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A novel capsid binding inhibitor displays potent antiviral activity against Enterovirus D68

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Abstract

Enterovirus D68 (EV-D68) is a respiratory viral pathogen that primarily infects children under the age of 8. Although EV-D68 infection typically leads to moderate to severe respiratory illnesses, recent years have seen increasing cases of EV-D68 triggered neurological complications such as the acute flaccid myelitis (AFM). There is currently no vaccine or antiviral available for EV-D68, we therefore aimed to develop potent and specific small molecule antivirals against EV-D68. In this study, we report our discovery of a viral capsid inhibitor **R856932** that inhibits multiple contemporary EV-D68 strains with single-digit to submicromolar efficacy. Mechanistic studies have shown that the tetrazole compound **R856932** binds to the hydrophobic pocket of viral capsid protein VP1, thereby preventing viral uncoating and releasing of viral genome in the infected cells. The mechanism of action of **R856932** was confirmed by time-of-addition, western blot, RT-qPCR, viral heat inactivation, serial viral passage and reverse genetics experiments. A single mutation located at VP1, A129V, confers resistance against **R856932**. However, recombination virus encoding VP1-A129V appeared to have compromised fitness of replication compared to the wild type EV-D68 virus as shown by the competition growth assay. Overall, the hit compound identified in this study, **R856932**, represents a promising starting point with a confirmed mechanism of action that can be further developed into EV-D68 antivirals.

Graphical Abstract

Keywords

EV-D68; antiviral; capsid inhibitor; enterovirus; pleconaril

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INTRODUCTION

Enterovirus D68 (EV-D68) is a non-enveloped, positive-sense, and single-stranded RNA virus that causes moderate to severe respiratory illnesses in children.^{1–3} EV-D68 belongs to the enterovirus genus of Picornaviridae family. The enterovirus genus also contains many other important human pathogens, including poliovirus, enterovirus A71 (EV-A71), rhinovirus, and coxsackievirus.^{2, 4} EV-D68 was previously mis-classified as rhinovirus 87 due to their similar route of infection and clinical manifestations. However, genetic studies have shown that it is more closely related to enterovirus. EV-D68 has two distinct features compared to other viruses in the enterovirus genus:⁵ 1) EV-D68 is cold-adapted and prefers a lower growth temperature (33°C) instead of the regular body temperature (37°C); 2) EV-D68 is acid sensitive,⁶ which explains why it cannot infect through the gastrointestinal tract as other enteroviruses, possibly due to the low pH in the gut.

EV-D68 was first isolated in California in 1962, and remained a neglected rare disease until 2014.⁷ EV-D68 came to the public attention when recent outbreaks coincided with neurological complications.^{1, 4, 8} Several outbreaks occurred in the United States as well as other parts of the world since 2014, and there appears to be a biennial pattern of EV-D68 outbreak since then.^{9, 10} This virus seems to be evolving over time, leading to more severe pathogenicity.^{8, 9, 11, 12} For example, infection with contemporary EV-D68 virus was linked to neurological complications, and children infected with EV-D68 had symptoms like muscle weakness, paralysis and even death in the severe cases.^{13, 14} Recent study has shown that contemporary EV-D68 strains (US/KY/14), but not historical EV-D68 strains (Fermon), were able to infect neuronal cells and cause cytopathic effect.¹¹ In mouse model studies, EV-D68 could be isolated from central nervous system (CNS) tissues including spinal cord, brain, and cerebral fluid.^{15–17} Although EV-D68 virus was rarely identified in the CNS tissues in human patients, there is nevertheless a strong correlation between EV-D68 infection and neurological complications as shown by a number of studies.^{8, 18}

Despite the severe disease outcome posed by EV-D68, there is currently no antiviral or vaccine available,² and current treatment is limited to supportive care. Several compounds were reported in the literature that inhibit EV-D68 amplification in cell culture, and many of the compounds tested were either originally developed as rhinovirus antivirals or repurposed from approved drugs.^{19–21} Unfortunately, many of the reported antivirals against EV-D68 either have moderate potency or concerning side effects.^{16, 21, 22} Therefore, it is imperative to develop potent and specific EV-D68 antivirals for the prophylaxis and treatment of EV-D68 infection.^{23, 24}

EV-D68 is a non-enveloped virus that is surrounded by viral capsid proteins.²⁵ The virion capsid consists of four proteins: VP1, VP2, VP3, and VP4. A concave pocket is formed in VP1 and it is important for cell receptor binding. The concave pocket is frequently referred as canyon. Small molecules such as pleconaril that are capable of binding to this canyon can stabilize the capsid protein and prevent viral uncoating, leading to inhibition of viral replication.^{25, 26} As such, the VP1 protein is a validated drug target. In this study, we report our discovery of an EV-D68 specific capsid inhibitor **R856932** that was identified through a

high-throughput screening. Mechanistic studies have shown that **R856932** inhibits the early stage of viral replication by blocking viral attachment to the host cell and protects virion against thermal denaturation. Additional evidences supporting the proposed mechanism of action include the results from serial viral passage and reverse genetics experiments in which a single mutation VP1-A129V located at the drug-binding site (canyon) led to reduced drug sensitivity. Although resistance could be selected against the capsid inhibitor **R856932** through serial viral passage experiments, the resistant mutant appears to compromise the fitness of viral replication compared to the wild-type (WT) EV-D68 virus as shown by the competition growth assay. Collectively, the compound **R856932** reported herein represents a novel EV-D68 capsid inhibitor, and further development of **R856932** might lead to promising EV-D68 antivirals. They can be used either alone or in combination with other direct-acting antivirals to combat EV-D68 infections.

