Targeting the Viral Nucleocapsid Protein in Anti-HIV-1 Therapy

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Abstract: The nucleocapsid protein (NC) plays seminal roles in HIV replication, thus representing a major drug target. NC functions rely on its two zinc-fingers and flanking basic residues. Zinc ejectors inhibit NC functions, but with limited specificity. New classes of molecules competing with NC or its viral nucleic acid and enzyme partners are reviewed here.

Key Words: NCp7, zinc ejector, library, HIV, structure, peptidomimetic, aptamer, intercalator.

I. INTRODUCTION

The human immunodefiency virus type-1 (HIV-1) belongs to the widespread family of Retroviruses. The viral particle is about 110 nm in diameter and is composed of an inner core with an outer envelope formed of a lipid bilayer derived from the infected cell, in which the viral surface (SU) and transmembrane (TM) glycoproteins are anchored. The inner core corresponds to a shell of capsid protein molecules (CA) surrounding the dimeric single stranded RNA genome coated by about 1500 molecules of the nucleocapsid protein NCp7 [1,2]. The core also contains molecules of the viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN) [3-5]. The HIV-1 replication cycle, as illustrated in Fig. (**1**), is divided into early and late phases, corresponding to virus entry and genome replication by RT, and virus biogenesis, production and maturation by PR, respectively [6-9] during which the nucleocapsid protein (NC) plays seminal roles (Fig. (**1**), steps 3, 4, 5 and 7). After virus entry, the genomic plus strand RNA is converted by RT into a double-stranded proviral DNA flanked by two long terminal repeats (LTR) (step 3) [10]. This process known as reverse transcription takes place in the incoming viral nucleocapsid substructure, where NC chaperones viral DNA synthesis by RT, that necessitates two NC-mediated obligatory strand transfers to generate the complete LTRs [3,11,12]. The proviral DNA is then integrated by IN into the cellular genome, in a reaction also assisted by NC [13] (step 5). During the late phase of virus replication in the infected cell, NC pilots genomic RNA selection and dimerization, and Gag oligomerization, and thus is considered to be an essential determinant of virus assembly (step 7). At the molecular level, the early and late functions of the viral NC protein appear to be mediated by its propensity to specifically bind the viral nucleic acids [14-16] and to chaperone their obligatory transconformation during the process of reverse transcription and virus assembly (for a recent review [17]).

HIV-1 NC is characterized by two highly conserved zinc finger motifs (ZF) flanked by basic residues. Point mutations in these fingers result in the production of totally defective virions [3,18-21] further underlining the importance of NC in the viral life cycle. This highlights the notion that HIV-1 NC represents a major target for the development of new anti-HIV-1 agents that could impair both early and late steps of HIV-1 replication. Anti-NC molecules could thus complement the so-called 'highly active anti-retroviral therapies' (HAART) based on drugs targeting the viral RT and PR. Due to the fact that NCp7 is highly conserved in all HIV-1 subtypes [17], a major benefit of anti-NC drugs should be to provide a sustained replication inhibition of a large panel of HIV-1 strains including those species that are resistant to anti-RT and anti-PR drugs. Different classes of anti-NC molecules have been developed or selected by means of a high throughput screening strategy (HTS). The most important ones are the zinc ejector agents, peptidomimetics, RNA aptamers and non zinc ejecting NC binders. These different classes of anti NC agents will be reviewed here.

II. ZINC EJECTORS

A common feature of a large number of DNA and RNA binding proteins in viruses and host cells is the presence of zinc finger motifs (ZF) [22-25]. Many classes of ZFs differing in their length and zinc ligands have been identified. ZFs of retroviral nucleocapsid proteins are the smallest fingers (or knuckles) yet reported. They coordinate zinc atoms through three Cys and one His residues in a Cys-X_2-Cys-X_4-His-X_4-Cys arrangement (also called CCHC motif). With the exception of spumaretroviruses, all retroviral nucleocapsid proteins are characterized by one or two copies of the CCHC motif. The nucleocapsid protein (NC) of HIV-1 contains two CCHC motifs separated by a short basic linker. The unusual high zinc binding affinity of 10^{13} to 10^{14} M^{-1} (several orders of magnitude higher than that of cellular zinc finger proteins)

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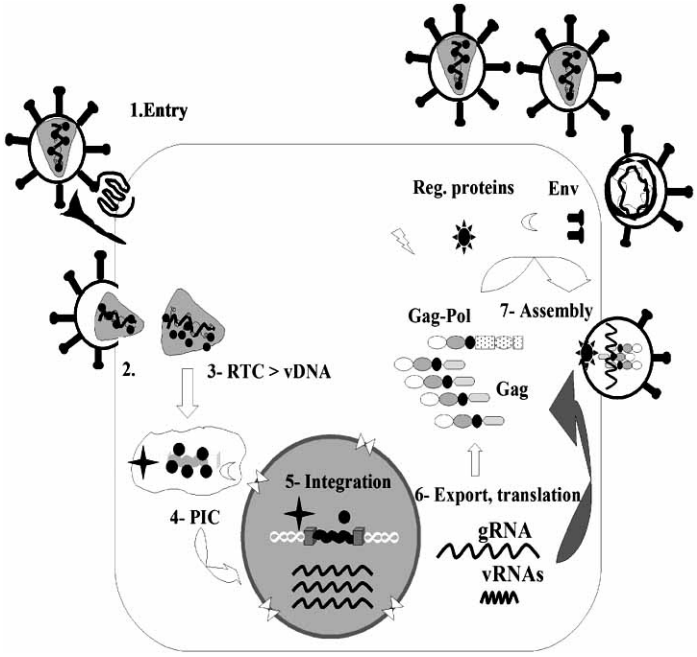


Fig. (1). Schematic illustration of HIV-1 replication in a human target cell.

