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Mechanism of Poliovirus Resistance to Host Phosphatidylinositol-4 Kinase III β Inhibitor

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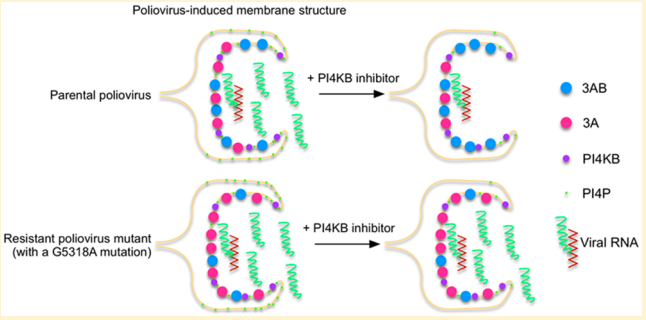
Supporting Information

ABSTRACT: Phosphatidylinositol-4 kinase III β (PI4KB) and oxysterol-binding protein (OSBP) family I have been identified as the major targets of anti-enterovirus drug candidates. Resistance mutations in poliovirus (PV) to these inhibitors have been identified in viral 3A protein, represented by a G5318A (3A-Ala70Thr) mutation, but the mechanism of viral resistance to host PI4KB/OSBP inhibitors remained unknown. In this study, we found that a G5318A mutation enhances the basal levels of phosphatidylinositol 4-phosphate (PI4P) and of the 3A protein and decreases the levels of the 3AB protein during PV replication. The 3A protein acted as a major effector responsible for the resistance to PI4KB inhibitor, but did not enhance the PI4KB activity in vitro in contrast to the 2C, 2BC, 3AB, and 3D proteins. The 3AB protein acted as the primary target of a G5318A mutation and also as an effector. We identified novel resistance mutations to a PI4KB inhibitor [C5151U (3A-T14M) and C5366U (3A-H86Y) mutations] and found that there is a positive correlation between the extent of the resistance phenotype and the levels of the 3A proteins. These results suggested that the 3A protein overproduced by enhanced processing of the 3AB protein with the resistance mutations overcomes the inhibitory effect of PI4KB inhibitor on PV replication independently of the hyperactivation of the PI4KB/OSBP pathway.



KEYWORDS: *poliovirus, enterovirus, PI4KB, resistance, inhibitor*

Poliovirus (PV) is a small nonenveloped virus with a single-strand positive genomic RNA of about 7500 nucleotides (nt) belonging to *Enterovirus* C species in the genus *Enterovirus*, the family Picornaviridae. PV is the causative agent of poliomyelitis, which is caused by the destruction of motor neurons by the direct infection of PV in cells.[1,2] In the global eradication program for poliovirus since 1988, antivirals for PV are anticipated to have some roles in the posteradication era of PV in control of a circulating vaccine-derived PV along with inactivated PV vaccine and for treatment of patients chronically infected with PV and for persons exposed to PV.[3] However, currently there is no antiviral available for PV infection.

Several independent large chemical screenings identified antienterovirus drug candidates.[4−8] The candidates of direct-acting antivirals target viral capsid proteins, 2A, 2C, 3C, and 3D.[9−16] For the candidate targets of host-targeting antiviral (HTA), many host factors for PV replication have been reported,[8,17−24] including druggable targets eIF4A, GBF1, and VCP/p97.[18,20,25−30] However, the targets of the identified candidate compounds seemed limited to phosphatidylinositol-4 kinase III β (PI4KB) or oxysterol-binding protein (OSBP) family I.[4,6,8,31−35] These suggested that the PI4KB/OSBP pathway might be the sole target pathway of HTA candidates identified in vitro cultured cells.[35]

PI4KB was originally identified as a host factor required for enterovirus replication.[36] Subsequently, it was also identified as the target of a group of compounds called enviroxime-like compounds [i.e., GW5074, T-00127-HEV1, enviroxime, BF-738735, pachypodol (Ro 09-0179), and oxoglaucine].[5,6,31,33,35,37−40] Enviroxime-like compounds share a common resistance mutation in the viral 3A-encoding region [a G5318A (3A-Ala70Thr) mutation in PV], which was originally identified as a resistant mutation against an anti-picornavirus compound, enviroxime.[4,41] OSBP family I was then identified as the target of the minor group of enviroxime-like compounds (i.e., AN-12-H5, T-00127-HEV1, 25-HC, and itraconazole).[8,34] OSBP transfers cholesterol between the endoplasmic reticulum and *trans*-Golgi by a phosphatidylinositol 4-phosphate (PI4P)-dependent manner and contributes to homeostasis of cholesterol and lipid.[42,43] On the basis of the fact that enviroxime-like compounds share a common resistance mutation, it was hypothesized that PI4KB and OSBP family I were located on the same biological pathway co-opted by PV replication.[6,8] The proposed model of the role of the PI4KB/OSBP pathway in PV replication is as follows: viral proteins modulate PI4KB activity to provide PI4P for the recruitment of OSBP to accumulate unesterified cholesterol (UC) on virus-induced membrane structure for the formation of a virus replication complex, rather than direct involvement in

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viral RNA synthesis.[30,34,44] Actually, a PI4KB inhibitor pachypodol (Ro 09-0179) did not suppress viral RNA synthesis of isolated viral replication complex despite its marked inhibitory effect on the virus replication in the cultured cells.[5] The viral replication complex formed by the PI4KB/OSBP pathway seemed to be involved preferentially in the synthesis of viral plus-strand RNA rather than minus-strand RNA.[41,45]

As the interactants of PI4KB, viral proteins 3A and 2BC have been identified.[30,36] These viral proteins act cooperatively in the formation of membrane structures derived from the endoplasmic reticulum, which were similar to those induced during PV replication (double-membrane structure) in terms of buoyant density and ultrastructure[46,47] and autophagy-like maturation of vesicles.[48] However, the role of the 3A and 2BC proteins in PI4P production remains enigmatic; ectopic expression of the 3A protein reduced PI4P levels in the cells in contrast to the 2BC protein, which increased PI4P levels.[30]

In unicellular protozoan, resistance to PI4KB inhibitors could be conferred by modulation of the copy number of the corresponding gene or by single-nucleotide mutation.[49] Obviously, this strategy is not applicable for a virus that depends on the host PI4KB activity. The mechanism of PV resistance conferred by a G5318A mutation against PI4KB inhibition is currently unknown. Recent study suggested that PI4KB inhibitors could interfere with viral polyprotein processing in vitro, which was alleviated in the presence of a G5318A mutation.[50] Analysis of PV replication in human primary fibroblast derived from individuals with Niemann−Pick type C diseases, which have enriched intracellular UC level, suggested that enriched UC specifically interferes with processing of viral 3CD protein.[51] These studies suggested that both PI4KB inhibition (i.e., resulting in less UC in viral replication site) and enriched UC could affect viral polyprotein processing. For coxsackievirus B3, a resistant mutant (3A-H57Y) to PI4KB inhibitor could replicate in the presence of PI4KB inhibitor without restoring high PI4P levels in the cells.[52] This suggested that the mutation in the 3A region conferred the resistance independently of PI4KB activation.

In the present study, we analyzed the mechanism of PV resistance to PI4KB inhibitor conferred by a G5318A mutation. Results suggested that the primary target of a G5318A mutation is the 3AB protein and that the major effector in the resistance is the 3A protein overproduced from the 3AB (G5318A) protein, which supports viral RNA synthesis independent of the hyperactivation of the PI4KB/OSBP pathway.

