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Short communication

Susceptibilities of enterovirus D68, enterovirus 71, and rhinovirus 87 strains to various antiviral compounds

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ABSTRACT

Compounds were evaluated for antiviral activity in rhabdomyosarcoma (RD) cells against a recent 2014 clinical isolate of enterovirus D68 (EV-D68), a 1962 strain of EV-68D, rhinovirus 87 (RV-87, serologically the same as EV-D68), and enterovirus 71 (EV-71). Test substances included known-active anti-picornavirus agents (enviroxime, guanidine HCl, pirodavir, pleconaril, and rupintrivir), nucleobase/nucleoside analogs (3-deazaguanine and ribavirin), and three novel epidithiodiketopiperazines (KCN-2,2′-epi-19, KCN-19, and KCN-21). Of these, rupintrivir was the most potent, with 50% inhibition of viral cytopathic effect (EC_{50}) and 90% inhibition (EC_{90}) of virus yield at 0.0022–0.0053 μM against EV-D68. Enviroxime, pleconaril and the KCN compounds showed efficacy at 0.01–0.3 μM; 3-deazaguanine and pirodavir inhibited EV-D68 at 7–13 μM, and guanidine HCl and ribavirin were inhibitory at 80–135 μM. Pirodavir was active against EV-71 (EC_{50} of 0.78 μM) but not against RV-87 or EV-D68, and all other compounds were less effective against EV-71 than against RV-87 and EV-D68. The most promising compound inhibiting both virus infections at low concentrations was rupintrivir. Antiviral activity was confirmed for the ten compounds in virus yield reduction (VYR) assays in RD cells, and for enviroxime, guanidine HCl, and pirodavir by cytopathic effect (CPE) assays in A549, HeLa-Ohio-1, and RD cells. These studies may serve as a basis for further pre-clinical discovery of anti-enterovirus inhibitors. Furthermore, the antiviral profiles and growth characteristics observed herein support the assertion that EV-D68 should be classified together with RV-87.

Enteroviruses, of which there are more than 100 known serotypes, are common causes of summer and fall illnesses, with up to 15 million infections annually in the United States. Enterovirus infections are associated with mild respiratory illness, febrile rash, and may also cause neurologic illness, such as aseptic meningitis and encephalitis (Solomon et al., 2010). According to the Centers for Disease Control and Prevention (CDC), non-polio enteroviruses are very common, and most people who get infected with these viruses do not get sick or have only a mild illness (Midgley et al., 2014). However, some people can have serious complications, especially infants and people with weakened immune systems.

Enterovirus D68 (EV-D68) is unique among non-polio enteroviruses in that it shares epidemiologic and biologic features with human rhinoviruses (Midgley et al., 2014; Oberste et al., 2004). It was first isolated in California in 1962 from 4 children with bronchiolitis and pneumonia. Since the original isolation of EV-D68, it has rarely been reported in the U.S. The National Enterovirus Surveillance System received only 79 EV-D68 reports during 2009–2013 (Schieble et al., 1967), but there was an increase in incidence in 2014 (Khan, 2015). EV-D68 is normally associated with mild to severe respiratory illness. However, infections with the 2014 virus strains caused more serious respiratory problems, especially in children who have asthma. The recent increase in the incidence of EV-D68 is also associated with an increase in unexplained polio-like cases in children, involving sudden onset of weakness in one or more limbs, referred to as acute flaccid myelitis in children (Greninger et al., 2015). Genetic sequencing of the virus found in respiratory secretions of children in California and Colorado who suffered from paralysis or muscle weakness in the fall of 2014 revealed that they were infected with a mutated strain of EV-D68 that is closer to polio than other strains common in previous years. This novel strain of EV-D68, called B1, appears to have emerged recently and has only 5 to 6 coding differences from previous strains commonly found in the United States. Of those six

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genetic polymorphisms in the B1 type of EV-D68 polyprotein, five are present in neuropathogenic poliovirus, enterovirus D70, or both (Greninger et al., 2015). Nucleotide sequencing, serology, and acid lability evidence suggest that EV-D68 is more similar to human rhinovirus 87 (RV-87) than to the enteroviruses (Blomqvist et al., 2002).

Another virus that has caused an increase in human infections is enterovirus 71 (EV-71). EV-71 is a major cause of hand, foot and mouth disease (HFMD) but also has the potential to induce severe neurological disease. EV-71 was first isolated from a two-month old patient with aseptic meningitis in California, USA in 1969 (Schmidt et al., 1974). Since that time, EV-71 has been identified as one of the causative agents for HFMD and is responsible for outbreaks in several countries including Taiwan, China, Australia, Singapore, Vietnam and Cambodia affecting up to millions of individuals at one time (Lin and Shih, 2014). These outbreaks often correlate with cohorts of children born that have not been previously exposed to the virus. While most cases of EV-71 present as a simple rash as seen in HFMD, approximately 10–20% of cases become neuropathogenic and cause severe neurological disease resulting in paralysis and death (McMinn, 2002; Ooi et al., 2010). According to the Global Disease Detection Program administered by the CDC, EV-71 is on the list of five infectious agents that are monitored worldwide for potential outbreaks (Christian et al., 2013). The CDC considers EV-71 a threat because of the potentially fatal nature of the disease, the ability to create large outbreaks through efficient spreading in daycare and school settings.