The mutifunctional nature of the viral nucleocapsid protein NC (black dots) is highlighted at the early and late steps, namely 1 to 7 for its role in the viral core structure, viral DNA synthesis by RT and integration by IN, and lately in virus assembly. Therefore, drugs targeting NC should in principle inhibit early and late steps of HIV-1 replication. Step1-Virus Entry is mediated firstly by interactions between SUgp120 and the cellular receptor CD4 and co-receptor CCR5 and followed by TMgp41-mediated membrane fusion; Step 2- core entry into the cytoplasm followed by virus uncoating ; Step 3- viral DNA synthesis takes place in the reverse transcription complex (RTC) and is chaperoned by NC; Step 4- viral DNA enters the nucleus in the form of a pre-integration complex (PIC) ; Step 5- the vDNA is integrated into the host genome by IN assisted by NC ; Step 6: the genomic RNA (gRNA) is synthesized by transcription of the integrated vDNA and undergoes balanced splicing to generate the 4 kb and 2 kb vRNAs and the gRNA found in the cytoplasm. Note that the roles of the viral factors TAT and REV are not shown here. The gRNA and vRNAs are translated by the host ribosome machinery to generate the virion polyproteins Gag, Gag-Pol, and Env and the regulatory proteins (large arrow). Step 7: selection of the gRNA by the NC domain of Gag is thought to start co-assembly of the gRNA with Gag and Gag-Pol (upper arrow); ultimately newly formed virions accumulate at the plasma membrane and undergo maturation by the viral protease. Viral particles are released by budding (top).

has foreseen the importance of this binding for the bona fide structure of the NC central domain and its implications in HIV replication [24,26-30]. Indeed, mutations that prevent zinc binding or cause a conformational disorder in the ZF result in the production of defective viral particles [18,19, 31,32] reviewed in [11,12,33]. Thus, in the search for new anti-HIV drugs, many efforts were performed to develop molecules able to specifically remove zinc from the NC ZF.

Structure of Zinc Ejectors

The proof of concept of zinc ejectors as anti-NC drugs was demonstrated in the early 90's with the 3-nitrosobenzamides (NOBA) (Fig. (**2**)) [34]. These C-nitroso derivatives were first identified as inhibitors of a cellular enzyme, the poly(ADP ribose) polymerase which possesses a large zinc finger sub-domain. Derivatives such as the 3-nitrosobenz-amide or 6-nitroso-1,2-benzopyrone were found to eject zinc from NC in solution and in the virus (either HIV-1 or SIV [35]) through oxidation of the cystein residues [36,37]. Surprisingly, these compounds elicit only small side effects though they are possibly targeting cellular proteins binding zinc. Later, disulfide-substituted benzamides (DIBA) were identified as zinc ejectors with a broad anti-viral activity towards laboratory and clinical HIV-1 isolates [38-40]. The finding that NC ZFs were the target of the DIBA compounds was definitively assessed by showing that DIBA did not modify the infectivity of the human foamy virus, a spumaretrovirus without a canonical NC domain [41]. In the DIBA family, the two benzamide moieties are linked through a disulfide bridge. Nevertheless, the monomeric form of DIBA (with free -SH) exhibits an antiviral activity similar to the parental molecule but does not eject zinc. In contrast, molecules where the disulfide bond or the -SH group are changed to -OH or -SO_2 group or alkylated do not eject zinc or show an antiviral activity [42]. Since in the intracellular reducing environment both reduced and oxidized species may exist [43], the anti-viral activity of the DIBA compounds is likely mediated by both species.

To avoid the S-S bond reduction observed with the DIBA family, substitution for a thioester link was developed leading to the generation of the pyrimidinioalkanoyl thioester (PATE) family [44]. The PATEs were found to exhibit an increased antiviral activity, water solubility, a lack of susceptibility to glutathione reductase and a reduced cell toxicity [44,45]. In an attempt to develop new anti-NC's, a combinatorial chemistry was performed using the PATE skeleton as a scaffold. Series of uncharged S-acyl-2-mercaptobenzamide thioester (SAMT) were generated, some of them showing virucidal activity [46]. Interestingly, no correlation was evi-

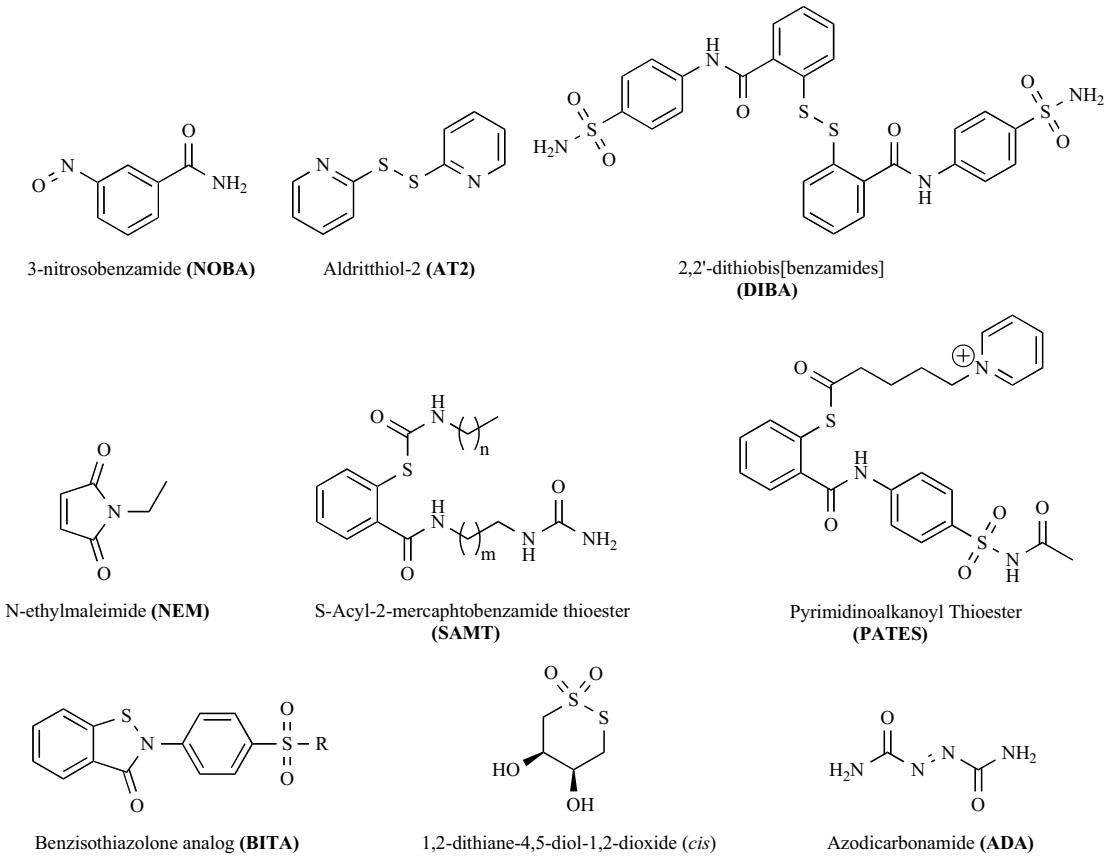


Fig. (2). Zinc ejector structures.

denced between the chemical stability of the thioesters in serum and their antiviral activity. Thus, the rapid release of free –SH groups in the serum does not appear as a solid criteria to discard a SAMT derivative for an *in vivo* study [47]. In fact, the released thiol groups of these compounds are likely re-acylated by acyl CoA, then available for a second round of reaction [48].