RESULTS AND DISCUSSION

Phenotypic Comparison of the Infection of Parental PV and the G5318A Mutant. To elucidate the mechanism of drug resistance conferred by a G5318A mutation against PI4KB inhibitor, we compared the phenotypes of parental PV infection with those of the G5318A mutant infection. First, we analyzed the range of concentration of PI4KB inhibitor (T-00127-HEV1) that could be overcome by a G5318A mutation (Figure 1). Parental $PV1_{pv}$ and $PV1_{pv}$(G5318A) showed almost similar EC_{50} values (0.66 and 0.84 μM, respectively). A G5318A mutation conferred significant resistance between 1.25 and 10 μM T-00127-HEV1, inclusive, but not at 20 μM. We compared the resistance phenotype of PV to PI4KB inhibitor with that to another host-targeting inhibitor, brefeldin A (BFA), which blocks membrane traffic between the *cis*- and *trans*-Golgi compartments by targeting a host cellular guanine nucleotide exchange factor GBF1. The G5318A mutant showed a narrow

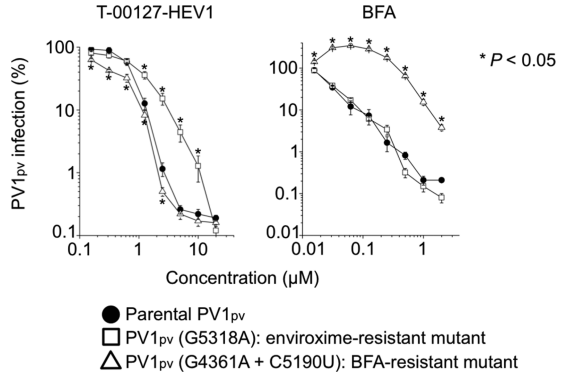

Figure 1. $PV1_{pv}$ infection in the presence of host-targeting inhibitors: inhibitory effect of PI4KB inhibitor (T-00127-HEV1) and BFA on $PV1_{pv}$ (parental or G5318A mutant or BFA-resistant mutant) infection in RD cells. $PV1_{pv}$ infection in the absence of the inhibitors was taken as 100%.

range of resistance to PI4KB inhibitors, making marked contrast to that of a BFA-resistant mutant to BFA[53] (Figure 1) (see Supporting Information, Figure S1). The BFA-resistant mutant also showed a drastic increase in the EC_{50} value (from 0.025 to 0.57 μM) (Figure 1). The proposed model for BFA resistance suggested that the biological activity of GBF1 is not the direct target of BFA for its anti-PV activity, but rather the formation of an abortive GBF1/ARF1−GDP complex;[54] BFA-resistance mutations in the viral proteins 2C and 3A are considered to adapt to use abortive GBF1/ARF1 complex as well as normal GBF1/ARF1 complex rather than to restore the biological activity of GBF1 suppressed by BFA. Observed difference in the resistance phenotypes suggested that the activity of PI4KB activity is essential for PV replication in contrast to that of GBF1 and is indispensable for the G5318A mutant as well as parental strain.

Next, we analyzed the expression levels of the viral proteins, PI4P, and UC in the cells infected with parental $PV1_{pv}$ or $PV1_{pv}$(G5318A) by flow cytometry (Figure 2). In the infected cells with $PV1_{pv}$(G5318A), fewer signals of anti-3B antibody and more signals of anti-PI4P antibody were detected than those infected with parental $PV1_{pv}$ in the absence of PI4KB inhibitor (1.2-fold increment, Figure 2A,B). Reduced signals of anti-3B antibody in the infected cells suggested decreased levels of an insoluble viral precursor protein 3AB. No significant change was observed for the signals of anti-2B, 2C, 3A, 3D antibodies and of UC. To analyze the effect of a G5318A mutation on the PI4P production in the presence of PI4KB inhibitor, where a G5318A mutation confers substantial resistance to PV, we transiently treated the infected cells with suboptimal concentration of PI4KB inhibitor (T-00127-HEV1, 2.5, and 5 μM) (Figure 1). A 2C inhibitor guanidine hydrochloride (GuHCl) was also added to suppress the replication of $PV1_{pv}$(G5318A) mutant as well as that of parental $PV1_{pv}$. Transient treatment with T-00127-HEV1 decreased the PI4P levels in the infected cells as previously observed.[30] The PI4P levels were retained higher in the cells infected with $PV1_{pv}$(G5318A) than those infected with parental $PV1_{pv}$, but were substantially reduced in the presence of suboptimal concentration of PI4KB inhibitor (Figure 2C,D). The 3A protein did not enhance the PI4KB activity in vitro in contrast to the 2C, 2BC, 3AB, and 3D proteins (see Supporting Information, Figure S2). These results suggested that a G5318A mutation confers the resistance to PI4KB inhibitor independent

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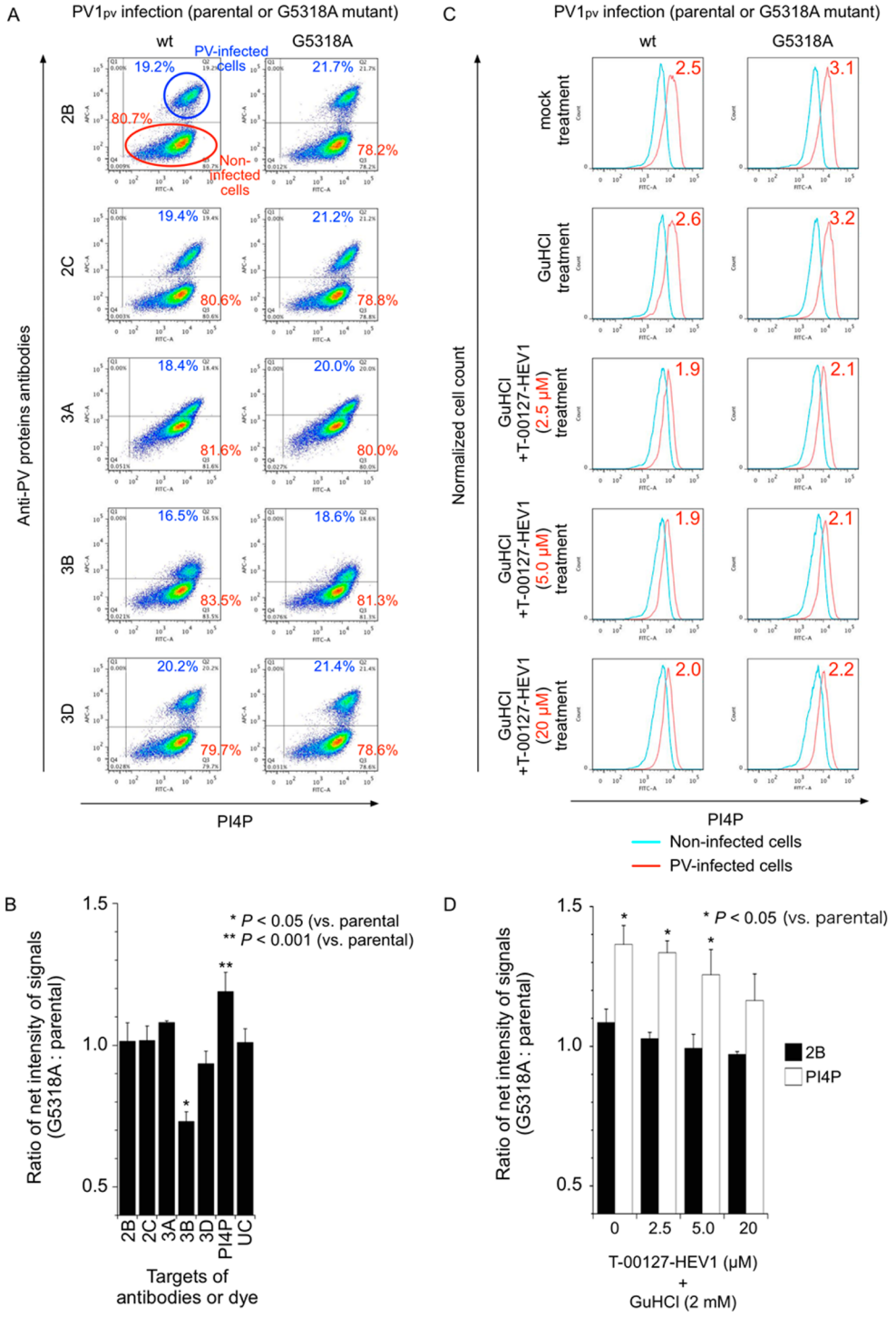

