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Potent inhibition of HIV-1 replication by backbone cyclic peptides bearing the Rev arginine rich motif

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Summary

Due to its essential role in the virus life cycle, the viral regulatory protein Rev constitutes an attractive target for the development of new antiviral molecules. In this work, a series of Backbone Cyclic Peptide (BCP) analogs that bear a conformationally constrained arginine rich motif (ARM) of Rev were tested for *in vitro* inhibition of HIV-1 replication. We observed a potent suppression of HIV-1 replication in chronically infected T lymphocytic cells treated with Rev-BCPs. We further investigated possible mechanisms of HIV-1 inhibition and showed that Rev-BCPs interfere slightly with the nuclear import process and are very efficient in blocking a mechanism that controls $Pr55^{gag}$ and $gp160^{env}$ synthesis. Interestingly, these protein precursors are known to be encoded by mRNAs that require Rev-binding for nuclear export. *In situ* hybridization using a Cy-3 conjugated HIV-1 gag oligonucleotide probe indicated that Rev-BCPs prevent the intracellular accumulation of unspliced viral RNA. As a model, the most promising analog, Rev-BCP 14, was studied by molecular modeling and dynamics in order to identify its binding site on the Rev Response Element (RRE). The annealing simulation suggests that upon binding on the RRE, Rev-BCP 14 widens the distorted major groove of the viral RNA. Numerous contacts between peptide and RNA were found within the complex and some were identified as key components for the interactions. Altogether, our data indicate that the use of conformationally constrained Rev-BCPs represents a promising strategy for the development of new peptide-based therapeutic agents against HIV-1.

Abbreviations: AZT – 3′-*azido-3′-deoxythymidine*; ARM – arginine rich motif; BCP – backbone cyclic peptide; BIV – bovine immunodeficiency virus; BTC – bis-(trichloromethyl)carbonate; Boc – *tert-butyloxycarbonyl*; DCM – dichloromethane; HIV-1 – human immunodeficiency virus type 1; Fmoc – 9-fluorenylmethoxycarbonyl; MBHA – methylbenzhydrylamine; MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NMP – *N-methylpyrrolidone*; RRE – Rev response element; NLS – nuclear localization sequence; RT – reverse transcriptase

Gene regulatory proteins such as Tat and Rev play a key role in the HIV-1 life cycle [1–3]. Tat, among other functions, enhances viral RNA synthesis by regulating the transcription elongation through interactions with its viral mRNA target sequence TAR and cellular cofactors cyclin T1 and cyclin-dependent kinase 9. Rev regulates the expression of the viral mRNAs. In the absence of Rev, HIV-1 cannot efficiently produce the precursors of viral structural proteins required for shaping new infectious viral particles and the viral envelope

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glycoproteins precursor. Recently, it has been shown that other cellular proteins like DEAD box (Asp-Glu-Ala-Asp) RNA helicases acting as nucleo-cytoplasmic shuttling proteins, were required for efficient Rev-mediated RNA export [4–6]. The C-terminal domain of Rev interacts with various human nucleoporins like hRIP [7] and Nup98 [8], suggesting that the Rev protein can associate in multifunctional complexes in which CRM1 plays an essential role. In this respect, both Tat and Rev represent potential targets for the design of new antiviral inhibitors that are urgently needed because of the toxicity of currently used antiviral compounds and the emergence of HIV mutant strains that resist the conventional antiviral treatment. Here, we focused our attention to the Rev target.

The HIV-1 Rev is a small (116 amino acids) protein organized into modular domains [9–12]. Mutational analyses of Rev protein have identified various functionally important regions: the C-terminal contains a leucine-rich motif (amino acids 75–84) that functions as a nuclear export signal (NES) whereas the N-terminal domain of Rev contains an arginine rich motif (ARM; amino acids 35–50) with dual functions as a nuclear and nucleolar localization signal (NLS/NOS) and RNA binding domain. The ARM binds with high affinity to the stem-loop IIB of the Rev-response element (RRE) while sequences flanking the ARM direct the oligomerization of Rev. The NES of Rev protein interacts with the cellular cofactors CRM1/Exportin 1 and Ran guanosine triphosphate (for a review see [13]). Thus, Rev is able to promote the selective export of unspliced and partially spliced mRNAs from nucleus to cytoplasm by binding to the RRE located in the *env* region of these transcripts [14, 15]. The RRE sequence is removed in the completely spliced HIV-1 mRNAs which therefore do not require Rev for cytoplasmic translocation and translation. The Tat and Rev proteins are encoded by the fully spliced viral *tat* and *rev* mRNAs. The *tat* and *rev* transcripts are obviously synthesized before the partially spliced and unspliced mRNAs during HIV replication cycle.

The dual functions of the ARM found in Rev, makes this domain particularly attractive for studying the structure–function relationships and for targeting new anti-HIV-1 molecules. Interestingly, Rev from HIV-1 and Tat from both HIV and BIV have been shown to bind specifically to their corresponding target RNA. This molecular recognition involves specific binding sequences located in the ARM of the viral proteins and a unique structural domain associated with bulges and/or loops on the RNA. Yet, the conformations of these regulatory proteins are extremely different. For example, the ARM of Tat from BIV adopts a β-sheet conformation [16] upon binding to its specific RNA target, whereas that of Rev folds in α-helix [17, 18]. These data point out the large structural diversity of the positively charged ARM domains and overall, highlight the large degree of freedom of the viral RNA sequences that afford the interaction with different peptidic folds [19, 20]. Therefore, a better knowledge of the 3-D structures of the regions within proteins that are involved in RNA binding is crucial to design new antiviral compounds aimed at blocking HIV replication.

Small molecules, such as peptides or peptidomimetics, are expected to bind specific domains within target proteins and thus modify their biological function. Conformationally constrained peptides, such as backbone cyclic peptides (BCPs), may therefore turn to be interesting candidates for approaching the design of new antiviral compounds. The use of BCPs presents a crucial advantage over their respective linear forms because they may be designed to mimic the structure of the corresponding active site within the protein. Moreover, the cyclization improves their metabolic stability. Backbone cyclization is a general method by which a conformational constraint is imposed to peptides by connecting the peptide N-backbone atoms to each other, to side-chains, or to one of the peptide's termini [21]. Backbone cyclization has been previously shown to convert peptides into selective and metabolically stable peptidomimetics with enhanced biological activity compared to the linear parent peptides [22, 23]. BCPs that mimic the ARM region of Tat (Tat-BCPs), have been already shown to inhibit the function of Tat *in vitro* [24].

Recently, we extended this promising strategy of small cyclized proteomimetic synthesis to the viral protein Rev by also targeting the ARM region [16]. We demonstrated that the Rev-BCPs used hereafter, penetrated into cytosol and nuclei of intact Colo-205 cells, have inhibitory effects on the binding of Rev-GFP to importin, and reduced Rev-dependent induction of a reporter gene in HeLa cells [25].

In the present work, we focused on the functional characterization of the Rev-BCPs series bearing the ARM of HIV-1 Rev and the evaluation of their antiviral activity in different cell lines. We showed for the first time, that Rev-BCPs were able to strongly inhibit the virus replicative cycle in culture of human T-lymphocytes. The mechanism by which such peptides promote HIV-1 inhibition was examined and we found that Rev-BCPs shutdown viral polyproteins precursors synthesis and reduce the intracellular amount of the full length viral RNA without affecting early stages of the virus replication cycle. In addition, a molecular modeling study was performed allowing a better understanding of the atomic forces that likely control the binding, avidity and affinity of these Rev-BCPs to their corresponding target domains on the viral mRNAs.

Materials and methods

Chemical reagents

Aphidicolin, β-galactosidase reporter gene activity detection kits and MTT [26] were purchased from Sigma-Aldrich. Protein concentrations were measured with the Coomassie protein assay reagent from Pierce Biotechnology, Inc., according to manufacturer instructions. Rabbit polyclonal anti-serum raised to $gp41^{env}$ was purchased from Fitzgerald Inc. and anti-$p6^{gag}$ was obtained after immunization of rabbits with GST-$p6^{gag}$ fusion protein. Cy-3 labeled oligonucleotide was kindly provided by M. Mougel (CNRS – UMR5236, Montpellier, France).

Peptide synthesis and backbone cyclization

The peptide synthesis procedure and chemical characterization of the Rev-BCPs used in this study were previously described by Hariton-Gazal et al. [25] and summarized hereafter. Briefly, peptide synthesis was performed on Rink amide MBHA resin (loading 0.6 mmol/g). Fmoc protecting group was removed using 20% piperidine in NMP (2×30 min) and the resin was washed with NMP (5×2 min) and DCM (2×2 min). Coupling of Fmoc-amino acids was performed using BTC [27]. Following cooling on ice bath, 2,4,6-collidine was introduced and the solution was added to the peptidyl-resin. The reaction mixture was shaken for 1 h and the peptidyl-resin was washed with DCM (5×2 min). Reaction completion was monitored by the chloranil test. Synthesis of the building unit (functionalized *N*-alkyl-Gly), cyclization, removal of the Boc protecting group and peptide cleavage and purification were performed as previously described [28]. The linear form of Rev ARM peptide hereafter called ''lin-Rev'' has the same sequence that the nuclear localization signal of Rev (residues 35–46 of the Rev protein) with an additional cysteine residue at the carboxy-terminus (RQARRNRRRRWRC). In addition to this linear form, 16 different backbone cyclic peptides, ''Rev-BCPs'' numbered from 1 to 16, have been synthesized using this procedure. The sequences of Rev-BCPs are shown in Figure 1. The peptide named ''oligo'' (LIKFLGQSN) was used as a negative control. Tat-11 and Tat-16 BCPs were used as internal control and derived from the ARM of HIV-1 Tat (residues 49–57 of the Tat protein) [28].

