The HIV-1 Nucleocapsid Zinc Finger Protein as a Target of Antiretroviral Therapy

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Abstract: Despite advances made in its therapeutic management, human immunodeficiency virus (HIV) infection has remained an intractable problem, and complete eradication of the virus an unrealized goal. Experience in the clinical application of combination therapy using a variety of reverse transcriptase and protease inhibitors have revealed a number of challenges, in spite of the observed albeit temporary success in reduction of patient viral loads. Problems with current protocols include poor patient compliance, and the presence of latent reservoirs of virus that ultimately result in the appearance of phenotypic resistance. These considerations necessitate continued research and development into alternative strategies to circumvent the aforementioned problems. One approach to minimizing and/or eliminating the appearance of escape mutants and latent viral reservoirs is the targeting of essential *and* mutationally intolerant enzymes such as the nucleocapsid protein, which contains two highly conserved zinc knuckles. Concerns have been raised regarding the targeting of this protein, since the ubiquitous occurrence of important mammalian zinc finger proteins implies that drug specificity towards the nucleocapsid protein may be difficult to attain. In this review, strong evidence supporting the hypothesis that this protein can be targeted to the exclusion of other cellular zinc finger proteins is presented. The effects of small molecule induced abrogation of nucleocapsid protein mediated activities, as well as efforts to develop nucleocapsid protein inhibitors as antiretrovirals are also discussed.

Key Words: Nucleocapsid, zinc knuckle, antiretrovirals, small molecule inhibitors, electrophiles.

INTRODUCTION

Currently, therapeutic management of HIV infection and pathogenesis is generally based upon administration of combination therapy with multiple inhibitors of the HIV type 1 (HIV-1) reverse transcriptase and protease [1]. Although this approach has generally resulted in significant suppression of viral loads in a substantial number of patients, the long-term treatment outlook is negatively impacted by a variety of issues including a high rate of drug protocol violations, drug toxicity, and the persistence of latent reservoirs of virus in long-lived populations of infected cells. Additionally, the extreme mutability of the virus, and the fact that fairly significant changes in the structures of key viral enzymes do not always translate into gross inefficiencies in their respective functions, renders combination therapy with a particular drug protocol eventually ineffectual in most cases.

These considerations imply that an appropriate target against which to develop anti-HIV, and by extension, other antiretroviral therapies, is a mutationally intolerant protein that plays essential *and* diverse roles in various phases of the viral replication cycle. A heretofore unexploited target that fits this general profile is the nucleocapsid protein of HIV-1. This small very basic and highly conserved protein participates in numerous obligate stages of the viral replication cycle, and mutations in its primary structure have been observed to result in the production of non-infectious virions. In this review, features of the viral replication cycle that are directly pertinent to an appreciation of the diversity of roles played by the nucleocapsid protein are highlighted, and the progress and challenges associated with the development of therapeutics directed against this zinc finger protein are discussed.

THE HIV-1 REPLICATION CYCLE: A GENERAL OVERVIEW

As has been observed for all orthoretroviruses, the HIV-1 replication cycle can be divided into “early” and “late” phases, and has been demonstrated to proceed through a series of steps that include viral attachment, fusion and uncoating, reverse transcription, integration (all early phase steps), RNA synthesis and processing, protein synthesis, virion assembly, viral budding, and virion maturation (late phase steps). In principle, all of these steps are potential targets for drug intervention. The proteins that mediate the required functions in the aforementioned steps (not including host cell derived enzymes that are recruited by the virus during the replication cycle as necessary) are encoded by three major coding domains within the RNA genome: *gag*, *gag-pro-pol*, and *env*. *Gag* encodes the internal structural proteins [namely, the matrix (MA), capsid (CA) and nucleocapsid (NC) proteins] that function in, among other things, the compartmentalization of viral components and maintenance of the morphology and structural integrity of the virus. *Gag-pol* encodes reverse transcriptase (RT) and integrase (IN) which are responsible for the transcription of viral RNA into viral DNA, and the integration of viral DNA into the host cell genome, respectively. The *pro* component

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of *gag-pro-pol* encodes the viral protease (PR). The *env* gene encodes surface and transmembrane protein components of the viral envelope that function in mediating adsorption to and penetration of the virus into susceptible cells. Once synthesized, the encoded Gag, Gag-pro-pol, and Env polypeptides are subjected to proteolytic cleavage to yield functional individual protein components [2]. For this reason, Gag, Gag-pro-pol and Env are referred to as polyprotein "precursors", or simply as precursors.