Figure 2. Quantitation of viral proteins and PI4P in PV-infected cells. (A) Flow cytometry analysis of HEK293 cells infected with $PV1_{pv}$ [parental or G5318A mutant, multiplicities of infection (MOI) = 1, at 6 h post-infection (pi)]. The cells were detected with anti-2B, 2C, 3A, 3B, 3D, and PI4P antibodies and filipin III (for detection of UC). (B) Quantitation of the ratio of net intensity of the signals of each antibody or filipin III. $n = 3$. (*) $P < 0.05$; (**) $P < 0.001$. (C) Flow cytometry analysis of HEK293 cells infected with $PV1_{pv}$ (parental or G5318A mutant, MOI = 1) after transient treatment (from 4 to 6 h pi) with 2C inhibitor (GuHCl) and PI4KB inhibitor (T-00127-HEV1). The cells were detected with anti-2B and PI4P antibodies. Ratios of geometric means of PI4P signals in $PV1_{pv}$-infected cells to noninfected cells are shown in the graphs. (D) Quantitation of the ratio of net intensity of the signals of each antibody. $n = 3$. (*) $P < 0.05$.

of the activation of the PI4KB/OSBP pathway, as previously observed for CVB3 resistant mutants.[52]

Next, we analyzed the relative amounts of the viral proteins in the infected cells with parental $PV1_{pv}$ or $PV1_{pv}$(G5318A) by Western blot analysis (Figure 3). Viral 3A or 3AB proteins with a G5318A mutation migrated slightly more quickly than parental proteins, suggesting a significant contribution of the Ala70Thr mutation to the electrostatic status of the proteins. The ratios of precursor proteins and viral proteins (2BC:2C and 3CD:3D) were about 0.20 and 0.72 for parental $PV1_{pv}$ and

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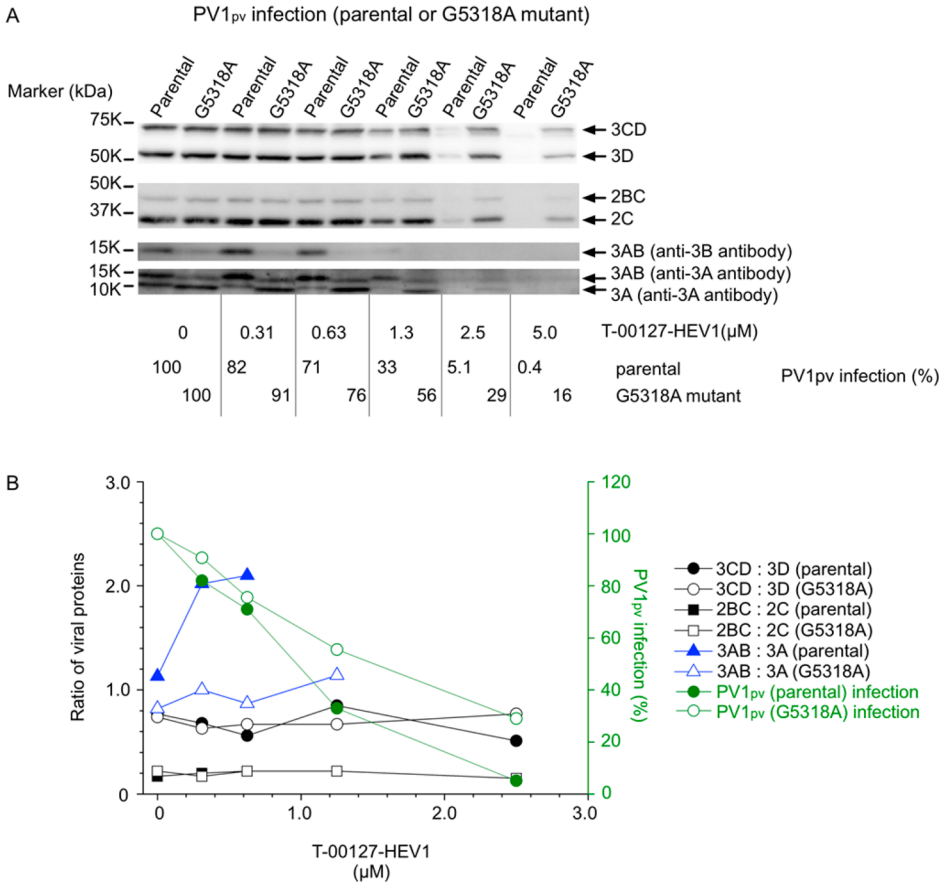

Figure 3. Processing of viral proteins in PV-infected cells in the presence of PI4KB inhibitor. (A) Western blot analysis of the RD cells infected with $PV1_{pv}$ (parental or the G5318A mutant, MOI = 5, at 7 h pi) in the presence of suboptimal concentrations of T-00127-HEV1. The viral proteins were detected with anti-2C, 3A, 3B, and 3D antibodies. $PV1_{pv}$ infection in the absence of T-00127-HEV1 was taken as 100%. (B) Quantitation of the viral proteins analyzed in (A).

about 0.21 and 0.68 for $PV1_{pv}$(G5318A), respectively, and remained consistent in the absence and presence of T-00127-HEV1. There were no significant differences in the relative levels of 2C, 2BC, 3C, and 3CD between parental $PV1_{pv}$ and $PV1_{pv}$(G5318A). Significant difference between parental $PV1_{pv}$ and $PV1_{pv}$(G5318A) was observed in the ratio of 3AB:3A. In the absence of T-00127-HEV1, the ratios of 3AB:3A were 1.1 and 0.82 for parental $PV1_{pv}$ and $PV1_{pv}$(G5318A), respectively. This indicated that $PV1_{pv}$(G5318A) produced 1.4-fold more 3A protein than parental $PV1_{pv}$ in the basal condition. The ratio of 3AB:3A was increased in the presence of T-00127-HEV1 for parental $PV1_{pv}$ (from 1.1 to 2.1), but mostly unchanged for $PV1_{pv}$(G5318A) (from 0.82 to 1.0). These results suggested that the basal level of the 3A protein was increased in the presence of a G5318A mutation.

