Retroinverso Analogue of the Antiviral Octapeptide C8 Inhibits Feline Immunodeficiency Virus in Serum

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Abstract: We described the antiviral activity of an octapeptide corresponding to a Trp-rich domain of feline immunodeficiency virus (FIV) transmembrane glycoprotein. To overcome the limited enzymatic stability of short peptides, the retroinverso analogue was prepared and tested for inhibitory activity of FIV in the presence or absence of normal cat serum. Differently from the unmodified peptide, the retroinverso analogue maintains strong inhibitory activity in serum. NMR studies showed that it displays crucial conformational features believed to be important for antiviral activity.

The fight against HIV-1 infection has been considerably boosted by the development of two important classes of antiviral drugs, i.e., the specific inhibitors of the viral enzymes reverse transcriptase and protease.[1] However, the extensive use of these inhibitors, alone or in combination, has raised several problems (mainly due to development of resistant mutants of HIV-1 and poor tolerability), thus emphasizing the demand for new classes of drugs. The cell entry step of the HIV-1 life cycle has been regarded as a promising target for the development of a new generation of inhibitors.[2] In fact, T-20, a fusion peptide inhibitor,[3] is currently under phase III clinical evaluation.[4] This 36-residue peptide corresponds to a C-terminal region of the ectodomain of the transmembrane (TM) glycoprotein gp41, a component of the HIV-1 envelope that plays a crucial role in membrane fusion and viral entry. T-20 is believed to owe its antiviral activity primarily to hindrance of the receptor-triggered conformation rearrangements of the TM gp that mediate virion and cell membrane fusion.[5] Other peptides, designed from different portions of gp41, are also active at this level.[6] Similar results have been reported with simian immunodeficiency virus-derived peptides, supporting the concept that the viral entry functions associated with the TM gp ectodomain are essentially conserved among lentiviruses.[7]

Feline immunodeficiency virus (FIV) is a naturally occurring pathogen that causes an AIDS-like syndrome in domestic cats[8] and is a valuable model system by which criteria for antiviral vaccine and drug development can be investigated.[9] We have previously described potent in vitro anti-FIV activity associated with synthetic peptides derived from the gp of FIV and in particular from the TM gp, which shares a common structural framework with the corresponding molecule of HIV and appears to play a similar role in cell entry.[10] In a subsequent recent study, we focused on the antiviral activity of an octapeptide, named **C8**, corresponding to a Trp-rich domain of FIV TM gp, residues 770−777. Peptide **C8** (Ac-*Trp*-Glu-Asp-*Trp*-Val-Gly-*Trp*-Ile-NH_2) exerted a powerful antiviral effect on all the FIV isolates tested, and this activity was dependent on an intact Trp motif. Additional investigations revealed that the structure of peptide **C8** is characterized by a well defined α-turn structure in the 773−776 segment. Additionally, **C8** shows a well-defined orientation of the side chains, including the Trp rings. The conformational properties of **C8** might explain why it is potently inhibitory despite its reduced size.[11]

One of the main concerns when testing a peptide (particularly a short one) as a drug candidate is its enzymatic stability, which normally makes its half-life in serum too short-lived for therapeutic application. To deal with this problem, we decided to synthesize and test the retroinverso analogue of **C8**. A retroinverso is formally a backbone-modified peptide in which each peptide bond is reversed.[12] Practically, it is an isomer of a linear peptide in which the direction of the sequence is reversed and the chirality of each amino acid residue is inverted. Accordingly, if a retroinverso peptide is superimposed onto the parent peptide in an antiparallel fashion, the overall topology of the side chains is maintained, even if the end groups are noncomplementary. There are several examples of retroinverso peptides maintaining the same biological activity as the parent peptide and showing greatly enhanced enzymatic stability.[13] However, the "end-group problem" was considered among the possible causes of inactivity of some retroinverso peptide hormones.[12d]

