AAC Journals.ASM.org

In Vitro Efficacy of Antiviral Compounds against Enterovirus D68

Eric Rhoden,[a] Mingyu Zhang,[a,b] W. Allan Nix,[a] M. Steven Oberste[a]

Division of Viral Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[a]; Immunization Planning Institute, Henan Provincial Center for Disease Control and Prevention, Zhengzhou, Henan, China[b]

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 α2,6-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-$\log_{10}$ dilutions of drug compounds (10 to 0.001 μM) were combined with 100 $CCID_{50}$ (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% CO_2 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 (EC_{50}) 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 EC_{50} of 0.38 ± 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 EC_{50}s of 0.0015 to 0.0051 μM (Table 1). Of the five influenza virus inhibitors tested, only DAS181 inhibited EV-D68, with EC_{50}s 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-

Received 30 March 2015 **Returned for modification** 27 April 2015
Accepted 3 July 2015

Accepted manuscript posted online 14 September 2015

Citation Rhoden E, Zhang M, Nix WA, Oberste MS. 2015. *In vitro* efficacy of antiviral compounds against enterovirus D68. Antimicrob Agents Chemother 59:7779–7781. doi:10.1128/AAC.00766-15.

Address correspondence to M. S. Oberste, soberste@cdc.gov.

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 EC_{50}s 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 EC_{50}s 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 10^5 $CCID_{50}$/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 EC_{50}s (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 EC_{50}s of the other compounds tested were observed across this same dose range. For example, the EC_{50} 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 EC_{50} 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 EC_{50}s of 0.13 to 0.36 μM for all four strains (Table 1), suggesting a cell-specific difference in drug susceptibility. Interestingly, the EC_{50}s 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.

ACKNOWLEDGMENTS

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 EC_{50} 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.

