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Short communication

Potent antiviral agents fail to elicit genetically-stable resistance mutations in either enterovirus 71 or Coxsackievirus A16

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ABSTRACT

Enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16) are the two major causative agents of hand, foot and mouth disease (HFMD), for which there are currently no licenced treatments. Here, the acquisition of resistance towards two novel capsid-binding compounds, NLD and ALD, was studied and compared to the analogous compound GPP3. During serial passage, EV71 rapidly became resistant to each compound and mutations at residues I113 and V123 in VP1 were identified. A mutation at residue 113 was also identified in CVA16 after passage with GPP3. The mutations were associated with reduced thermostability and were rapidly lost in the absence of inhibitors. *In silico* modelling suggested that the mutations prevented the compounds from binding the VP1 pocket in the capsid. Although both viruses developed resistance to these potent pocket-binding compounds, the acquired mutations were associated with large fitness costs and reverted to WT phenotype and sequence rapidly in the absence of inhibitors. The most effective inhibitor, NLD, had a very large selectivity index, showing interesting pharmacological properties as a novel anti-EV71 agent.

Hand, foot and mouth disease (HFMD) usually manifests as a mild self-limiting childhood infection, causing sores on the hands, feet, mouth and buttocks, but can be associated with more serious symptoms, including fatal neurological/cardiovascular disorders. HFMD is usually caused by Enterovirus A species picornaviruses, especially enterovirus 71 (EV71) and Coxsackievirus A16 (CVA16), with EV71 more commonly associated with severe disease (Mcminn, 2003). EV71-mediated HFMD is therefore the major picornavirus-related public health problem in a post-poliovirus era and there are currently no clinically-approved therapeutic or prophylactic treatments.

WIN compounds and related molecules prevent receptor attachment/uncoating of a number of enteroviruses (Pevear et al., 1999). These compounds bind to a cavity in the capsid (the pocket in one of the viral capsid proteins, VP1) displacing hydrophobic lipids termed pocket factors. These are expelled upon receptor binding or uncoating, allowing the capsid to undergo a conformational change resulting in release of the RNA genome (Dang et al., 2014; Ren et al., 2013; Wang et al., 2012). The relatively high affinity between the pocket and WIN compounds prevents the conformational changes necessary for uncoating, increasing capsid stability (Rotbart et al., 1998) and can be effective at preventing infection in culture (Pevear et al., 1999; Shia et al., 2002; Benschop et al., 2015), and murine models (Groarke and Pevear, 1999; Liu et al., 2012). One compound, Vapendavir (BTA798), has shown efficacy in asthmatic patients with human rhinovirus (HRV) infections in phase II trials (Feil et al., 2012).

A related compound, Pleconaril, was used as a model to design a new class of pyridyl imidazolidinones, (IC_{50} values against EV71 of 0.001–25 μM), the most potent of which was termed GPP3 (Ke and Lin, 2006; Shia et al., 2002). Crystallographic analysis of EV71 (Plevka et al., 2012; Wang et al., 2012) in complex with four different pyridyl imidazolidinones, combined with computational

Abbreviations: VP1, Viral Protein 1; IC_{50}, Half-maximal inhibitory concentration; CC_{50}, Half maximal cytotoxic concentration; $TCID_{50}$, Half-maximal tissue culture infective dose; PV, poliovirus; CVB, Coxsackie virus B; WT, Wild Type; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; EV71, Enterovirus 71; CVA16, Coxsackie virus A16.

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methods including quantum mechanics–enhanced ligand docking were used to develop two new compounds (NLD and ALD), based upon GPP3 (De Colibus et al., 2014). NLD was shown to have more than an order of magnitude greater potency against EV71 than GPP3, $IC_{50} = 25$ pM. GPP3 and the new derivative compounds also had anti-CVA16 activity.

To identify mutations associated with resistance, EV71/CVA16 were passaged eight times in the presence of these compounds at concentrations that reduced the $TCID_{50}$ values by over 99.9% (see supplementary information). Virus titres in the presence of the compounds rapidly rose to WT-equivalent levels (~1×10^7 $TCID_{50}$/ml for EV71 and ~1×10^5 $TCID_{50}$/ml for CVA16), indicating the acquisition of resistance (Fig. 1). Sequencing revealed three different VP1 mutants in EV71 (I113L, I113M, I113M/V123I) and one in CVA16 (L113F) (Table 1). Virus passaged in a combination of NLD/GPP3 over 30 passages maintained the I113M/V123I mutations ($n = 1$).

The crystal structure of EV71 in complex with NLD enabled visualisation of the NLD binding site within the protomeric unit of the capsid, this showed that all interactions of NLD are with VP1 (Fig. 2A) (De Colibus et al., 2014). Fig. 2B and C shows the interactions close-up, with key stabilising interactions highlighted and the location of the resistance mutations in EV71, respectively. Mutations I113M and V123I are located on the inside of the VP1 pocket and I113 is one of the residues involved in compound binding (Fig. 2B). Fig. 2D shows the location of the resistance mutation for CVA16 in the context of the adjacent GPP3 molecule.