Virus

A stock solution of infectious virus (HIV-1 Bru strain [29]) was prepared from the supernatant of a HIV-1-chronically infected H9 T-lymphocytic cell culture and used for all infections. The virus titre was 1000 $TCID_{50}$/ml (50% tissue culture infective dose).

Cell culture and transfections

The HeLa MAGIC-5-LTR-βGal indicator cell line [30, 31] expressing the β-galactosidase reporter gene cloned downstream of the HIV-1 LTR promoter and stably expressing the CD4, CXCR4 and CCR5 receptors was used for the HIV challenge experiments (for clarity, this cell line is noted hereafter ''HeLa MAGIC''). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) from Invitrogen containing ultraglutamine and complemented with 10% of heat-inactivated fetal calf serum and 1% antibiotics: penicillin (200 U/ml), streptomycin (50 μg/ml). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere. For cells synchronization, aphidicolin (5 μg/ml) was added to the cell culture medium 24 h before incubation with inhibitors. Rev-BCPs were added to the cells without remov-

ing aphidicolin. The same conditions were used with the human H9 T-lymphocytic cells except that the medium used was RPMI 1640.

For the FISH experiments, transient transfections were performed on HeLa MAGIC cells plated onto glass coverslips in 24-well culture plates. Cells were incubated in the presence of the plasmid encoding for the full genome of HIV-1 Bru mixed with the ExGen 500 transfection reagent (Euromedex) at a ratio of 2 μl of ExGen per μg of DNA. Addition of the Rev-BCPs was done simultaneously and experiment was stopped after 24 h for fluorescence microscopy observations. Transfection experiments were systematically performed in duplicate in order to control that the infectious particles production was efficiently blocked by Rev-BCPs before FISH treatment and fluorescence microscopy analysis.

Effect of Rev-BCP on Tat-TAR interaction: transient transfections experiments were performed on HeLa cells using *firefly* luciferase expression under the control of LTR promoter and transactivation by endogenous Tat_{101} protein (expressed by co-transfecting HeLa cells with a plasmid encoding for Tat_{101}) as previously described [32]. Briefly, HeLa cells (10^5 cells cultured in 24-well plates) were transfected with 1 μg of DNA (pGL3-LTR/pRL-TK/pcDNA3-Tat101 or pCI 7:1:5) and 2 μl of transPEI (purchased from Eurogentec), then incubated for 3.5 h at 37 °C, washed extensively with DMEM and 2 h later, Rev-BCPs were added once directly into the culture medium. Twenty-one hours later, cells were washed three times with PBS and incubated with Promega passive lysis buffer (Dual-Luciferase® Reporter Assay System kit from Promega). Specifically Tat-induced luciferase activity was normalized by the *renilla* luciferase signal expressed under the control of CMV promoter (indifferent to Tat_{101} expression, used as transfection control and for normalization).

Antiviral assays with HIV-1 infected cells

The antiviral effect of Rev-BCPs 1 and 14 was first investigated over 3 weeks in the human H9 T-lymphocytic cell line. For infection, 10^6 H9 cells were incubated for 30 min at 4 °C with 100 μl of HIV-1 Bru at a concentration of 1000 $TCID_{50}$/ml. Then, cells were washed five times and cultured at 5×10^5 cells/ml in 24-well plates with peptides. Viral production was monitored twice a week by measuring the reverse transcriptase (RT) activity from 0.5 ml of cell-free culture supernatants. At the same time, culture medium and Rev-BCPs concentrations were readjusted by addition of peptide and medium after each culture supernatant collected (meaning addition of freshly prepared solutions of Rev-BCPs every 3–4 days). After centrifugation of the supernatants at 95,000 rpm for 5 min, the virus pellet was recovered and resuspended in 10 μl of a buffer containing 10 mM Tris/HCl pH 8.00, 100 mM NaCl, 1 mM EDTA and 0.1% (v/v) Triton X-100. RNA-dependent DNA polymerase activity on poly(rA)/oligo$(dT)_{12–18}$ substrates was measured by a standard assay previously described [33, 34] using 10 μl of virus lysate and an incubation time extended to 1 h at 37 °C.

The antiviral activity of all Rev-BCPs was also evaluated with an indirect β-galactosidase activity assay. HeLa MAGIC cells were plated in 96-well plates at 12,500 cells/well and incubated with 5 or 20 μl of HIV stock preparation in the presence or absence of Rev-BCPs 1–16, lin-Rev or oligo, in the concentration range 0.25–100 μM. After 2 days of culture, virus infectivity was monitored by measurement of ONPG (*o*-nitrophenyl β-D-galactopyranoside) hydrolysis by β-galactosidase from the cell lysates as previously described [35]. Briefly, 100 μl of total cellular extracts were incubated for 45 min at 37 °C with 100 μl of 2× assay buffer containing 200 mM Na_2HPO_4 pH 7.3, 2 mM $MgCl_2$, 100 mM β-mercaptoethanol and 4.4 mM ONPG. β-galactosidase activity was measured by reading absorbance at 410 nm using an ELISA plate reader (Tecan Sunrise). The absorbance was normalized according to the total protein content of the cell lysates. All experiments were performed in duplicate.

Cytotoxicity assay

Cytotoxicity was evaluated by the MTT colorimetric assay [26]. After 2 days of culture in the presence or in the absence of peptides, MTT (0.5 mg/ml) was added to the cell culture medium and held for 3 h at 37 °C and 5% CO_2. The medium was then removed and the insoluble MTT products dissolved in 100 μl of isopropanol and 0.1 N HCl. The absorbance was measured at 570 nm using an ELISA plate reader. Results

shown correspond to the average of three independent experiments.

Western blot analysis

To quantify the changes in the level of viral protein expression, HIV-infected HeLa MAGIC cells were treated or not with the Rev-BCPs and infected simultaneously by HIV-1 Bru (5 µl of stock preparation at 1000 $TCID_{50}$/ml). One day later, cells were harvested directly into lysis buffer containing 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Deoxycholate, 1% NP40, 2 mM DTT and 1 mM PMSF for 20 min at 4 °C. Proteins were separated by 10% SDS-PAGE, transferred, blotted with an anti-$p6^{gag}$ polyclonal serum (1:1000) or with a rabbit polyclonal anti-serum raised against $gp41^{env}$ (1:5000) and appropriate HRP conjugated secondary antibodies. Proteins were detected by chemiluminescence.

Fluorescence in situ hybridization (FISH)

One day after transfection or infection, HeLa MAGIC cells growing on glass coverslip were washed three times with PBS and fixed for 10 min at room temperature in 3% *p*-formaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100 in PBS for 3 min at room temperature and rinsed with PBS. Detection of the full length viral RNAs was performed by FISH [36, 37] with a Cy-3 conjugated oligonucleotide corresponding to position 1524–1563 of the HIV-1 gag sequence (TAC 3GG GAT AGG TGG A3T ATG TGT CA3 CCA TCC TAT T3G T) where "3" represents the Cy3 dye locations within the oligonucleotide sequence. Image acquisition and analysis was performed using the Montpellier RIO Imaging (MRI) microscopy facility. Images were taken with a Leica DMRA wide-field microscope using 10 or 100 planapochromatic oil immersion objectives (NA 1.32) with Y3 filter (Leica, Ex. BP545/30 nm, dichroic 565 nm, Em. BP 610/75 nm) for Cy3. Acquisition was performed with a CoolSnap HQ (Photometrics) camera driven by Metamorph software (Universal Imaging Corporation). Deconvolution was performed using the Maximum Likelihood Estimation algorithm and Huygens 2.6 software (Science Volume Imaging). 3D image manipulation was done using Imaris 3.0 software (Bitplane).

Molecular modeling

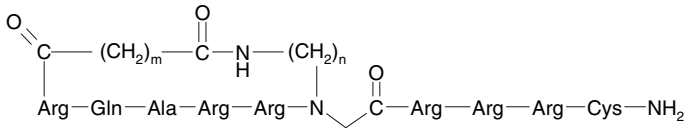
Models were generated using the Insight II package software from MSI Technologies Inc (Accelrys, San Diego, CA), installed on Silicon Graphics Octane workstation. The structures of Rev-BCP 1 and Rev-BCP 14 were built with the Biopolymer module, and structural refinement of the peptides was performed by energy minimization. This step was achieved by using the steepest descent and conjugate gradient algorithms, down to a maximum derivative of 0.01 kcal/Å with the consistent valence force field of discover and a forcing constant of 100 kcal/mol. The validity of the models was assessed by structural check using the Eval123D suite program from the structural bioinformatics web server available in Montpellier (http://abcis.cbs.cnrs.fr/atome) [38, 39].