The retroinverso analogue of **C8** (**riC8**: Ac-D-Ile^{777}-D-Trp-Gly-D-Val-D-Trp-D-Asp-D-Glu-D-Trp^{770}-NH_2) was prepared by conventional manual solid-phase peptide synthesis, purified to homogeneity by reverse-phase semipreparative HPLC, and characterized by analytical HPLC and ESI-MS (see Supporting Information). Inhibitory activity on FIV replication was tested with the standard procedure in the presence or absence of normal cat serum (NCS, 50% final concentration) to reproduce a biological environment. Briefly, test peptides were diluted in tissue culture medium or in pooled NCS to final concentrations ranging from 0.0005 to 50 μg/mL and assayed for virus inhibition against 10 50% tissue culture infectious doses of the fresh isolate FIV-M2, using lymphoid MBM cells as substrate and p25 antigen quantification as an end point (see Supporting Information for details).[11] Similar to **C8**, **riC8** had no detectable toxicity for cultured cells.

The results of virus inhibition testing are reported in Table 1. The first observation is that peptide **C8** was about 35-fold less active when assayed in 50% NCS,

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Table 1. Effect of NCS on the Inhibition of FIV Replication by Peptide **C8** and Its Retroinverso Analogue, **riC8**

	IC_{50} [a] (μg/mL)	
peptide	medium	NCS
C8	0.15 ± 0.07	5.21 ± 2.10
riC8	0.63 ± 0.38	0.80 ± 3.70

[a] Values shown are the mean ± SD of the IC_{50} obtained in two to three independent assays.

compared to normal tissue culture medium. This result was not unexpected because of the well-known poor stability of peptides in serum. Although **C8** was partly protected by terminal protections (N-terminal acetylation and C-terminal amidation), most likely it was still sensitive to endopeptidase degradation and therefore markedly inhibited in its activity by NCS.

A second important observation concerns the inhibitory activity of the retroinverso analogue **riC8** when tested in regular tissue culture medium. Under these conditions, **riC8** showed an IC_{50} of 0.63 μg/mL; i.e., it was only 4.2-fold less active than the corresponding unmodified peptide (IC_{50}(**C8**) = 0.15 μg/mL). This is an important finding because the complete retroinversion of an active peptide does not necessarily imply conservation of biological activity.

Finally, the third relevant information obtained from the experiment is the substantial stability of **riC8** in serum, demonstrated by the good inhibitory activity observed in 50% NCS. In fact, the retroinverso peptide was about 6-fold more potent than the corresponding unmodified peptide (IC_{50} = 0.80 vs 5.21 μg/mL) when assayed in NCS.

Also of importance, while the inhibitory activity of **C8** was evident only if the input virus were removed from the test cultures after completion of the virus adsorption phase,[11] **riC8** was active even if the input virus was left in the cultures throughout (data not shown), thus arguing in favor of a higher stability of the latter.

That the retroinverso analogue conserved inhibitory activity can be interpreted assuming that the peptide maintains the same overall side chain topology as the unmodified counterpart, despite the backbone modification. To investigate this important issue, we performed a conformational analysis of the **riC8** solution structure by means of NMR and molecular modeling techniques and compared it with the one previously published for **C8**.[11]

