

In Vitro Efficacy of Antiviral Compounds against Enterovirus D68

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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 the antiviral activity of compounds developed for EV/rhinovirus infection or other indications. Three of 15 compounds were highly active against all four strains tested (the prototype and three 2014 strains), with 50% effective concentrations of 0.0012 to 0.027 μM . Additional studies are needed to assess their *in vivo* efficacy against EV-D68.

Enterovirus (EV) D68 (EV-D68), a rare EV 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 >1,100 cases (<http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html>). The outbreak was characterized by a severe disease, often requiring intensive care and noninvasive ventilatory support, that particularly affected children with a history of asthma or reactive airway disease (4, 30). Cases were also reported in Canada and Europe (5, 6). Exacerbation of pre-existing asthma or reactive airway disease, similar to that associated with rhinovirus (RV) infection (7), was noted in a high proportion of cases, though some patients with no history of asthma also had asthma-like symptoms (4; Midgley et al., submitted).

Despite decades of development, a significant disease burden, and more than 100,000 hospitalizations per year (8, 9), there are currently no approved antiviral drugs for the treatment of diseases associated with EV or RV infections (10). 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 [11]), and several drugs that are FDA approved for other indications. The compounds tested included the picornavirus capsid inhibitors pleconaril (12), pocapavir (V-073; ViroDefense, Washington, DC) (13), and vapendavir (BTA-798; Biota Holdings, Alpharetta, GA) (12); the picornavirus protease inhibitors rupintrivir (AG-7088; Pfizer, Groton, CT) (14) and V-7404 (ViroDefense) (15); and the viral polymerase inhibitor favipiravir (T-705; Toyama Chemical Co., Toyama, Japan) (16). DAS181 is an inhibitor of influenza virus binding to α 2,6-linked sialic acids (Ansun Biopharma, San Diego, CA) (17). 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 EVs or RVs. These include fluoxetine (selective serotonin reuptake inhibitor antidepressant) (18), formoterol (bronchodilator) (19), and itraconazole (antifungal) (20). Two additional drugs, mefloquine (anti-malarial) and nitazoxanide (antiprotozoal), have also been reported to have activity against several virus families, though not necessarily picornaviruses (21, 22). These five drugs were purchased from Sigma-Aldrich, St. Louis, MO.

Antiviral activity was assessed in a homogeneous, cell-based assay that measured viral cytopathic effect (CPE) inhibition in human

rhabdomyosarcoma (RD) cells (ATCC CCL-136). The viruses included three representative EV-D68 strains from the 2014 outbreak (USA-MO/18947, USA-MO/18949, USA-IL/18956) (23), as well as the 1962 prototype strain (Fermon) (1). For the CPE inhibition assay, half-log₁₀ dilutions of drug compounds (10 to 0.001 μM) were combined with 100 CCID₅₀ (50% cell culture infectious doses) of virus and added to monolayers of RD cells (5,000 per well) in 384-well, white, flat-bottom microplates. Plates were incubated at 33°C and 5% CO₂ for 5 days, and cell viability was assessed with ATPLite (PerkinElmer, Waltham, MA) by adding 15 μl of cell lysis buffer and then 15 μl of substrate solution in accordance with the manufacturer's recommendations. Luminescence was read in a plate reader, and the 50% effective concentration (EC₅₀) of each compound was calculated by four-parameter curve fitting with GraphPad Prism (version 5.0.3; GraphPad Software, La Jolla, CA).

Pleconaril inhibited the Fermon strain with an EC₅₀ of 0.38 \pm 0.01 μM , but activity against the 2014 strains was detected only at concentrations of >4 μM (Table 1). Two other capsid inhibitors, pocapavir and vapendavir, were inactive against all four EV-D68 strains. Rupintrivir and V-7404 were highly active against all four EV-D68 strains, with EC₅₀s of 0.0015 to 0.0051 μM (Table 1). Of the five influenza virus inhibitors tested, only DAS181 inhibited EV-D68, with EC₅₀s comparable to those of the protease inhibitors (0.0012 to 0.004 μM ; Table 1). Fluoxetine (Prozac; a selective serotonin reuptake inhibitor) inhibited the EV-D68 strains at concentrations of 0.34 to 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 7 are already FDA approved for other indications. Fluoxetine was the only FDA-approved drug that had significant activity against EV-D68. However, fluoxetine's psy-

