In vitro efficacy of antiviral compounds against enterovirus D68

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Abstract

In 2014, the United States experienced a large outbreak of severe respiratory illness associated with enterovirus D68 (EV-D68). We used a homogeneous, cell-based assay to assess antiviral activity of compounds developed for enterovirus/rhinovirus infection or other indications. Three of 15 compounds were highly active against all four strains tested (prototype and three 2014 strains), with EC₅₀ of 0.0012 to 0.027 µM. Additional studies are needed to assess their in vivo efficacy against EV-D68.
Enterovirus D68 (EV-D68), a rare enterovirus until recently, has been associated with severe respiratory illness in children, often resulting in hospitalization (1-3). During late summer and fall of 2014, a large outbreak of severe respiratory disease in young children occurred across the United States, with laboratory-confirmation of EV-D68 infection in over 1100 cases (http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html). The outbreak was characterized by severe disease, often requiring intensive care and non-invasive ventilatory support, and particularly affected children with a history of asthma or reactive airway disease (4, 5). Cases were also reported in Canada and Europe (6, 7). Exacerbation of pre-existing asthma or reactive airway disease, similar to that associated with rhinovirus (RV) infection (8) was noted in a high proportion of cases, though some patients with no history of asthma also had asthma-like symptoms (4, 5).

Despite decades of development, a significant disease burden, and more than 100,000 hospitalizations per year (9, 10), there are currently no approved antiviral drugs for the treatment of diseases associated with enterovirus or rhinovirus infections (11). To identify potential therapeutic compounds to treat EV-D68 disease, we tested compounds developed specifically for RV or EV indications, drugs that inhibit influenza virus (given that EV-D68 was recently shown to also bind sialic acids on the cell surface (12)), and several drugs that are FDA-approved for other indications. The compounds tested included picornavirus capsid inhibitors pleconaril (13), pocapavir (V-073; ViroDefense, Washington, DC) (14), and vapendavir (BTA-798; Biota Holdings, Alpharetta, GA) (13); picornavirus protease inhibitors rupintrivir (AG-7088; Pfizer, Groton, CT) (15) and V-7404 (ViroDefense) (16); and the viral polymerase inhibitor favipiravir (T-705; Toyama Chemical Co., Toyama, Japan) (17). DAS181 is an inhibitor of influenza virus binding to α2,6-linked sialic acids (Ansun Biopharma, San Diego, CA) (18). In addition to these antiviral compounds, we also tested several compounds that were originally developed and approved for other indications but have been shown subsequently to have
antiviral activity against one or more EV or RV. These include fluoxetine (selective serotonin reuptake inhibitor anti-depressant) (19), formoterol (bronchodilator) (20), and itraconazole (antifungal) (21). Two additional drugs, mefloquine (anti-malarial) and nitazoxanide (anti-protozoal) have also been reported to have activity against several virus families, though not necessarily picornaviruses (22, 23). These five drugs were purchased from Sigma Aldrich, St. Louis, MO.

Antiviral activity was assessed in a homogeneous cell-based assay that measured inhibition of viral cytopathic effect in human rhabdomyosarcoma cells (RD; ATCC CCL-136). The viruses included three representative EV-D68 strains from the 2014 outbreak (USA-MO/18947, USA-MO/18949, USA-IL/18956) (24), as well as the 1962 prototype strain (Fermon) (1). For the CPE inhibition assay, half-log10 dilutions of drug compound (10 µM to 0.001 µM) were combined with 100 CCID50 (50% cell culture infectious dose) of virus and added to monolayers of RD cells (5000 cells per well) in 384-well, white, flat-bottom microplates. Plates were incubated at 33°C and 5% CO2 for five days, and cell viability was assessed using ATPLite® (Perkin Elmer, Waltham, MA) by adding 15 µL of cell lysis buffer and then 15 µL of substrate solution, following the manufacturer’s recommendations. Luminescence was read in a plate reader and the 50% effective concentration (EC50) of each compound was calculated by 4-parameter curve-fitting with GraphPad Prism® (version 5.0.3; GraphPad Software, La Jolla, CA).

Pleconaril inhibited the Fermon strain with an EC50 value of 0.38 ± 0.01 µM but activity against the 2014 strains was detected only at concentrations greater than 4 µM (Table 1). Two other capsid inhibitors, pocapavir and vapanavir, were inactive against all four EV-D68 strains. Rupintrivir and V-7404 were highly active against all four EV-D68 strains, with EC50 values of 0.0015 – 0.0051 µM (Table 1). Of five influenza inhibitors tested, only DAS181 inhibited EV-D68, with EC50 values comparable to those of protease inhibitors (0.0012 – 0.004 µM; Table 1). Fluoxetine (Prozac®; a selective serotonin reuptake inhibitor) inhibited the EV-D68 strains at concentrations of 0.34 – 1.05 µM (Table 1). Four other
compounds that have been reported to have antiviral activity had no activity against the EV-D68 strains, even at the highest concentration tested (10 µM) (Table 1).

Fourteen of the 15 compounds tested have completed at least Phase II clinical trials and seven are already FDA-approved for other indications. Fluoxetine was the only FDA-approved drug that had significant activity against EV-D68. However, fluoxetine’s psychoactive properties, and its intended use to treat depression and other psychological disorders, suggest that the potential risk of unintended effects may outweigh the benefit of using it to treat EV-D68 infection. Furthermore, given typical fluoxetine dosing and maximal plasma levels (<200 nM), it is unlikely that virus inhibitory concentrations can be achieved in vivo.