NMR spectra of **riC8** were recorded on a Bruker DRX-600 spectrometer. Samples were prepared by dissolving the appropriate amount of the peptide in 0.5 mL of 8 mM ^1H_2O sodium phosphate buffer (pH 6.6) to obtain a 1 mM solution, lyophilized, and dissolved in phosphate buffer containing 80% DMSO. To check the absence of an aggregation state of the peptide, spectra were acquired in the range 0.5−15 mM. No significant changes were observed in the distribution and in the shape of the ^1H resonances, indicating that no aggregation phenomena occurred in this concentration range. One-dimensional NMR spectra were recorded in the Fourier mode with quadrature detection, and the water signal was suppressed by low-power selective irradiation in the homogated mode. DQF-COSY, TOCSY, and NOESY experiments[14] were run in the phase-sensitive mode using quadrature detection by time-proportional phase increase of the initial pulse. Data block sizes were 2048 addresses in t2 and 512 equidistant t1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. NOESY experiments were run at 270 K in DMSO/water. A mixing time of 70 ms was used for the TOCSY experiments, while mixing times in the range 150−300 ms were used for NOESY experiments. The qualitative and quantitative analyses of DQF-COSY, TOCSY, and NOESY spectra were obtained using the interactive program package SPARKY.[15] The NOE-based distance restraints were obtained from NOESY spectra collected with a mixing time of 250 ms. For structure calculation, the NOE cross-peaks were integrated with the SPARKY program and converted into upper distance bounds using the CALIBA module of the DYANA software.[16] Cross-peaks overlapping by more than 50% were treated as weak restraints in the DYANA calculation. An ensemble of 50 structures was generated with a distance geometry simulated annealing program DYANA using 75 NOE-based distance constraints. No dihedral angle restraints and no hydrogen bond restraints were used. The necessary pseudoatom corrections were applied for nonstereospecifically assigned protons at prochiral centers and for the methyl group. The best 20 structures, having low values of target functions (0.83−1.19) and small residual violations (maximum violation of 0.3 Å), were refined by in vacuo minimization in the AMBER 1991 force field, using the program SANDER of the AMBER 5.0 suite.[17] To mimic the effect of solvent screening, all net charges were reduced to 20% of their real value, and moreover, a distance-dependent dielectric constant ($\epsilon = f(r)$) was used. The cutoff for nonbonded interactions was 12 Å. The NMR-derived upper bounds were imposed as semiparabolic penalty functions, with force constants of 16 kcal/(mol Å^2). The function was shifted to linear when the violation exceeded 0.5 Å. The final structures were analyzed using the Insight 98.0 program. Computations were performed on SGI Indigo II computers.

We recently reported a conformational analysis of the peptide **C8** in DMSO/water solution.[11] The inherent flexibility of peptides requires the choice of a medium suitable to make the conformational properties of these molecules evident. For this purpose, mixtures of water and organic solvents are generally used. These mixtures, endowed with high viscosity, have the property of affecting the equilibrium among isoenergetic conformers, thus selecting the more ordered ones. Furthermore, these media have the ability to produce physicochemical conditions compatible with those of biological fluids.[18] Accordingly, the NMR investigation of **riC8** was undertaken in DMSO/water solution as well. The chemical shift assignment was performed according to the conventional Wüthrich procedures.[19] The values of the proton chemical shifts of **riC8** are reported in the Supporting Information. Figure 1 shows amide and fingerprint regions of the NOESY spectra (270 K, 600 MHz, tm = 300 ms). Several sequential cross-peaks, relevant for assignment and structure calculation, are labeled. The presence of a high number of well-resolved cross-peaks, unusual for peptides as short as the ones under scrutiny, is evident. Particularly notable is a series of sequential NH_i-NH_{i+1} NOEs that are diag-

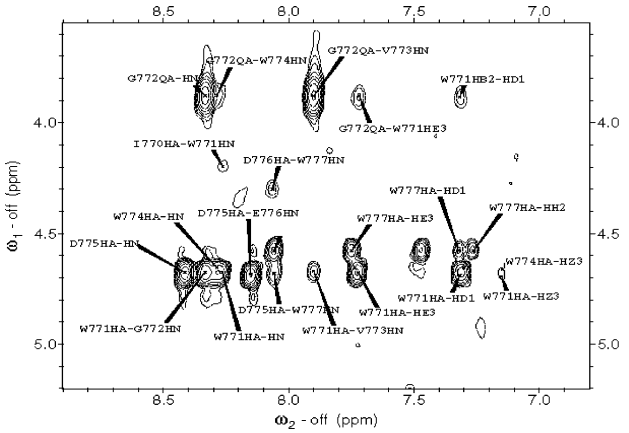

Figure 1. Amide region of the NOESY spectra of **riC8**.

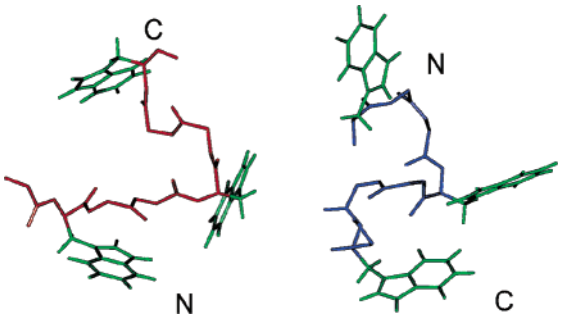

Figure 2. Solution structures of **C8** (right) and **riC8** (left).

nostically critical, strongly indicating the predominance of ordered conformers. In the segment 773−776, the presence of the NN(i, i + 2) and αN(i, i + 2) accounts for the presence of a turn conformation.

