Blocking human enterovirus 71 replication by targeting viral 2A protease

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Objectives: Human enterovirus 71 (EV-71), a member of the Enterovirus genus, constitutes a major public health issue in the Asia-Pacific region, where it is associated with several severe neurological complications. There is currently no effective vaccine or antiviral against EV-71. The aim of this study was to determine whether the six amino acid peptide LVLQTM, which was previously shown to inhibit human rhinovirus (HRV) 2A protease (2Apro) activity in vitro and HRV replication in vivo in mice, could be of more general use against enteroviruses and more particularly against EV-71.

Methods: To investigate whether the LVLQTM peptide was a pseudosubstrate of EV-71 2Apro, a recombinant luciferase containing the LVLQTM sequence was designed so that recognition of this sequence by 2Apro led to luciferase activation. Direct interaction between EV-71 2Apro and the LVLQTM peptide was further confirmed by isothermal titration calorimetry. We then tested the effects of the peptide on EV-71 2Apro cleavage activity and EV-71 replication in HeLa cells.

Results: We showed that the LVLQTM peptide behaved as an effective substrate analogue of EV-71 2Apro, a recombinant luciferase containing the LVLQTM sequence was designed so that recognition of this sequence by 2Apro led to luciferase activation. Direct interaction between EV-71 2Apro and the LVLQTM peptide was further confirmed by isothermal titration calorimetry. We then tested the effects of the peptide on EV-71 2Apro cleavage activity and EV-71 replication in HeLa cells.

Conclusions: This study demonstrates that the LVLQTM peptide that has previously been shown to inhibit HRV replication is also an effective inhibitor of EV-71 2Apro and therefore of EV-71 replication, opening new doors in the development of new antivirals against EV-71.

Keywords: picornavirus, antiviral, inhibitory peptide, cysteine protease

Introduction

Human enterovirus 71 (EV-71), a member of the Enterovirus genus, is a causative agent of hand, foot and mouth disease, aseptic meningitis, brainstem encephalitis and poliomyelitis-like acute flaccid paralysis.1 EV-71 is the most pathogenic non-polio enterovirus in the Asia-Pacific region, where several outbreaks have been reported. The largest EV-71 epidemic so far occurred in 1998 in Taiwan, where ~1.5 million people and >100000 children were infected. Among them, 405 severe cases of neurological and cardiopulmonary complications were described, of which 78 were fatal.1 Moreover, the latest large Asian epidemic was in China in 2008, where ~490000 infections and 126 deaths in children were reported.2 There is no effective vaccine against EV-71 available on the market, despite the investigation of various types of vaccines, including inactivated whole virus, live-attenuated, subviral particle and DNA vaccines.3,4 In addition, several antiviral strategies have been employed, including compound library screening and revisiting of some antivirals developed against poliovirus and rhinovirus, but none of them has been approved yet. The lack of vaccine or antiviral options against EV-71 remains a worrying situation, as EV-71 has been found to circulate endemically. Thus, the aim of this study was to describe a new inhibitor of EV-71 infection.
We previously showed that some peptide ligands that were selected from a human placenta cDNA library by the yeast two-hybrid procedure inhibited the activity of the human rhinovirus (another member of the Enterovirus genus) 2A protease (2A\textsuperscript{pro}) by docking into the active site of the enzyme.\textsuperscript{5} Among them, the dominant peptide LVLQTM was also shown to inhibit subsequent viral replication \textit{in vitro} in A549 cells and \textit{in vivo} in mice, and could therefore be an interesting lead compound for a therapeutic agent against rhinoviruses. In the present study, we demonstrated that the LVLQTM peptide is also a strong inhibitor of EV-71 2A\textsuperscript{pro} cleavage activity as well as EV-71 replication, opening new prospects in the development of anti-EV-71 drugs.

**Materials and methods**

Most protocols used in this study have already been described in detail in Falah et al.\textsuperscript{7} Specific methods are detailed in Supplementary data (available at JAC Online).