Rescue of PV Replication in the Presence of PI4KB Inhibitor by *trans*-Complementation with Viral Proteins. To identify the viral protein that acts as the effector in the resistance to PI4KB inhibitor, we performed a *trans*-complementation assay with each viral protein (Figure 4).[55] In this assay, each viral protein was overexpressed in HEK293 cells, and then the effect of viral protein levels on $PV1_{pv}$ infection in the presence of PI4KB inhibitor (1.3 or 2.5 μM) was analyzed. In the presence of 1.3 or 2.5 μM T-00127-HEV1, $PV1_{pv}$ infection was about 19−44% or 0.2−1.1%, respectively. About 10−40% of the cells expressed the viral proteins (Figure 4A). We found that overexpression of the 3A and 3AB proteins (without or with histidine tag) restored viral replication in the presence of T-00127-HEV1 (1.3% in mock-transfected sample to 9.6, 4.7, and 7.1%, respectively). Overexpression of the 2B, 2C, 2BC, and 3CD proteins did not restore PV replication in the presence of T-00127-HEV1; expression of the 2B and 2C proteins rather suppressed PV replication.

Next, to identify the direct target of a G5318A mutation, we performed a *trans*-complementation assay with the 3A and 3AB proteins with or without the mutation (Figure 4B). The expression level of the 3AB protein, but not of the 3A protein, was slightly reduced in the presence of a G5318A mutation (60% of the parental 3AB protein). We also examined C-terminally histidine-tagged 3AB protein (3AB-HIS) that has partially restored expression level with a G5318A mutation (80% of parental histidine-tagged 3AB protein) in the assay. The presence of a G5318A mutation in the 3AB proteins, but not in the 3A protein, restored the PV replication in the presence of T-00127-HEV1 to higher levels than those by parental 3AB proteins (by 2.8- or 2.5-fold increase compared to parental 3AB proteins). Next, we analyzed the importance of processing of the 3AB protein by using a 3AB protein with noncleavable mutation between the 3A and 3AB protein regions (mutation at Q/G cleavage site to A/G) (Figure 4C). We found that both parental and the mutant 3AB protein could restore PV replication in the presence of PI4KB inhibitor in *trans*-complementation. Interestingly, the presence of a G5318A mutation in noncleavable 3AB protein also restored

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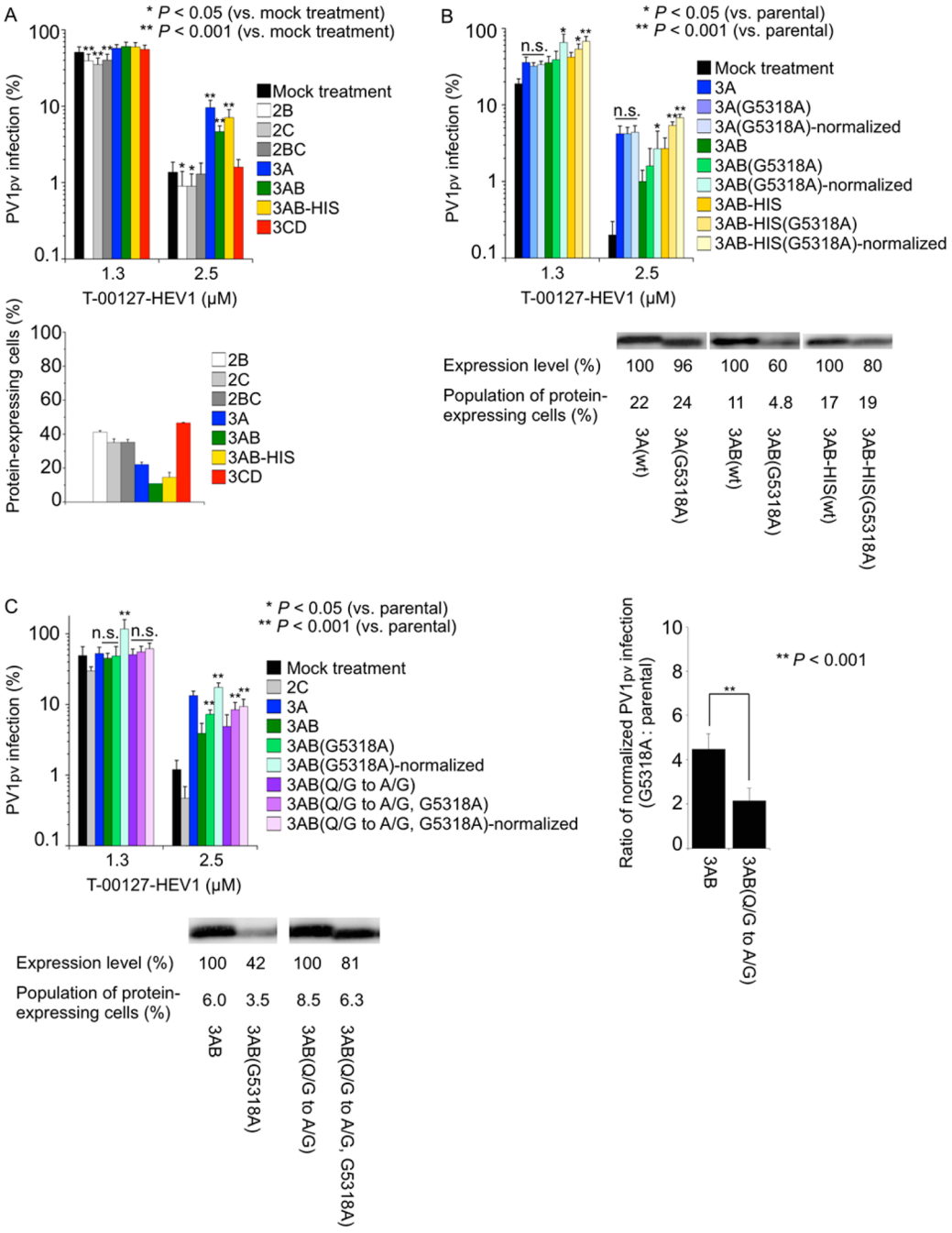

Figure 4. Rescue of PV replication in the presence of PI4KB inhibitor by *trans*-complementation with viral proteins. (A, upper panel) *trans*-Complementation assay with viral proteins. HEK293 cells expressing each viral protein were infected with $PV1_{pv}$ (MOI = 0.1) in the presence of suboptimal concentrations of T-00127-HEV1. $PV1_{pv}$ infection in the absence of T-00127-HEV1 was taken as 100%. (A, lower panel) Populations of viral protein-expressing cells (%) determined by flow cytometry. $n = 6$. (∗) $P < 0.05$, (∗∗) $P < 0.001$; (n.s.) not significant. (B, C, upper panels) *trans*-Complementation assay with the 3A, 3AB, and 3AB-HIS proteins. $PV1_{pv}$ infection normalized by the relative expression levels of the 3AB(G5318A) protein to the 3AB(parental) protein is also shown. (B, C, lower panels) Protein expression levels determined by Western blot analysis, and populations of viral protein-expressing cells (%) determined by flow cytometry. Protein expression levels of parental proteins were taken as 100%. (C, right panel) Ratio of the normalized $PV1_{pv}$ infection of the parental protein: G5318A mutant for the 3AB protein and the noncleavable 3AB protein mutant (Q/G to A/G mutation). $n = 6$ or 3. (∗) $P < 0.05$, (∗∗) $P < 0.001$; (n.s.) not significant.

the PV replication in the presence of T-00127-HEV1 to higher levels than those by parental noncleavable 3AB protein (2.1-fold enhancement), but with a lesser extent compared to the cleavable 3AB protein (4.5-fold enhancement) (Figure 4C, right panel). This suggested that both the 3A and 3AB proteins could serve as effectors in the resistance to PI4KB inhibitor. The importance of the 3A protein in positive-strand RNA synthesis has been suggested with PV mutants defective in viral RNA synthesis,[56] consistent with the target step of PI4KB inhibitors.[41,45] Considering (1) the abundance of the 3A protein in the infected cells with the G5318A PV mutant (Figure 3) and (2) the stronger *trans*-complementation effect of a G5318A mutation in the cleavable 3AB protein than that in the noncleavable 3AB protein, the 3A protein seems to act as