RESULTS AND DISCUSSION

R856932 inhibits various EV-D68 strains, but not EV-A71 in vitro

In searching for potent EV-D68 antivirals, we carried out a cell-based cytopathic effect (CPE) screening assay against EV-D68 US/MO/14–18947 strain in rhabdomyosarcoma (RD) cell. US/MO/14–18947 was chosen because of its clinical significance: it is a member of the B1 clade, the predominant circulating EV-D68 clade in 2014 that has been shown to be associated with AFM.¹³ Compound **R856932** (Fig. 1) showed complete protection of RD cells against EV-D68 infection at 15 μ M. To confirm the results, **R856932** was re-synthesized and titrated to determine the antiviral EC₅₀ value and cellular cytotoxicity CC₅₀ value. It was found that **R856932** inhibited EV-D68 US/MO/14–18947 in RD cells with an EC₅₀ value of 0.46 μ M and a CC₅₀ value of 32.0 μ M (Table 1). The antiviral efficacy of **R856932** was further confirmed in four additional contemporary EV-D68 strains with EC₅₀ values ranging from 1.40 to 4.36 μ M. Previously reported EV-D68 antivirals pleconaril^{25, 27} and telaprevir²³ (Fig. 1) were included as controls and were found to be active against all the EV-D68 strains as well. However, when tested against two EV-A71 strains, compound **R856932** was not active, neither were the two control compounds. These results suggest that the antiviral activity of **R856932** is specific to EV-D68.

R856932 inhibits viral RNA and protein synthesis

To confirm the antiviral effect of **R856932** on EV-D68 replication, we quantified the viral RNA and protein levels with and without **R856932** treatment by RT-qPCR, immunofluorescence assay and western blot, respectively. In this experiment, RD cells were infected with EV-D68 US/MO/14–18947 virus at a multiplicity of infection (MOI) of 1 with and without compound treatment (Fig. 2). At 9 hours post-infection (hpi), RD cells were harvested and cellular viral RNA level was quantified by real-time quantitative PCR (RT-qPCR). Compound **R856932** greatly reduced viral RNA level, similarly as rupintrivir and pleconaril (Fig. 2A and 2B). Rupintrivir is a known EV-D68 3C protease inhibitor and pleconaril is an EV-D68 virus capsid VP1 binding inhibitor.^{19, 25, 27} Next, the viral protein expression levels of the capsid protein VP1 were monitored by western blot (Fig. 2C and 2D) and immunofluorescence imaging (Fig. 2E). Compound **R856932** significantly reduced the viral VP1 protein level at 9 hpi in a dose-dependent manner (Fig. 2D), similarly to

pleconaril treatment (Fig. 2C). Immunofluorescence imaging also showed a similar reduction of VP1 protein fluorescence signal with compound **R856932** treatment (Fig. 2E).

Time-of-addition and viral attachment assays indicate that **R856932** blocks viral attachment and entry

To dissect the antiviral mechanism of **R856932**, we first checked which stage(s) of the viral replication cycle was affected by **R856932** in a drug time-of-addition experiment. In this experiment, RD cells were infected with EV-D68 US/MO/14–18947 with a MOI of 0.01, and **R856932** was added at different time points either before, during or after viral infection (Fig. 3). Viruses in the cell supernatant were harvested at 14 hpi and the titer was quantified by plaque assay. The life cycle of EV viruses ranges from 9 to 14 hrs,²⁸ and this experiment mimics one cycle of viral replication. A known viral capsid inhibitor, pleconaril, was included as a control. As shown in Fig. 3, when compound **R856932** was applied 1 hpi or afterwards, its antiviral efficacy was completely abolished. In contrast, the antiviral efficacy of **R856932** remained when it was added either before (–1h–14h) or during infection (0h–14h). Significantly, the antiviral efficacy of **R856932** during the 0h–1h treatment alone was similar to that of –1h–14h and 0h–14h treatments, suggesting **R856932** exerted its antiviral activity solely at the viral attachment or entry process. The time-of-addition experiment results of **R856932** matched with that of pleconaril, suggesting these two compounds might have a similar mechanism of action.

The time-of-addition experiments described above quantify the progeny viruses in the cell supernatant. To independently confirm the results, we performed another set of time-of-addition experiments by quantifying the intracellular viral protein level using immunofluorescence imaging (Fig. 4). In this experiment, RD cells were infected with EV-D68 US/MO/14–18947 at MOI of 1, and compound **R856932** was added at different time points either before, during, or after viral infection. Cells were fixed at 8 hpi and viral VP1 protein was stained with anti-VP1 antibody. As shown in Fig. 4, **R856932** treatment at –1h–8h, 0h–8h, and 0h–1h time periods all led to complete inhibition of viral protein synthesis, while treatment at 2h–8h time period had almost no effect on viral protein synthesis (Fig. 4B). The immunofluorescence pattern of **R856932** treatment (Fig. 4B) was once again similar to that of pleconaril (Fig. 4A). Taken together, these two sets of time-of-addition experiments suggest that **R856932** inhibits the early stage of viral replication during either the attachment or entry step.

