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Discovery of Potent RSV Peptides Antivirals

https://doi.org/10.17952/38EPS.2026.P312

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INTRODUCTION

Respiratory Syncytial Virus (RSV) is very contagious and represents the main cause of severe acute respiratory tract illness in young children worldwide and in adults over the age of 65.

RSV F is a fusion protein located on the surface of the virion that mediates RSV entry into the respiratory tract epithelial cells. RSV F is activated upon cell entry into a metastable prefusion form named “preF”, which contains a hydrophobic fusion peptide, two heptad repeats (HR1 and HR2), and a viral transmembrane domain. Following the insertion of the fusion peptide into the target cell membrane, preF undergoes a dramatic conformational rearrangement to yield a postfusion six-helix bundle hairpin structure named “postF”, whereby 3 HR2 sequences bind in an antiparallel manner to a trimeric HR1 coiled coil. This hairpin structure brings the viral and cell membranes into close apposition, thereby facilitating membrane fusion and subsequent viral entry.

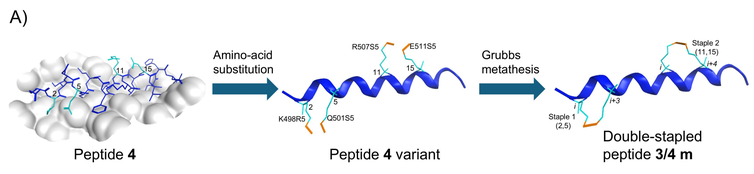
Previously, we reported antiviral peptides inhibiting RSV fusion, named 4bb and 4ca (1). These peptides are derived from the HR2 domain of RSV F, and function as dominant negative inhibitors by binding to the transiently exposed HR1 coiled coil in the pre-hairpin fusion intermediate. Through performing a stapled peptide walk across the HR2 sequence, we identified a minimal domain named peptide 4 ((RSV F (485-515)).

Here, we have expanded our screen and identified 3 potent RSV peptides leads.

DISCOVERY OF POTENT DOUBLE STAPLED PEPTIDES, FUSION INHIBITORS OF RSV

We performed an extended double staple scan across peptide 4. Four amino acids of peptide 4 were substituted with crosslinking unnatural residues (Scheme 1B) with the aim to yield a peptide containing two staples cross-linking one α-helical turn each (Scheme 1A). Only the amino acids that are not in hydrophobic contact with trimeric HR1 (color coded in black in Scheme 1D) were substituted. Of all peptides that were tested only peptide 4/4g, 3/4i and 3/4m were significantly more potent than peptides 4bb and 4ca, displaying EC_{50} values of 120, 83 and 53 nM, respectively (Scheme 1C).

Interestingly, these 3 lead peptides appear to use only two staple combinations, that is, staple 1 (2,5) or (4,8) combined with staple 2 (11,15) or (12,16).



Peptide	Staple 1	Staple 2	EC_{50}(μM)	Peptide	Staple 1	Staple 2	EC_{50}(μM)
4bb	4,8	11,15	0.74±0.27	**4ca**	2,5	8,12	0.59±0.13
4/4a	1,5	15,19	> 33	**3/4a**	2,5	15,19	8.89±3.21
4/4b	8,12	14,18	186.69±117	**3/4b**	9,12	14,18	4.94±1.88
4/4c	7,11	14,18	6.52±0.58	**3/4c**	8,11	14,18	33.95±10.6
4/4d	4,8	14,18	5.68±0.65	**3/4d**	5,8	14,18	17.15±1.86
4/4e	1,5	14,18	6.77±6.51	**3/4e**	4,7	14,18	5.87±0.83
4/4f	5,9	12,16	0.95±0.66	**3/4f**	2,5	14,18	73.42±53.3
4/4g	4,8	12,16	0.12±0.14	**3/4g**	5,8	12,16	19.69±10.2
4/4h	2,6	12,16	0.53±0.04	**3/4h**	4,7	12,16	4.48±3.52
4/4i	1,5	12,16	0.36±0.21	**3/4i**	2,5	12,16	0.083±0.078
4/4j	5,9	11,15	34.32±8.89	**3/4j**	1,4	12,16	>33
4/4k	2,6	11,15	54.63±68.70	**3/4k**	5,8	11,15	3.95±5.19
4/4l	1,5	11,15	2.61±2.35	**3/4l**	4,7	12,15	10.92±5.19
4/4m	1,5	8,12	9.13±6.94	**3/4m**	2,5	11,15	0.053±0.041
4/4n	1,5	7,11	>33	**3/4n**	1,4	11,15	2.61±1.79
Reference				**3/4o**	1,4	8,12	66.50±47.38
Ziresovir			0.0003±0.0002	**3/4p**	2,5	7,11	1.88±0.46
				3/4q	1,4	7,11	2.42±0.20