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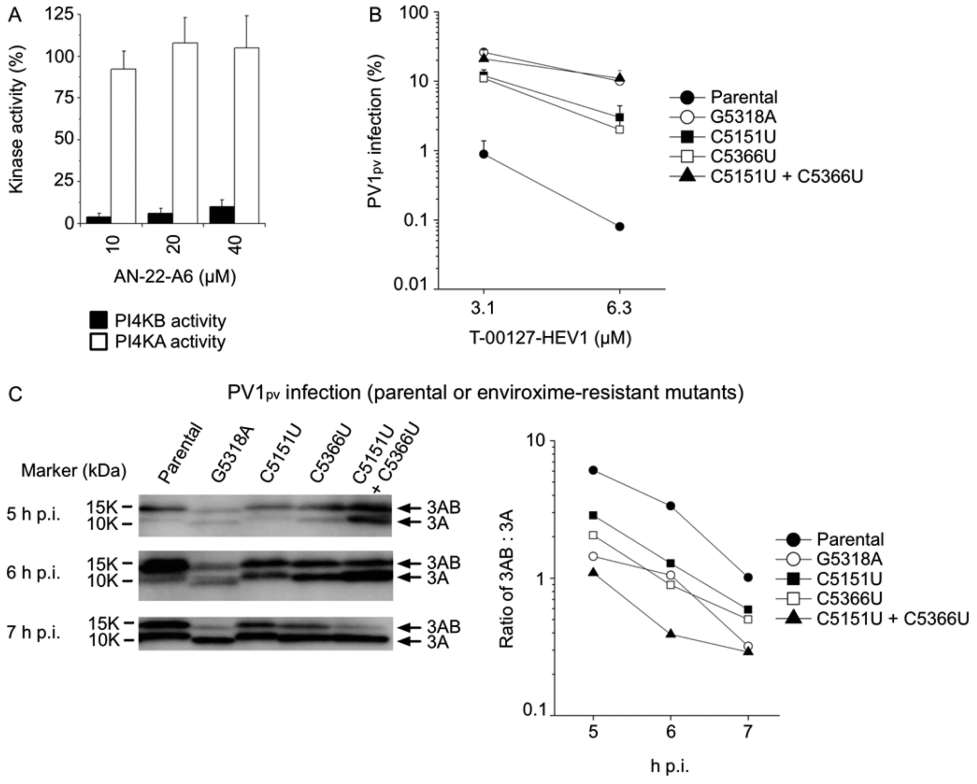

Figure 5. Effect of other resistance mutations to PI4KB inhibitor on the 3A protein production. (A) Effect of AN-22-A6 on in vitro PI4KB activity. (B) Resistance phenotypes of the AN-22-A6-resistant mutants against T-00127-HEV1. (C, left panel) Western blot analysis of the RD cells infected with PV1pv (parental or the resistant mutants, MOI = 5, at 5, 6, and 7 h pi). The 3A and 3AB proteins were detected with anti-3A antibody. (C, right panel) Quantification of the ratio of 3AB:3A in the left panel.

the major effector in the resistance to PI4KB inhibitor along with activated 3AB protein with a G5318A mutation.

Effect of the Resistance Mutations to PI4KB Inhibitor on the 3A Protein Production. Previously, we identified another enviroxime-like compound AN-22-A6 along with the resistance mutations in the 3A protein [C5151U (3A-T14M) and C5366U (3A-H86Y) mutations].[32] However, the target of AN-22-A6 remained unknown. We analyzed the effect of AN-22-A6 on in vitro PI4KB activity and found that AN-22-A6 is a novel PI4KB inhibitor (Figure 5A). Next, we analyzed the effect of these novel resistant mutations to PI4KB inhibitor on the resistance phenotype and on the production of the 3A protein. The effects of C5151U and C5366U mutations on the resistant phenotype to T-00127-HEV1 were weaker than that of a G5318A mutation (Figure 5B). Combination of the C5151U and C5366U mutations conferred a level of resistance similar to that of a G5318A mutation. Next, we analyzed the expression levels of the 3A and 3AB proteins in the cells infected with $PV1_{pv}$ with the resistance mutations (Figure 5C). Processing of the 3AB protein occurred as the replication proceeded (5−7 h pi), and the ratio of 3AB:3A was consistently lower for $PV1_{pv}$ mutants with the resistance mutations than that of the parental $PV1_{pv}$. There was a positive correlation between the extent of the resistance phenotype and the relative levels of the 3A proteins. The 3A and 3AB proteins with these mutations migrated slightly more rapidly than parental proteins, as well as those with a G5318A mutation (Figures 3 and 5), suggesting the electrostatic change of the 3AB protein caused by these mutations might enhance the processing by the 3C protein. This suggested that overproduction of the 3A protein is a general strategy for the resistance of PV against PI4KB inhibitor.

In summary, our results suggested that the viral 3AB protein is the primary target of a G5318A mutation, and the 3A protein is the major effector responsible for the resistance to PI4KB inhibitor. Our results provide a novel insight into the mechanism of virus resistance against host-targeting antivirals.

METHODS

Cells, Viruses, Antibodies, Plasmids, and Chemical Library. RD cells (human rhabdomyosarcoma cell line) were obtained from the U.S. Centers for Disease Control. HEK293 cells (human embryonic kidney cell line) were obtained from American Type Culture Collection. The cells were cultured as monolayers in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). RD cells were used for the titration of viruses and pseudoviruses. HEK293 cells were used for the preparation of PV1 pseudovirus ($PV1_{pv}$), which encapsidated luciferase-encoding PV replicons with capsid proteins derived from PV1(Mahoney).[57] $PV1_{pv}$ mutants with drug-resistance mutations, including G5318A mutation (enviroxime or PI4KB inhibitor-resistance mutation, 3A-Ala70Thr)[6,41] or G4361A and C5190U mutations (BFA resistance, 2C-Val80Ile and 3A-Ala27Val),[53] were used for characterization of the effect of PI4KB inhibitor. Rabbit hyperimmune serum against PV 2C protein was a kind gift from Tomoichiro Oka (Department of Virology II, National Institute of Infectious Diseases, Japan). Antibodies against PV 2B, 3A, 3AB, and 3D proteins were raised in rabbits with peptides WLRKKACDVLEIPYVIKQ (amino acids 80−97 of PV 2B protein), CDLLQAVDSQEVRDY (amino acids 23−36

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of PV 3A protein), CNKKPNVPTIRTAKVQ (amino acids 8−22 of PV 3B protein), and GEIQWMRPSKEVGYPIINA (amino acids 1−19 of PV 3D protein), respectively.[20,30]

Expression vectors for PV proteins (2B, 2C, 2BC, 3A, 3AB, 3AB, 3CD, 3A, or 3AB with a G5318A mutation and C-terminally hexahistidine-tagged 3A and 3AB proteins) were constructed with pKS435, where expression of viral proteins was controlled under the HEF-1α promoter. N-terminally FLAG-tagged viral proteins (indicated as N-FLAG-viral proteins) were constructed with pHEK293 Ultra Expression Vector I (TaKaRa Bio Inc.). H40E mutation in 3CD was introduced to avoid autocleavage of 3CD protein into 3C and 3D, but to retain RNA binding activity.[58]

A specific PI4KB inhibitor T-00127-HEV1,[6] (3-(3,4-dimethoxyphenyl)-2,5-dimethyl-*N*-[2-(4-morpholinyl)ethyl]-pyrazolo[1,5-*a*]pyrimidin-7-amine), was purchased from Pharmeks Ltd. (Moscow) (purity > 99%).