Next, to determine whether **R856932** blocks viral attachment or entry, we performed viral attachment assay. In this experiment, RD cells were cooled to 4 °C and mixed with EV-D68 US/MO/14–18947 at MOI of 30 at 4 °C for 2 h, then unbound viruses were washed away, and the attached viruses on cell surface were quantitated by real-time PCR (Fig. 5A) or immunostaining (Fig. 5B). The purpose of keeping the cells at 4 °C for the attachment assay was to prevent viral entry or internalization. The viral RNA levels with **R856932** and pleconaril treatments were less than 10% of the DMSO control (Fig. 5A). Similarly, viruses were detected at the surface of RD cells that were treated with DMSO control, but not with **R856932** and pleconaril treatments (Fig. 5B).

Overall, the results from the two sets of time-of-addition experiments as well as the viral attachment assay collectively suggest that **R856932** inhibits viral attachment to the host cell, and the mechanism of action of **R856932** is similar to that of pleconaril, a known viral capsid VP1 inhibitor.

Compound **R856932** protects EV-D68 virus from heat inactivation

It has been shown before that viral capsid inhibitors can protect virus from heat inactivation.²⁹ To determine whether **R856932** directly binds to the EV-D68 capsid proteins, we performed thermal protection assay. In this assay, EV-D68 US/MO/14–18947 virus was heated for 2 minutes at temperatures ranging 37 to 57 °C in the absence or presence of 50 μM compound **R856932** or 12.5 μM of pleconaril (Fig. 6A). At temperatures of 54 °C or above, compound **R856932** significantly reduced the virus inactivation by heat, and pleconaril displayed similar pattern, though less effective (Fig. 6A). Next, the thermal protection assay was repeated with another EV-D68 strain US/MO/14–18949. Although US/MO/14–18949 was more sensitive to heat inactivation than US/MO/14–18947 strain, the overall protection pattern was very similar to that of US/MO/14–18947 strain with compound treatment (Fig. 6B). These data indicate that compound **R856932** directly binds to the virus capsid protein(s) and stabilize viral capsid from heat inactivation.

Selection of EV-D68 mutants that are resistant to compound **R856932** via serial viral passage experiment

Based the above results, it appears that compound **R856932** directly interacts with EV-D68 virus capsid proteins. In order to determine which viral capsid protein(s) compound **R856932** interacts with, we carried out a serial passage experiment in the presence increasing concentration of compound **R856932** with an attempt to select drug-resistant mutants.²³ Since the purpose was to select resistant mutants, we used high drug selection pressure ($8.7 \times EC_{50}$ to $34.8 \times EC_{50}$) to speed up this process (Table 2).^{23, 30} At passage 3 (P3), the EC_{50} value increased about 80-fold in the presence of **R856932** selection pressure, while for the DMSO control, the EC_{50} value remained the same (Table 2), suggesting the observed resistance was not due to cell culture passage adaption.

We then sequenced the whole viral genome of both DMSO and **R856932** P3 viruses by Sanger sequencing. No mutation was identified in DMSO P3 virus; however, a few mutations were identified in the **R856932** P3 virus: A45V and A129V in VP1 protein, T139A in VP2 protein, and I44V in non-structural 3A protein.

VP1-A129V and VP2-T139A mutants confer **R856932** resistance

Based on the results from time-of-addition, viral attachment, and thermo-protection assay results, we hypothesized that compound **R856932** acts on the EV-D68 capsid proteins. Therefore, we focused our efforts on examining the mutations in viral capsid proteins: VP1-A45V, VP1-A129V, and VP2-T139A. To rationalize the selected resistant mutants, we examined the X-ray crystal structure of the EV-D68 capsid proteins in complex with pleconaril (PDB: 4WM7) (Fig. 7A).²⁵ Residues VP1-A45 (numbered A33 in the crystal structure) was located at the surface of VP1, and there was no drug-binding site close to this region, therefore the VP1-A45V mutant was probably unrelated to **R856932** resistance. In

contrast, residue VP1-A129 was located right above the pleconaril drug-binding site in the canyon region, and it was close to the pleconaril resistant mutant VP1-V81A (numbered V69 in the crystal structure). It was reported that VP1-V81A confers resistance against pleconaril.¹⁹ Residue VP2-T139 was located at a flexible loop that was near the entrance of the pleconaril drug-binding site, and it might affect drug entrance in the canyon region. We therefore hypothesized that **R856932** might have an overlapping drug-binding site as pleconaril and mutants VP1-A129V and VP2-T139A might lead to **R856932** resistance. To test this hypothesis, we first performed molecular docking of **R856932** in the pleconaril drug-binding site using Schrödinger Glide. Pleconaril was set as the centroid of the grid box and the docking was performed using the Glide standard precision. In the docking model, **R856932** fitted snugly in the pleconaril drug-binding site and was surrounded by hydrophobic residues V69, W93, I95, T97, F115, I119, F147, I184, Y193, I217, L220, V239, and M241 (numbering in the X-ray crystal structure) (Fig. 7B & 7C). It was noted that the tetrazole from **R856932** was 3.4 Å apart from the resistant mutant residue VP1-A129 (corresponds to A117 in the crystal structure). Mutation of VP1-A129 to V129 was therefore likely to create a steric clash with **R856932**, leading to drug resistance. In addition, in the overlay structure of **R856932** with pleconaril (Fig. 7D), the 1-phenyl ring from **R856932** overlapped with the isoxazole ring from pleconaril, and the distal phenyl ring from **R856932** overlapped with the central benzene ring from pleconaril. Overall, molecular docking suggests that **R856932** might bind to the same canyon site as pleconaril and the selected resistant mutant VP1-A129V might lead to **R856932** resistance. In addition, based on the docking model (Fig. 7D), it is reasonable to speculate that replacing the 1, 5-tetrazole linker in **R856932** with either the 2,5-disubstituted tetrazole linker or the 1,4-disubstituted 1H-1,2,3-triazole linker would lead to analogs with increased antiviral potency since it will extend the molecule to occupy the trifluoromethyl oxadiazole pocket.