Peptide	497			500					505					510						515	
4	E	K	**I**	N	Q	**S**	L	A	F	**I**	R	K	**S**	D	E	L	**L**	H	N	**V**
Residue #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Scheme 1. Double stapled peptide walk across peptide 4 and identification of lead peptides inhibitors (Reproduced from Pidoux et al., J. Med. Chem. 2026, 69, 12910–12924. DOI: 10.1021/acs.jmedchem.5c02932, under CC BY 4.0, (2)).

X-RAY STRUCTURE OF PEPTIDE 3/4m AND 4/4g IN COMPLEX WITH TRIMERIC HR1

HR1 (RSV F (159-209)) was synthesized by SPPS using pseudoproline analogs and mixed with an equimolar amount of peptide 3/4m or 4/4g. The complexes were purified to homogeneity by size exclusion chromatography, and crystals diffracting to 1.6 Å and 1.3 Å were obtained using the vapor diffusion method for HR1−3/4m and HR1−4/4g complexes, respectively.

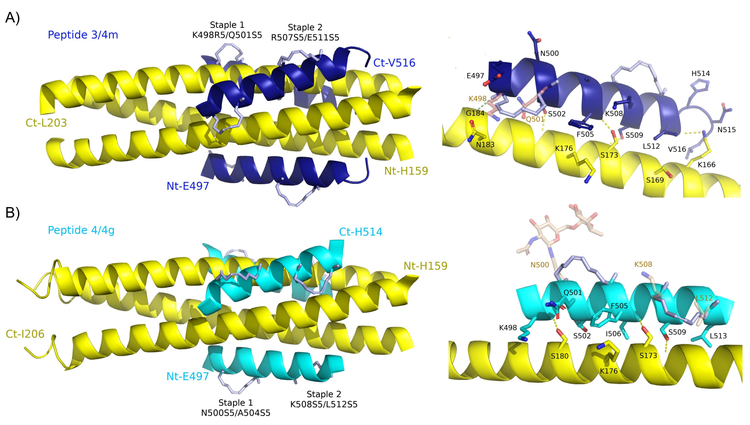


Figure 1. Crystal structure of HR1-3/4m and HR1-4/4g complexes. Left panel, ribbon diagram of the HR1−3/4m complex, top, and of the HR1−4/4g complex, bottom. The HR1 helices are shown in yellow, and the 3/4m and 4/4g peptides are depicted in dark blue and cyan, respectively. Staples 1 and 2 are shown in tubular structures in light blue. Top right panel, view of the 3/4m staple 1 binding site. Bottom right, view of the 4/4g staple 2 binding site. (Reproduced from Pidoux et al., J. Med. Chem. 2026, 69, 12910–12924. DOI: 10.1021/acs.jmedchem.5c02932, under CC BY 4.0).