Western Blot Analysis. Samples were subjected to 5−20% gradient polyacrylamide gel electrophoresis (e-PAGEL; Atto Corp.) in a Laemmli buffer system. The proteins in the gel were transferred to a polyvinylidene difluoride filter (Immobilon; Millipore) and blocked by using 0.5% bovine serum albumin (BSA) in HBS [21 mM HEPES buffer (pH 7.4), 0.7 mM disodium hydrogen phosphate, 137 mM NaCl, 4.8 mM KCl]. The filters were incubated with primary antibodies [antiviral proteins antibodies or anti-penta-His antibody (Qiagen)] and then with secondary antibodies [goat anti-rabbit or anti-mouse IgG antibodies conjugated with horseradish peroxidase (Pierce), 1:200 dilution] in iBind Western System (Thermo Fischer Scientific Inc.). The signals were detected with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce) and then analyzed with LAS3000 (Fujifilm).

Flow Cytometry. HEK293 cells (8.0×10^5 cells) infected with $PV1_{pv}$ (parental or G5318A mutant) at multiplicities of infection (MOI) of 1 or transfected with viral proteins expression vectors were collected at 6 h postinfection (pi) or at 24 h post-transfection (pt) in 0.8 mL of 10% FCS−DMEM in the presence or absence of indicated concentrations of T-00127-HEV1. To evaluate the effect of PI4KB inhibition on PI4P production and UC accumulation during PV replication, T-00127-HEV1 (0, 2.5, 5.0, 20 μM) and GuHCl (2 mM) were added to the cells at 4 h pi. The cells were fixed with 3% paraformaldehyde for 10 min at room temperature and then permeabilized with 20 μM digitonin in HBS for 5 min. The cells were incubated with primary antibodies for 30 min at 37 °C. Cells were washed two times by 0.5% BSA in HBS and then incubated with secondary antibodies conjugated with Alexa Fluor 647 and 488 dyes (Molecular Probes) for 20 min at 37 °C. UC in the cells was stained with filipin III (Cayman) at room temperature for 30 min. The cells were suspended in 250 μL of HBS. About 5.0×10^4 cells were analyzed per sample with a BD FACSCanto II flow cytometer (BD Biosciences) and FlowJo software (FLOWJO, LLC). The relative intensity of the signals was determined as

$$\text{relative intensity of signals} = \text{signals in viral protein-expressing or PV-infected cells} / \text{signals in non-expressing or non-infected cells}$$

The net intensity of the signals was determined as

$$\text{net intensity of signals} = \text{relative intensity of signals} - 1$$

***trans*-Complementation Assay.** HEK293 cells (7.3×10^3 cells) were transfected with viral protein expression vectors and then were infected with $PV1_{pv}$ at an MOI of 0.1 in the presence of T-00127-HEV1 (0, 1.3, or 2.5 μM) at 24 h pt. Luciferase activity in the infected cell was measured at 7 h pi. For 3AB(G5318A) proteins, $PV1_{pv}$ infection normalized by the relative expression levels of 3AB(G5318A) to 3AB(parental) proteins determined by Western blot analysis is also determined.

In Vitro PI4KB Activity. Inhibitory effect of AN-22-A6 on in vitro PI4KB activity was assessed by SelectScreen Kinase Profiling Service with ATP concentration of 10 μM and phosphatidylinositol of 100 μM (Invitrogen).

Statistical Analysis. The results of experiments are shown as the averages with standard deviations. A one-tailed *t* test was performed with data obtained from three or four independent experiments as indicated. *P* values of <0.05 were considered significantly different and are indicated by asterisks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00122.

Figures S1 and S2; methods (PDF)

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■ ABBREVIATIONS

BFA, brefeldin A; GuHCl, guanidine hydrochloride; HTA, host-targeting antiviral; OSBP, oxysterol-binding protein; PI4KB, phosphatidylinositol-4 kinase III beta; PI4P, phosphatidylinositol 4-phosphate; PV, poliovirus; UC, unesterified cholesterol

■ REFERENCES

(1) Bodian, D. (1949) Histopathologic basis of clinical findings in poliomyelitis. *Am. J. Med. 6*, 563−578.