Docking of the peptides to their respective RNA binding element was performed in vacuum using the affinity program of the Docking module. The RRE used in this study was obtained from the crystal structure of the Rev binding element of HIV-1 (PDB code: 1ETF) [17]. Affinity is an energy-based method, using a Monte Carlo procedure in conjunction either with energy minimization [40] or with molecular dynamics and simulated annealing. First, we applied energy minimization and screened the initial complexes for their capacity to converge to a final structure offering both the best stacking interaction and the minimum energy. The parameters of the minimization were 20 cycles of 1000 minimization steps using the Cell-multipole method as a non-bond summation procedure and convergence criteria to 10 kcal/mol as the energy test and 1 Å as the r.m.s.d. (root mean square deviation) tolerance threshold. This run was performed in a two steps process: first a Monte-Carlo search without any Coulombic contribution and van der Waals scaled down to 0.1, followed by another run including all the contributions in the newly generated file. Simulated annealing was then applied for each selected complex. No major change was observed after 50 stages of 100 fs; the initial and final temperatures being 500 and 300 K, respectively. A final step of minimization (conjugate gradient, 2000 steps) was applied to the last 3D models. The Ligplot program was used in order to determine the intermolecular hydrogen bonds an van der Waals contacts formed within the complexes obtained after docking [41].

Results

Design of the Rev-BCPs

The sequence, synthesis and chemical characterization of the Rev-BCPs used in this study were previously reported in reference [25], and summarized in Figure 1. This peptides series derives from the ''arginine rich motif'' (ARM) of the HIV-1 Rev protein and was subjected to backbone cyclization. The Rev-BCPs present a variation in the number of carbons (alkyl chains) encountered in the ring. The bridge length depends on the number of carbons inserted both at the amino-terminus and in the middle of the sequence (see ''m'' and ''n'' in Figure 1, for details) that imposes different conformational constraints to each Rev-BCP compound. Sixteen Rev-BCPs have been synthesized in which the rings contain between 2 and 5 carbons for the ''m'' chain and between 2 and 6 carbons for the ''n'' chain. In addition, the sum ''m + n'' giving a relevant estimation of the global constraint applied onto peptides is shown and will be useful for the later comparison of structure–function relationships of the peptides. The peptide ''lin-Rev'' corresponds to the linear



Peptide Name	m	n	sum (m + n)	IC_{50} (μM)	+/- SD
Rev-BCP 1	2	2	4	8.5	1.93
Rev-BCP 2	3	2	5	12.6	0.68
Rev-BCP 3	2	3	5	11.2	1.29
Rev-BCP 4	4	2	6	9.7	0.45
Rev-BCP 5	3	3	6	11.5	0.76
Rev-BCP 6	2	4	6	9.7	0.99
Rev-BCP 7	5	2	7	11.8	0.15
Rev-BCP 8	4	3	7	13.2	0.68
Rev-BCP 9	3	4	7	14.6	1.32
Rev-BCP 10	5	3	8	7.6	0.16
Rev-BCP 11	4	4	8	7.1	0.43
Rev-BCP 12	2	6	8	10.0	0.89
Rev-BCP 13	5	4	9	8.0	0.10
Rev-BCP 14	3	6	9	6.2	0.36
Rev-BCP 15	4	6	10	13.9	0.52
Rev-BCP 16	5	6	11	16.2	0.22
Lin-Rev	-	-	-	24.3	0.62

Figure 1 Illustration of the Rev-ARM backbone cyclic peptide library or Rev-BCPs. Upper part of the figure shows the general scheme for the design of the Rev ARM-mimetic library (aminoacids are noted using the 3-letter code). Detailed description of the sequence of cyclic peptides in the library is shown in the lower part where m and n depict the lengths of the alkyl chains comprised in the cyclic ring. The last column of the table indicates the IC_{50} (concentration at which 50% of inhibition is observed) calculated from the hyperbolic fit of the data presented in the Figure 3. Each value is the mean of two independent experiments ± standard deviation

form of the Rev ARM and is used for comparing the antiviral activity of the linear and the cyclic forms of the Rev-derived peptides. As demonstrated hereafter, all the Rev-BCPs are potent antiviral compounds, as summarized in the last column of Figure 1 (IC_{50}). Consequently, we assume later that each peptide can be considered as representative of the series when used at concentrations 4-fold higher than the IC_{50} ($\sim$50 μM), although slight differences in efficiency can be observed at lower Rev-BCP concentrations.

Rev-BCPs 1 and 14 inhibit HIV-1 replication in either human H9 T-lymphocytes or HeLa MAGIC cells and lack cytotoxic effect

To evaluate the ability of such peptides to inhibit HIV-1 replication in cells from lymphoid origin, H9 T-lymphocytic cells were infected with HIV-1 and challenged with Rev-BCPs. The two most representative Rev-BCPs of the series, Rev-BPC1 and Rev-BCP14, which significantly diverge in the sum "m + n" but demonstrate similar IC_{50} (see Figure 1) were selected as Rev-BCP model. As shown in Figure 2A), both peptides were able to block completely the HIV-1 replication when used at 50 μM (Rev-BCPs concentrations into the cell culture wells were systematically readjusted after culture supernatant samples had been collected for RT monitoring, by addition of peptides freshly prepared) and no virus production was detected up to 21 days post-infection. Similar results were obtained using the nucleoside inhibitor AZT (10 μM). Under these experimental conditions, Rev-BCPs did not modulate the cellular proliferation, indicating the lack of cytotoxic or cytostatic effect of these peptides on the cell culture (data not shown). For a lower concentration of Rev-BCP such as 12.5 μM, viral replication was delayed and viral production remained low in the cell culture supernatant, as evidenced by RT assay (Figure 2A) and confirmed by ELISA p24 dosage (data not shown). This result indicates that Rev-BCPs 1 and 14 are functionally active against HIV-1 and can be used to inhibit virus replication in CD4+, CXCR4+ human T cells.

The ability of the Rev-BCPs 1 and 14 to block HIV-1, at various concentrations, was further tested using the HIV-1 induced β-galactosidase expression cell line HeLa MAGIC (Figure 2B). Compared to the human H9 T-lymphocytes, this HIV-1 permissive CD4+, CXCR4+ and CCR5+ cell line presents the advantage to provide a more rapid readout of the antiviral activity of anti-HIV-1 molecules. While no effect was observed at very low concentration in Rev-BCP 1 or 14 (1 μM), increasing peptide concentrations induced an intermediate (at 10 and 25 μM) and next a full inhibition (at 50 μM) of the reporter gene expression. Similar effects were observed in presence of AZT (10 μM).

According to the MTT-based assay that allows estimating the cell viability by measuring the amount of metabolized MTT produced by the cells, only reduced cytotoxicity was observed when Rev-BCPs 1 and 14 were incubated in the concentration range varying from 0.1 to 50 μM with HIV-1 infected HeLa MAGIC cells (Figure 2C). This result clearly indicates that the inhibition of HIV promoter activation observed in the presence of Rev-BCPs was not due to a cytotoxic effect of the peptides.

All Rev-BCPs inhibit HIV-1 induced reporter gene expression in HIV-1 infected HeLa MAGIC cells and lack cytotoxic effect

Since HIV-1 infected HeLa MAGIC cells were shown to represent a relevant cellular model system for the evaluation of the antiviral activity of Rev-BCP 1 and 14, this cell line was reused for testing all Rev-BCPs of the series (Figure 3, panels A to D). While the negative control, oligo, did not promote any inhibition of the HIV-promoter driven reporter gene expression up to 50 μM, all Rev-BCPs tested induced a strong inhibition with an IC_{50} between 6 and 16 μM (see the IC_{50} values in Figure 1). From the thorough analysis of the data, it appears that some of these peptides, among which Rev-BCPs 10, 11 and 14, showed the strongest effect, suggesting that the length of their alkyl chains may modulate their interaction with the target RRE. The efficiency of the best cyclic peptide analog was significantly higher (up to 4-fold) than that of the linear form (note that the IC_{50} of lin-Rev is 24.3 μM). For instance, lin-Rev induced a 5% inhibition at 10 μM whereas, at the same concentration, Rev-BCP14 was able to inhibit up to 70% of the HIV-promoter driven reporter gene expression. In parallel of all biological evaluation assays, cytotoxic effects of Rev-BCPs were determined and are presented in

Figure 3 (panels E–H). As previously observed with Rev-BCPs 1 and 14, for most peptides no significant cell toxicity was measured up to 25 μM with occasionally an increase up to 40% cytotoxicity at higher concentration in peptides (50 μM) especially in the case of Rev-BCP 1–4, 9 and 11.