As expected, the peptides pack with trimeric HR1 into a six helix bundle (Figure 1 left). **The peptides are totally helical although the 3 last residues 514-HNV-516 adopt a 3_{10} helix structure in 3/4m, allowing Val516 to deeply insert into HR1**. This Cterm region is not resolved in 4/4g, which ends at H514. The two peptides make a very tight interaction with the two helices making the groove of the HR1 trimeric coiled coil. **For 3/4m, the interaction of staple 1 with trimeric HR1 contributes significantly to the enhancement of inhibitory activity of peptide 3/4m** (Figure 1, top right panel).

In contrast to peptide 3/4m, staple 1 of 4/4g remains exposed and does not contribute to interactions with HR1 (Figure 1, bottom left panel), whereas staple 2 engages in hydrophobic contacts with residues of trimeric HR1 (Figure 1, bottom right panel).

Although crystallization trials with peptide 3/4i were unsuccessful, this peptide incorporates staple 1 from 3/4m and staple 2 from 4/4g, enabling the construction of a structural model for this third potent peptide (Figure 2). In this model, both staples participate in hydrophobic interactions with residues of the trimeric HR1, consistent with observations from the X-ray structures of the 3/4m and 4/4g complexes.

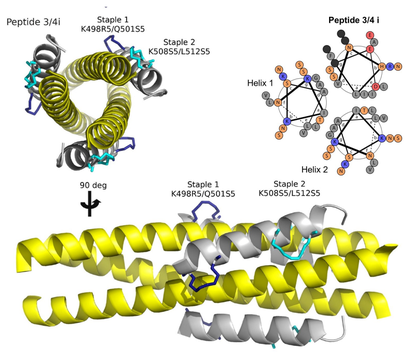


Figure 2. Ribbon diagram of the HR1-3/4i complex. Left panel, rotated 90deg. relative to the view shown in Figure 1. The HR1 helices are shown in yellow, and the 3/4i peptides in gray. Staples 1 and 2 are shown in a tubular structure in dark blue and cyan for 3/4i peptide. Right panel, α-helical wheel representations depicting the interactions of peptide 3/4i with helix 1 and helix 2 of trimeric HR1. Bottom panel, model of the HR1-3/4i complex.(Adapted from Pidoux et al., J. Med. Chem. 2026, 69, 12910–12924. DOI: 10.1021/acs.jmedchem.5c02932, under CC BY 4.0).

DOUBLE STAPLED PEPTIDE LEAD INHIBIT SMALL-MOLECULE ESCAPE MUTANTS

Small molecules fusion inhibitors bind to a 3-fold symmetry cavity in trimeric preF (3), and will yield the same viral escape mutants. Peptide 3/4m was capable of inhibiting infection by RSV mCherry S398L/K394R double mutant (Figure 3). The S398L small-molecule escape mutant was observed in a phase 2a human challenge study against presatovir (Gilead) and the K394R mutation was identified in an *in vitro* cellular resistance study against BMS-433771 (Bristol-Myers).

In contrary the inhibition of presatovir (GS-5806) was completely abrogated against the double resistant mutant (> 38’000-fold change relative to wild type rRSV-mCherry).

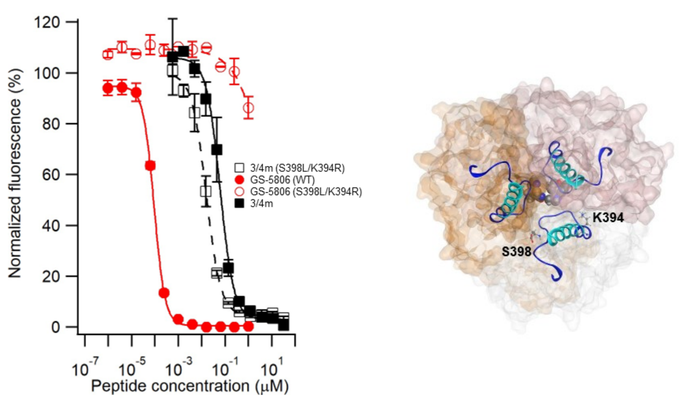


Figure 3. Inhibition of RSV F small-molecule escape mutant. A) Inhibition of rRSV-mCherry WT and S398L/K394R variant infection by lead double stapled peptide 3/4m and GS-5806 in HEp-2 cells. B) Top view of X-ray structure of BSM -433771 bound to preF (PDB code 5EA7). The 3 RSV F protomers are shown as surface representation in grey, gold and pink. The 3 HR2 domains are shown in a ribbon representation with peptide 4 color coded in cyan. BMS-433771 is shown in a sphere model, K394 and S398 in ball and stick.