DOI: 10.1021/acsinfecdis.5b00122
ACS Infect. Dis. 2016, 2, 140−148

(2) Couderc, T., Christodoulou, C., Kopecka, H., Marsden, S., Taffs, L. F., Crainic, R., and Horaud, F. (1989) Molecular pathogenesis of neural lesions induced by poliovirus type 1. *J. Gen. Virol. 70*, 2907−2918.
(3) Committee on Development of a Polio Antiviral and Its Potential Role in Global Poliomyelitis Eradication, NRC. (2006) *Exploring the Role of Antiviral Drugs in the Eradication of Polio: Workshop Report*, National Academies Press, Washington, DC, USA.
(4) Wikel, J. H., Paget, C. J., DeLong, D. C., Nelson, J. D., Wu, C. Y., Paschal, J. W., Dinner, A., Templeton, R. J., Chaney, M. O., Jones, N. D., and Chamberlin, J. W. (1980) Synthesis of syn and anti isomers of 6-[[(hydroxyimino)phenyl]methyl]-1-[(1-methylethyl)sulfonyl]-1*H*-benzimidaz ol-2-amine. Inhibitors of rhinovirus multiplication. *J. Med. Chem. 23*, 368−372.
(5) Ishitsuka, H., Ohsawa, C., Ohiwa, T., Umeda, I., and Suhara, Y. (1982) Antipicornavirus flavone Ro 09-0179. *Antimicrob. Agents Chemother. 22*, 611−616.
(6) Arita, M., Kojima, H., Nagano, T., Okabe, T., Wakita, T., and Shimizu, H. (2011) Phosphatidylinositol 4-kinase III beta is a target of enviroxime-like compounds for antipoliovirus activity. *J. Virol. 85*, 2364−2372.
(7) Spickler, C., Lippens, J., Laberge, M. K., Desmeules, S., Bellavance, E., Garneau, M., Guo, T., Hucke, O., Leyssen, P., Neyts, J., Vaillancourt, F. H., Decor, A., O'Meara, J., Franti, M., and Gauthier, A. (2013) Phosphatidylinositol 4-kinase III beta is essential for replication of human rhinovirus and its inhibition causes a lethal phenotype in vivo. *Antimicrob. Agents Chemother. 57*, 3358−3368.
(8) Arita, M., Kojima, H., Nagano, T., Okabe, T., Wakita, T., and Shimizu, H. (2013) Oxysterol-binding protein family I is the target of minor enviroxime-like compounds. *J. Virol. 87*, 4252−4260.
(9) Fox, M. P., Otto, M. J., and McKinlay, M. A. (1986) Prevention of rhinovirus and poliovirus uncoating by WIN 51711, a new antiviral drug. *Antimicrob. Agents Chemother. 30*, 110−116.
(10) Molla, A., Hellen, C. U., and Wimmer, E. (1993) Inhibition of proteolytic activity of poliovirus and rhinovirus 2A proteinases by elastase-specific inhibitors. *J. Virology 67*, 4688−4695.
(11) Caliguiri, L. A., and Tamm, I. (1968) Action of guanidine on the replication of poliovirus RNA. *Virology 35*, 408−417.
(12) Shimizu, H., Agoh, M., Agoh, Y., Yoshida, H., Yoshii, K., Yoneyama, T., Hagiwara, A., and Miyamura, T. (2000) Mutations in the 2C region of poliovirus responsible for altered sensitivity to benzimidazole derivatives. *J. Virol. 74*, 4146−4154.
(13) De Palma, A. M., Heggermont, W., Lanke, K., Coutard, B., Bergmann, M., Monforte, A. M., Canard, B., De Clercq, E., Chimirri, A., Purstinger, G., Rohayem, J., van Kuppeveld, F., and Neyts, J. (2008) The thiazolobenzimidazole TBZE-029 inhibits enterovirus replication by targeting a short region immediately downstream from motif C in the nonstructural protein 2C. *J. Virol. 82*, 4720−4730.
(14) Ulferts, R., van der Linden, L., Thibaut, H. J., Lanke, K. H., Leyssen, P., Coutard, B., De Palma, A. M., Canard, B., Neyts, J., and van Kuppeveld, F. J. (2013) Selective serotonin reuptake inhibitor fluoxetine inhibits replication of human enteroviruses B and D by targeting viral protein 2C. *Antimicrob. Agents Chemother. 57*, 1952−1956.
(15) Patick, A. K., Binford, S. L., Brothers, M. A., Jackson, R. L., Ford, C. E., Diem, M. D., Maldonado, F., Dragovich, P. S., Zhou, R., Prins, T. J., Fuhrman, S. A., Meador, J. W., Zalman, L. S., Matthews, D. A., and Worland, S. T. (1999) In vitro antiviral activity of AG7088, a potent inhibitor of human rhinovirus 3C protease. *Antimicrob. Agents Chemother. 43*, 2444−2450.
(16) Furuta, Y., Takahashi, K., Fukuda, Y., Kuno, M., Kamiyama, T., Kozaki, K., Nomura, N., Egawa, H., Minami, S., Watanabe, Y., Narita, H., and Shiraki, K. (2002) In vitro and in vivo activities of anti-influenza virus compound T-705. *Antimicrob. Agents Chemother. 46*, 977−981.
(17) Hellen, C. U., Witherell, G. W., Schmid, M., Shin, S. H., Pestova, T. V., Gil, A., and Wimmer, E. (1993) A cytoplasmic 57-kDa protein that is required for translation of picornavirus RNA by internal ribosomal entry is identical to the nuclear pyrimidine tract-binding protein. *Proc. Natl. Acad. Sci. U. S. A. 90*, 7642−7646.
(18) Wessels, E., Duijsings, D., Niu, T. K., Neumann, S., Oorschot, V. M., de Lange, F., Lanke, K. H., Klumperman, J., Henke, A., Jackson, C. L., Melchers, W. J., and van Kuppeveld, F. J. (2006) A viral protein that blocks Arf1-mediated COP-I assembly by inhibiting the guanine nucleotide exchange factor GBF1. *Dev. Cell 11*, 191−201.
(19) Bedard, K. M., Daijogo, S., and Semler, B. L. (2007) A nucleo-cytoplasmic SR protein functions in viral IRES-mediated translation initiation. *EMBO J. 26*, 459−467.
(20) Arita, M., Wakita, T., and Shimizu, H. (2012) Valosin-containing protein (VCP/p97) is required for poliovirus replication and is involved in cellular protein secretion pathway in poliovirus infection. *J. Virol. 86*, 5541−5553.
(21) Rozovics, J. M., Chase, A. J., Cathcart, A. L., Chou, W., Gershon, P. D., Palusa, S., Wilusz, J., and Semler, B. L. (2013) Picornavirus modification of a host mRNA decay protein. *mBio 3*, e00431.
(22) Sasaki, J., Ishikawa, K., Arita, M., and Taniguchi, K. (2012) ACBD3-mediated recruitment of PI4KB to picornavirus RNA replication sites. *EMBO J. 31*, 754−766.
(23) Virgen-Slane, R., Rozovics, J. M., Fitzgerald, K. D., Ngo, T., Chou, W., van der Heden van Noort, G. J., Filippov, D. V., Gershon, P. D., and Semler, B. L. (2012) An RNA virus hijacks an incognito function of a DNA repair enzyme. *Proc. Natl. Acad. Sci. U. S. A. 109*, 14634−14639.
(24) Nchoutmboube, J. A., Viktorova, E. G., Scott, A. J., Ford, L. A., Pei, Z., Watkins, P. A., Ernst, R. K., and Belov, G. A. (2013) Increased long chain acyl-CoA synthetase activity and fatty acid import is linked to membrane synthesis for development of picornavirus replication organelles. *PLoS Pathog. 9*, e1003401.
(25) Bordeleau, M. E., Mori, A., Oberer, M., Lindqvist, L., Chard, L. S., Higa, T., Belsham, G. J., Wagner, G., Tanaka, J., and Pelletier, J. (2006) Functional characterization of IRESes by an inhibitor of the RNA helicase eIF4A. *Nat. Chem. Biol. 2*, 213−220.
(26) Irurzun, A., Perez, L., and Carrasco, L. (1992) Involvement of membrane traffic in the replication of poliovirus genomes: effects of brefeldin A. *Virology 191*, 166−175.
(27) Maynell, L. A., Kirkegaard, K., and Klymkowsky, M. W. (1992) Inhibition of poliovirus RNA synthesis by brefeldin A. *J. Virol. 66*, 1985−1994.
(28) Cuconati, A., Molla, A., and Wimmer, E. (1998) Brefeldin A inhibits cell-free, de novo synthesis of poliovirus. *J. Virol. 72*, 6456−6464.
(29) Belov, G. A., Feng, Q., Nikovics, K., Jackson, C. L., and Ehrenfeld, E. (2008) A critical role of a cellular membrane traffic protein in poliovirus RNA replication. *PLoS Pathog. 4*, e1000216.
(30) Arita, M. (2014) Phosphatidylinositol-4 kinase III beta and oxysterol-binding protein accumulate unesterified cholesterol on poliovirus-induced membrane structure. *Microbiol. Immunol. 58*, 239−256.
(31) Arita, M., Wakita, T., and Shimizu, H. (2008) Characterization of pharmacologically active compounds that inhibit poliovirus and enterovirus 71 infectivity. *J. Gen. Virol. 89*, 2518−2530.
(32) Arita, M., Takebe, Y., Wakita, T., and Shimizu, H. (2010) A bifunctional anti-enterovirus compound that inhibits replication and early stage of enterovirus 71 infection. *J. Gen. Virol. 91*, 2734−2744.
(33) Delang, L., Paeshuyse, J., and Neyts, J. (2012) The role of phosphatidylinositol 4-kinases and phosphatidylinositol 4-phosphate during viral replication. *Biochem. Pharmacol. 84*, 1400−1408.
(34) Strating, J. R., van der Linden, L., Albulescu, L., Bigay, J., Arita, M., Delang, L., Leyssen, P., van der Schaar, H. M., Lanke, K. H., Thibaut, H. J., Ulferts, R., Drin, G., Schlinck, N., Wubbolts, R. W., Sever, N., Head, S. A., Liu, J. O., Beachy, P. A., De Matteis, M. A., Shair, M. D., Olkkonen, V. M., Neyts, J., and van Kuppeveld, F. J. (2015) Itraconazole inhibits enterovirus replication by targeting the oxysterol-binding protein. *Cell Rep. 10*, 600−615.
(35) Arita, M., Philipov, S., and Galabov, A. S. (2015) Phosphatidylinositol 4-kinase III beta is the target of oxoglaucine and pachypodol (Ro 09-0179) for their anti-poliovirus activities, and is