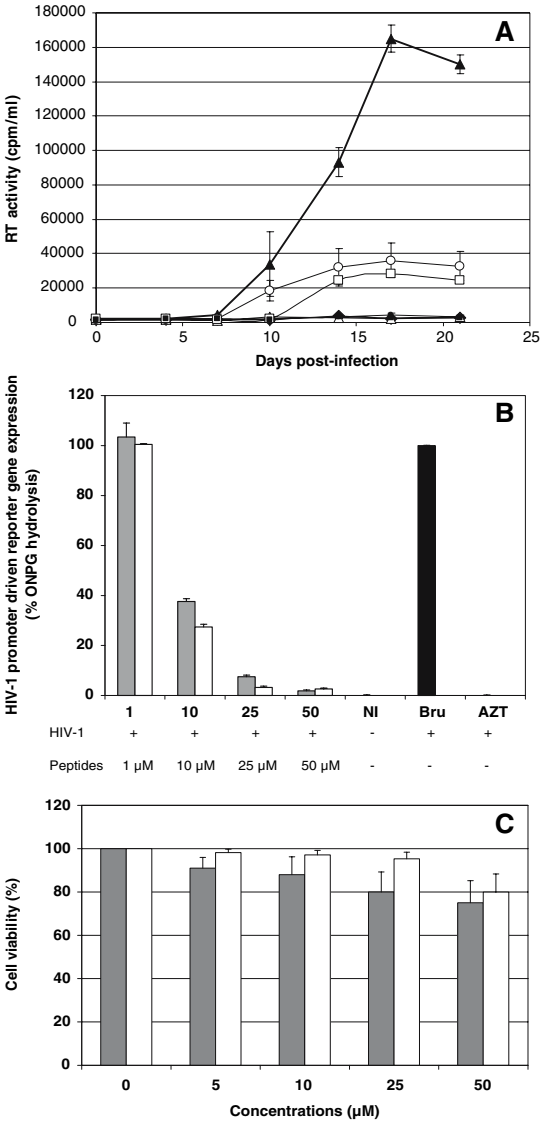


Figure 2 Antiviral activity and cytotoxicity evaluation of Rev-BCP 1 and 14 in different HIV-1 infected cell lines. (A) Human H9 T-lymphocytes were exposed to 100 $TCID_{50}$ of HIV-1 Bru and cultured in absence of inhibitor (▲) or in presence of AZT at 10 μM (△) or Rev-BCP 1 at 12.5 μM (○), 50 μM (●) or Rev-BCP 14 at 12.5 μM (□) and 50 μM (■). Virus production was monitored by measuring reverse transcriptase (RT) activity every 3 or 4 days. Culture supernatants from virus-free H9 cells were tested as a control. Rev-BCPs concentrations into the cell culture wells were readjusted after culture supernatant samples had been collected for RT monitoring, by addition of freshly prepared peptide solutions. Values plotted are the mean of two independent experiments. (B) HeLa MAGIC cells were incubated with Rev-BCP 1 (gray bars) or 14 (white bars) and were infected 2 min later by HIV-1 (5 μl of HIV-1 Bru at 1000× $TCID_{50}$/ml). Viral replication was monitored after 48 h of cell culture by quantification of ONPG hydrolysis from the cell lysates. The results are expressed as the percentage of ONPG hydrolysis measured in HIV-1 infected cells cultured in the absence of peptides. Each value is the mean of two independent experiments, AZT (10 μM) is used as known antiviral control. (C) Cytotoxicity of the Rev-BCP 1 and Rev-BCP 14 in HIV-1 infected HeLa MAGIC cells. Cell viability was monitored after 48 h of cell culture by quantification of the metabolized MTT produced from cells (see Materials and Methods). Results are expressed as the percentage of metabolized MTT measured in the absence of peptide

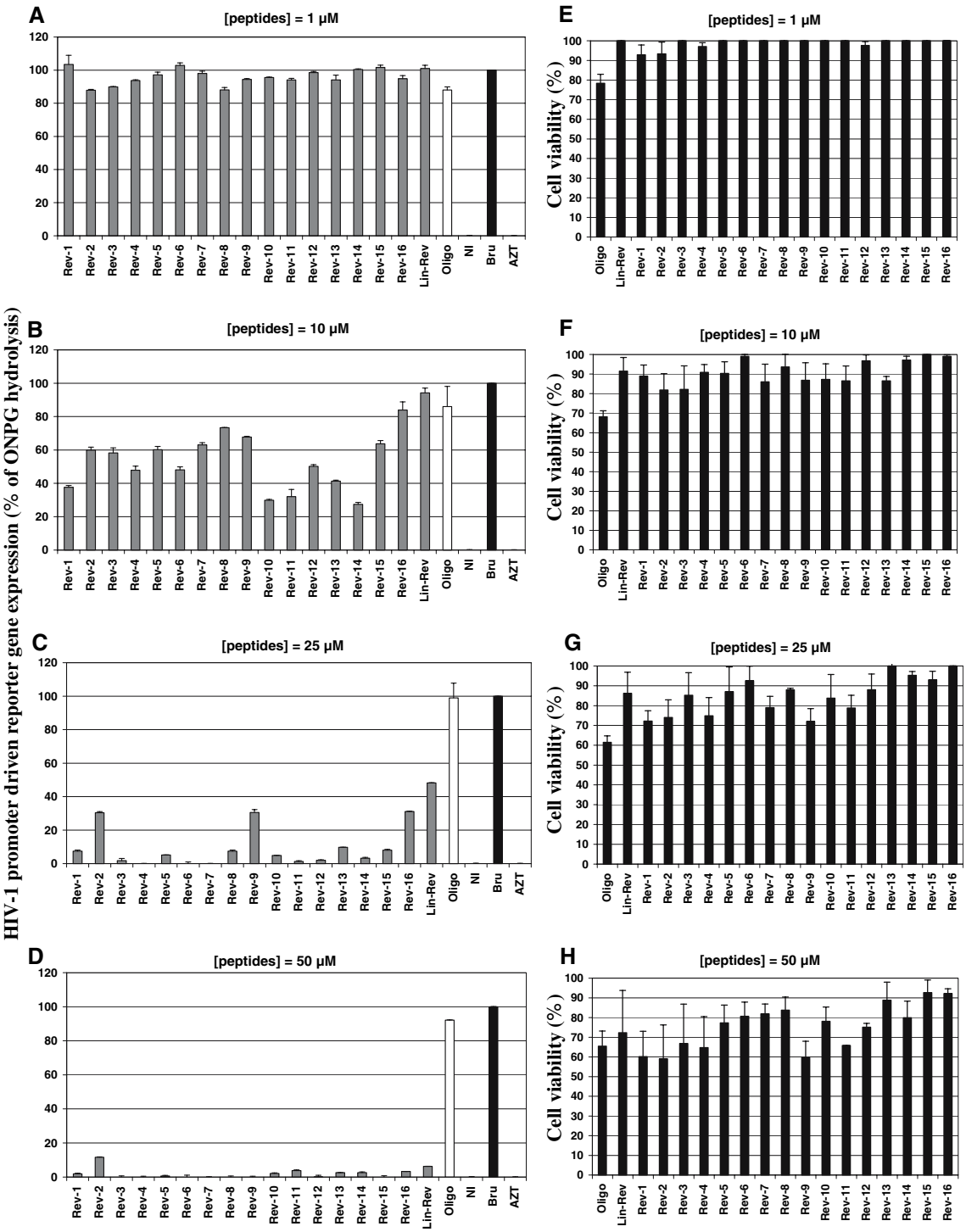


Figure 3 Antiviral activity (A, B, C and D) and cytotoxicity evaluation (E, F, G and H) of all Rev-BCPs in HIV-1 infected HeLa MAGIC cells. Peptides were added to the cells at different concentrations (A and E: 1 µM; B and F: 10 µM; C and G: 25 µM; D and H: 50 µM) just before infection by HIV-1 (5 µl of HIV-1 Bru at 1000× $TCID_{50}$/ml). Viral replication was monitored after 48 h of cell culture by quantification of ONPG hydrolysis from the cell lysates. The results are expressed as the percentage of ONPG hydrolysis measured in HIV-1 infected cells cultured in the absence of peptide. Background level of ONPG hydrolysis in non-infected cells (NI) is also shown. AZT (10 µM) is used as known antiviral control. Cell viability was monitored after 48 h of cell culture by quantification of the metabolized MTT produced from cells. Each value is the mean of four experiments (two independent experiments performed in duplicate) ± standard deviation

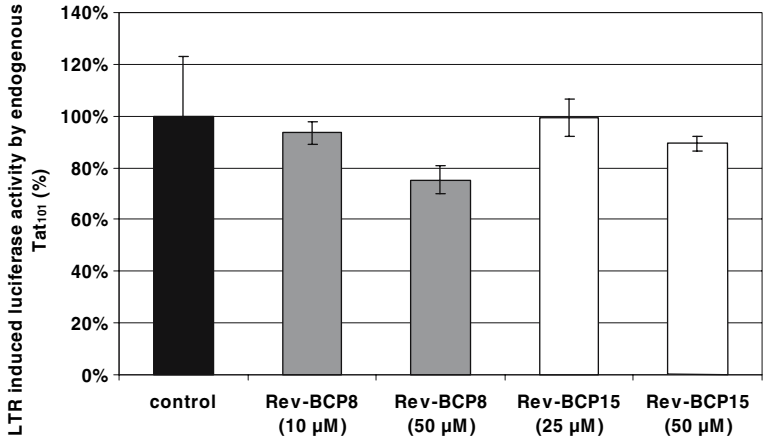


Figure 4 TAR transactivation by recombinant Tat protein and effect of Rev-BCPs. Transient transfections experiments were performed in HeLa cells using *firefly* luciferase expression under the control of LTR promoter and transactivation by endogenous Tat_{101} protein (see Materials and Methods for details) in the absence or in the presence or of Rev-BCPs at various concentrations. Luciferase signal induced by Tat_{101} protein was monitored and quantified after 21 h. Each value is the mean of two experiments ± standard deviation

Cytotoxicity was found to be more pronounced for the control peptide "oligo" (around 40% at 25 µM). Very similar results were obtained with non-infected cells (data not shown). Furthermore, the efficiency of these peptides was not affected by the viral load used for the infection of cells. The antiviral activity observed was similar when the virus input was increased from 5 to 20 µl of the 1000 $TCID_{50}$/ml virus stock solution (data not shown).