To experimentally validate the relevance of VP1-A129V and VP2-T139A mutations in **R856932** resistance, we used reverse genetics approach to generate recombinant EV-D68 viruses that encode either the VP1-A129V mutant or the VP2-T139A mutant and compared their drug sensitivity with the WT EV-D68 virus. Specifically, we introduced VP1-A129V or VP2-T139A into EV-D68 US/MO/14–18947 genome individually via a reverse genetics approach using a pHH21 vector.²³ We named the resulting virus rMO VP1-A129V and rMO VP2-T139A, respectively. The drug sensitivity of these recombinant viruses against **R856932** was evaluated with a plaque reduction assay (PRA), and recombinant rMO WT virus was included as a control (Fig. 8). The introduction of VP1-A129V mutation alone increased EC₅₀ value of compound **R856932** from 0.39 μM to 16.00 μM; while the VP2-T139A mutation increased EC₅₀ value from 0.39 μM to 1.15 μM (Table 3). The plaque assay results were also independently confirmed by the cytopathic effect assay (CPE). As shown in Table 3, CPE assay also showed that the antiviral efficacy of compound **R856932** was greatly reduced against rMO VP1-A129V virus, and slightly reduced against rMO VP2-T139A virus. Overall, both plaque reduction assay and cytopathic effect assay indicate that VP1-A129V is the major mutation that confers **R856932** resistance, while VP2-T139A had a partial role in **R856932** resistance. Taken together, the molecular docking and reverse genetics collectively suggested that **R856932** had an overlapping drug-binding site with

pleconaril in the canyon region of VP1, and resistant mutants VP1-A129V and VP2-T139A both conferred drug resistance against **R856932**.

Recombinant rMO VP1-A129V virus had compromised fitness of replication compared to rMO WT virus

We compared the fitness of replication of recombinant rMO VP1-A129V virus with the rMO WT virus by a co-cultivation competition assay in RD cells.³⁰ We started the co-cultivation by mixing the rMO VP1-A129V virus with the rMO WT virus in a 100 to 1 ratio, and applied the virus mixture to infect the RD cells. Every 2 days after infection, culture media supernatant was harvested and viruses were quantified with plaque assay, and 28,000 PFU progeny viruses were used for the next round of infection. The VP1 coding sequences of the resulting viruses of each passage were determined by reverse transcription-PCR, and followed by Sanger sequencing.

After passage P3, no VP1-A129V mutation could be detected in the virus mixture (Fig. 9), suggesting that rMO VP1-A129V was overtaken by rMO WT. Comparing to the VP1 sequences in P1 and P2, it was found that mutant rMO VP1-A129V virus was gradually replaced by rMO WT virus from P1 to P3. These results indicate that recombinant rMO VP1-A129V had compromised fitness of replication than rMO WT virus in cell culture.

CONCLUSION

Recent years of upswing of EV-D68 virus infections and its association with polio-like neurological disorder such as acute flaccid myelitis (AFM), coupled with the lack of effective vaccine and antivirals,^{14, 15, 31} prompted us carry out a high-throughput screening assay to identify novel EV-D68 antivirals. Compound **R856932** was identified to have potent antiviral efficacy in this screening. Further testing showed that compound **R856932** was effective against all the contemporary EV-D68 strains tested, but not the EV-A71 strains (Table 1).

To elucidate the mechanism of action by compound **R856932**, we performed drug time-of-addition, viral attachment, thermal protection, and drug-resistance selection assays. The time-of-addition and viral attachment assays showed that compound **R856932** inhibited viral attachment (Figs. 2–5). Moreover, compound **R856932** displayed very similar patterns as the known capsid-binder pleconaril in all of the assays performed. Furthermore, the thermal protection assay revealed that compound **R856932** could protect EV-D68 viruses from heat inactivation (Fig. 6). In the independent serial viral passage experiments, the drug-resistant mutant viruses were obtained and the mutations were located in the capsid proteins VP1 and VP2, namely VP1-A129V and VP2-T139A. Molecular docking and reverse genetics suggested that the binding site of **R856932** overlapped with pleconaril (Figs. 7–8), and VP1-A129V had a direct effect on **R856932** binding, while VP2-T139A might have an indirect effect by affecting the drug entry into the binding site.

