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.

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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, (IC50 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; IC50, Half-maximal inhibitory concentration; CC50, Half-maximal cytotoxic concentration; TCID50, Half-maximal tissue culture infective dose; PV, poliovirus; CVB, Coxsackie virus B; WT, Wild Type; MTI, 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, IC₅₀ = 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 TCID₅₀ values by over 99.9% (see supplementary information). Virus titres in the presence of the compounds rapidly rose to WT-equivalent levels (~1x10⁷ TCID₅₀/ml for EV71 and ~1x10⁵ TCID₅₀/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 (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 (ΔΔG_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 ΔΔG_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.](image-url)
pocket factor. Thus, the distance between the carbon atom of the methyl group of the methionine side chain and a carbon atom in the linker of NLD is $2.1\,\text{Å}$ (Fig. 2C), smaller than the sum of the Van der Waals radii and leading to a repulsive interaction. However, the $\Delta\Delta G_{\text{folding}}$ for the CVA16 mutant was $1.7\,\text{kcal/mol}$, suggesting that the mutant is more stable than WT (not taking pocket factor into account). The Rosetta structures are shown in grey in Fig. 2C and D.

It has been reported that pocket-binding compounds increased thermostability of WT PV and HRVs, while inhibitor-resistant isolates with mutations in the pocket were often more thermolabile than their untreated WT counterpart (Katpally et al., 2007; Mosser et al., 1994; Shepard et al., 1993). The thermostabilities of WT EV71 and inhibitor-resistant mutant virus selected in the presence of NLD/GPP3 were therefore evaluated. WT EV71 (heated with NLD) was tolerant of temperatures $6\,\text{°C}$ higher before detectable inactivation and $2.5\,\text{°C}$ higher before complete inactivation occurred, compared to virus in the absence of NLD (Fig. 3A). In contrast, the thermostability of inhibitor-resistant virus was not increased in the presence of NLD or by a combination of GPP3 and NLD. In addition, the inhibitor-resistant mutant virus was more thermolabile than

<table>
<thead>
<tr>
<th>Virus/Compound</th>
<th>Mutation(s)</th>
<th>Number sequenced</th>
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<tbody>
<tr>
<td>EV71/NLD</td>
<td>I113M (62%), I113L (31%), I113M/V123I (7%)</td>
<td>$n = 13$</td>
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<tr>
<td>EV71/GPP3</td>
<td>I113M/V123I</td>
<td>$n = 4$</td>
</tr>
<tr>
<td>EV71/ALD</td>
<td>I113M/V123I</td>
<td>$n = 1$</td>
</tr>
<tr>
<td>EV71/NLD + GPP3</td>
<td>I113M/V123I</td>
<td>$n = 12$</td>
</tr>
<tr>
<td>CVA16/GPP3</td>
<td>L113F</td>
<td>$n = 10$</td>
</tr>
</tbody>
</table>

Table 1

Resistance mutations. Mutations identified in EV71/CVA16 after passage in the presence of NLD, GPP3, ALD or a combination of NLD/GPP3 (see Fig. 1). CVA16 isolates resistant to NLD or ALD were not selected.
carried out in triplicate and error was measured using standard deviation. Diluted to a level at which the inhibitor has no effect before titration. Titrations were carried out to a level at which the inhibitor has no effect before titration. Titrations were carried out in triplicate and error was measured using standard deviation.

The V123I mutation reduces this further. The pocket, reducing the space available for inhibitor binding and predicting that there is a difference in the stability of viruses carrying the resistance mutations (in the absence of pocket factor), however, it is likely that the mutations also have a deleterious effect on the binding of the natural pocket factor, which is known to be a virion stabiliser. Similar results have been reported for WIN51711-resistant PV3 and WIN52035-2-resistant HRV14, with mutations identified in the VP1 pocket (Mosser et al., 1994; Shepard et al., 1993).

To assess whether the mutations affected growth kinetics, one-step growth curves were performed with WT EV71 and a resistant isolate. These showed no significant difference between the WT and the resistant virus (Fig. 4). The phenotype of the mutant viruses described here is consistent with previous observations with other picornaviruses (Groarke and Pevear, 1999; Heinz et al., 1989; Lacroix et al., 2014; Liu et al., 2012; Mosser et al., 1994; Salvati et al., 2004). However, it should be noted that a small difference in kinetics of virus resistant to BPR0Z-19 has been reported (Shih et al., 2004).