Another strategy to prevent disulfide reduction was based on the use of dithiane compounds [49]. The S-S bond in this series is tethered to a ring structure which confers an antiviral activity to the compound even in the presence of a high concentration of glutation reductase. Synthesis of the enantiomers of the first published molecule did not significantly improved the antiviral activity [50]. Last, Vandevelde *et al.* described azodicarbonamide (ADA) derivatives (Fig. (**1**)) as being potent anti-HIV drugs. These compounds were put in clinical trials phases I and II although their mechanism of action was not fully understood [51]. Interestingly, there is no disulfide bond in ADA which could lead to a loss of activity after reduction. The target of ADA was disclosed by Rice and collaborators as being the NC protein [52] even though these results were not confirmed in cell culture experiments (Berthoux L and Darlix JL, personal communication).

Mechanism of Zinc Ejection

Zinc ejectors, such as DIBA, were shown to penetrate into cells and virions [53]. The mechanism of zinc ejection was mainly studied using DIBAs, PATEs, N-ethylmaleimide (NEM) and SAMTs. As mentioned above, NCp7 zinc depletion begins at the level of the C-terminal ZF, which is consistent with its lower thermal stability and lower zinc affinity in comparison with the N-terminal ZF [27,32,54,55]. The nucleophilic attack of the cysteins (Cys39 and Cys 49) of the second ZF leads to a covalent modification of NCp7 (Fig. (**3**)) with the formation of either a disulfide bridge with DIBAs [43] or a thioester bond with SAMTs [48] or a thioether link with NEM [56]. This initial covalent link promotes the reaction of additional reactants with the other Cys residues of the same motif, and finally leads to zinc ejection and the loss of NCp7 folding. The Cys49 thiolate appears to be the major reaction site with DIBAs [40,43], PATES [57] and NEM [56] while Cys 39 seems to be the primary target of the SAMT analogues [58]. The reactivity of various electrophilic agents was found to depend on the ability of these agents to function as soft electrophiles [59]. Steric factors are also important since selective ligand binding regions within the conserved ZF are thought to promote the reaction. The presence of such regions in the ZFs is substantiated by the strong differences in the reactivity patterns to NEM between the native NCp7 and its isolated fingers [56]. In addition, several benzamides structurally related to DIBA failed to extrude the zinc from the ZFs [40,42,56] since they probably do not adopt the right conformation to fit the specific conformation of the putative binding pocket. Differences in the binding pockets may also explain the low sensitivity of large

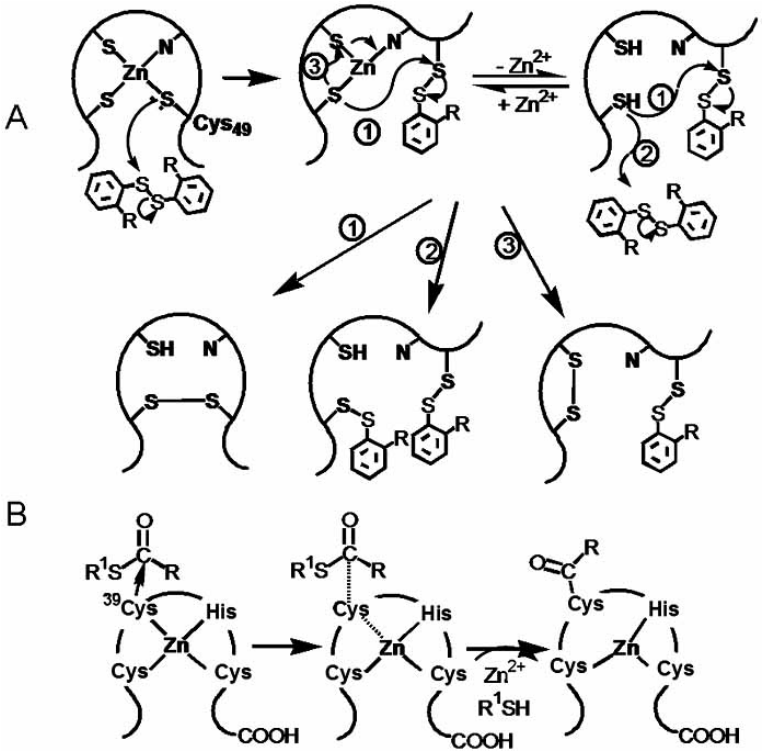


Fig. (3). Proposed mechanism of zinc ejection by DIBA (A) and SAMT (B).

Both zinc ejectors react with the Cys residues of the distal ZF of NC. Cys 49 and Cys 39 residues are the preferential targets for DIBA [43] and SAMT [48], respectively. Covalent reaction of both zinc ejectors with these Cys residues favors the reaction of additional zinc ejector molecules with the remaining Cys. These reactions decrease the zinc binding constant of the ZFs, leading to a release of zinc and NC unfolding.

nonviral zinc fingers to DIBAs [44,60]. However, the NC binding pockets have never been characterized due to the rapid structural changes undergone by the ZFs during the zinc removal process that follows binding of the zinc ejectors.

Are Zinc Ejectors Specific for Retroviral Zinc Fingers?

DIBA as well as dithiane ejectors were suggested to be unable to alter cellular ZF proteins [44,49,52,60]. However, this claim contrasts with the sensitivity of the ZFs of cellular transcription factors such as Sp1 and the steroid-binding glucocorticoid receptor (GR) to thiol-reducing agents [61,62]. This sensitivity towards reducing agents has prompted Wang *et al.* [63] to test DIBA derivatives on oestrogen-receptors in a breast cancer model. Interestingly, DIBA derivatives were found to inhibit the oestrogen receptor-mediated growth of breast cancer cells in a dose dependent manner and to decrease the binding of other nuclear receptors to their responsive elements [63]. Recently, various viral and cellular proteins have been challenged with thioesters [58]. The ZFs of GAGA and protein kinase C-delta as well as the C-terminal finger of mouse mammary tumor virus (MMTV) NCp9 were not sensitive to thioesters. In contrast, thioesters were able to efficiently eject zinc from the N-terminal ZF of NCp9, the CCHC motif of FOG-1 and both CCCC motifs of GATA-1. Taken together, these results indicate that the drug response does not depend on the origin of the ZF protein but more probably on the zinc accessibility and the ZF structure [34,58,64].