IN VITRO STABILITY OF PEPTIDE LEADS

Lead peptides were incubated with trypsin, chymotrypsin and mice serum, and the amount of remaining peptide was quantified at different time points by LC/MS-MS.

Wild-type peptide 4 was rapidly degraded by trypsin and chymotrypsin (Fig. 4A-B) with a half-life of 4.91 min and 4.25 min, respectively. In contrast, the stapled peptides were much less susceptible to trypsin and chymotrypsin than peptide 4.

The resistance of stapled peptides to degradation by trypsin decreased in the order of 4/4g, 3/4i and 3/4m ($t_{1/2}$ of 91.98, 40.91 and 18.15 min, respectively, Fig. 4A).

The resistance of stapled peptides to degradation against chymotrypsin was of 36.36, 20.13 and 108.3 minutes for peptides 4/4g, 3/4i and 3/4m, respectively (Fig. 4B).

Stapled peptides did not show any degradation over the entire time course (>24 hours; Fig. 4C), when they were incubated in the presence of mouse plasma at 37°C.

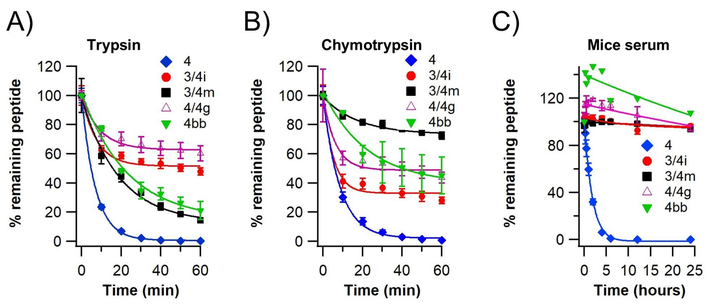


Figure 4. Proteolytic stability assay. Susceptibility of double-stapled peptides 3/4i, 3/4m, and 4/4g in comparison to peptides 4bb and 4 toward degradation by A) trypsin, B) chymotrypsin, and C) mouse serum, in comparison to peptide 4, i.e., RSV F (497−516). (Reproduced from Pidoux et al., J. Med. Chem. 2026, 69, 12910–12924. DOI: 10.1021/acs.jmedchem.5c02932, under CC BY 4.0).

FEASIBILITY STUDY IN BALB/c MOUSE MODEL

Balb/c mice were treated by intranasal (IN) administration of peptide 3/4i or vehicle, followed by IN inoculation of a recombinant human RSV encoding the gene for firefly luciferase, rHRSV-Luc. The mice were treated once daily and were anesthetized at 2- and 4-days post infection (dpi) to quantify viral replication using an *in vivo* imaging system (IVIS). As shown in Fig. 5, a significant reduction of bioluminescence signal was detected at both 2 and 4 dpi in mice treated with peptide 3/4i in comparison of the untreated control group

