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Structure-Guided Improvement of a Dual HPIV3/RSV Fusion Inhibitor

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Abstract

Human parainfluenza virus 3 (HPIV3) and respiratory syncytial virus (RSV) are leading causes of lower respiratory tract infections. There are currently no vaccines or antiviral therapeutics to treat HPIV3 or RSV infections. We recently reported a peptide (VIQKI), derived from the C-terminal heptad repeat (HRC) domain of the HPIV3 fusion (F) glycoprotein that inhibits infection by both HPIV3 and RSV. The dual inhibitory activity of VIQKI is due to its unique ability to bind to the N-terminal heptad repeat (HRN) domains of both HPIV3 and RSV F, thereby preventing the native HRN-HRC interactions required for viral entry. Here we describe the structure-guided design of dual inhibitors of HPIV3 and RSV fusion with improved efficacy. We show that VIQKI derivatives possessing one (I456F) or two (I454F/I456F) phenylalanine substitutions near the N-terminus exhibit more stable assemblies with RSV-HRN domain and enhanced antiviral efficacy against both HPIV3 and RSV infection. Co-crystal structures of the new Phe-substituted inhibitors co-assembled with HPIV3 or RSV-HRN domains reveal that the I456F substitution makes intimate hydrophobic contact with the core trimers of both HPIV3 and RSV F.

Graphical Abstract

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Respiratory syncytial virus (RSV) and human parainfluenza virus 3 (HPIV3) are major causes of lower respiratory tract infections in young children, resulting in thousands of deaths each year.¹³⁻¹⁴ There is no vaccine for RSV or HPIV3 and no treatment for these infections, which present with similar symptoms although the viruses are quite different. To be most effective, treatment would have to start soon after the appearance of symptoms, and before the causative agent had been identified. Therefore, a single agent that could address both RSV and HPIV3 is desirable.

Inhibition of viral fusion is a potential therapeutic strategy⁴⁻¹⁴, and we recently described a 36-residue peptide derived from the HPIV3 fusion (F) glycoprotein that displays significant inhibition of RSV infection while maintaining anti-HPIV3 potency.¹⁵ This peptide, designated VIQKI, appears to block viral infection by interfering with F-mediated fusion of the HPIV3 or RSV envelope and the target cell membrane. Fusion is essential for infection and is driven by the formation of a six-helix bundle assembly within the F protein trimer (Figure 1B,C).¹⁶⁻¹⁸ The three inner helices are formed by the N-terminal heptad repeat (HRN) domain of F, and the three outer helices are formed by the C-terminal heptad repeat (HRC) domain. VIQKI is a variant of HPIV3-HRC. Crystallographic data revealed that the N-terminal portion of VIQKI displays an unexpected ability to adapt itself to two distinct assemblies: a native-like extended conformation when co-assembled with HPIV3-HRN, but an unexpected helical conformation upon co-assembly with RSV-HRN. Presumably this structural adaptability underlies the dual activity of VIQKI against HPIV3 and RSV.

Here we ask whether efficacy against RSV can be enhanced without sacrificing efficacy against HPIV3 by modifying the N-terminal region of VIQKI. Because this portion of VIQKI uses two different sets of residues to engage HPIV3-HRN vs. RSV-HRN, it was not clear at the outset that our goal could be achieved. Nevertheless, an experimental design based on careful inspection of F protein structures from both viruses delivered enhanced dual inhibitors.

Our recent crystal structure of VIQKI co-assembled with HPIV3-HRN¹⁶ revealed a six-helix bundle that corresponds closely to the HRC/HRN assembly in the post-fusion form of the HPIV3 F protein (Figure 2A,B).¹⁹ Co-crystallization of VIQKI with the RSV-HRN peptide led to a comparable hexameric assembly, but the N-terminal portion of VIQKI displayed substantial conformational variation between these two structures (Figure 2B,D). In the HPIV3-HRN co-assembly, the N-terminal segment of VIQKI is extended and projects three

aliphatic side chains (L451, I454 and I456; numbering based on the full-length HPIV3 F protein) to make native hydrophobic contacts with the HRN trimer core. In the RSV-HRN co-assembly, the N-terminal segment of VIQKI forms an additional helical turn, and the side chain carboxylates of D452 and D455 form salt bridges with the RSV-HRN trimer core. The corresponding region of the RSV-HRC domain in the post-fusion state of the RSV F protein does not form salt bridges with the HRN trimer; instead, the Phe483 and Phe488 side chains make hydrophobic contacts with the HRN trimer (Figure 2C).^{20,21}