Do Rev-BCPs interfere with Tat-TAR transactivation in the HeLa MAGIC cellular model?

The use of a cellular model in which the β-galactosidase gene expression depends entirely on TAR transactivation raised one question about the possible interference of Rev-BCPs with TAR. As already described, both protein domains are rich in arginine residues and both target an RNA sequence. In order to check this particular point, another HeLa cell line was used allowing the direct assessment of Tat-dependent transactivation of TAR (luciferase expression under the control of TAR and transactivation by endogenous Tat_{101} protein). It was found that Rev-BCPs did not strongly interfere with Tat-dependent transactivation (Figure 4). Yet, at a concentration of 50 µM, some Rev-BCPs demonstrated up to 20% (e.g., Rev-BCP 8) of reduction of the tat-TAR-dependent luciferase expression. At this latter concentration a full inhibition of the β-galactosidase expression was observed when using the indirect antiviral assay in HeLa MAGIC cells indicating that the antiviral effect of Rev-BCPs was not due to the blocking of Tat-TAR interaction.

Antiviral activity of Rev-BCPs in aphidicolin-synchronized HeLa MAGIC cells

Both Tat and Rev proteins should translocate into the nucleus to perform their main functions. Previous data [42, 43] have suggested that Rev NLS may inhibit the nuclear import of Rev in uninfected cells by a direct binding to importin β. Furthermore, we have recently reported that the Rev-BCPs used in the present work inhibit the binding of Rev-GFP to importin β [25]. Finally, it had also been reported [24] that Tat ARM mimetic backbone peptides (Tat-BCP11 and Tat-BCP16, hereafter called Tat11 and Tat16, respectively) act as active NLS undergoing importin receptor-based nuclear import.

Since the importins pathway is bypassed during mitosis, we used HeLa MAGIC cells synchronized for 24 h with aphidicolin, a DNA polymerase inhibitor promoting cell cycle arrest in the G1/S boundary, to reduce the initial number of cells progressing to mitosis. Then, synchronized cells were infected by HIV-1 for 20 h (corresponding likely to a single round of infection) in presence or absence of the different Rev-BCPs. Under these

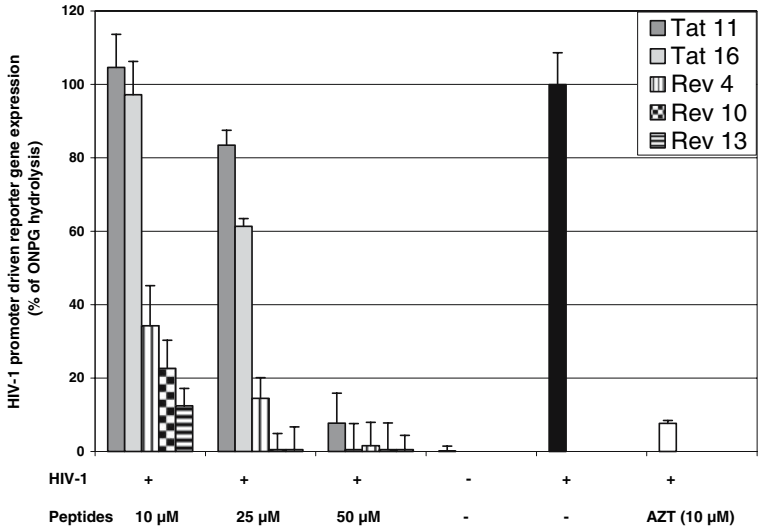


Figure 5 Antiviral activity of the Rev-BCPs in aphidicolin treated cells. HeLa MAGIC cells were synchronized by addition of aphidicolin at 5 µg/ml for 24 h. The peptides were added to the cells immediately before infection by the HIV-1 Bru strain. Viral replication was monitored after 20 h (single round of infection) of cell culture by quantification of ONPG hydrolysis from the cell lysates. The results are expressed as the percentage of ONPG hydrolysis measured in HIV-1 infected cells cultured in the absence of peptide. Background level of ONPG hydrolysis in non-infected cells (NI) is also shown. AZT (10 µM) is used as known antiviral control. The Tat-BCPs 11 and 16 (or Tat11 and Tat16) were used as internal controls because previously tested in asynchronous cells. Each value is the mean of four experiments (two independent experiments performed in duplicate) ± standard deviation

experimental conditions, the importin β dependent pathway that is involved in the active nuclear addressing of viral proteins Tat and Rev, should be totally efficient. Although LTR activation was very low (because of the short time of culture and also probably because virions production was significantly reduced by aphidicolin treatment), Tat transactivation and the subsequent galactosidase reporter gene expression following HIV-1 infection remained detectable in the absence of antiviral compound. Consequently, the ability of several Rev-BCPs to block the HIV-1 promoter activation in aphidicolin-treated HeLa MAGIC cells was investigated. As shown in Figure 5, over 90% inhibition of the HIV promoter-driven reporter gene expression was observed at 50 µM for the three Rev-BCPs model peptides. Rev-BCPs 10 and 13 appeared to be slightly more efficient than Rev-BCP 4 when used at 10 µM or 25 µM since the inhibition was almost total for Rev-BCPs 10 and 13 but not for Rev-BCP 4. The previously studied Tat peptides, Tat11 and Tat16 were assayed simultaneously for comparison of the two different families of BCPs. They were found to inhibit the HIV promoter activation much less efficiently than Rev-BCPs. This result was in good agreement with the previous work carried out on Tat analogs even so the efficiency was not as strong as observed in asynchronous cells (L. Briant, unpublished observations). The concentration required for a complete inhibition of the viral activation was around 50 µM with Tat-BCPs whereas it was around 20 µM with Rev-BCPs. These results suggest that Rev-BCPs might also affect the virus replicative cycle by interfering with the nuclear translocation of Tat and/or the Tat-TAR interaction. However, this antiviral mechanism should not be very efficient in culture of cells that undergo mitosis.

Inhibition of $Pr55^{gag}$ and $gp160^{env}$ polyproteins precursors synthesis in HIV-1 infected cells treated by Rev-BCPs

Since we found that Rev-BCPs inhibit virus particles production and release in supernatant of HIV-1 infected cells, we investigated whether HIV-1 structural Gag polyprotein precursor that is encoded by viral mRNA under control of Rev, can be produced in cells treated by Rev-BCPs. In this

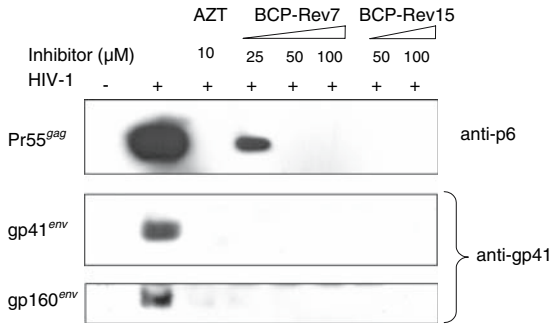


Figure 6 Inhibition by the Rev-BCPs of the Gag polyprotein precursor $Pr55^{gag}$ (upper panel), the Env glycoprotein precursor $gp160^{env}$ (lower panel) and the $gp41^{env}$ (middle panel) synthesis. Cellular extracts were obtained from HeLa MAGIC cells after one day of infection by HIV-1 Bru in the presence of Rev-BCPs (7 and 15) at different concentrations (25, 50 and 100 μM). After normalization by their total protein content, extracts were analyzed by Western blot using a rabbit anti-$p6^{gag}$ or a rabbit anti-$gp41^{env}$ polyclonal serum and detected by chemiluminescence. Extracts from uninfected cells (first vertical lane), HIV-1 infected cells cultured in the absence of antiviral compounds (second lane) and HIV-1 infected cells cultured in the presence of AZT (10 μM), were used as controls

respect, we examined the expression of the polyprotein precursor protein $Pr55^{gag}$, by Western blotting experiments in HeLa MAGIC cells infected by HIV-1 and treated or not with Rev-BCPs. As shown in Figure 6, Rev-BCPs (e.g. Rev-BCPs 7 and 15) were shown to reduce considerably the expression of the $Pr55^{gag}$ polyprotein (detected by an anti-$p6^{gag}$ polyclonal serum) in a dose-dependent manner. Similarly, we found that the envelope glycoprotein precursor $gp160^{env}$, another protein encoded by a Rev-regulated singly spliced mRNA, and its proteolytic cleavage product, the viral transmembrane $gp41^{env}$ (both detectable by an anti-$gp41^{env}$ polyclonal serum) were absent in infected cells treated by Rev-BCPs.