How do the Zinc Ejectors Block Virus Replication?

In vitro, zinc ejectors were tested on the complex formed between NCp7 and the genomic RNA packaging signal ψ. This specific signal is located in the 5' leader of the HIV-1 genome and composed of four stem-loops (SL1 to SL4). Specific interactions between NCp7 and the SLs govern genomic RNA selection, dimerization and packaging during virus assembly (Fig. (**1**)) [3,12,14,16,65-67]. Interestingly, DIBA, NOBA, SAMTs and ADA (Fig. (**1**)) can impair the binding of NCp7 to its nucleic acid targets [48]. When NCp7 was pre-incubated with various zinc ejectors, a reduction of NCp7 binding to the ψ RNA was observed [40,60]. In contrast, zinc ejection was reduced when zinc ejectors were incubated with a preformed NCp7-SL3 complex [48]. This protection likely results from the involvement of the distal ZF (which is the most susceptible to zinc ejection) in viral RNA recognition notably through the stacking of its Trp37 residue with the G residues of the RNA target [68-70]. Nevertheless, this protection depends on the nucleic acid sequence, probably reflecting different affinities of NCp7 for its targets and varying accessibilities of the reactive Cys in the nucleoprotein complexes [68-70].

The protection of NCp7 ZFs by RNA was also investigated with Acr37 NCp7, a derivative where Trp37 was substituted by an acridine (Acr) moiety. This derivative exhibits the same structure and biological activities as the wild type protein *in vitro* [71]. Addition of zinc to the apoform of Acr37 NCp7 induces a three fold decrease of the Acr fluo-

rescence [71]. In contrast, addition of 5 equivalents of DIBA-1 causes a time-dependent increase of Acr fluorescence (Fig. (**3**), curve A), which is fully consistent with a progressive oxidation of the distal ZF and zinc ejection [39]. Addition of the genomic RNA(1-415) region to the holoprotein in the absence of DIBA-1 results in a fluorescence decrease (Fig (**4**), curve B at t= 0), most probably due to the intercalation of the Acr moiety into the RNA chain. Interestingly, no significant fluorescence change was observed when DIBA-1 was added to the NCp7/RNA complex, indicating that NCp7 was protected by the RNA from oxidation by DIBA-1. To confirm this, sodium dodecylsulfate (SDS), a detergent which disrupts macromolecular complexes, was added. As expected, this restored the fluorescence level of the holoprotein. Thereafter, the fluorescence intensity increased smoothly, as in curve A. It thus appears that the effect of the zinc ejectors on viruses and infected cells is probably due to their interaction with free NC protein or Gag polyprotein, but does not target those molecules bound to their RNA or DNA partners.

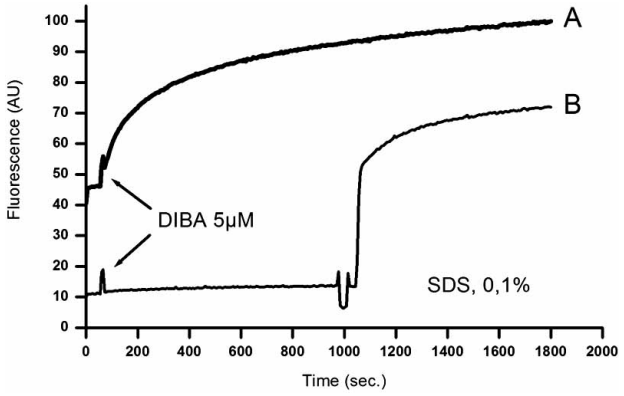


Fig. (4). Protection of NCp7 from DIBA-1 attack by HIV-1 RNA (1-415).

The protective effect of the RNA was evaluated by comparing the effect of DIBA on 1µM of Acr37-NCp7 alone (trace A) or complexed to HIV-1 RNA(1-415) (trace B). Addition of DIBA-1 (5 µM) to NC is indicated by the arrows. Addition of 0.1% SDS (final concentration) in trace B was carried out after 15 min. The SDS-induced dissociation of the protein/RNA complex leads to a sharp increase of the fluorescence due to the DIBA-induced zinc ejection.

In cellular assays, all zinc ejectors inhibit a wide range of HIV-1 isolates [53]. Since NCp7 as a free protein or in the context of Gag is required all along the virus replication cycle, it was interesting to evaluate which steps are affected by the zinc ejectors. Given the importance of the NC ZF structure for the specific packaging of genomic RNA, the effect of the zinc ejector on this recognition should have resulted in low amounts of genomic RNA packaged into newly formed particles [18,21,33]. Surprisingly, a significant amount of genomic RNA was found in the treated viruses [72]. This rather high encapsidation level of genomic RNA could be due to the role of the basic residues surrounding the zinc fingers in RNA recognition or to the protection afforded by the RNA from the attack by the zinc ejectors, as mentioned above [44,48,56,58,72]. This high level of genomic RNA in viruses could also be due to the stability of the first ZF apparently less sensitive than the second one to the attack by zinc ejectors, and which plays a more critical role than the distal ZF in genome packaging [11,16,21,31,43,48,56]. Interestingly, DIBA-1 also causes formation of heterogeneous populations of immature viral particles [72]. This likely results from a DIBA-induced alteration of the viral assembly pathway, in line with recent data showing that the C-terminus of Gag is essential for Gag assembly[73]. Despite a frozen-like core morphology, the surface of the virion keeps the wild type conformation with fusion properties similar to the wild-type virus [56,74,75] but proviral DNA synthesis was strongly impaired. This may result from the inability of oxidized NCp7 to chaperone initiation and elongation of cDNA synthesis [11,76]. Moreover, the oxidized NCp7 is probably unable to interact with RT, which should impair completion of a bona fide proviral DNA [77-79]. NCp7 is not the only protein targeted by DIBA, NEM or Aldrithiol-2 (2-AT) since the free sulfhydryl groups of the capsid protein can also be modified [80]. In contrast, the cysteins of the envelope glycoproteins form disulfide bridges, explaining their poor susceptibility to zinc ejectors. The complete inactivation of virus infectivity together with the preservation of the structure and functions of the viral envelope make these particles a potential attractive vaccine [56,81,82]. This approach was tested with the simian immunodeficiency virus (SIV), which is closely related to HIV-2 and infects monkeys. The AT-2-inactivated SIV was not infectious and elicited both humoral and cellular immune responses [83]. The resulting immunization facilitated effective containment of pathogenic homologous challenge viruses but failed to protect against heterologous SIV isolates.