**Results and discussion**

**Expression and purification of EV-71 2A\textsuperscript{pro}**

Production of soluble and active EV-71 2A\textsuperscript{pro} in the cytoplasm of Escherichia coli was achieved through the Staby plasmid system (Eurogentec). Purification of the 2A protein from the cell extract was achieved by metal-chelate affinity chromatography followed by size-exclusion chromatography (SEC). The SEC elution profile depicted in Figure 1(a) shows that EV-71 2A\textsuperscript{pro} (peak D1) was purified to >95% purity, as estimated by SDS–PAGE analysis. However, the enzyme presented very abnormal chromatographic behaviour, since it was eluted very late from the SEC column together with the salt, which made it impossible to accurately determine its molecular weight using column calibration. To resolve this problem, the combined technology SEC-multilange light scattering (SEC-MALS) was used. This analysis, presented in Figure 1(b), revealed that 2A\textsuperscript{pro}, which eluted in a main peak from 17.0 to 18.8 mL, had a calculated molecular mass of 17.7 kDa and thus behaved essentially as a monomer. Noticeably, this monomeric organization is very similar to that of other 2A\textsuperscript{pro}, as previously described for Coxackie virus B4 (CVB4) and human rhinovirus B14 (HRV-B14) 2A\textsuperscript{pro}, which also behave as monomers.\textsuperscript{6,7}

**The LVLQTM peptide is a pseudosubstrate of EV-71 2A\textsuperscript{pro}**

The ability of the LVLQTM peptide to act as a pseudosubstrate of EV-71 2A\textsuperscript{pro} was evaluated \textit{in vitro} in the Protease-Glo\textsuperscript{TM} Assay (Promega). In this assay, the LVLQTM-G peptide was inserted in frame into the linker region of the GloSensor\textsuperscript{TM} luciferase. This sequence was chosen to reconstruct a potent site of cleavage by the protease, with LVLQTM corresponding to P1–P6 residues on the N-terminal side of the scissile bond and glycine corresponding to the P1′ residue, as this amino acid is found in the equivalent position in the authentic cis-cleavage site of the EV-71 polyprotein. As shown in Figure 1(c), significant activation of the luciferase containing the LVLQTM-G sequence was detected when incubation was performed in the presence of EV-71 2A\textsuperscript{pro}. This cleavage was specific, since no luminescence was measured when using a luciferase protein containing the peptide sequence ENLYFQ-S, which was only cut by the TEV protease (positive control).

**Measuring LVLQTM peptide binding to EV-71 2A\textsuperscript{pro} by isothermal titration calorimetry**

We used isothermal titration calorimetry to investigate the interaction between EV-71 2A\textsuperscript{pro} and the LVLQTM peptide (Figure 1d). The results showed that the binding was exothermic and analysis of the data yielded a dissociation constant (K\textsubscript{d}) of 9.6 μM. Concerning thermodynamic parameters, binding of LVLQTM to EV-71 2A\textsuperscript{pro} was associated with favourable entropy (ΔS = 19.7 cal/mol/deg, i.e. ΔTS = 5.8 kcal/mol) and favourable enthalpy (ΔH = −956 cal/mol). The favourable enthalpy indicated that the peptide established tight interactions through hydrogen bonds and Van der Waals contacts, and that these interactions were strong enough to compensate for the unfavourable enthalpy associated with desolvation. On the other hand, the highly favourable entropy showed that the binding mainly involved hydrophobic interactions and was consistent with the displacement of water molecules from the active site and/or peptide hydration shell. Moreover, this high ΔTS value, indicating that the interaction between EV-71 2A\textsuperscript{pro} and the LVLQTM peptide was predominantly entropic, might also reflect low conformational entropy change, as a consequence of the loss of conformational degrees of freedom of both the drug molecule and the protein molecule upon binding.