There is a need to identify inhibitors of enterovirus infections that may be developed for treatment. As we were in the process of preparing this manuscript for publication, two reports emerged describing antiviral activities of various anti-picornavirus agents against EV-D68 in vitro. Sun et al. reported efficacies of seven compounds against EV-D68 clusters A, B, and C viruses, with rupintrivir and SG85 being the most active compounds (Sun et al., 2015). Rhoden and colleagues evaluated 15 compounds from various classes against four EV-D68 strains (Rhoden et al., 2015). They also found the antipicornavirus agent rupintrivir to be highly active, but also similarly potent was the sialidase cleaving enzymatic compound DAS181. In this report we describe the antiviral activities of ten antiviral substances that have the potential to treat conditions caused by these viruses. Some of the compounds we investigated were recently reported (Rhoden et al., 2015; Sun et al., 2015), and other compounds are reported here for the first time, such as epidithiodiketopiperazine inhibitors. In addition to the information in the two recent publications, we show the cytotoxicity and selectivity indices of the compounds, their effects on virus yield, and comparative inhibitory activity of certain compounds in three human cell lines.

The following viruses were obtained from the American Type Culture Collection (ATCC, Manassas, VA) for evaluation: Enterovirus D68 (Fermon strain, VR-1076), Enterovirus 71 (Tainan/4643/98 strain, NR-471), and Rhinovirus 87 (F02-3607 Corn strain, VR-1197). Enterovirus D68 (US/KY/14-18953 strain, NR-49132) was from BEI Resources (Manassas, VA). Assays were performed in the following cell lines: A549 human lung carcinoma (CCL-185) and RD human rhabdomyosarcoma (CCL-136) cells from ATCC; and HeLa-Ohio-1 human cervical epithelioid carcinoma cells from Frederick Hayden (University of Virginia, Charlottesville, VA). Most of the studies were performed using RD cells.

Test compounds were obtained from various sources. Enviroxime was from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), Ft. Detrick, Frederick, MD through the NIAID antiviral screening program. Guanidine HCl was purchased from Sigma Aldrich (St. Louis, MO). Pirodavir was purchased from AdooQ Bioscience, (Irvine, CA). Pleconaril and rupintrivir were obtained from the former Biota Pharmaceuticals (Notting Hill, Victoria, Australia). 3-deazaguanine and ribavirin were from the former ICN Pharmaceuticals (Costa Mesa, CA). Epidithiodiketopiperazine compounds KCN-2,2′-epi-19, KCN-19, and KCN-21 were prepared at The Scripps Research Institute (La Jolla, CA) as previously described (Nicolaou et al., 2012). Each compound was dissolved in DMSO or MEM then diluted in half-$\log_{10}$ increments, and added to cells immediately prior to addition of virus-containing medium. The compounds represented different types of inhibitors, such as those inhibiting viral uncoating (so-called virus canyon binders), viral protease, viral RNA polymerase, and other modes of action as discussed below.

In vitro antiviral studies were conducted by two well-established methods, the inhibition of virus-induced cytopathic effect (CPE) and virus yield reduction (VYR). CPE assays were conducted in triplicate wells of 96-well microplates of infected cells as described previously (Barnard et al., 1997, 2004). EV-D68 and RV-87 viruses were evaluated in RD cells at 33 °C with 25 mM $MgCl_2$ and 2% FBS in minimal essential medium (MEM), typical conditions for evaluating rhinoviruses. EV-71 was tested in RD cells in MEM with 2% FBS but no $MgCl_2$ and incubated at 37 °C. Quantitation of percent virus destruction was made by neutral red dye uptake. The multiplicity of infection (MOI) used was the minimum inoculum that caused >80% CPE in 3–4 days of incubation for each virus (~16 cell culture infectious doses ($CCID_{50}$) per microwell for EV-D68 (US/KY/14-18953), ~270 for EV-D68 (Fermon), ~430 for EV-71, and ~46 for RV-87). The MOI was varied to obtain equivalent CPE for comparisons. To verify that MOI differences did not confound the results, selected assays were also performed using equal MOIs; these yielded similar results as in Table 1 with the same comparisons and conclusions (data not shown).

Compounds were evaluated at 8 half-$\log_{10}$ concentrations designed to bracket the 50% virus inhibitory concentration (EC_{50}). The 50% cytotoxic concentration (CC_{50}) of the compounds was determined in duplicate uninfected wells on the sample microtiter plate. Cells were in the confluent, stationary monolayer stage at the time of infection and treatment. VYR assays were conducted by first replicating the viruses in the presence of inhibitor, then after 3 days collecting the supernatant from each concentration of test compound and storing at −80 °C. Later, virus yield was determined by endpoint dilution method (Reed and Muench, 1938). Briefly, supernatant virus was serially diluted in $\log_{10}$ increments then plated onto quadruplicate wells of 96-well plates seeded with RD cells. The presence or absence of CPE for determining a viral endpoint was evaluated by microscopic examination of cells 6 days after infection. From these data, 90% virus inhibitory concentrations (EC_{90}) were determined by regression analysis.

The ten compounds were evaluated against EV-D68, RV-87, and EV-71 strains in CPE assays to determine viral inhibition (Table 1). Compounds that were potent inhibitors of EV-D68 and RV-87 included rupintrivir, pleconaril, enviroxime, and three KCN compounds. However, these same compounds were less active or ineffective against EV-71. Of the compounds tested, rupintrivir exhibited the most potent effects against EV-D68, RV-87, and EV-71 viruses. Guanidine HCl, 3-deazaguanine, pirodavir, and ribavirin also exhibited slight antiviral activity against EV-D68, RV-87 and/or EV-71. Pirodavir was the only compound that was more active against EV-71 than it was against EV-D68. Importantly, the antiviral sensitivity profile of EV-D68 more closely resembled that of RV-87 than of EV-71, supporting the assertions by Blomqvist et al. (2002) that RV-87 and EV-68 are highly similar and should be reclassified as a single serotype, perhaps as rhinoviruses rather than enteroviruses.

Dose-responsive effects of compounds on virus yield are shown graphically in Fig. 1. Rupintrivir and KCN-21 were the most potent