In Vivo Activity of the Zinc Ejectors

In a chemotherapeutic approach, SAMT derivatives were shown to reduce the levels of infectious viruses in a murine transgenic model, where an integrated provirus was reactivated [47]. More recently, a SAMT derivative was used to treat SIV infected monkeys [84]. This derivative was well tolerated by all monkeys and did not alter the liver, kidney and immunological functions. It showed no clear effect on the virus load but significantly reduced the levels of infectious viruses in peripheral blood mononuclear cells. Thus, the antiviral potency of these zinc ejectors is mainly due to the production of non infectious particles rather than to a decrease in the viral production per se.

In the case of ADA, administration into mice was found to blunt their response to polyclonal T-cell activation induced by the injection of monoclonal antibody against CD3 and to delay rejection of skin allografts. These effects were related to the strong inhibition by ADA of the calcium mobilization machinery in T lymphocytes [85,86] and indicated that, due to its immunosuppressive properties, ADA could be used as a therapeutic agent in allograft rejection, autoimmune diseases and allergic disorders. ADA has also been used for the first, and to our knowledge unique, clinical trial using zinc ejectors. This clinical investigation mainly focused on the safety and tolerability of ADA in patients with advanced AIDS [86]. Preliminary results showed an increase in the number of CD4 cells similar to that obtained with other antiretroviral agents and a decrease in the viral load, but only in 1/3 of the treated patients. Unfortunately, the

ratio of infectious to non infectious particles was not determined in this assay. A combination with other antiretrovirals could be possible, but the renal toxicity of the biurea metabolite of ADA would prevent a combination with PR inhibitors that are nephrotoxic [87].

III. NON ZINC EJECTING NC BINDERS

The work carried out on zinc ejectors demonstrated a straightforward link between the loss of NCp7 activity and the virucidal effect. However, their limited specificity for HIV NC hampered their use in therapy. These results prompted the scientific community to identify compounds able to bind NCp7 but with no zinc ejecting property. To reach this aim, a first medium throughput screening [88] was developed to identify antagonists of the strong binding of NCp7 to oligonucleotides containing TG repeats [89,90]. To that end, a chemical library of approximately 2000 small molecules (the NCI Diversity Set) was screened.

From the 26 active inhibitors that were identified, five contained a xanthenyl ring structure (Table **1**). Further analysis of structurally related compounds led to the identification of tetrachlorogallein, which stoichiometrically binds NCp7 and exhibits a significant anti-HIV activity *in vitro* with an IC50 of about 20 μM. Nevertheless, the correlation was not absolute since several compounds were poor competitors for NC binding to the TG-rich oligonucleotide but exhibited good anti-HIV activity. All the active compounds contain a xanthene ring substituted by two hydroxyls in positions 4' and 5'. These two hydroxyls were found critical for binding to NCp7 and to provide protection against HIV infection. Molecular modelling predicted that these hydroxyl groups bind to the amide nitrogen of Gly35 with additional contacts at the carbonyl oxygens of Gly40 and Lys33 of NC (Fig. (**5**)). The same compounds were then tested for their ability to inhibit NCp7 chaperone properties on λ-DNA, taken as model sequences [91]. The more active compounds such as gallein were found to be active in the low nanomolar range. However, these compounds did not show any activity when added after DNA titration with NCp7, suggesting that they did not compete for NC binding with the large λ-DNA molecule and thus exhibited an antagonist effect against free NC only. It is thought that these compounds might interfere with NC's ability to stack its aromatic residues with the bases of its target nucleic acids. The proposed method based on λ-DNA molecules can be used to further refine the positive hits but, due to its limited speed, it cannot substitute for initial HTS assays.

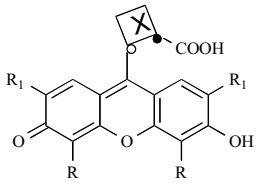
A new screening assay has recently been developed based on NCp7 ability to destabilize the HIV-1 transactivation response element in the form of cTAR, through an activation of end fraying [92-94]. This assay is thought to be highly specific since the NCp7-promoted destabilization is entirely mediated by the properly folded ZF domain [76]. As a consequence, a HTS based on this NCp7–promoted fraying should select compounds able to block specific interactions between the ZF domain and cTAR. The assay uses a cTAR molecule labeled at its 5' and 3' ends by a fluorophore and a quencher, respectively. In the closed form of cTAR, the emission of the fluorophore is quenched. Addition of NCp7 partly melts the stem, increasing the distance between the two dyes, thus causing a large fluorescence increase. Positive hits in this HTS are detected through the decrease of the fluorophore emission. This assay was used to screen an "in-house" chemical library of 5000 molecules [95]. About ten compounds with IC50 values in the low micromolar range have been selected (Mély *et al.*, unpublished). These compounds were shown to interact with NC but did not eject zinc. Interestingly, this HTS confirmed the importance of the hydroxyl groups in the positive hits. Though non zinc ejecting NC binders are potentially interesting, it is necessary to further understand their mechanism of action, improve their binding to NC and demonstrate their antiviral activity.

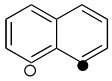
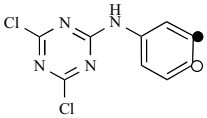
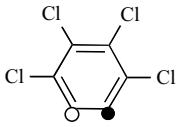
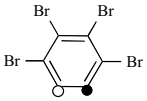
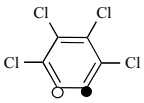
IV. NUCLEIC ACID INTERCALATORS AND BINDERS