This result clearly indicates that Rev-BCPs inhibit HIV-1 replication cycle at a stage before the translation of viral mRNA encoding the structural polyprotein precursor and the envelope glycoproteins of the virions.

Rev-BCPs altered the intracellular distribution of the full length genomic viral RNA in transfected or infected HeLa MAGIC cells

Rev-BCPs inhibit the molecular pathway resulting in $Pr55^{gag}$ and $gp160^{env}$ polyproteins synthesis. Therefore, one can wonder whether Rev-BCPs can interfere with the functioning of Rev related to RNA export. In infected cells, Rev binding to the RRE sequence is required to export unspliced or singly spliced viral RNA from the nucleus through the CRM1-mediated RNA export machinery. It is likely that the presence of several molecules of Rev-BCP bound to the RRE of viral transcripts will interfere with their export process or their integrity. To address this question, the intracellular localization of unspliced genomic viral RNA was analyzed in HIV-1 transfected or infected HeLa MAGIC cells by FISH using a fluorescent probe specific to the full length genomic unspliced viral RNA. Experiments were performed both in infected and transfected cells for two reasons: firstly, in order to differentiate between newly synthesized full length RNA (exported from nucleus during the virus life cycle) and viral genomic RNA delivered in the cytoplasm soon after fusion of viral particles with the target cell (coming from the infectious dose applied onto cells). Secondly, comparing transfection versus infection experiments allow us to determine whether or not early stages of virus infection (adsorption of the virus on the cell membrane, fusion, virus entry, capsid release, reverse transcription and viral DNA integration into host genes) are targeted by Rev-BCPs. These steps are obviously absent in transfection experiments.

After one day of transfection with HIV-1 (that likely corresponds to a single round of infection), unspliced viral RNA was detected into most of the cells (Figure 7, panel B) while no detectable *in situ* signal was recorded for the control (mock transfected cells, panel A). In the presence of Rev-BCP 2, 9, 12 or Lin-Rev at 50 μM, the fluorescence signal for full length viral mRNA was dramatically reduced in transfected cells (panels C, D, E and F, respectively) indicating that the Rev-BCPs strongly affect the normal process of unspliced RNA synthesis, transport or integrity. Additionally, these results obtained from transfected cells suggest that the Rev-BCPs do not interfere with an early stage of the virus replication cycle (e.g. virus adsorption and fusion, capsid release and viral DNA incorporation into host gene) but most likely at a stage where viral RNA are produced.

Similar observations were performed from HIV-1 infected HeLa MAGIC cells (Figure 7, panels G to L). Twenty-four hours after infection, an intense fluorescence signal was detected that correspond to the full length unspliced viral RNAs present in the cells after one cycle of

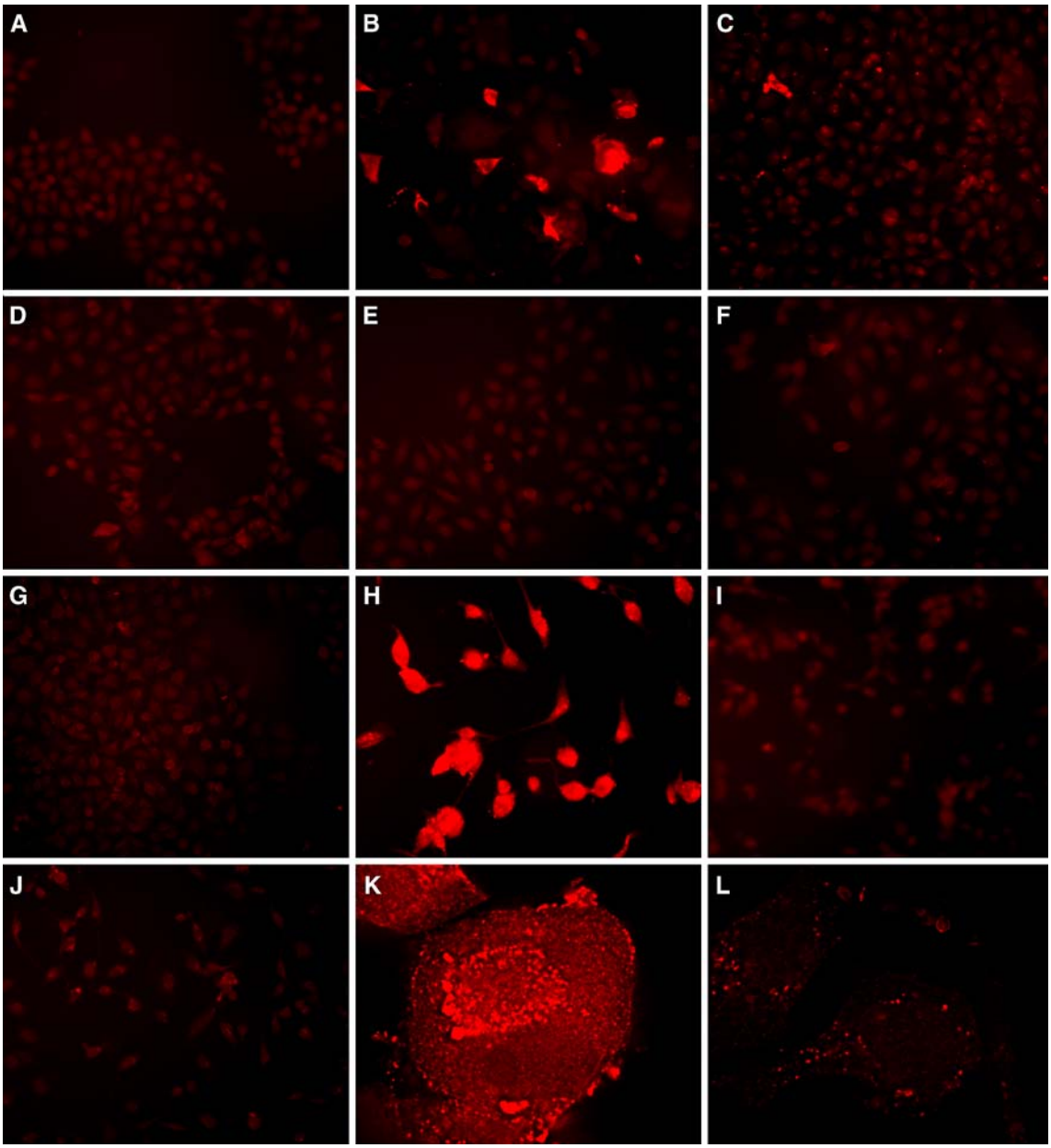


Figure 7 Cellular localization of unspliced viral RNA detected by FISH using a Cy-3 labeled oligonucleotide (position 1524–1563 of the HIV-1 Gag sequence). Experiments were performed in either HIV-1 transfected (top) or infected HeLa MAGIC cells (bottom) and the different pictures are presented as follow: non transfected cells used as control (A); HIV-1 transfected cells analyzed after 24 h (B); HIV-1 transfected cells treated with Rev-BCP 2 (C), Rev-BCP 9 (D), Rev-BCP 12 (E) or Lin-Rev (F) at 50 μM for each peptide. Bottom part of the figure refers to infection experiments: background signal obtained for non-infected cells used as control (G); HIV-1 infected cells (H); HIV-1 infected cells after treatment with 10 μM AZT (I) or 50 μM of Rev-BCP14 (J). The last two pictures (K and L) recorded at a higher magnification and deconvoluted, represent untreated HIV-1 infected cells (K), and HIV-1 infected cells treated with 50 μM of Rev-BCP 14 (L), respectively. Pictures presented are representatives of five different experiments

infection (panel H). After improving the picture resolution by a deconvolution procedure (panels K), a high fluorescence signal was detected from both the nucleus and the cytoplasm of HIV infected cells indicating the accumulation of unspliced viral genomic RNA in both compartments. Moreover, fluorescence was also found localized close to the cellular membrane as normally occurring during the maturation process before virus budding. As expected, when infected cells maintained in the presence of AZT (10 μM) were analyzed (panel I) fluorescence intensity was lowered to the background level detected from uninfected control cells (panel G). When HIV-infected cells were cultured in the presence Rev-BCP 14 (50 μM) the intracellular genomic viral RNA was imperceptible (panels J and L) and almost no fluorescence was detected indicating that Rev-BCP affects considerably the expression of unspliced HIV-1 RNAs. It is worth noting that syncitia formation, that consists of multinucleated giant cells resulting from a cellular fusion process initiated between HIV-1-infected cells expressing the viral envelope glycoprotein (HIV-1 $gp120^{env}$) at their surface and cells expressing the CD4 surface receptor identified to act as a ligand for the HIV-1 $gp120^{env}$ as previously described by Rey and collaborators [44], could be only observed in the culture of untreated HIV-1 infected cells. The fusion process is also known to involve the transmembrane envelope protein (TM)-$gp41^{env}$ [45]. Thus, the presence of BCP-Rev prevents the apparition of syncitia indicating that $gp120^{env}$ and $gp41^{env}$ are not expressed at the cell surface and in the membrane, respectively. This is in good agreement with the inhibition of $gp160^{env}$ polyprotein precursor synthesis observed in presence of Rev-BCPs by Western blot analysis.