Taken together, these results demonstrated that the LVLQTM peptide exhibits an overall conformation that fits well with the substrate-binding pocket of EV-71 2A\textsuperscript{pro} and may thus represent a potent inhibitor of this enzyme.

**LVLQTM peptide inhibits eIF4G-cleavage activity of EV-71 2A\textsuperscript{pro}**

We then investigated the effects of the LVLQTM peptide on 2A\textsuperscript{pro} activity in a cellular system. Based on our previous results, the benzylloxycarbonyl-LVLQTM-fluoromethylketone (z-LVLQTM-fmk) was preferably used in an \textit{in cellulo} cleavage assay that relied on the ability of the EV-71 2A\textsuperscript{pro} to hydrolyse the initiation factor of translation, eIF4G. The results depicted in Figure 2(a) indicated that EV-71 2A\textsuperscript{pro}-induced inhibition of cap-R-luciferase activity in the presence of the control peptide z-FA-fmk was completely dismissed in the presence of z-LVLQTM-fmk. Similarly, EV-71 2A\textsuperscript{pro}-induced enhancement of internal ribosome entry site (IRES)-R-luciferase activity was inhibited when cells were treated with z-LVLQTM-fmk. Altogether, these results demonstrated that z-LVLQTM-fmk specifically inhibited EV-71 2A\textsuperscript{pro} activity in HeLa cells.

**z-LVLQTM-fmk peptide inhibits EV-71 replication**

To investigate whether the z-LVLQTM-fmk peptide could inhibit EV-71 replication, HeLa cells were infected with EV-71 (multiplicity of infection = 1) and treated with 200 μM z-FA-fmk or z-LVLQTM-fmk at different times before and after infection. Total virus titres were determined 24 h post-infection. As indicated in Figure 2(b), the presence of z-LVLQTM-fmk reduced virus multiplication by >50-fold when cells were infected and treated at the same time. Noticeably, delayed administration of the peptide at 2 and 4 h post-infection had a more...
Figure 1. Characterization of the interaction between EV-71 2A^pro and the LVLQTM peptide. (a) Purification of recombinant EV-71 2A^pro. After partial purification by immobilized metal ion affinity chromatography (IMAC), pooled fractions enriched in 2A^pro were further fractionated on a Superdex™ 200 column. The column was equilibrated with a buffer containing 20 mM Tris-HCl, pH 7.4, 50 mM NaCl and 1 mM dithiothreitol, and calibrated with ferritin (A, 440 kDa), ovalbumin (B, 43 kDa) and ribonuclease A (C, 13.7 kDa). Insert, SDS–PAGE analysis of the SEC fractions stained with Coomassie brilliant blue. (b) SEC-MALS analysis of EV-71 2A^pro. SEC elution pattern and molar mass (MM) plot. 500 mL of 0.7 mg/mL EV-71 2A^pro eluted at a different retention time than in SEC. The weight-averaged molecular mass determined by MALS corresponds to a monomer of EV-71 2A^pro for the peak at 17.8 mL elution volume. (c) Protease-Glo™ Assay in the presence of EV-71 2A^pro. In vitro-synthesized GloSensor ENLYFQ-S and GloSensor LVLQTM-G proteins were incubated with purified TEV protease or EV-71 2A^pro for 45 min. Luminescence was measured using a luminometer after addition of Bright-Glo assay reagent for 5 min. RLU, relative light units. Data are expressed as the means of three independent experiments and standard deviations are indicated. The asterisk indicates *P < 0.05. (d) Isothermal titration calorimetry for the interaction of EV-71 2A^pro and LVLQTM peptide. The upper panel shows a thermogram, and the lower panel shows the corresponding binding isotherm and best-fitted curve. Stoichiometry (N), dissociation constant (K_d), binding enthalpy (ΔH) and binding entropy (ΔS) were calculated, and are presented as an inset in the lower panel.
pronounced inhibitory effect on virus replication, since the viral titre dropped by ~107- and 417-fold, respectively.