Targeting the proper nucleic acids of pathogens is a classical way to fight bacterial infections. In fact, most antibiotics interact with bacterial ribosomal RNA rather than with proteins [96]. Through their binding to the bacterial rRNAs, antibiotics impede the interaction of the rRNAs with their cognate partners and facilitate miscoding during protein synthesis. The 5' untranslated leader of HIV-1 RNA is a multifunctional region composed of several SLs required for cDNA synthesis, viral translation and virus assembly (Fig. (**1**)) [66,67]. These SLs being unique to the virus and being engaged in interactions with NC represent targets in order to block NC functions [11,12,33]. In this context, actinomycin D was first identified [97-99]. Actinomycin D is a widely used anti-cancer drug that inhibits the DNA-dependent DNA/RNA synthesis through DNA intercalation and binding to the minor groove. Early reports showed that the replication of both RSV and MLV retroviruses was sensitive to this drug [100-102]. Actinomycin D was found to inhibit the DNA-dependent DNA synthesis catalyzed by RT, only at very high concentrations. In contrast, actinomycin D inhibits efficiently HIV-1 minus strand transfer during reverse transcription. In spite of its strong ability to promote minus-strand transfer NC cannot overcome the inhibitory effect of actinomycin D on the annealing of (-) SSDNA to the acceptor RNA. Since NC (but not RT) is required for efficient annealing, actinomycin D likely inhibits the minus-strand transfer by blocking the nucleic acid chaperone activity of NC. Nevertheless, the main problem of actinomycin D is that it interacts with a large range of nucleic acids, giving a poor specificity. As a consequence, a clinical use of such a molecule will likely generate critical side effects and the emergence of resistant strains as for bacteria [103].

More recently, mass spectroscopy was used to investigate the effects of a small library of nucleic acid binders on the interaction of NCp7 with the SL2, SL3 and SL4 structures of the specific ψ packaging signal [104]. This library included intercalators, minor groove-binders, mixed-mode intercalator/minor groove-binders, and multifunctional polycationic aminoglycosides. Only aminoglycosides were capable of dissociating preformed NC/SL3 and NC/SL4 complexes, but not NC/SL2 complexes. Among the aminoglycosides, a correlation was found between their binding constants to SL3 and SL4 and their ability to dissociate the NC/RNA complexes. Further studies revealed that the competition mechanism on SL3 and SL4 resulted from an extensive overlap of the aminoglycoside and NC binding sites [105]. Indeed, both

Table 1. Structure Activity Relationship of Xanthenyl Derivatives on the Inhibition of NCp7 Chaperone Properties



Name	X	R	R1	K_d (M) (SPR)	K_d (M) (Fl)	Antiviral Activity
Gallein		OH	H	ND	8×10^{-8}	A
119911/158917		OH	H	3.5×10^{-7}	5×10^{-8}	A
157411		OH	H	1.5×10^{-5}	I	A
122391		OH	H	1.1×10^{-5}	5×10^{-7}	A
119913		OH	H	3.8×10^{-5}	1×10^{-7}	A
378139		H	H	1.45×10^{-4}	5×10^{-8}	A
Tetrachlorogallein 723402		OH	H	ND	$>2.5\times10^{-6}$	A
Tetrabromogallein 119889		OH	H	2.5×10^{-7}	1×10^{-8}	A
Tetrachlorofluorescein		H	H	ND	$>8.5\times10^{-6}$	I
119910 Fluorescein		H	H	4.1×10^{-7}	1.5×10^{-6}	M
Eosin Y		Br	Br	ND	$>3.2\times10^{-6}$	I
Erytrosine B		I	I	ND	1×10^{-6}	I

K_d values have been measured by surface plasmon resonance (SPR) and fluorescence (Fl) measurements. In the first row are presented the names and/or the NSC numbers (http://dtp.nci.nih.gov) of the tested compounds. In the structure of the common scaffold (top of the table), X represents the connecting moiety between the xanthenyl ring and the carboxylic group. X maintains the carboxylic group over the xanthenyl cycle. The A, M, I abbreviations refer to the anti-HIV-1 activity, defined as the percentage of cells from the human T-cell line CEM-SS that survived after incubation with HIV-1_{RF} virus. The antiviral activity of these compounds was tested for protection against the HIV-1 induced cytopathic effects. Active (A) compounds provide more than 80% protection, moderate active compounds provide 50-80% protection, while inactive (I) compounds provide less than 50% protection. Data are from [88,91].

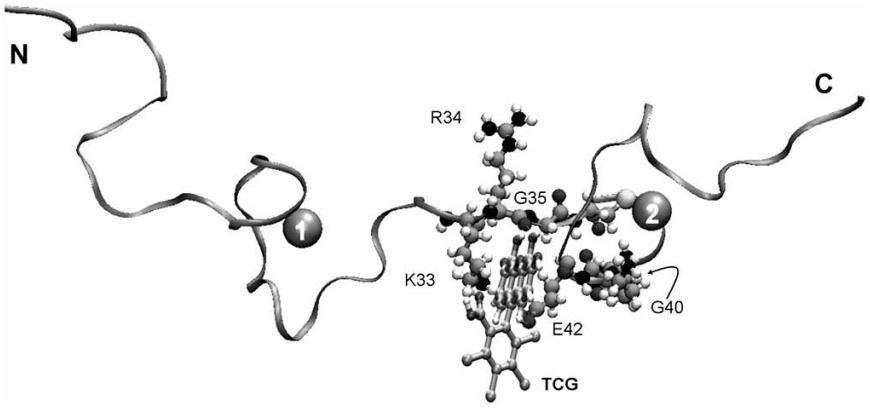


Fig. (5). Molecular modeling of the binding of tetracholorogallein to NCp7.

Tetrachlorogallein (TCG) is thought to bind to a putative binding pocket located between the basic ^{29}RAPRKK^{34} linker and the C-terminal ZF (2) of the native NC. Amino acids thought to interact with the tetrachlorogallein molecule are indicated [88].

the antibiotic and the protein were found to preferentially bind the upper stem and the loop of SL3 and SL4 [30,69, 106,107]. In contrast, the inability of the aminoglycosides to dissociate the NC/SL2 complexes resulted from the minimal overlap between the aminoglycoside binding site (in the middle of SL2 stem) with the NC binding site (at the SL2 loop). Importantly, aminoglycosides inhibit also the TAT-TAR complex [108], highlighting common features between HIV-1 NCp7 and TAT proteins [109]. This observation paves the way for the search of molecules targeting simultaneously these two essential proteins of HIV-1.

V. PEPTIDOMIMETICS

An alternate strategy to inhibit NCp7 functions is to design peptides that can directly compete with NCp7 for the binding to its RNA and DNA substrates. In this context, one approach is to design peptides mimicking part of the NC structure. NCp7 contains two ZFs held in close proximity by a short basic linker containing a Pro residue. The globular conformation of the ZF domain (Fig. (**6**)) is stabilised by hydrophobic and aromatic interactions between the two ZFs. Residues Val13, Phe16, Thr24 and Ala25 of the proximal finger and residues Trp37, Gln45 and Met46 of the distal finger form a hydrophobic plateau on the top of the folded fingers [32,110,111]. Interestingly, a similar hydrophobic plateau was also found for SIV NCp8 and MLV NCp10 [112,113]. As illustrated in the NC/SL3 complex (Fig. (**7**)), the role of this plateau is to provide specific hydrophobic and H-bonding interactions with the RNA or DNA bases and notably, to promote the stacking of Trp37 with a guanine residue [69,70,111,114,115] for recent review see [132]. This complex is further stabilised by electrostatic interactions with the flanking basic amino acids.