Molecular modeling of the Rev-BCP/RRE complexes

Two Rev-BCPs were used as models for this study: Rev-BCP 1 because it is among the most efficient at inhibiting HIV-1 and is the most rigid peptide ($m + n = 4$) of the series and Rev-BCP 14 because it is the most efficient in blocking HIV-1 replication and is also the most flexible ($m + n = 9$). Indeed, the bridge lengths in these peptides are quite different: the length of the alkyl chains m and n are 2 and 2 in Rev-BCP 1 and 3 and 6 in Rev-BCP 14, respectively. We have studied the binding capacity of these peptides to the RRE domain using molecular dynamics simulations *in vacuum* keeping the whole RNA molecule flexible. After having performed a simulated annealing in the presence of the RRE, both peptides were found to bind the RNA molecule within the same area. This is a good indication of the specificity of the binding because the interaction area defined at the beginning of the simulation included the whole RRE molecule (Figure 8). Therefore, the difference in length of the alkyl chains did not seem to play a major role in the determination of the final RNA binding site. As observed in both cases, the binding of the peptides did not change the overall conformation of the RNA molecule but rather induced a larger shape distortion within the binding site. Indeed, the opening of the major groove was extended by the binding of the peptides. From the 20 calculated complexes obtained by the Monte-Carlo search, the final ones, retained according to their lowest energy, delineated the same binding pocket, located near a purine-rich internal loop in the RRE. The analysis of the interaction interface indicates that Rev-BCP 14 covers a larger binding surface than Rev-BCP 1 due to a more extended conformation. These results demonstrate the capacity of both peptides to bind to the RRE in a widened RNA major groove which is characteristic of an adaptive binding.

The different interactions involved in the peptide–RNA complexes were further analyzed using the "Ligplot" algorithm that allows the calculation of hydrogen bonds and van der Waals interactions (Figure 9, panels A and B). In addition to obvious electrostatic interactions between the phosphate backbone and positive charges of the peptides, numerous hydrogen bonds were identified. These bonds represent a very important contribution of the complex interactions for Rev-BCP 1 (Arg1, 4, 5, 6, 7, 8, Cys9) and for Rev-BCP 14 (Arg1, 4, 6, 7, 9, Gln2 and Ala3). For both Rev-BCPs, the whole peptide seems to participate in the RNA binding and the carboxy-amidated terminus of the peptide was found to form hydrogen bond with the backbone phosphate of RNA.

However, several points differed between the two peptides. Gln2 and Ala3 were involved in the

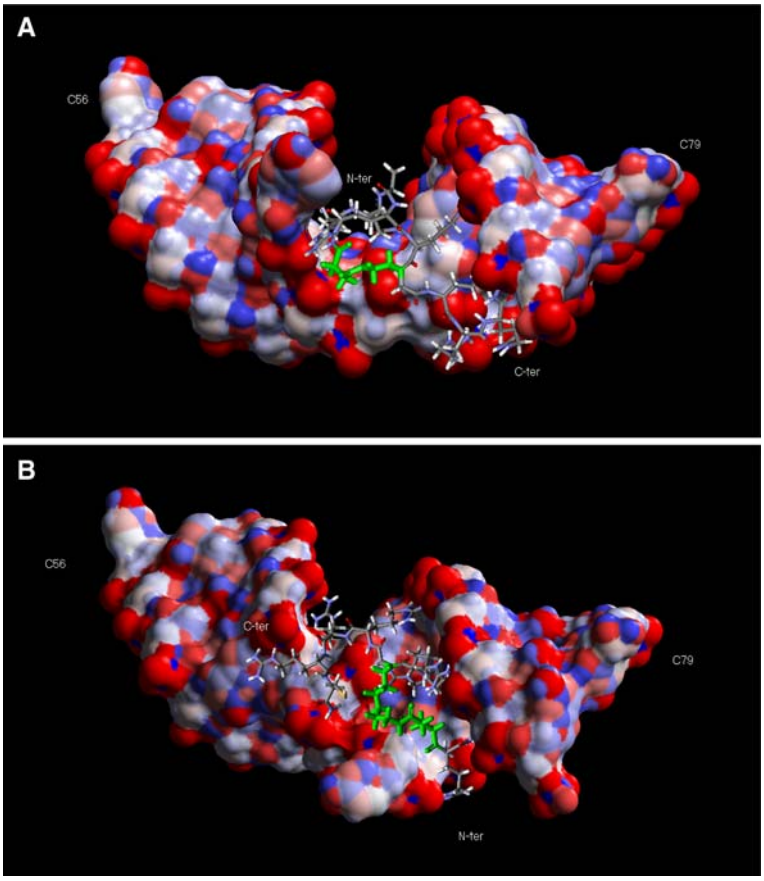


Figure 8 3D representation of the RRE/Rev-BCP 1 (A) and the RRE/Rev-BCP 14 (B) docked complexes obtained by molecular dynamics simulations (SA docking). The alkyl chains of the cycle of the BCPs are shown in green. The RRE is shown with its solvent accessible surface of RRE which was generated by the Connolly algorithm. The nucleotide C56 and C79 from the RRE are labeled and the abbreviations N-ter and C-ter depict the amino and carboxy termini of the Rev-BCP

RRE interactions only with Rev-BCP 14 but not with Rev-BCP 1. When focused on Rev-BCP 14, Gln2 acted as both donor and acceptor group for hydrogen bonding. The other feature that must be emphasized is the role of Ala3 that made specific interactions with three different bases (C44, U45 and A75 with Rev-BCP 14). Two main hydrophobic contacts were also determined. They involved the same residues Gln2 and Ala3. In contrast, Rev-BCP 1/RRE complex showed less specific base-residue contacts (U72, A75 and G76 directed on one residue Cys9). By examining the non-bonded contacts, hydrophobic interactions could be also determined for both complexes with a less extent in the case of Rev-BCP 1/RRE. In this latter case, several contacts were observed between the ring and the side chains of Arg1, 4, 5 and 6, stabilizing the overall peptide conformation. Additional hydrophobic interactions were detected between C69, U72 and A73 with Arg1, 5 and 7. Concerning the Rev-BCP 14/RRE complex, the hydrophobic contacts were also observed between the bridge and side chains of several arginine residues. In contrast with Rev-BCP 1, Rev-BCP 14/RRE showed many more intermolecular hydrophobic contacts. Gln2 and Ala3 were found to form this type of interactions as previously mentioned but also Arg7 and Arg8 with the ribose of the RNA (see Figure 9B).

Discussion

In the present study, we demonstrated for the first time that backbone cyclic ARM Rev peptides were able to totally block HIV-1 replication in cultures

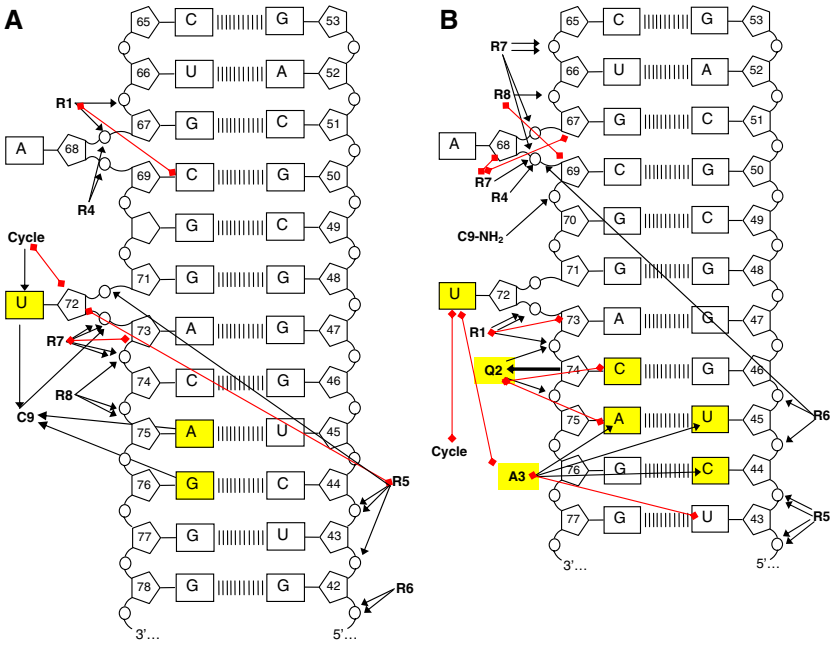


Figure 9 Schematic representation of the RNA–peptide interactions. Hydrogen bonds and van der Waals contacts were calculated with the Ligplot algorithm for the Rev-BCP 1/RRE (A) and the Rev-BCP 14/RRE (B) complexes. Black arrows indicate hydrogen bonds between peptide and RNA. The most important amino acid residues for conferring specificity are highlighted in yellow as well as the corresponding bases involved in the contact. Red arrows indicate van der Waals contacts (note that only intermolecular contacts have been represented for clarity)

of CD4+, CXCR4+ human T cells during the 21 days of the experiment, using peptide concentrations in the low micromolar range (Rev-BCPs concentrations into cell culture wells were systematically readjusted by addition of freshly prepared peptide solutions every 3 or 4 days). This result point out the relative high stability of the Rev-BCPs to proteolysis in respect to their amino acid composition and corroborates previous observations made on the metabolic stability of other backbone cyclic peptides [28].