Three-dimensional structures of **riC8** were calculated by simulated annealing procedures based on sequential and medium-range NOE-derived restraints. The best 20 structures out of 50 calculated were chosen according to the lowest values of the penalty (f) for the target function.[16] These structures were energy-minimized using the distance restraints with progressively smaller force constants. The minimization procedure yielded an improved helical geometry and a lower total energy of the structures. To validate the resulting structures, the PDB files were submitted to an online PROCECK procedure.[20]

The backbone torsion angles of **riC8** are available as Supporting Information. The analysis of the Rhamachandran plot shows that the backbone torsion angles of D-Val^{774}-D-Trp^{773}-D-Asp^{772}-D-Glu^{771} are in a region corresponding to a left-handed α-helix. The turn of the α-helix is a type III turn stabilized by the presence of an H bond between the C═O of Gly^{775} and the NH of DGlu^{771}. The calculated structure is shown in Figure 2 (left). Similar to **C8**, the **riC8** conformation is characterized by a well-defined orientation of the side chains, including those of the relevant Trp residues. Inspection of the side chain arrangement shows that the aromatic rings of these residues have the same topochemical location in the low-energy structures of **C8** and **riC8** (Figure 2).

The structural relationship between retroenantiomer peptides has been much discussed in the literature.[12c] The respective activity profiles have been analyzed in a number of different systems,[21] while several theoretical papers speculated on the topological similarity of retroenantiomers.[22] The common result of these investigations is that retro, retroinverso, or partially modified retroinverso analogues maintain or even improve the biological activity of the parent peptides if the topological arrangement of the molecule is conserved. Indeed, comparison of the conformational properties of **C8** and **riC8** shows a very similar topochemical assessment of the structures, although the backbone conformations of the two peptides are different. In fact, **C8** is arranged in an α-turn in region 772−775, which forms a turn of a 3_{10} helix. On the other hand, **riC8** is characterized by a segment of a left-handed α-helix involving the same amino acids in the opposite stereochemistry and directionality (Figure 2). As reported and as is obvious for a different backbone arrangement, different patterns of hydrogen bonds stabilize the structures of **C8** and **riC8**, (in particular, C═O of Asp^{772} and the NH of Gly^{775}, as well as between the C═O of Trp^{773} and the NH of Trp^{776}). Despite the differences in the backbone conformations, the pairwise superimposition of the corresponding residues in **C8** and **riC8** low-energy structures shows that the side chains for each couple of the three essential Trp residues are similarly oriented with respect to the backbone (Figure 3).

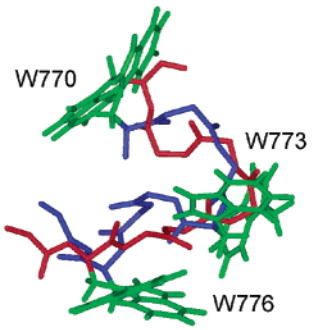

Figure 3. Pairwise superimposition of the corresponding residues in **C8** (blue) and **riC8** (red) low-energy structures.

As discussed in a previous paper,[11] the conformational properties of **C8** suggest that the marked antiviral activity of this peptide is most likely attributable to the presence in its active site of three equally spaced Trp residues, which determine a remarkable conformational stability. Moreover, the presence of the Trp motif induced a well-defined folded structure that appears to be the key molecular determinant of the inhibitory activity. In this perspective, the common orientation of the aromatic side chains of Trp^{773} and Trp^{776} observed in the structure of both **C8** and **riC8** confirms the pivotal role played by these aromatic side chains in hydrophobic interactions with the putative target site.

In conclusion, the retroinverso analogue of the antiviral peptide **C8** maintains crucial conformational features believed to be important for antiviral activity and, additionally, displays a remarkable serum stability, thus appearing as a promising candidate for in vivo testing.

Supporting Information Available: Experimental details . This material is available free of charge via the Internet at http://pubs.acs.org.

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