Development of capsid inhibitors targeting the hydrophobic pocket in the canyon region is a promising strategy in combating picornavirus infection. Pirodavis, pleconaril and vapendavis successfully advanced to clinical trials, though none of them was approved.^{2, 26} Pleconaril is

one of the best studied capsid-binding inhibitor against picornaviruses. It has broad-spectrum antiviral activity against several human enteroviruses and rhinoviruses. It should be noted that pleconaril does not inhibit enterovirus A71, as we confirmed in our study (Table 1). Pleconaril originally was developed to treat common cold caused by human rhinoviruses, and made all the way to clinical trial Phase III.³² However FDA rejected pleconaril as a drug to treat rhinoviruses caused common cold, citing pleconaril's concerning side effect and inducing drug resistant mutations.³³ In the clinical trial pleconaril caused some women to bleed between menstrual periods and reduced the effectiveness of oral contraceptives.³³ Although compound **R856932** had overlapping drug-binding site as pleconaril, compound **R856932** possesses a different scaffold from pleconaril and other known capsid inhibitors, such as pirodavir and vapendavir. Therefore, compound **R856932** possibly represents a new starting point to the development of effective capsid-binding inhibitor with high efficacy against all temporary EV-D68 strains, favorable drug-like properties, and limited side effects. Future SAR campaigns will help optimize the potency and pharmacological properties of this class of capsid-binding antivirals. Such study is ongoing and will be reported when available.

Another concern with viral capsid inhibitors is the drug resistance. Although resistance could be raised for **R856932** in the in vitro cell culture with a high drug selection pressure, it should be noted that the resistant mutant virus encoding VP1-A129V had a compromised fitness of replication compared to the WT EV-D68 virus. Moreover, EV-D68 is an acute infection and treatment might not need to last more than a couple of weeks, so the chances of resistance remain low. In addition, it is expected that combining capsid inhibitors with other EV-D68 antivirals, such as the protease inhibitors,²³ might further decrease the pace of resistance evolution.

MATERIAL AND METHODS

Chemicals, cells and virus

R856932 was purchased from Sigma-Aldrich (Cat # R856932); Pleconaril was purchased from Sigma-Aldrich (Cat # SML0307); Telaprevir was purchased from Achemblock (Cat #F-4593).

Human rhabdomyosarcoma (RD; ATCC CCL-136) was maintained at 37°C in a 5% CO₂ atmosphere and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin antibiotics. The following reagents were obtained through BEI Resources, NIAID, NIH: Human Enterovirus D68, US/MO/14-18949, NR-49130; Enterovirus D68, US/MO/14-18947, NR-49129; Enterovirus D68, US/IL/14-18952, NR-49131; Enterovirus D68, US/KY/14-18953, NR-49132; Enterovirus D68, US/IL/14-18956, NR-49133; Human Enterovirus 71 (HEV-71), Tainan/4643/1998, NR-471; and Human Enterovirus 71 (HEV-71), MP4, NR-472. All viruses were amplified in RD cells prior to infection assays.

Cytopathic effect assay (CPE) and plaque reduction assay (PRA)

RD cells for antiviral assays were seeded and grown overnight at 37°C in a 5% CO₂ atmosphere to ~90% confluence on the next day. For EV-D68 virus infections, cells were washed with PBS saline containing magnesium and calcium and infected with virus diluted in DMEM with 2% FBS and 30 mM MgCl₂. Viruses were incubated for at least 1 h at 33°C in a 5% CO₂ atmosphere, followed by addition of compound as well as 1% penicillin-streptomycin. The CC₅₀ was measured similarly but in the absence of viral infection. For cytopathic effect (CPE) assays, cells were stained with 66 µg/ml neutral red for 2 h, and neutral red uptake was measured at an absorbance at 540 nm using a Multiskan FC microplate photometer (Thermo Fisher Scientific). The EC₅₀ and CC₅₀ values were calculated from best-fit dose-response curves using GraphPad Prism software. For plaque reduction assays, a 1.2% Avicel microcrystalline cellulose (FMC BioPolymer, Philadelphia, PA) overlay in DMEM media supplemented with 2% FBS and 30 mM MgCl₂ was used, and the cells were stained after 3 days at 33 °C as previously described.^{23, 27} For EV-A71 virus infection, the procedures are identical as EV-D68 virus, except that 30 mM MgCl₂ was omitted in all the media and viruses were infected and incubated at 37°C instead of 33 °C.

High-throughput screening (HTS)

High-throughput screening was performed in 96-well plate using the CPE assay as described above. The library was purchased from Sigma (MyriaScreen, product number T990000) which contains 10,000 drug-like compounds. All compounds were initially screened at 15 µM, and hits showing more than 50% inhibition were repurchased from Sigma and tested in the serial titration experiment to determine the EC₅₀ values. The calculated Z-factor was 0.68, indicating this was an excellent assay for HTS.

Western blot

RD cells infected with EV-D68 strain US/MO/14–18947 (MOI = 1) were treated with indicated compound for 9 hours. Then total proteins were extracted using RAPI lysis buffer (50 mM Tris [pH 8.0], 1% NP-40, 0.1% SDS, 150 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 10 mM NaF, 10 mM NaPPi, 2 mM phenylmethylsulfonyl, 1 mM PMSF). Equal amounts of total proteins were separated by electrophoresis and transferred to PVDF membrane. Target protein was detected using protein-specific antibody [anti-VP1 antibody: GTX132313 (GeneTex) or anti-GAPDH antibody: MAB374 (EMD Millipore); 1:3,000 dilution] and corresponding Horseradish peroxidase (HRP)-conjugated secondary antibody (catalog number 32430 or 656120; Thermo Fisher Scientific; 1:3,000 dilutions) and Supersignal West Femto substrate (Thermo Fisher Scientific).