Inhibition of HIV-1 cycle by Rev-BCPs was also evidenced in the HIV-promoter induced reporter gene expression cell line (HeLa MAGIC) validating this cellular model for the current study. Moreover, the potential interaction of Rev-BCPs directly with the TAR sequence was controlled using another cell line and showed no significant interference making relevant the HeLa MAGIC cellular model. Although we observed slight differences in the capability of the 16 studied Rev-BCPs to inhibit HIV-1 (IC_{50} varying from 6.2 to 16.2 μM), all peptides from this series demonstrated an antiviral potential. In addition, all Rev-BCPs tested were more active than the linear form of the Rev-ARM peptide (Lin-Rev) confirming the importance of the cyclization for their function.

The relative high concentration required for inducing a full inhibition of HIV-1 replication constitutes an important drawback of the BCP strategy and will have to be improved for future therapeutic developments. Different explanations can be proposed especially in human T lymphocytic cells in which the transfection efficiency or the cellular uptake is known to be very poor for many peptides or small charged molecules, in general. Indeed, it must be emphasized that the concentration required for a total inhibition is different when testing BCP-Revs in H9 or in HeLa cells (50 μM for H9 lymphocytes should be compared to 15 μM in the case of HeLa cells). The cell types, the membrane composition (proteins and lipids), the volume of intracellular compartments, are some of the differences between these two cell lines that can be responsible of the present results. The high concentration requirement could also be ascribed to the degradation of peptide. However, the stability of these cyclic peptides was previously measured and Rev-BCPs showed longer half-life in comparison with their linear counterparts. It must

be noted that the incubation time of Rev-BCPs is longer in T-lymphocytes (4 days) than that used in HeLa cells (2 days) corroborating the difference in the needed concentration. The tight binding of the viral Rev protein on RRE constitutes likely another important reason to use large amount of Rev-BCPs in order to competitively displace the natural ligand. In order to improve the efficacy of these peptides, the future developments should include a shuttle system that will increase both the cellular uptake and the stability of the Rev-BCPs. Many non-viral based systems for drug delivery have been developed during the past decade especially for nucleic acids transfection, RNA interference or protein transduction but none of these systems is fully adapted for carrying small positively charged molecules such as Rev-BCPs (for a review see [46]). However, it may be assumed that the addition of a stabilizing agent like poly(ethylene glycol) already used for cancer treatment [47], improve the antiviral efficiency of the peptides.

The mechanisms driving Rev-BCPs inhibition of HIV-1 replication have been investigated. We demonstrate here that the main mechanism by which Rev-BCPs inhibit HIV-1 replication is related to Rev-dependent regulation of full length/singly spliced viral mRNAs. Indeed, we found that Rev-BCPs can inhibit the intracellular accumulation of the full length genomic viral RNA and, in this way, block the viral replication at a stage before the translation of viral mRNA encoding the structural polyprotein precursor and envelope glycoproteins of the virions. Accordingly, it was not surprising to find that $Pr55^{gag}$ and $gp160^{env}$ could not be detected in Rev-BCPs-treated cells. This result corroborates the observations made on the syncitia formation which could occur only for the untreated HIV-1 infected cells, meaning that the expression of envelope proteins ($gp120^{env}$ and $gp41^{env}$) was flawed in the case of HIV-1 infected cells treated with Rev-BCPs (compare panels K and L of Figure 7). Besides inhibition of genomic viral RNA and protein precursors synthesis, it cannot be excluded that Rev-BCPs trigger some other viral targets. Indeed, a faint reduction of the Tat-TAR-driven gene expression was observed for some cyclic peptides used in the present study but this effect is not sufficient to explain a full inhibition of the virus replication cycle. Previous data [42] have suggested that Rev NLS may inhibit the nuclear translocation of viral Rev protein in uninfected cells by a direct binding to importin β. Then, we used aphidicolin-synchronized cells in which the importin β-dependent pathway is totally efficient for the active nuclear addressing of viral Rev protein. The inhibition of HIV-1 induced reporter gene expression observed only in the presence of Rev-BCPs suggests that peptides might interfere with nuclear translocation of the different viral regulatory proteins such as Tat and Rev. This mechanism has to be particularly considered in the aphidicolin-treated HeLa MAGIC cell line but should not be really efficient in culture of cells that undergo mitosis like T-lymphocytes (where nuclear membrane is disrupted at each mitosis).

On the other hand, performing transfections and infections experiments in parallel could allow us to determine whether or not the early stages of the virus infection cycle (from adsorption of the virus to the cell surface to viral DNA integration into host genes) were affected by the Rev-BCPs treatment in HeLa MAGIC infected cells. From transfection experiments (where these early steps do not obviously occur) Rev-BCPs were shown to inhibit the viral particles production and the genomic viral RNA expression (FISH), therefore these results signify that a later stage (than viral DNA integration) of the virus cycle is targeted by the Rev-BCPs. Assuming that there could be several stages for the observed inhibition, this can not happen at a stage before DNA integration since all these former steps (virus adsorption, fusion, capsid release, reverse transcription) do not exist in transfected cells with pNL4.3.

The bifunctionnality of the ARM domain of the viral Rev protein (also present in the Rev-BCPs sequence) has been well established [48]. Indeed, a direct interaction between a peptide bearing the Rev ARM domain and RRE has been demonstrated elsewhere [17, 42, 49]. The ARM of Rev binds specifically to its RNA site when the peptide is in an α-helical conformation [50]. However, it is still unclear if Rev binds viral mRNAs as a preformed oligomeric complex or if oligomerization occurs after binding of a first monomer to the stem-loop IIB of the RRE sequence [51]. The natural biological function of the Rev protein is the regulation of the export of unspliced/singly spliced viral RNA from the nucleus to the cell cytoplasm. The absence of

Rev strongly affects intracellular accumulation of viral RNAs [15]. Hence, Rev-ARM peptides may inhibit the Rev–RRE interactions that are required for the export or the stability of unspliced/singly spliced viral RNA, either directly by steric hindrance at the stem-loop IIB of the RRE or indirectly by acting on the oligomeric Rev complex formation. Consequently, the stability of the full length/singly spliced viral RNA should be drastically reduced. At least, fluorescence *in situ* RNA hybridization results clearly showed the disappearing of genomic viral RNA (full length) probably being the result of degradation or lost of stability.

Among the 16 Rev-BCPs that differ only in the length of the alkyl chain rings, 4 of the quite flexible Rev-BCPs demonstrated a good inhibition potential, namely Rev-BCPs 10, 11, 13 and 14. On the other hand, some were slightly less active, Rev-BCPs 2, 9 and 16 (compare the IC_{50} values in Figure 1). The limited degree of conformational freedom permitted by such alkyl cycle can therefore be considered as an important factor to be taken into account in optimizing peptides for increasing their antiviral properties against HIV-1. A comparative analysis allowed the identification of the "m" and "n" numbers corresponding to the alkyl chain lengths that allow peptide folding with the best antiviral properties. The results indicate that optimum values for the sum m + n are 8 and 9. This suggests that the peptide must preserve a certain conformational flexibility in order to accommodate in the RNA binding pocket. However, there is one exception to this rule, since Rev-BCP 1 (m + n = 4) falls into the same category than Rev-BCPs with m + n = 8 or 9, for antiviral activity. Molecular docking study showed that the surface covered by Rev-BCP 14 (m + n = 9) on the RNA molecule was greater than that observed for Rev-BCP 1 (m + n = 4). The difference in the covered surface and the numerous interactions identified (van der Waals and hydrogen bonds) are most likely the parameters that govern the biological activity and the efficacy of such peptides and it must be noted that strong electrostatic interactions are required but not sufficient for specific recognition with the target. It must be also highlighted that the complex Rev-BCP 14/RNA exhibited a higher number of hydrophobic contacts and hydrogen bonds than those determined for Rev-BCP 1/RNA complex.

From a structural point of view, the binding of arginine rich peptides to RNA has been extensively studied over the last decade [20, 52, 53] and the structure flexibility that can be induced by such binding has been extensively documented [54, 55]. One important feature is that structurally constrained cyclic peptides can still promote an adaptive binding to a widened major groove (as observed for the natural Rev peptide [17, 18] and also for the BIV Tat peptide). In this way, the peptide can penetrate more deeply into the RNA major groove. The flexibility of the RNA structure also plays a critical role because the A form helix can accommodate a disordered loop allowing the peptide to bind easier and deeper. This mode of binding is a typical example of an induced fit mechanism for RNA–protein interactions. This phenomenon also seems to be required for the biological function of several RNA binding protein, although the general mechanism is not yet clearly understood [53].

Altogether, these data suggest that Rev-BCPs represent an interesting approach for the design of novel antiviral compounds. Even this strategy needs yet to be improved by taking a particular attention in reducing the concentration required for a total inhibition, it has to be considered as promising for the future peptide-based therapeutic developments. In conclusion, this work not only demonstrates the possible use of structurally constrained Rev-derived cyclic peptides to interfere with HIV replication in chronically infected cells but also sheds light on the possible mechanism by which these peptides are able to inhibit HIV-1 life cycle.

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