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

Drug or antiviral compound	Mean EC ₅₀ ± SD (μM)			
	USA-MO/18947	USA-MO/18949	USA-IL/18956	Fermon
EV or RV capsid inhibitors				
Pleconaril ^{a,b}	4.44 ± 0.55	6.09 ± 0.26	6.11 ± 1.05	0.38 ± 0.01
Pocapavir ^a	>10	>10	>10	>10
Vapendavir ^a	>10	>10	>10	>10
EV or RV protease inhibitors				
Rupintrivir ^a	0.0046 ± 0.0016	0.0015 ± 0.003	0.0037 ± 0.007	0.002 ± 0.0005
V-7404 ^c	0.026 ± 0.004	0.027 ± 0.008	0.024 ± 0.007	0.0035 ± 0.0006
Influenza virus inhibitors				
Amantidine ^d	>10	>10	>10	>10
Arbidol ^{a,e}	>10	>10	>10	>10
DAS181 ^a	0.0036 ± 0.0015	0.0026 ± 0.0012	0.004 ± 0.0016	0.0012 ± 0.0009
Favipiravir ^a	>10	>10	>10	>10
Oseltamivir ^d	>10	>10	>10	>10
Approved for other indications				
Fluoxetine ^d	0.53 ± 0.15	0.64 ± 0.17	1.05 ± 0.2	0.34 ± 0.04
Formoterol fumarate ^d	>10	>10	>10	>10
Itraconazole ^d	>10	>10	>10	>10
Mefloquine ^d	>10	>10	>10	>10
Nitazoxanide ^d	>10	>10	>10	>10

^a Completed a phase II clinical trial but not yet FDA approved.

^b In HeLa H1 cells, the EC₅₀s of pleconaril for the four strains were 0.131 ± 0.024, 0.358 ± 0.036, 0.321 ± 0.094, 0.36 ± 0.021 μM, respectively. 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 or RV infection.

^e Licensed for human use in Russia and China.

choactive 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 infections. Furthermore, given the typical fluoxetine dosing and maximal levels in plasma (<200 nM), it is unlikely that virus-inhibitory concentrations can be achieved *in vivo*.

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 EC₅₀s of 0.32 to 0.43 μM for the Fermon strain (20, 24). In both studies, the methods were somewhat different from our approach. Gao et al. (24) used the virus titer as their readout and observed an only 1.5-log titer reduction, to 10⁵ CCID₅₀/ml, even at drug concentrations of >1 μM. Strating et al. (20) infected with “the lowest MOI [multiplicity of infection] that resulted in [a] full CPE within 3 days” and used a CPE reduction assay similar to ours. Itraconazole activity appears to be very sensitive to the virus dose, such that very different EC₅₀s (0.29 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). Similar EC₅₀s of the other compounds tested were observed across this same dose range. For example, the EC₅₀ of pleconaril only varied from 0.3 to 0.5 μM. We believe our assay represents a more stringent test of activity and is more likely to predict the clinical relevance of the compounds tested.

Pleconaril was originally developed for the treatment of EV and RV infections and has broad activity against a wide range of RV and EV serotypes (25). In RD cells, the activity of pleconaril against the Fermon strain was similar to that recently reported by Liu

et al. (26); however, its EC₅₀ was about 10-fold higher against the 2014 strains (Table 1). Upon repeat testing with the HeLa H1 cells used by Liu et al., we obtained EC₅₀s of 0.13 to 0.36 μM for all four strains (Table 1), suggesting a cell-specific difference in drug susceptibility. Interestingly, the EC₅₀s of the other compounds were similar for 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 the treatment of poliovirus infections, especially in immunodeficient persons who are chronically infected and at risk of paralysis, in support of the global polio eradication endgame strategy (27, 28). DAS181 is a sialidase that cleaves α2,6-linked sialic acids on the surface of cells, thus inhibiting the binding of neuraminidase, and is being developed to treat influenza and parainfluenza virus infections (29). There are no animal models of 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.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the CDC and other contributing agencies. The use of trade names is for identification purposes only and does not constitute an endorsement by the CDC or the U.S. Government.

ADDENDUM IN PROOF

After the acceptance of our paper, we became aware of a manuscript (L. Sun et al., *Antimicrob Agents Chemother* 59:7782–7785, 2015, <http://dx.doi.org/10.1128/AAC.01375-15>) that also assesses the susceptibility of several EV-D68 strains to a range of antiviral compounds, with some overlap in strains and compounds between the two papers. Our two groups have reached similar conclusions, that protease inhibitors are highly effective against all strains tested. Differences in absolute EC₅₀ values are probably due to differences in cell lines and experimental conditions used by the two groups of investigators, as we have found in our own testing.

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