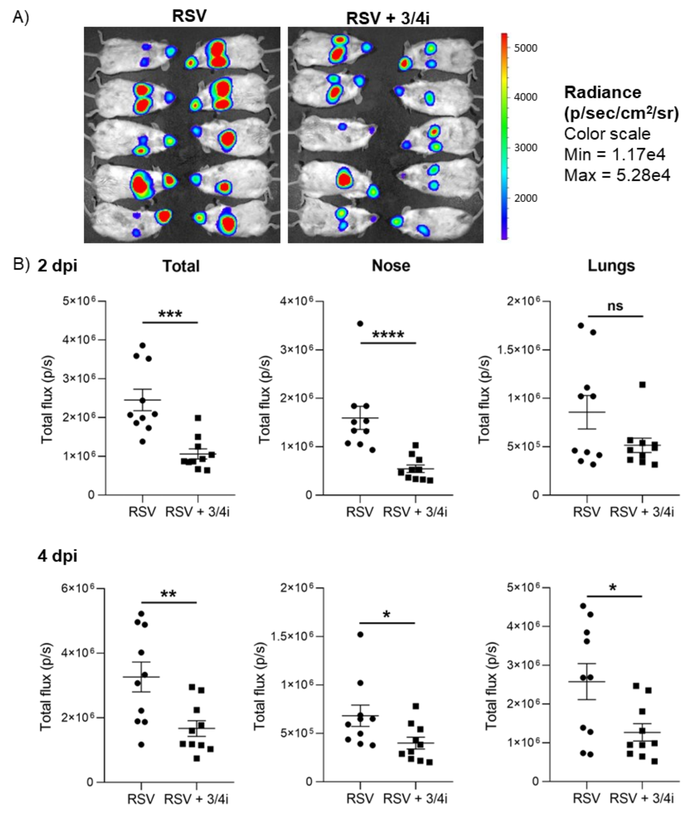


Figure 5. **Protective effect of peptide 3/4i on RSV infection in mice.** 8 weeks-old female Balb/c mice were treated intranasally with 1.17 mg/kg peptide 3/4i or vehicle. Groups of 10 mice were treated at day 0 individually with either 50 µL PBS or 50 µL peptide 3/4i (200 µM) in PBS containing 0.8% DMSO and infected 10 min later by 60 µL rHRSV-Luc (8 x 10^4 PFU per mouse). The administration of peptide 3/4i was repeated daily until day 3. A) Bioluminescence was measured at day 4 post inhalation (dpi) by intranasal administration of 50 µL of D-luciferin. Luciferase activities were quantified for each mouse using Living Image software. Luciferase activity is expressed as photons per second (p s^{-1}); B) Total bioluminescence, or bioluminescence in the nose and lungs was quantified for each animal at 2 and 4 dpi. Each dot represents one animal.*(Adapted from Pidoux et al., J. Med. Chem. 2026, 69, 12910–12924. DOI: 10.1021/acs.jmedchem.5c02932, under CC BY 4.0).*

CONCLUSION

Novel double stapled peptides capable of inhibiting RSV fusion in cellular antiviral assays were discovered. Intranasal administration of 3/4i to Balb/c RSV infected mice resulted in approximately one log decrease of viral load. Recent SAR studies resulted in the identification of highly potent peptides displaying single digit nanomolar EC_{50} values in Hep-2 cellular inhibition assay. To identify the most appropriate route of administration for optimal targeted delivery of these peptides to the lungs, their ability to penetrate the mucus layer is currently being evaluated in Calu-3D cells. The most suitable administration route will subsequently be selected and validated using the Epithelix MucilAir™ and SmallAir™ epithelial tissue models.

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ACKNOWLEDGMENTS

This work was partly funded by the HES-SO and domaine Ingénierie et Architecture (HES-SO 78166/IA-INTERDISC17-2, 135271/IA RECHERCHE24-14). We thank Dr. Jean-François Eleouet for sharing recombinant RSV. We thank the animal facilities of Infectiology of Fishes and Rodents (IERP, INRAE, doi: 10.15454/1.5572427140471238E12) and the Emerg’in platform for access to IVIS200 that was financed by the Region Ile De France (SESAME). This work has benefited from the facilities and expertise of @BRIDGe (Universite Paris-Saclay, INRAE, AgroParisTech, GABI, Jouy-en-Josas, France).

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