RNA extraction and RT-qPCR

RD cells infected with EV-D68 strain US/MO/14–18947 (MOI = 1) were treated with indicated compound for 9 hours. Then total RNA was extracted using TRIzol reagents (Thermo Fisher Scientific). After digestion of genomic DNA with RQ1 RNase-free DNase (Promega), 1.5 µg of total RNA was used to synthesize the first strand of cDNA of viral RNA and host mRNA using SuperScript III reverse transcriptase (Thermo Fisher Scientific) and oligo(dT)₁₈. Target gene was amplified on a QuantStudio 5 Real- Time PCR System

(Thermo Fisher Scientific) using FastStart Universal SYBR Green Master (Rox) (Roche) and virus-specific primers: D68-F (5'-CGCTGAAGTGGCGTGGTCC-3') and D68-R (5'-GGCTGCCCTGCTAAGAAAATTCTCC-3'). GAPDH was amplified to serve as a control using GAPDH-specific primers (GAPDH-F: 5'-ACACCCACTCCTCCACCTTTG-3' and GAPDH-R: 5'-CACCACCCTGTTGCTGTAGCC-3'). The amplification conditions were: 95 °C for 10 min; 40 cycles of 15 s at 95 °C and 60 s at 60 °C. The melting curve analysis was performed to verify the specificity of each amplification. Details regarding the PCR amplification method and sequencing primers are available upon request.

Immunostaining

RD cells were infected with EV-D68 strain US/MO/14-18947 (MOI = 1). At 9 hpi, cells were fixed with 4% formaldehyde for 10 min, followed by permeabilization with 0.2% Triton X-100 for another 10 min. After blocking with 10% bovine serum, cells were stained with rabbit anti-VP1 antibody and followed by staining with anti-rabbit immunoglobulin secondary antibody conjugated to Alexa Fluor 488 (Thermo Fisher Scientific). The nuclei were stained with 300 nM DAPI (Thermo Fisher Scientific) after secondary antibody incubation. Fluorescent images were acquired using a Leica SP5-II spectral confocal microscope (Leica).³⁴⁻³⁶

Virus attachment assay

RD cells were pre-cooled at 4 °C for 30 min, followed by infection with EV-D68 strain US/MO/14-18947 (MOI of 30). After 2 h incubation at 4 °C, unbound viruses were removed by two times of washing with ice-cold PBS. The attached viruses on cell surface were harvested for quantitated by real-time PCR or fixed for visualized by immunostaining.

Time-of-addition assay

Approximately 90% confluent RD cells were treated with compound **R856932** and pleconaril at 1 h before infection (-1 hpi), at the time of infection (0 hpi), or at 1-7 hpi with EV-D68 US/MO/14-18947. For quantification the progeny virus in the supernatant via plaque assay, MOI of 0.01 was used to infect the RD cells, the supernatant was harvested at 14 hpi, and the progeny viruses were quantified via plaque assay. For quantification the progeny virus inside the RD cells via immunostaining, MOI of 1 was used to infect the RD cell, and the cells were fixed at 8 hpi, and immunostaining was carried as described in immunostaining section.

Thermo-protection assay

12.5 μM Pleconaril or 50 μM compound **R856932** or same volume DMSO was incubated with viruses at 30 °C for 30 min. then the viruses were heated for 2 min temperature ranging from 37 to 57 °C, followed a rapid cool down to 4 °C on PCR machine. The resulting live virus was quantified via regular plaque assay on RD cells.

Serial passage experiment and viral genome sequencing

Serial passage experiment and viral genome sequencing were performed using an approach similar to that described previously.^{30, 36, 37} Briefly, serial passages were carried out in the presence of increasing concentration of compound **R856932** or DMSO as shown in Table 2. The virus was harvested after approximately 3 days after infection with EV-D68 US/MO/14–18947 at MOI=0.1 when significant cytopathic effect was observed, and the viral titers were determined by a plaque assay. The passage P3 virus drug sensitivity was determined via CPE assay, and their genome sequences were determined by first purifying viral RNA using a QIAamp viral RNA minikit (Qiagen, Hilden, Germany), followed by reverse transcription using a SuperScript III first-strand reverse transcriptase (Invitrogen) with an oligo(dT) primer and PCR amplification. The whole viral genome was sequenced via 14 sequencing reactions by Eton Bioscience, Inc. Details regarding the PCR amplification method and the sequencing primers are available upon request.

Reverse genetics of EV-D68 virus

A plasmid-based reverse genetic system for EV-D68 US/MO/14–18947 was generated in a pHH21 vector, based on the study by Pan et al.^{23, 38} The mutations were introduced via site-directed Mutagenesis with Agilent Technologies QuikChange II XL kit according to manufacturer protocol.