We predicted that the anti-RSV potency of VIQKI could be enhanced via replacement of one residue or more in the N-terminal region with Phe, if an appropriately positioned Phe side chain engaged the complementary pocket on the RSV-HRN trimer that is occupied by the F483 or F488 side chain in the post-fusion RSV F structure (Figure 2C). By mimicking these native interactions, we hypothesized that we could maintain specificity for the RSV-HRN trimeric core while limiting non-specific engagement of unintended targets. A key uncertainty, however, was the impact of new Phe residue(s) on anti-HPIV3 potency; enhancing activity against RSV would be most valuable if activity against HPIV3 were not compromised.

We conducted a “Phe scan” of the first eight positions of VIQKI, corresponding to HPIV3 F residues V449-I456, to identify favorable Phe substitutions. The impact of each Phe modification was assessed by circular dichroism (CD) measurements. Combining VIQKI with a peptide corresponding to the RSV-HRN domain or the HPIV3-HRN domain leads to a strong α -helical CD signature, which is attributed to helix-bundle formation. Thermal disruption of the helix bundle can be detected by monitoring the characteristic CD minimum at 222 nm as the sample is heated, and the midpoint of the transition provides an apparent melting temperature ($T_{m,app}$). Table 1 shows $T_{m,app}$ values for VIQKI and each of the eight single-site Phe variants in combination with RSV-HRN. At most positions, Phe substitution led to a destabilization of the helix-bundle assembly ($T_{m,app} < 0$). However, increases in assembly stability were observed for modifications L451F, I454F or I456F, with the most substantial enhancement for the last of these three variants. Two double Phe substitutions were examined, L451F/I456F and I454F/I456F. $T_{m,app}$ values suggested that only the latter combination was favorable.

We employed a plaque-reduction assay to measure the efficacies of selected Phe-containing variants of VIQKI at inhibiting RSV infection. Monolayers of Hep2 cells were exposed to an engineered recombinant RSV that expresses green fluorescent protein (GFP), which allows the extent of infection to be quantified via fluorescence high content image screening. Previous data demonstrate that VIQKI is approximately as effective as RSV-HRC and much more effective than HPIV3-HRC at inhibiting RSV infection.¹⁵ The substitutions in VIQKI enhance inhibition of HPIV3 infection relative to HPIV3 HRC.^{10,14,15} Here, we asked whether we could improve upon VIQKI's anti-RSV potency without decreasing its efficacy against HPIV3. Four peptides were evaluated, new compounds VIQKI-I456F and VIQKI-I454F/I456F, along with RSV-HRC and VIQKI as reference compounds (Figure 3A); peptide was added to the cells along with the virus. RSV-HRC and VIQKI displayed comparable efficacies in this assay, with IC_{50} values of $\sim 1 \mu M$. Both Phe-containing variants, VIQKI-I456F and VIQKI-I454F/I456F, were significantly more effective inhibitors

than either RSV-HRC or VIQKI, with IC₅₀ values 10-fold lower for the Phe-containing variants (Table S1). Thus, the stabilization of six-helix bundle assembly suggested by CD measurements for the two new peptides relative to VIQKI (Table 1) correlates with enhanced efficacy in blocking RSV infection.