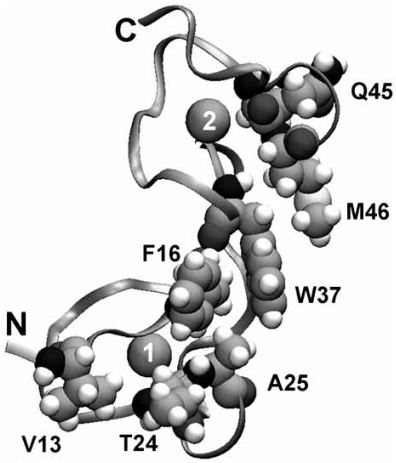


Fig. (6). Structure of the hydrophobic plateau of the NC zinc finger domain.

The polypeptide backbone of the ZF domain of NC adopts a globular structure in which the two ZFs are in close proximity (1 and 2). The peptide backbone is represented as a ribbon. The hydrophobic residues from the two fingers that form the hydrophobic plateau are indicated. This plateau is thought to be involved in the interaction with the DNA and RNA substrates of NC.

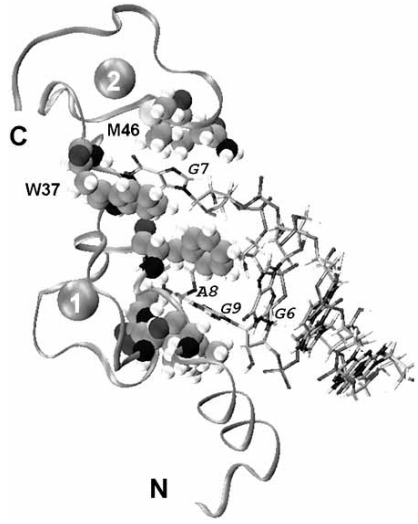


Fig. (7). NMR structure of the NCp7/SL3 complex.

The ZFs of the native NC protein bind the exposed G^6-G^7-A^8-G^9 residues of the SL3 loop. The complex is stabilized by interactions between the side chains of the hydrophobic plateau and the G^7 and G^9 residues of the loop. As illustrated here, the N-terminal region of NC adopts a 3_{10} helix [69]. Only the loop of SL3 and the top of its stem are indicated on the graph. The bases are indicated in italics. The peptide backbone is represented as a ribbon.

NC structure and hydrophobic plateau are remarkably conserved in all 3D structures of NCp7/oligonucleotide complexes that have been solved [69,70,111,114,115]. Moreover, modification or loss of the hydrophobic plateau through mutations of the two zinc fingers resulted in NC proteins with reduced binding specificity and chaperone properties [76, 89]. Interestingly, the same mutations resulted in a total loss of viral infectivity [18-20,32,97,116], indicating that the NC hydrophobic plateau plays a key role in HIV-1 replication and can thus be used as a structural basis for the design of specific NCp7 inhibitors without zinc ejection. Based on this concept, cyclic peptides mimicking the spatial orientation of Phe16 and Trp37 residues in the hydrophobic plateau have been designed [117]. To further strengthen the interaction of this peptide with the RNA and DNA substrates of NCp7, two basic residues thought to mimic Arg 26 and Arg 32 have been introduced into the cyclic peptide. The leader of this series of peptides was referred to as RB2121. An excellent overlap was found between the structure of RB2121 and the corresponding residues in the folded zinc fingers. In spite of its two Cys residues, RB2121 does not directly interact with NCp7 nor eject zinc. In contrast, RB2121 competes with NCp7 for its RNA and DNA substrates. This competition results in the inhibition of the NCp7-dependent annealing of the primer $tRNA_3^{Lys}$ to the HIV-1 PBS. Moreover, RB2121 also inhibits the interaction between NCp7 and RT while peptides without Trp are inactive [77,78]. Both inhibitory activities lead to a dose-dependent reduction in cDNA levels that may explain the observed inhibition of HIV-1 replication in infected CEM-4 cells. These results clearly indicate that small molecules mimicking structural determinants of NC can impair virus replication [118]. However, though RB2121 shows no cytotoxicity up to 150 μM, its IC50 is still high (about 30 μM), showing that its structure should be improved to augment its antiviral potential. Attempts are actually done to better mimic the NC hydrophobic plateau, in order to increase the activity of these peptidomimetics.

In a different approach, small peptides able to specifically recognize NCp7 RNA targets were identified using a phage-displayed library [119]. This technology provides a large diversity of peptides on the surface of filamentous phages [120]. This strategy has been used for selecting peptides that specifically recognize the ψ encapsidation sequence [119]. All selected peptides are composed of a cluster of Trp residues surrounded by basic residues and bind to the ψ sequence with micromolar affinities. The optimized peptide HKWPWW was found to adopt a symmetric cis-trans equilibrium at the level of the Pro residue where it is structured (Fig. (**8**)) [121]. These two conformations are characterised by a close proximity between Trp5 and 6 in the cis conformation and between Trp 3 and 5 in the trans conformation. The two conformations of the peptide bind both TAR and PBS sequences with low micromolar affinities, mainly through stacking interactions between the Trp residues and the oligonucleotide bases. Moreover, the HKWPWW peptide stabilizes the cTAR secondary structure and inhibits the NC-directed melting of the cTAR sequence. Interestingly, HKWPWW was found to inhibit RNA encapsidation and HIV-1 replication in cellular assays (Dietz *et al.*, submitted), probably due to a competition with NC to its target nucleic sequences. Like RB2121, HKWPWW probably partially mimics the NC hydrophobic plateau, notably the two aromatic residues of this plateau, confirming that the NC hydrophobic plateau is a promising starting point to develop agents with potent antiviral activities.