A mature virion is composed of shells of individual Gag proteins [3]: MA proteins form an icosahedral-like outer shell that lies just beneath the host cell derived lipid membrane, making contact with it *via* amino-terminal myristylated and positively charged segments. Encapsulated within the MA layer is a shell of CA proteins that form the cone-shaped capsid [3]. Enclosed in the capsid, at the center of the virus, is a complex comprised of NC proteins and two identical positive strands of genomic HIV RNA molecules (the RNA dimer) held together by a limited number of base pairs [3,4]. This complex is known as the nucleocapsid. Associated with the complex are smaller numbers of RT and IN molecules [3]. The capsid, together with the components it encloses, is referred to as the core. The exact location of the third enzyme in virions, PR, is not precisely known, but it is thought to be present both within and outside the nucleocapsid [3,4].

Gag proteins are subjected to many modifications both during and after their synthesis, the most dramatic of which is PR mediated proteolytic processing which occurs late in viral assembly, to generate the cleavage products [5]. The cleaved proteins are said to be "mature". Because proteolytic cleavage occurs late in assembly, during or after the last stages of budding, virions contain equimolar mixtures of the mature proteins [3]. According to older reports, there are approximately 1500-2000 molecules of each Gag protein in a virion, which together represent about three-quarters of the protein component of the virus [3]. Although this is a conventionally accepted number, the exact number of copies of Gag in a virion has not been definitively established [3].

The replication cycle begins with attachment of HIV to a cell receptor. Its envelope fuses with the cell membrane, releasing the matrix and core into the cytoplasm. Subsequent shedding of the matrix proteins leaves the core. While still in the cytoplasm, RT converts the viral RNA to viral DNA. The viral DNA is then translocated to the nucleus where it is inserted into the genome of the cell. This irreversible joining of virus DNA and host cell DNA is termed integration, and leaves the cell permanently infected.

The integrated proviral DNA is transcribed into full-length viral RNA, which is subsequently capped at the 5'-end and polyadenylated at the 3'-end by the cellular machinery [6]. This full-length RNA serves as the mRNA template for the ribosomal synthesis of the structural (Gag) and polymerase (Gag-pol) viral proteins [6]. Full-length viral RNA is also packaged into budding particles during virus assembly [6]. HIV-1 genome recognition is mediated by specific interactions between the nucleocpasid protein domain of the Gag polyprotein and a ~120 nucleotide (nt) segment (packaging signal) of the RNA located near the 5'-end of the genome known as the -site [6-11]. The -site contains four stem-loops (SL1-SL4) connected by relatively short linkers of 4 to 13 nucleotides, that possess overlapping and possibly redundant functions [6,7].

The export of viral RNA from the nucleus is followed by the synthesis of Gag, Gag-Pro-Pol and Env polyproteins. Subsequently, these polyproteins come together, along with two copies of viral RNA and tRNA primers, at a common site on the inner cell membrane to assemble viral particles [5]. It is the Gag protein, in groups of approximately 2000 interacting molecules, that alone provides the driving force for the release of uniform particles from the plasma membrane [5]. The viral particles bud from the cell surface becoming cloaked in cell membrane, which forms the fatty envelope for the virus. Concomitant with or shortly after budding, the nascent virions undergo a striking change in internal morphology, termed maturation, that results in the formation of an infectious particle. Intimately involved in this process are PR mediated proteolytic cleavage of the Gag and Gag-Pro-Pol enzymes, and a change in the conformation of the dimeric genomic RNA [5].