Mutations are often associated with a fitness cost, therefore the genetic stability of inhibitor-resistant EV71 was tested by passage in the absence of inhibitors. The titre in the presence of inhibitor dropped dramatically after just one passage. After two further passages the titre became equivalent to the WT in the presence of the inhibitors (Fig. 5). Sequencing confirmed that the virus had reverted to a WT genotype (n = 1), indicating a strong selection pressure for reversion. However, it should be noted that as this experiment was performed with a pool of viruses, it is not formally possible to distinguish between the amplification of residual WT virus in the population and back-mutation of selected mutant virus.

Fitness costs appear to be common in pocket inhibitor-resistant enteroviruses, with Pleconaril-resistant CVB3 and V-073-resistant PV causing reduced virulence in murine models (Groarke and Pevear, 1999; Kouiavskaia et al., 2011). Similarly, Pleconaril-resistant HRV B isolated from patients was shown to be non-pathogenic and was associated with a greatly reduced viral load (Pevear et al., 2005). The avirulent PV Sabin strains are also thermolabile and other thermolabile EV71 mutants have been shown to have reduced pathogenesis in Cynomolgus monkeys (Arita et al., 2005). Given the apparent fitness cost of these mutations, resistance may not pose an obstacle to the therapeutic use of pocket-binding compounds. Indeed, assessment of the toxicity of the three compounds showed NLD to have a large selectivity index (Fig. 6, Table 2). However, further work will be necessary in order to evaluate if inhibitor-resistant EV71/CVA16 mutants are less virulent.

In conclusion, we have described the selection and characterisation of resistance mutations of EV71 in the presence of three pocket-binding inhibitory compounds. We modelled these

**Fig. 3.** Inhibitor-resistant mutants are more thermolabile than WT virus. (A) Thermolability curves of EV71 WT (filled circle) and inhibitor-resistant EV71 (open square), WT EV71 in the presence of 2 nM NLD (open circle), inhibitor-resistant EV71 in the presence of 0.9 nM GPP3 and 0.1 nM NLD (open triangle) and 2 nM NLD (filled triangle), and (B) Thermolability curves of WT CVA16 (filled circle) and inhibitor-resistant CVA16 (open square). All samples were heated at a range of temperatures for 30 min using a thermocycler, prior to titration by TCID₅₀ assay. Samples containing NLD were first diluted to a level at which the inhibitor has no effect before titration. Titrations were carried out in triplicate and error was measured using standard deviation.

**Fig. 4.** One step growth curves comparing inhibitor-resistant and WT EV71. WT (filled circle) and inhibitor-resistant EV71 selected in the presence of 0.1 nM NLD and 0.9 nM GPP3 (open square) were used to infect six wells each of a 96 well plate at an MOI of 10. Every 3 h the supernatant and cells of a well from each isolate were removed, and freeze-thawed to lyse the cells. These samples were then titrated by TCID₅₀ assay.

**Fig. 5.** Inhibitor-resistant isolates are genetically unstable. A NLD/GPP3 inhibitor-resistant isolate selected with a combination of 0.1 nM NLD and 0.9 nM GPP3 was passaged four times in the absence of inhibitor. Virus from each passage was titrated in the presence (filled circles) or absence of inhibitors (open squares) by TCID₅₀ assay.
respectively. DMSO (1%) had no apparent cytotoxic effect.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>NLD</th>
<th>GPP3</th>
<th>ALD</th>
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<tr>
<td><strong>CC50 (Cell toxicity)</strong></td>
<td>1600 nM</td>
<td>1000 nM</td>
<td>700 nM</td>
</tr>
<tr>
<td><strong>IC50 (Virus inhibition)</strong></td>
<td>0.025 nM</td>
<td>0.39 nM</td>
<td>8.7 nM</td>
</tr>
<tr>
<td><strong>Selectivity Index (CC50:IC50)</strong></td>
<td>$6 \times 10^4$</td>
<td>$2.6 \times 10^3$</td>
<td>$8 \times 10^2$</td>
</tr>
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mutations in the VP1 pocket to explain their effect on the binding mode of the pocket factor and calculated the $\Delta \Delta G_{\text{folding}}$ of these mutants. The in silico predictions were in agreement with some of the observed properties of the viruses in vitro. Furthermore we found that the most effective EV71 pocket-binding inhibitor, NLD, has the greatest selectivity index, showing interesting pharmacological properties as a novel anti-EV71 agent.

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Transparency declarations

Nothing to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2015.10.006.

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