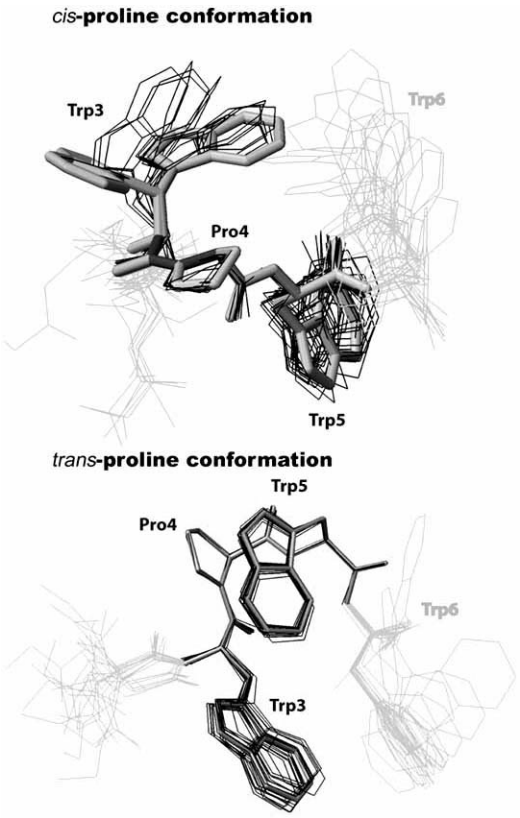


Fig. (8). NMR-derived structures of HKWPWW.

The HKWPWW peptide showed an equilibrium between *cis* and *trans* conformers around the central Pro residue. The *cis*-Pro conformation (top) exhibits a folded structure while the *trans*-Pro conformation (bottom) is more extended. In the *cis* conformation, Trp3 adopts two orientations while Pro4 and Trp5 can be superimposed. In the *trans* conformation, these three moieties adopt a unique conformation. In both conformations, Trp6 shows some preferred conformation and is therefore shaded. In contrast, the side chains of His1 and Lys2 are not represented since they do not exhibit any preferred conformation [121].

VI. APTAMERS

Though the specific recognition of HIV-1 ψ packaging signal by Gag *via* its NC domain has been firmly established [14-16,122,123], the reasons for this specificity are still poorly understood. Indeed, only modest differences in NC affinity were observed between the stem loops of the ψ sequence in comparison with control RNAs. Nevertheless, the direct link between the specific recognition by NC of this region and the production of infectious particles stimulated the search for small RNA aptamers with antiviral activity. Different libraries of RNA were produced using the SELEX method [124], generating aptamers of about 40 nucleotides in length [125,126]. Their secondary structures correspond to stem-loops in which the stems are rich in GC base pairs and the loops contain G and U residues. Several aptamers exhibit

a higher affinity (K_d in the low nM range) for NC than the ψ sequence itself and are thus able to fully abolish the binding of NC to ψ and TAR *in vitro*. The proof of concept to use an RNA aptamer as an inhibitor of NC function was assessed by cotransfecting a plasmid expressing the aptamer and one expressing the HIV-1 DNA. This resulted in a partial inhibition of genomic RNA packaging, without modifying the amount of genomic RNA within transfected cells. Thus, the aptamer probably interferes with the recognition of ψ by NCp7 in HIV-1 producing cells [127]. One possible explanation for this limited decrease of genomic RNA packaging into virions caused by NC-specific RNA aptamers could be that genomic RNA recruitment by the Gag polyprotein could also be under the control of the matrix protein (MA) at the Gag N-terminus. In fact, SELEX carried out using HIV-1 Gag polyprotein has clearly evidenced two classes of aptamers: those directed against NC and those against MA [128] . Binding of a NC ligand to Gag was unable to inhibit the binding of a MA ligand and vice-versa, suggesting that aptamers directed against NC can not prevent the binding of MA to the genomic RNA. More importantly, differences in the binding and chaperone properties of NC alone as compared with Gag-NC have been recently reported [129] Thus, one should be careful when using free NCp7 as opposed to Gag-NC to identify inhibitory ligands capable of disrupting Gag assembly and thus the formation of infectious HIV-1. Accordingly, the proof of concept for the use of aptamers as anti-NC agents still awaits full validation.

CONCLUSIONS

Due to its central role in both the early and late steps of the HIV-1 replication cycle, the nucleocapsid protein should be viewed as a major target for the development of new antiviral agents. This conclusion is further substantiated by the strong conservation of the central zinc finger domain of NC and the inability of the virus to escape from mutations that affect the proper folding of this ZF domain. The importance of the proper folding appears to be related to the role played by the hydrophobic residues at the top of the folded ZF domain. In fact, these residues form a hydrophobic plateau that is required for the proper recognition of the viral nucleic acids by NC and thus for NC functions. Nevertheless, NC exhibits several drawbacks that do not facilitate the search for antiviral agents directed against it. Indeed, NC has no enzymatic activity and therefore HTS assays are not easy to design. In this respect, one should keep in mind that all the drugs used in HAART are directed against the HIV enzymes, except for the T20 agent, which appears to be a moderate inhibitor as compared with anti-RT and anti-PR. Screening for anti-NCs is further complicated by the fact that the early and late functions of NC also rely on the basic residues flanking the ZFs. Due to its large number of basic residues, NC can bind a large array of DNA and RNA molecules in addition to the viral RNA and DNA molecules [11,130,131]. , Moreover, the extensive coating of the genomic RNA by about 1500 copies of NC in the viral particle and during the early steps of the viral cycle (Fig. (**1**); RTC) is thought to generate a very high local concentration of NC and consequently, would need a high local concentration of anti-NC. Last, during virus assembly, NC needs to selectively recruit the genomic RNA among a large excess of cellular RNAs. However, such a selective mechanism is carried out by the NC domain of Gag and not by the free NC protein and is still poorly understood. [129].

In spite of these difficulties, several strategies have already been designed to inhibit NC. Various classes of zinc ejectors have been developed. These molecules covalently react with the Cys ligands of the ZFs, causing zinc ejection and NC unfolding. These compounds are efficient and result in a loss of virus infectivity. However, they suffer from a lack of selectivity but may be promising in inactivating viruses for HIV vaccine trials. More recent strategies are directed at inhibiting the two main functions of NC, namely the chaperoning of RT during viral DNA synthesis and the specific recognition of the ψ packaging signal necessary during virus assembly [73]. These strategies include the use of small molecules (selected by screening), peptidomimetics, DNA binders and intercalators, as well as RNA aptamers. Though promising preliminary results have been obtained, more extensive studies are required to better understand their mechanism and structure activity relationships to improve their bioavailability and demonstrate their potential use in clinical trials against HIV-1 infection and pathogenesis.

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