THE NUCLEOCAPSID PROTEIN: STRUCTURE AND FUNCTION

The nucleocapsid has as its most abundant constituent the NC (Fig. (**1**)). The NC is generated upon processing of the Gag polyprotein by the viral protease. It is a 55-amino acid, highly basic, nucleic acid binding protein that contains two copies of the conserved sequence $CX_2CX_4HX_4C$, where X is a conservatively substituted amino acid. This conserved sequence has been designated a CCHC motif [12] and termed a zinc finger, although it is also described as a zinc knuckle. The term knuckle derives from the fact that whereas the spacing between zinc coordinating residues in the most commonly observed zinc finger proteins varies from ~8 to as many as ~30 amino acids (thus giving rise to finger-like protrusions), the CCHC motif in the NC has amino acid spacers that are only two to four amino acids long. Thus, instead of 'fingers', one observes what may more appropriately be referred to as 'knuckles'. The CCHC motif in the NC binds Zn^{2+} with high affinity (K_a ~$10^{12} – 10^{14}$ M^{-1}) [13]. Its N- and C-terminal regions are rich in basic amino acids, with the protein containing 15 Arg and Lys residues in total. The 3D structure of the NC, which has been solved by NMR methods [14], reveals two zinc knuckles in close proximity separated by a flexible 7 amino acid linker (RAPRKKG), and poorly structured and flexible N- and C-terminal domains [6,14-17]. Indeed, the protein shows little recognizable secondary structure, its key constraints being largely associated with the metal sites themselves [18]. Although each of the two conserved NC zinc fingers share the same retroviral zinc finger motif, the core of the C-terminal finger is substantially more reactive than that of the N-terminal finger [19-21] with experiment [19-20] and theory [22] indicating that Cys49 is the most labile site of the NC protein [23].

Mutagenesis studies on virus infectivity have established the participation of the NC in multiple activities during both early (reverse transcription, integration) and late (protease processing and genomic RNA selection) stages of HIV

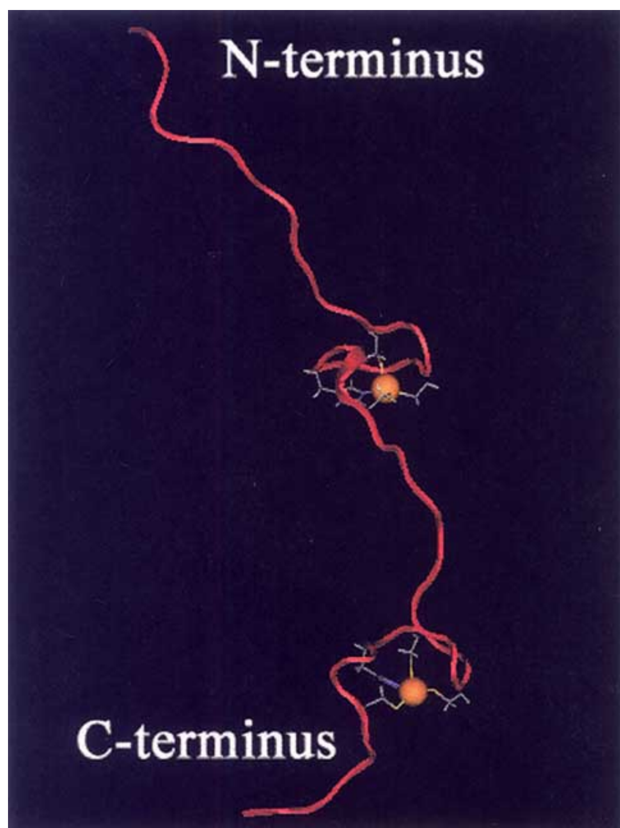


Fig. (1). The 3D structure[14] of NC rendered as a tube tracing. The orange spheres represent zinc, whilst the yellow and blue coordinating ligands represent cysteines and histidines respectively. The structure is based on the amino acid sequence H_2N-MQRGNFRNQRKIIK**C**FN**C**GKEG**H**IAKN**C***RAPRKRG***C**WK**C**G KEG**H**QMKD**C**TERQAN-COOH,[14] with the zinc coordinating ligands indicated in bold type and the amino acid linker between the two fingers indicated in italics.

replication. The structural integrity of the conserved CCHC arrays is important to virus replication, such that subtle modifications in or destruction of the zinc binding sites have the dramatic effect of inhibiting multiple phases of the viral replication cycle. Described herein are elements of the numerous and varied roles played by the NC in reverse transcription, integration, and genome recognition and packaging. The feasibility of targeting the NC is discussed, and the effects of inhibition of NC mediated activities are highlighted.

Involvement of the NC in Reverse Transcription

Reverse transcription—the reverse or "retro" flow of genetic information from RNA to DNA—is a hallmark of the viral replication cycle [24]. The process, which is catalyzed by RT, occurs in the cytoplasm *via* an intricate series of steps that ultimately result in the conversion of single stranded genomic RNA to a linear DNA duplex [24]. Two distinct enzymatic activities characteristic of RT that are essential to successful accomplishment of reverse transcription are DNA polymerase activity using an RNA or DNA template, and nuclease activity (termed RNase H activity) that is specific for the RNA strand of DNA/RNA duplexes [24].