In our hands, itraconazole failed to inhibit any EV-D68 strain in our standard assay at any concentration tested (Table 1), contrary to two published reports that determined EC\textsubscript{50} values of 0.32 µM to 0.43 µM for the Fermon strain (25, 26). In both studies, the methods were somewhat different from our approach. Gao et al. (25) used virus titer as their readout and observed a titer reduction of only 1.5 log, to 10\textsuperscript{5} CCID\textsubscript{50}/ml, even at drug concentrations >1 µM. Strating et al. (26) infected with “the lowest MOI that resulted in full CPE within 3 days” and used a CPE reduction assay similar to ours. Itraconazole activity appears to be very sensitive to virus dose, such that very different EC\textsubscript{50} values (0.29 µM to >10 µM for the Fermon strain) are obtained within a relatively narrow range of virus doses (100-fold dose range, using five half-log dilutions; data not shown). For the other compounds tested, similar EC\textsubscript{50} values were observed across this same dose range. For pleconaril, for example, the EC\textsubscript{50} varied only from 0.3 µM to 0.5 µM. We believe our assay represents a more stringent test of activity and is more likely to predict clinical relevance of the compounds tested.

Pleconaril was originally developed for treatment of EV and RV infections and it has broad activity against a wide range of RV and EV serotypes (27). In RD cells, the activity of pleconaril against the Fermon strain was similar to that recently reported by Liu et al. (28); however, its EC\textsubscript{50} value was
about 10-fold higher against the 2014 strains (Table 1). Upon repeat testing in the HeLa H1 cells used by
Liu et al., we obtained EC<sub>50</sub> values of 0.13 – 0.36 µM for all four strains (Table 1), suggesting a cell-
specific difference in drug susceptibility. Interestingly, the EC<sub>50</sub> values for the other compounds were
similar in both cell lines; the nature of the difference in pleconaril susceptibility remains unknown but is
under investigation.

The three most promising compounds strongly inhibited all four EV-D68 strains tested, at low
nanomolar concentrations (Table 1). Two of these are in active development for other viral infections;
rupintrivir is not currently being developed further. V-7404 is being developed in combination with
pocapavir for treatment of poliovirus infections, especially in immunodeficient persons who are
chronically infected and at risk for paralysis, in support of the global polio eradication endgame strategy
(29, 30). DAS181 is a sialidase that cleaves α2,6-linked sialic acids on the surface of cells, thus inhibiting
binding of neuraminidase, is being developed to treat influenza and parainfluenza infections (31). There
are no animal models for EV-D68 infection or disease. However, if EV-D68 continues to circulate and
cause severe illness, it will be important to assess the efficacy of these or other antiviral drugs in vivo,
either in animals or in human clinical studies.


broad-spectrum antiviral activity of rupintrivir, a novel human rhinovirus 3C protease inhibitor.  


Table 1. Efficacy of 15 drugs and antiviral compounds against four EV-D68 strains.

<table>
<thead>
<tr>
<th>Mean EC₅₀ ± SD (µM)</th>
<th>USA-MO/18947</th>
<th>USA-MO/18949</th>
<th>USA-IL/18956</th>
<th>Fermon</th>
</tr>
</thead>
<tbody>
<tr>
<td>EV/RV Capsid inhibitors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pleconarilᵃᵇ</td>
<td>4.44 ± 0.55</td>
<td>6.09 ± 0.26</td>
<td>6.11 ± 1.05</td>
<td>0.38 ± 0.01</td>
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<td>Pocapavirᵃ</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Vapendavirᵃ</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10</td>
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<tr>
<td>EV/RV Protease inhibitors</td>
<td></td>
<td></td>
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<tr>
<td>Rupinriviʳᵃ</td>
<td>0.0046 ± 0.0016</td>
<td>0.0015 ± 0.003</td>
<td>0.0037 ± 0.007</td>
<td>0.002 ± 0.0005</td>
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<tr>
<td>V-7404ᶜ</td>
<td>0.026 ± 0.004</td>
<td>0.027 ± 0.008</td>
<td>0.024 ± 0.007</td>
<td>0.0035 ± 0.0006</td>
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<tr>
<td>Influenza inhibitors</td>
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<tr>
<td>Amantidineᵈ</td>
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<td>Arbidolᵃ,e</td>
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<tr>
<td>DAS181ᵃ</td>
<td>0.0036 ± 0.0015</td>
<td>0.0026 ± 0.0012</td>
<td>0.004 ± 0.0016</td>
<td>0.0012 ± 0.0009</td>
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<td>Favipiravirᵃ</td>
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<tr>
<td>Approved for other indications</td>
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<tr>
<td>Fluoxetineᵈ</td>
<td>0.53 ± 0.15</td>
<td>0.64 ± 0.17</td>
<td>1.05 ± 0.2</td>
<td>0.34 ± 0.04</td>
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<td>Formoterol fumarateᵈ</td>
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<td>Itraconazoleᵈ</td>
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</tbody>
</table>

ᵃ Completed a Phase II clinical trial but not yet FDA-approved.
ᵇ In HeLa H1 cells, the EC₅₀ values for the four strains were 0.131 ± 0.024, 0.358 ± 0.036, 0.321 ± 0.094, 0.36 ± 0.021, respectively, for pleconaril. For other compounds, the values were not significantly different in the two cell lines (data not shown).
ᶜ Completed a Phase I clinical safety trial.
ᵈ FDA-approved for an indication other than EV/RV infection.
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