In summary, the LVLQTM peptide, which was previously characterized as a new inhibitor of HRV 2Apro, also impairs EV-71 2Apro activity and EV-71 replication in HeLa cells. (a) z-LVLQTM-fmk impairs EV-71 2Apro-mediated inhibition of capped mRNA translation. HeLa cells were treated with 100 μM z-LVLQTM-fmk or z-FA-fmk for 1 h and were subsequently transfected with an mRNA coding for EV-71 2Apro or green fluorescent protein (GFP) for 2 h. Cells were then transfected for 3 h with a capped mRNA or an IRES-containing mRNA, both coding for the Renilla luciferase. The first contained the 5′ untranslated region (UTR) of the β-globin gene and the second contained the 5′ UTR of the encephalomyocarditis virus RNA. Cells were then lysed and luciferase activity was measured by luminometry. Error bars denote the standard deviations from the mean values obtained from three independent experiments. (b) z-LVLQTM-fmk inhibits EV-71 replication. HeLa cells were infected with EV-71 at a multiplicity of infection of 1 and treated with 200 μM z-LVLQTM-fmk or z-FA-fmk at different times before and after infection. Virus titres were determined 24 h post-infection. (c) Sequence alignments of HRV-A2 (GenBank accession number X02316) and EV-71 2Apro (GenBank accession number AEF32490). Sequence alignment was performed using the ClustalW program and plotted with the ESPript program. Similar residues are highlighted in grey and identical residues are in black. Secondary structure elements of HRV-A2 are shown above the sequences, arrows indicate residues involved in the binding of the VLQTM peptide at subsites S5–S1 in HRV-A2 2Apro and stars indicate point mutations in 2Apro found for 40 EV-71 strains (see Figure S1 available as Supplementary data at JAC Online for GenBank accession number). The s- and b-marked stars represent surface residues and buried residues, respectively. Residues are numbered according to the amino acid sequence of EV-71 2Apro.

**LVLQTM peptide represents a potential inhibitor of multiple EV-71 2Apro variants**

The 2Apro sequences from 40 EV-71 strains were compared to evaluate whether the LVLQTM peptide could be of general use against different EV-71 genotypes. Genotyping of EV-71 has so far been based on structural genes VP1 and VP4. Recently, Chan et al. proposed a new classification into four genotypes, A–D, based on EV-71 complete genomes (taking into account 2Apro variants). Point mutations occur at 22 positions along the 2Apro sequence (Figure 2c and Figure S1 available as Supplementary data at JAC Online), which may induce local or long-range conformational rearrangement. More interestingly, four mutations occur at particular positions corresponding to substrate-binding pocket residues defined for HRV-A2 2Apro. In
particular, the S5 Asn129 residue is extremely conserved among nearly all EV-71 serotypes analysed in this study and is consistent with the presence of an aspartate in HRV-A2 or a glutamate in EV-6 and poliovirus-1. Only EV-71 strain BrCr displays a serine at this position. The shorter side chain of serine compared with that of aspartate or asparagine likely reflects some flexibility of the substrate at P5. This could suggest a lesser affinity of this strain for the LVLOTM peptide, as previously shown for HRV-B14 displaying also a serine at position 129. The apolar depression defining S4 is maintained, as mutations I82V and V84G/I are both conservative. Finally, mutation P107S in strain MS742387 occurs at S1 in a very well conserved stretch of residues. HRV-B14 2Apro, which also displays a mutation at this position (proline to leucine), is still inhibited by LVLOTM, suggesting that the peptide nonetheless inhibits strain MS742387.

The general conclusion from these analyses supports the view that the peptide inhibitor LVLOTM may interact with a large spectrum of EV-71 2Apros, especially from subgenotypes B4, B5, C1, C2, C5 and D currently circulating in the world and associated with severe CNS infection of young children.

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Transparency declarations
None to declare.

Supplementary data
Figure S1 and more details of the methods are available at JAC Online (https://jac.oxfordjournals.org/).

References