REFERENCES

1. **Schieble JH, Fox VL, Lennette EH.** 1967. A probable new human picornavirus associated with respiratory diseases. Am J Epidemiol **85:**297–310.
2. **Imamura T, Fuji N, Suzuki A, Tamaki R, Saito M, Aniceto R, Galang H, Sombrero L, Lupisan S, Oshitani H.** 2011. Enterovirus 68 among children with severe acute respiratory infection, the Philippines. Emerg Infect Dis **17:**1430–1435.
3. **Ikeda T, Mizuta K, Abiko C, Aoki Y, Itagaki T, Katsushima F, Katsushima Y, Matsuzaki Y, Fuji N, Imamura T, Oshitani H, Noda M, Kimura H, Ahiko T.** 2012. Acute respiratory infections due to enterovirus 68 in Yamagata, Japan between 2005 and 2010. Microbiol Immunol **56:**139–143. http://dx.doi.org/10.1111/j.1348-0421.2012.00411.x.
4. **Midgley CM, Jackson MA, Selvarangan R, Turabelidze G, Obringer E, Johnson D, Giles BL, Patel A, Echols F, Oberste MS, Nix WA, Watson JT, Gerber SI.** 2014. Severe respiratory illness associated with enterovirus D68—Missouri and Illinois, 2014. MMWR Morb Mortal Wkly Rep **63:**798–799.
5. **Meijer A, Benschop KS, Donker GA, van der Avoort HG.** 2014. Continued seasonal circulation of enterovirus D68 in the Netherlands, 2011–2014. Euro Surveill **19:**209835. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20935.
6. **Drews SJ, Simmonds K, Usman HR, Yee K, Fathima S, Tipples G, Tellier R, Pabbaraju K, Wong S, Talbot J.** 2015. Characterization of enterovirus activity, including that of enterovirus D68, in pediatric patients in Alberta, Canada, in 2014. J Clin Microbiol **53:**1042–1045. http://dx.doi.org/10.1128/JCM.02982-14.
7. **Cox DW, Bizzintino J, Ferrari G, Khoo SK, Zhang G, Whelan S, Lee WM, Bochkov YA, Geelhoed GC, Goldblatt J, Gern JE, Laing IA, Le Souef PN.** 2013. Human rhinovirus species C infection in young children with acute wheeze is associated with increased acute respiratory hospital admissions. Am J Respir Crit Care Med **188:**1358–1364. http://dx.doi.org/10.1164/rccm.201303-0498OC.
8. **Khetsuriani N, LaMonte A, Oberste MS, Pallansch MA, Centers for Disease Control and Prevention.** 2006. Enterovirus surveillance United States, 1970–2005. MMWR Surveill Summ **55:**1–20. http://www.cdc.gov/mmwr/preview/mmwrhtml/ss5508a1.htm.
9. **Miller EK, Lu X, Erdman DD, Poehling KA, Zhu Y, Griffin MR, Hartert TV, Anderson LJ, Weinberg GA, Hall CB, Iwane MK, Edwards KM.** 2007. Rhinovirus-associated hospitalizations in young children. J Infect Dis **195:**773–781. http://dx.doi.org/10.1086/511821.
10. **Abzug MJ.** 2014. The enteroviruses: problems in need of treatments. J Infect **68**(Suppl 1)**:**S108–S114. http://dx.doi.org/10.1016/j.jinf.2013.09.020.
11. **Imamura T, Okamoto M, Nakakita S, Suzuki A, Saito M, Tamaki R, Lupisan S, Roy CN, Hiramatsu H, Sugawara KE, Mizuta K, Matsuzaki Y, Suzuki Y, Oshitani H.** 2014. Antigenic and receptor binding properties of enterovirus 68. J Virol **88:**2374–2384. http://dx.doi.org/10.1128/JVI.03070-13.
12. **Thibaut HJ, De Palma AM, Neyts J.** 2012. Combating enterovirus replication: state-of-the-art on antiviral research. Biochem Pharmacol **83:**185–192. http://dx.doi.org/10.1016/j.bcp.2011.08.016.
13. **Oberste MS, Moore D, Anderson B, Pallansch MA, Pevear DC, Collett MS.** 2009. *In vitro* antiviral activity of V-073 against polioviruses. Antimicrob Agents Chemother **53:**4501–4503. http://dx.doi.org/10.1128/AAC.00671-09.
14. **Binford SL, Maldonado F, Brothers MA, Weady PT, Zalman LS, Meador JW, III, Matthews DA, Patick AK.** 2005. Conservation of amino acids in human rhinovirus 3C protease correlates with broad-spectrum antiviral activity of rupintrivir, a novel human rhinovirus 3C protease inhibitor. Antimicrob Agents Chemother **49:**619–626. http://dx.doi.org/10.1128/AAC.49.2.619-626.2005.
15. **Rhoden E, Liu HM, Wang-Chern SW, Oberste MS.** 2013. Anti-poliovirus activity of protease inhibitor AG-7404, and assessment of in vitro activity in combination with antiviral capsid inhibitor compounds. Antiviral Res **98:**186–191. http://dx.doi.org/10.1016/j.antiviral.2013.03.003.