Studies with other enteroviruses have documented many mutations associated with resistance to a variety of pocket-binding compounds (see Supplementary Information). Generally, resistant viruses have acquired mutations that interfere with the correct placement of the inhibitors in the binding pocket (Liu et al., 2012). To test this in the EV71/CVA16 resistant-isolates, *in silico* folding energy predictions resulting from the mutations were performed using Rosetta (Fowler et al., 2010; Kellogg et al., 2011; Tyka et al., 2011) on VP1 subunits. The difference in the lowest free energy of folding ($\Delta\Delta G_{folding}$) between the WT and EV71 I113M or V123I mutants was +0.73 and + 0.98 kcal/mol, respectively. The combination of both mutations gave $\Delta\Delta G_{folding}$ of ~+1.1 kcal/mol per VP1 (note that this value should be multiplied by 60 to reflect the number of VP1 molecules per capsid), suggesting that the mutant virus capsid is less stable than WT. The mutations I113M and V123I appeared to cause a shrinking of the VP1 pocket, with the methionine residue pointing inside, suggesting a steric clash with the

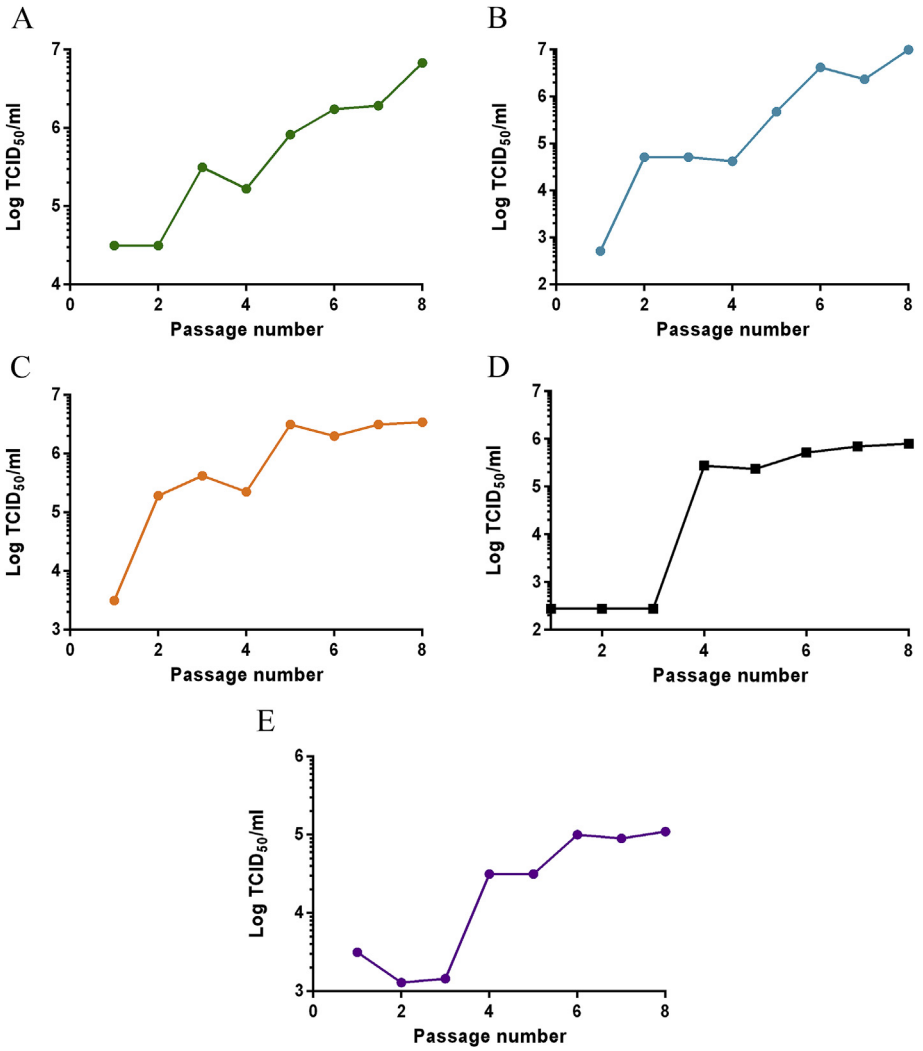

Fig. 1. Generation of resistant isolates. WT EV71 was passaged in the presence of either (A) 0.1 nM NLD, (B) 0.9 nM GPP3, (C) 80 nM ALD or, (D) a combination of 0.1 nM NLD and 0.9 nM GPP3, (E) WT CVA16 was passaged in the presence of 20 nM GPP3. Each isolate was passaged a total of 8 times and after each passage a sample was titrated in the presence of the selecting concentration of compound. CVA16 isolates resistant to NLD or ALD were not selected.