Hartley, C. L.; Palmenberg, A. C., Dipyridamole reversibly inhibits mengovirus RNA replication. *J. Virol.* **2005,** *79* (17), 11062-11070. DOI: 10.1128/JVI.79.17.11062-11070.2005.
2. Nakatsu, Y.; Ma, X.; Seki, F.; Suzuki, T.; Iwasaki, M.; Yanagi, Y.; Komase, K.; Takeda, M., Intracellular transport of the measles virus ribonucleoprotein complex is mediated by Rab11A-positive recycling endosomes and drives virus release from the apical membrane of polarized epithelial cells. *J. Virol.* **2013,** *87* (8), 4683-4693. DOI: 10.1128/JVI.02189-12.