An analogous plaque-reduction assay was performed to compare the anti-HPIV3 activities of the two new Phe-containing VIQKI variants with the activity of VIQKI itself. We have previously demonstrated that RSV-HRC does not block infection by HPIV3.¹⁵ Monolayers of Hep2 cells were exposed to an engineered recombinant HPIV3 that expresses GFP along with variable concentrations of VIQKI, VIQKI-I456F or VIQKI-I454F/I456F (Figure 3B). Both of the new Phe-containing peptides were modestly superior to VIQKI as inhibitors of HPIV3 infection. Thus, both peptides are potent dual inhibitors of RSV and HPIV3.

In order to elucidate Phe side chain locations within helix-bundle assemblies containing the new dual inhibitors, we sought to co-crystallize VIQKI-I456F and VIQKI-I454F/I456F with either RSV-HRN or HPIV3-HRN. Two of the four pairings produced high-quality crystals that led to atomic-resolution structures, VIQKI-I456F+RSV-HRN and VIQKI-I454F/I456F+HPIV3-HRN (Figure 4).

The VIQKI-I456F+RSV-HRN co-crystal structure revealed that the newly introduced Phe residue induced a reorientation of the N-terminal region of the VIQKI derivative relative to the trimeric HRN core, as intended. Although salt bridges involving D455 and D452 form when VIQKI co-assembles with the RSV-HRN trimer (Figure 2D), comparable salt bridges are not found in the new complex; instead, the side chain of F456 occupies the pocket that accommodates the F488 side chain in the structure of post-fusion RSV F (Figure 4D,E, Figure S30).^{20,21} Other critical residues of VIQKI that engage the RSV-HRN trimer, such as L467, I474, and L481, maintain their contacts in the new VIQKI-I456F+RSV-HRN structure (Figure S31).

The VIQKI-I454F/I456F+HPIV3-HRN co-crystal structure displayed a reorientation of the VIQKI derivative N-terminal region comparable to that observed for VIQKI-I456F+RSV-HRN (Figure 4E,F). Helical secondary structure extends further toward the N-terminus of VIQKI-I454F/I456F than was observed in the VIQKI+HPIV3-HRN co-crystal structure. The side chain of F456 occupies the pocket that accommodates I456 in the structure of VIQKI+HPIV3 (Figure 4F vs. 2B).¹⁹ We were surprised, however, to observe that the side chain of F454 is oriented away from the HPIV3-HRN trimeric core; we had speculated that this side chain would occupy the pocket that is naturally filled by the side chain of I454. Inspection of interactions involving neighboring six-helix bundles within the co-crystal lattice revealed that F454 from one assembly stacks against W473 from an adjacent assembly (Figure S32). It is possible that this lattice contact distorts the positioning of F454 relative to an isolated six-helix bundle in solution. On the other hand, the $T_{m,app}$ values are indistinguishable for co-assemblies with HPIV3-HRN formed by VIQKI-I456F and VIQKI-I456F/I454F, which suggests that the side chain of F454 does not contribute to helix-bundle stability in the latter case. This observation is consistent with the location of the F454 side chain in the VIQKI-I454F/I456F+HPIV3-HRN co-crystal structure (Figure 4C,F).

We have shown that a structure-guided strategy can deliver new dual inhibitors of HPIV3 and RSV that manifest enhanced anti-RSV potency relative to the previously reported peptide VIQKI, which is very effective against HPIV3 but less effective against RSV. Comparisons involving available crystal structures led us to hypothesize that introduction of a Phe residue in the N-terminal segment of VIQKI would improve binding to the RSV-HRN trimer if the new Phe residue could mimic Phe488 in the RSV-HRC domain. Because we could not predict the best modification site in VIQKI, we evaluated a set of variants in which each of the eight residues nearest the N-terminus was replaced, individually, by Phe. Three sites of modification stabilized the assembly with RSV-HRN; the largest benefit observed was for substitution at I456. VIQKI-I456F is substantially superior as an inhibitor of RSV infection relative to VIQKI itself or the RSV-HRC domain. In addition, VIQKI-I456F is comparable or possibly superior to VIQKI as an inhibitor of HPIV3 infection. Crystal structures of six-helix bundles formed between new inhibitors and either RSV-HRN or HPIV3-HRN show that in both assemblies the Phe456 side chain makes intimate hydrophobic contacts with the core trimer. VIQKI-I456F is a promising therapeutic candidate for both RSV and HPIV3 infections.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Domain architecture and structures of HPIV3 and RSV F. (A) Diagram of HPIV3 and RSV F with fusion peptide (FP), N-terminal (HRN) and C-terminal (HRC) heptad repeat, and transmembrane (TM) domains. Structures of pre-fusion (B, PDB:4MMV) and post-fusion (C, PDB:3RRR) conformations of RSV F. (D) Peptide sequences for HPIV3-HRC, RSV-HRC, VIQKI-I456F, and VIQKI-I454F/I456F (Phe-substitutions in red).