Virus growth competition assay³⁰

To compare the relative viral fitness of rMO VP1-A129V with rMO WT virus, rMO WT virus was mixed with rMO VP1-A129V virus at a 1:100 ratio (280 PFU rMO WT and 28,000 PFU rMO A129V). Every 2 days after infection, culture media supernatant was harvested and viruses were quantified with plaque assay, and 28,000 PFU progeny viruses were used to next round of infection. The VP1 coding sequences of the resulting viruses of each passage were determined by reverse transcription-PCR, and followed by sequencing reaction as described in the serial passage experiment and virus genome sequencing section. The percentage of each viruses was estimated by measuring the height of the nucleotide sequence electropherogram peak.^{30, 39}

Molecular Modeling

Molecular docking was performed using Schrödinger Glide standard precision. The X-ray crystal structure of EV-D68 capsid proteins in complex with pleconaril was used as the template (PDB: 4WM7).²⁵ The centroid of the grid box was set as the pleconaril. Figures were generated in Pymol.

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R856932 **Pleconaril** **Telaprevir****Figure 1.**

Chemical structures of **R856932**, pleconaril, and telaprevir. Pleconaril is a known EV-D68 viral VP1 capsid binding inhibitor,²⁵ and telaprevir is a EV-D68 2A protease inhibitor.²³

Figure 2. Compound R856932 reduced the EV-D68 viral RNA and protein levels.

(A-D) **R856932** reduced the EV-D68 viral RNA and protein levels. RD cells were infected with EV-D68 strain US/MO/14-18947 (MOI = 1) with treatment of the indicated compound. At 9 hpi, cells were harvested for viral RNA quantification by RT-qPCR (A and B) or viral protein quantification by western blot (C and D). Asterisks indicate a statistically significant difference in comparison with the DMSO control (one-way analysis of variance analysis by Prism 5, *** $P < 0.001$). The images were representatives of three independent experiments. (E) **R856932** inhibited EV-D68 VP1 synthesis in immunofluorescence assay. RD cells infected with EV-D68 strain US/MO/14-18947 (MOI = 1) were fixed at 9 hpi and stained

with anti-VP1 and DAPI for detecting VP1 and nucleus, respectively. Drug concentrations used were shown in the figure.

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Figure 3. Time-of-addition experiments by quantify progeny virus yield in cell culture supernatant.

RD cells were infected with EV-D68 US/MO/14-18947 at MOI = 0.01 at 0h time-point. At 1 hpi, inoculant virus was removed and washed with PBS buffer, progeny viruses in cell culture supernatant were harvested at 14 hpi and were quantified with plaque assay. Red arrow represents the period of time that drug (compound **R856932** or pleconaril) was present in the cell culture. The concentrations used for **R856932** and pleconaril were 25 μ M and 12.5 μ M, respectively. Asterisks indicate a statistically significant difference in comparison with the DMSO control (one-way analysis of variance analysis by Prism 5, *** $P < 0.001$). The value is the mean of two independent experiments $\pm$ S.D.

Figure 4: Time-of-addition experiments by immunostaining VP1 protein inside the host cells. RD cells were infected with EV-D68 US/MO/14–18947 at MOI = 1 at 0 h time-point, and at 1 hpi, inoculant virus was removed and washed with PBS buffer. **A)** Pleconaril or **B)** Compound **R856932** was applied at –1, 0 or 2 hpi to 8 hpi; for the 0h–1h condition, Pleconaril or compound **R856932** was only present during the infection stage for one hour, then pleconaril or compound **R856932** was washed away and fresh media was applied. The concentrations used for **R856932** and pleconaril were 50 μ M and 12.5 μ M, respectively. Cells were fixed at 8 hpi and viruses were detected via staining VP1 protein with anti-VP1. The images were representatives of three independent experiments.

Figure 5. R856932 reduced the EV-D68 viral attachment to host cells (A and B)

Pre-cooled RD cells were infected with EV-D68 strain US/MO/14-18947 (MOI of 30) and were incubated at 4 °C for 2 h. After removing unbound viruses by washing with ice-cold PBS, the attached viruses on cell surface were quantitated by real-time PCR (A) or visualized by immunostaining (B). Asterisks indicate a statistically significant difference in comparison with the DMSO control (one-way analysis of variance analysis by Prism 5, *** $P < 0.001$). The value is the mean of three independent experiments $\pm$ S.D. The images were

representatives of three independent experiments. Drug concentrations used were shown in the figure.

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Figure 6. Thermo-protection assay showing that R856932 prevented EV-D68 US/MO/14-18947 (A) and US/MO/14-18949 viruses (B) from heat inactivation.

Protective effects of pleconaril or compound **R856932** on heat inactivation of EV-D68 US/MO/14-18947 (A) and US/MO/14-18949 viruses (B). 12.5 μ M Pleconaril or 50 μ M compound **R856932** or DMSO was incubated with viruses (2×10^6 pfu/ml) for 30 minutes. The virus/compound mixture was then heated for 2 minutes at temperature ranging from 37 to 57 °C, followed by a rapid cool down to 4 °C. The resulting infectious virus was quantified via plaque assay using RD cells. The values are the mean $\pm$ standard deviation from three replicates.

Figure 7. Molecular docking of R856932 in EV-D68 viral capsid protein VP1.