DOI: 10.1021/acsinfecdis.5b00122
ACS Infect. Dis. 2016, 2, 140−148

located at upstream of the target step of brefeldin A. *Microbiol. Immunol. 59*, 338−347.
(36) Hsu, N. Y., Ilnytska, O., Belov, G., Santiana, M., Chen, Y. H., Takvorian, P. M., Pau, C., van der Schaar, H., Kaushik-Basu, N., Balla, T., Cameron, C. E., Ehrenfeld, E., van Kuppeveld, F. J., and Altan-Bonnet, N. (2010) Viral reorganization of the secretory pathway generates distinct organelles for RNA replication. *Cell 141*, 799−811.
(37) Galabov, A. S., Nikolaeva, L., and Philipov, S. (1995) Aporphinoid alkaloid glaucinone: a selective inhibitor of poliovirus replication. *Antiviral Res. 26*, A347.
(38) Arita, M., Wakita, T., and Shimizu, H. (2009) Cellular kinase inhibitors that suppress enterovirus replication have a conserved target in viral protein 3A similar to that of enviroxime. *J. Gen. Virol. 90*, 1869−1879.
(39) De Palma, A. M., Thibaut, H. J., van der Linden, L., Lanke, K., Heggermont, W., Ireland, S., Andrews, R., Arimilli, M., Altel, T., De Clercq, E., van Kuppeveld, F., and Neyts, J. (2009) Mutations in the non-structural protein 3A confer resistance to the novel enterovirus replication inhibitor TTP-8307. *Antimicrob. Agents Chemother. 53*, 1850−1857.
(40) MacLeod, A. M., Mitchell, D. R., Palmer, N. J., Van de Poel, H., Conrath, K., Andrews, M., Leyssen, P., and Neyts, J. (2013) Identification of a series of compounds with potent antiviral activity for the treatment of enterovirus infections. *ACS Med. Chem. Lett. 4*, 585−589.
(41) Heinz, B. A., and Vance, L. M. (1995) The antiviral compound enviroxime targets the 3A coding region of rhinovirus and poliovirus. *J. Virol. 69*, 4189−4197.
(42) Ridgway, N. D. (2010) Oxysterol-binding proteins. *Subcell. Biochem. 51*, 159−182.
(43) Mesmin, B., Bigay, J., Moser von Filseck, J., Lacas-Gervais, S., Drin, G., and Antonny, B. (2013) A four-step cycle driven by PI(4)P hydrolysis directs sterol/PI(4)P exchange by the ER-Golgi tether OSBP. *Cell 155*, 830−843.
(44) Roulin, P. S., Lotzerich, M., Torta, F., Tanner, L. B., van Kuppeveld, F. J., Wenk, M. R., and Greber, U. F. (2014) Rhinovirus uses a phosphatidylinositol 4-phosphate/cholesterol counter-current for the formation of replication compartments at the ER-Golgi interface. *Cell Host Microbe 16*, 677−690.
(45) Rodriguez, P. L., and Carrasco, L. (1992) Gliotoxin: inhibitor of poliovirus RNA synthesis that blocks the viral RNA polymerase 3Dpol. *J. Virol. 66*, 1971−1976.
(46) Suhy, D. A., Giddings, T. H., Jr., and Kirkegaard, K. (2000) Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. *J. Virol. 74*, 8953−8965.
(47) Rust, R. C., Landmann, L., Gosert, R., Tang, B. L., Hong, W., Hauri, H. P., Egger, D., and Bienz, K. (2001) Cellular COPII proteins are involved in production of the vesicles that form the poliovirus replication complex. *J. Virol. 75*, 9808−9818.
(48) Jackson, W. T., Giddings, T. H., Jr., Taylor, M. P., Mulinyawe, S., Rabinovitch, M., Kopito, R. R., and Kirkegaard, K. (2005) Subversion of cellular autophagosomal machinery by RNA viruses. *PLoS Biol. 3*, e156.
(49) McNamara, C. W., Lee, M. C., Lim, C. S., Lim, S. H., Roland, J., Nagle, A., Simon, O., Yeung, B. K., Chatterjee, A. K., McCormack, S. L., Manary, M. J., Zeeman, A. M., Dechering, K. J., Kumar, T. R., Henrich, P. P., Gagaring, K., Ibanez, M., Kato, N., Kuhen, K. L., Fischli, C., Rottmann, M., Plouffe, D. M., Bursulaya, B., Meister, S., Rameh, L., Trappe, J., Haasen, D., Timmerman, M., Sauerwein, R. W., Suwanarusk, R., Russell, B., Renia, L., Nosten, F., Tully, D. C., Kocken, C. H., Glynne, R. J., Bodenreider, C., Fidock, D. A., Diagana, T. T., and Winzeler, E. A. (2013) Targeting Plasmodium PI(4)K to eliminate malaria. *Nature 504*, 248−253.
(50) Ford Siltz, L. A., Viktorova, E. G., Zhang, B., Kouiavskaia, D., Dragunsky, E., Chumakov, K., Isaacs, L., and Belov, G. A. (2014) New small-molecule inhibitors effectively blocking picornavirus replication. *J. Virol. 88*, 11091−11107.
(51) Ilnytska, O., Santiana, M., Hsu, N. Y., Du, W. L., Chen, Y. H., Viktorova, E. G., Belov, G., Brinker, A., Storch, J., Moore, C., Dixon, J. L., and Altan-Bonnet, N. (2013) Enteroviruses harness the cellular endocytic machinery to remodel the host cell cholesterol landscape for effective viral replication. *Cell Host Microbe 14*, 281−293.
(52) van der Schaar, H. M., van der Linden, L., Lanke, K. H., Strating, J. R., Purstinger, G., de Vries, E., de Haan, C. A., Neyts, J., and van Kuppeveld, F. J. (2012) Coxsackievirus mutants that can bypass host factor PI4KIIIbeta and the need for high levels of PI4P lipids for replication. *Cell Res. 22*, 1576−1592.
(53) Crotty, S., Saleh, M. C., Gitlin, L., Beske, O., and Andino, R. (2004) The poliovirus replication machinery can escape inhibition by an antiviral drug that targets a host cell protein. *J. Virol. 78*, 3378−3386.
(54) Belov, G. A., Kovtunovych, G., Jackson, C. L., and Ehrenfeld, E. (2010) Poliovirus replication requires the N-terminus but not the catalytic Sec7 domain of ArfGEF GBF1. *Cell. Microbiol. 12*, 1463−1479.
(55) Teterina, N. L., Zhou, W. D., Cho, M. W., and Ehrenfeld, E. (1995) Inefficient complementation activity of poliovirus 2C and 3D proteins for rescue of lethal mutations. *J. Virol. 69*, 4245−4254.
(56) Teterina, N. L., Rinaudo, M. S., and Ehrenfeld, E. (2003) Strand-specific RNA synthesis defects in a poliovirus with a mutation in protein 3A. *J. Virol. 77*, 12679−12691.
(57) Arita, M., Nagata, N., Sata, T., Miyamura, T., and Shimizu, H. (2006) Quantitative analysis of poliomyelitis-like paralysis in mice induced by a poliovirus replicon. *J. Gen. Virol. 87*, 3317−3327.
(58) Andino, R., Rieckhof, G. E., Achacoso, P. L., and Baltimore, D. (1993) Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. *EMBO J. 12*, 3587−3598.

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