Figure 2. HRC domain N-terminal segments in six-helix bundle assemblies. X-ray structures of (A) the full 6HB HPIV3-HRN+VIQKI (PDB:6NRO) with magnified images of (B) HPIV3-HRN+VIQKI, (C) post-fusion RSV F (PDB:3RRR), and (D) RSV-HRN+VIQKI (PDB:6NTX).

Figure 3. Antiviral efficacy of VIQKI-I456F and VIQKI-I454F/I456F against RSV or HPIV3. Peptide activity against (A) RSV or (B) HPIV3 was determined by plaque-reduction assay in infected Hep-2 cell monolayers. Data are expressed as mean $\pm$ standard deviation ($n=3$ separate experiments).

Figure 4.

X-ray structures of (A) 6HB segment of RSV F in post-fusion conformation (PDB:3RRR) and co-crystal structures of (B) RSV-HRN+VIQKI-I456F (PDB:6OJ7) and (C) HPIV3-HRN+VIQKI-I454F/I456F (PDB:6O40). The phenylalanine-substituted VIQKI analogs adopt a binding mode similar to that of RSV-HRC. Colors correspond to RSV-HRN (salmon), HPIV3-HRN (orange), RSV-HRC (yellow), and VIQKI/VIQKI-I456F/VIQKI-I454F/I456F (green with Phe-substitutions in violet). Electrostatic potential maps at the binding site of (D) Phe488 (RSV F), (E) Phe456 (RSV-HRN+VIQKI-I456F), and (F) Phe456 (HPIV3-HRN+VIQKI-I454F/I456F) with anionic (red), neutral (gray), or positive (blue) charge density.

Table 1.

Apparent Melting Temperatures ($T_{m,app}$) of Co-assemblies Formed Between VIQKI Variants and HPIV3-HRN or RSV-HRN

Inhibitor	HPIV3-HRN		RSV-HRN	
	$T_{m,app}^a$ (°C)	$T_{m,app}^{\dagger b}$	$T_{m,app}^a$ (°C)	$T_{m,app}^{\dagger \ddagger c}$
VIQKI	88.6	-	64.3	-
VIQKI-V449F	-	-	41.9	-22.4
VIQKI-A450F	-	-	45.3	-19.0
VIQKI-L451F	-	-	65.9	+1.6
VIQKI-D452F	-	-	42.2	-22.1
VIQKI-P453F	-	-	63.0	-1.3
VIQKI-I454F	-	-	65.1	+0.8
VIQKI-D455F	-	-	55.6	-8.7
VIQKI-I456F	84.9	-3.7	70.0	+5.7
VIQKI-L451F/I456F	83.1	-5.5	67.2	+2.9
VIQKI-I454F/I456F	85.1	-3.5	72.8	+8.5

^a 1:1 mixture of Inhibitor (50 μ M) and HRN (50 μ M) peptides.

^b $T_{m,app}^{\dagger} = T_{m,app}(\text{HPIV3-HRN+Inhibitor}) - T_{m,app}(\text{HPIV3-HRN+VIQKI})$.

^c $T_{m,app}^{\dagger \ddagger} = T_{m,app}(\text{RSV-HRN+Inhibitor}) - T_{m,app}(\text{RSV-HRN+VIQKI})$.