(A) X-ray crystal structure of EV-D68 (Fermon CA62-1) capsid proteins in complex with pleconaril (PDB: 4WM7).²⁵ VP1, VP2 and VP3 were colored as green, cyan, and grey, respectively. **R856932**-selected resistant mutants VP1-A129V, VP1-A45V, and VP2-T139A, as well as the pleconaril-resistant mutant VP1-V81A, were labeled and the side chains were shown as spheres. (B) Ligand interaction diagram of **R856932** in the pleconaril binding site in VP1. The figure was generated in Schrödinger Glide. Note that A117 in the crystal structure corresponds to A129 in the conventional numbering. (C) Docking model of **R856932** in VP1. VP1-A129 side chain was shown as spheres. (D) Overlay of **R856932** and pleconaril in the canyon region of VP1. Docking was performed using Schrödinger Glide standard precision.

Figure 8. Representative plaque reduction assay with compound R856932 against recombinant rMO WT, rMO VP1-A129V and rMO VP2-T139A viruses.

Approximately 100 pfu/well of recombinant rMO WT, rMO VP1-A129V or rMO VP2-T139A viruses were applied to RD cell monolayers. 0.1 to 10 μ M compound **R856932**, or 0.1 or 1.0 μ M pleconaril was present in the 1.2% avicel overlay. RD cells were stained with crystal violet 3 days after infection. The images were representatives of three independent experiments.

Figure 9. Competition growth assay to assess the replication fitness of rMO VP1-A129V virus. 280 PFU rMO WT and 28,000 PFU rMO VP-A129V viruses (ratio = 1/100) were used to infect RD cells in a T25 flask. Every 2 days after infection, culture media supernatant was harvested and viruses were quantified with plaque assay, and 28,000 PFU progeny viruses were used for the next round of infection. The VP1 coding sequences were determined in each round of passage. The percentage of each viruses was estimated by measuring the height of the nucleotide sequence electropherogram peak as shown in the bar graph.

Table 1.Antiviral efficacy and cellular cytotoxicity of **R856932** against EV-D68 and EV-A71 in RD cells.^a

Viruses	R856932 CC ₅₀ = 32.0 ± 3.4 (μM)		Pleconaril CC ₅₀ = 14.1 ± 4.1 (μM)		Telaprevir CC ₅₀ = 46.2 ± 14.3 (μM)	
	EC ₅₀ (μM)	SI	EC ₅₀ (μM)	SI	EC ₅₀ (μM)	SI
EV-D68 US/MO/14–18947 (clade B1)	0.46 ± 0.09	70	0.03 ± 0.01	470	0.86 ± 0.07	54
EV-D68 US/MO/14–18949 (clade B1)	1.40 ± 0.04	22.9	0.15 ± 0.01	94	0.50 ± 0.04	92
EV-D68 US/IL/14–18952 (clade B2)	4.39 ± 0.21	7.29	0.14 ± 0.03	101	1.54 ± 0.07	30.0
EV-D68 US/IL/14–18956 (clade B2)	1.77 ± 0.05	18.1	0.19 ± 0.02	74	0.44 ± 0.00	105
EV-D68 US/KY/14–18953 (clade D)	3.34 ± 0.31	9.58	0.09 ± 0.02	157	0.39 ± 0.05	118
EV-A71 Tainan/4643/1998	>50	N.A.	>5	N.A.	>15	N.A.
EV-A71 MP4	>50	N.A.	>5	N.A.	>15	N.A.

^aThe EC₅₀ values of different strains of EV-D68 and EV-A71 in RD cells were determined using a cytopathic effect (CPE) assay. The CC₅₀ values were determined by a CPE assay in RD cells after 3 days of drug incubation. EC₅₀ = mean ± standard deviation. SI: Selection Index; N.A.: Not Available; The values are the mean ± standard deviation from three replicates.

Table 2:

Serial viral passage experiment of **R856932** against EV-D68 US/MO/14–18947.^a

Passage #	DMSO		R856932	
	Selection pressure (μM)	EC ₅₀ (μM)	Selection Pressure (μM)	EC ₅₀ (μM)
0	0	0.46 ± 0.09	0	0.46 ± 0.09
1	0	N.D.	4	N.D.
2	0	N.D.	8	N.D.
3	0	0.77 ± 0.44	16	35.60 ± 5.10

^aUS/MO/14–18947 (MOI = 0.1) were treated with DMSO or compound **R856932** at the indicated concentration (μM); EC₅₀ (μM) = mean ± standard deviation; The values are the mean ± standard deviation from three replicates. N.D.: Not determined.

Table 3:

Drug sensitivity against recombinant EV-D68 US/MO/14–18947 viruses

	R856932		Pleconaril	
	CPE EC₅₀ (μM)	PRA EC₅₀ (μM)	CPE EC₅₀ (μM)	PRA EC₅₀ (μM)
rMO WT	0.86 ± 0.14	0.39 ± 0.03	0.07 ± 0.02	0.03 ± 0.01
rMO VP1-A129V	16.6 ± 1.2	16.0 ± 7.4	0.44 ± 0.04	0.23 ± 0.04
rMO VP2-T139A	1.46 ± 0.39	1.15 ± 0.14	0.13 ± 0.05	0.13 ± 0.02

CPE: cytopathic effect assay; PRA: Plaque reduction assay; EC₅₀ (μM) = mean ± standard deviation. The values are the mean ± standard deviation from three replicates.