16. **Furuta Y, Takahashi K, Fukuda Y, Kuno M, Kamiyama T, Kozaki K, Nomura N, Egawa H, Minami S, Watanabe Y, Narita H, Shiraki K.** 2002. In vitro and in vivo activities of anti-influenza virus compound T-705. Antimicrob Agents Chemother **46:**977–981. http://dx.doi.org/10.1128/AAC.46.4.977-981.2002.
17. **Moss RB, Hansen C, Sanders RL, Hawley S, Li T, Steigbigel RT.** 2012. A phase II study of DAS181, a novel host directed antiviral for the treatment of influenza infection. J Infect Dis **206:**1844–1851. http://dx.doi.org/10.1093/infdis/jis622.
18. **Ulferts R, van der Linden L, Thibaut HJ, Lanke KH, Leyssen P, Coutard B, De Palma AM, Canard B, Neyts J, van Kuppeveld FJ.** 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. http://dx.doi.org/10.1128/AAC.02084-12.
19. **Bochkov YA, Busse WW, Brockman-Schneider RA, Evans MD, Jarjour NN, McCrae C, Miller-Larsson A, Gern JE.** 2013. Budesonide and formoterol effects on rhinovirus replication and epithelial cell cytokine responses. Respir Res **14:**98. http://dx.doi.org/10.1186/1465-9921-14-98.
20. **Strating JR, van der Linden L, Albulescu L, Bigay J, Arita M, Delang L, Leyssen P, van der Schaar HM, Lanke KH, Thibaut HJ, Ulferts R, Drin G, Schlinck N, Wubbolts RW, Sever N, Head SA, Liu JO, Beachy PA, De Matteis MA, Shair MD, Olkkonen VM, Neyts J, van Kuppeveld FJ.** 2015. Itraconazole inhibits enterovirus replication by targeting the oxysterol-binding protein. Cell Rep **10:**600–615. http://dx.doi.org/10.1016/j.celrep.2014.12.054.
21. **Brickelmaier M, Lugovskoy A, Kartikeyan R, Reviriego-Mendoza MM, Allaire N, Simon K, Frisque RJ, Gorelik L.** 2009. Identification and characterization of mefloquine efficacy against JC virus *in vitro*. Antimicrob Agents Chemother **53:**1840–1849. http://dx.doi.org/10.1128/AAC.01614-08.
22. **Rossignol JF.** 2014. Nitazoxanide: a first-in-class broad-spectrum antiviral agent. Antiviral Res **110:**94–103. http://dx.doi.org/10.1016/j.antiviral.2014.07.014.
23. **Brown BA, Nix WA, Sheth M, Frace M, Oberste MS.** 2014. Seven strains of enterovirus D68 detected in the United States during the 2014 severe respiratory disease outbreak. Genome Announc **2:**e01201-14.
24. **Gao Q, Yuan S, Zhang C, Wang Y, Wang Y, He G, Zhang S, Altmeyer R, Zou G.** 2015. Discovery of itraconazole with broad-spectrum *in vitro* antienterovirus activity that targets nonstructural protein 3A. Antimicrob Agents Chemother **59:**2654–2665. http://dx.doi.org/10.1128/AAC.05108-14.
25. **Pevear DC, Tull TM, Seipel ME, Groarke JM.** 1999. Activity of pleconaril against enteroviruses. Antimicrob Agents Chemother **43:**2109–2115.
26. **Liu Y, Sheng J, Fokine A, Meng G, Shin WH, Long F, Kuhn RJ, Kihara D, Rossmann MG.** 2015. Structure and inhibition of EV-D68, a virus that causes respiratory illness in children. Science **347:**71–74. http://dx.doi.org/10.1126/science.1261962.
27. **Collett MS, Neyts J, Modlin JF.** 2008. A case for developing antiviral drugs against polio. Antiviral Res **79:**179–187. http://dx.doi.org/10.1016/j.antiviral.2008.04.002.
28. **De Palma AM, Pürstinger G, Wimmer E, Patick AK, Andries K, Rombaut B, De Clercq E, Neyts J.** 2008. Potential use of antiviral agents in polio eradication. Emerg Infect Dis **14:**545–551. http://dx.doi.org/10.3201/eid1404.070439.
29. **Belser JA, Lu X, Szretter KJ, Jin X, Aschenbrenner LM, Lee A, Hawley S, Kim do H, Malakhov MP, Yu M, Fang F, Katz JM.** 2007. DAS181, a novel sialidase fusion protein, protects mice from lethal avian influenza H5N1 virus infection. J Infect Dis **196:**1493–1499. http://dx.doi.org/10.1086/522609.
30. **Midgley CM, Watson JT, Nix WA, Curns AT, Rogers SL, Brown BA, Conover C, Dominguez SR, Feikin DR, Gray S, Hassan F, Hoferka S, Jackson MA, Johnson D, Leshem E, Miller L, Bezdeck Nichols J, Nyquist AC, Obringer E, Patel A, Patel M, Rha B, Schneider E, Schuster JE, Selvarangan R, Seward JF, Turabelidze G, Oberste MS, Pallansch MA, Gerber SI, EV-D68 Working Group.** Lancet Respir Med, in press.