As has been observed in all retroviral particles, the genomic RNA is actually present as a dimer that is composed of two identical positive strand genomic RNA molecules held together non-covalently by a limited number of base pair interactions [6,7]. The ends of the genomic RNA template contain various sequence elements important for reverse transcription. These include the direct repeats, termed R for "repeated sequences" that lie at the 5'- and 3'-ends of the RNA, and sequences that are unique to each end of the viral RNA and are adjacent and internal to the R sequences. These have been called U5 (for "unique to the 5'-end") and U3 (for unique to the 3'-end). Additional sequences include the primary binding site (PBS) and the polypurine tract (PPT), which are required to either position or become the RNA primers for minus and plus strand DNA synthesis [24]. All of these sequence elements are maintained, and in the case of U5 and U3, duplicated, in the viral DNA. The duplication of the U5 and U3 sequences that are found at both ends of the retroviral DNA are termed long terminal repeats (LTRs).

Synthesis of minus-strand DNA is initiated when the 3'-end of a partially unwound host-derived tRNA is annealed to the genomic RNA PBS as a primer. RT then synthesizes a single strand of minus DNA that is complementary to and extends from the PBS of the genomic RNA, to the LTR at its 5'-end. This generates a viral RNA-DNA duplex, the DNA component of which is termed minus-strand strong-stop DNA (-sssDNA). Since the PBS of the tRNA primer is near the 5'-end of the viral RNA, -sssDNA is relatively short, comprising 100-150 bases [4]. At this stage, continued synthesis of the complete complementary minus-strand DNA requires that the 3'-end of the genomic RNA serve as a template. This is accomplished through RNase H digestion of the RNA strand of the RNA/-sssDNA duplex and consequent dissociation of the tRNA/RT/ssDNA ternary complex from the HIV-RNA. The liberation of the ternary complex from the PBS end of the genomic RNA affords it the opportunity to then attach itself to the opposite (i.e. 3'-end) of the genome RNA strand *via* annealing of its R segment to the complementary R segment at the 3'-end of the genomic RNA. This attachment to the 3'-end is termed strand transfer, and in this case, is referred to as the 'first strand transfer'. Once the -sssDNA has been transferred to the 3'-end of the RNA genome, minus-strand DNA synthesis resumes, accompanied by RNase H digestion of the corresponding RNA template strand. When the PPT sequence, which is resistant to RNase H digestion, is reached, RT begins synthesis of a plus strand of DNA that complements the code of the previously synthesized minus-strand DNA, using the viral RNA PPT as a primer. This plus-strand DNA synthesis is halted after a portion of the primer tRNA is reverse-transcribed, to form plus-strand strong-stop DNA (+sssDNA) of discrete length. The copying of a portion of the primer tRNA produces a DNA copy of the PBS, or more accurately, a copy of the end of the tRNA used to initiate minus-strand DNA synthesis at the 3'-end of the +sssDNA. Through exercise of its RNase H activity, RT digests both the tRNA, which until this point was still

attached to the newly synthesized -sssDNA, and the PPT, which was used to prime the newly synthesized +sssDNA. Removal of the primer tRNA exposes sequences in the +sssDNA that are complementary to sequences at or near the 3'-end of the +sssDNA. The DNA then circularizes so that the complementary PBS segments in +sssDNA and -sssDNA can anneal. This constitutes the 'second strand transfer'. Elongation of plus and minus strands then continues. The DNA copy of the viral genome is completed when RT copies the plus and minus strands entirely. The final product is a blunt-ended linear duplex DNA.

The NC is known to play at least three distinct roles in the process of reverse transcription. Firstly, it facilitates annealing of the tRNA primer to the PBS of the viral RNA template [25-32]. Enhancement of tRNA/PBS annealing has been shown to occur with either mature NC, or NC in the context of the Gag precursor. Experiments with HIV-1 NC mutants and genomic RNA sequences have shown that although the zinc fingers are required for NC enhanced tRNA:viral RNA PBS annealing, the presence of NC basic residues is absolutely essential to maintenance of annealing enhancement ability [26,28,33-36]. A second role of the NC in reverse transcription is the facilitation of viral DNA synthesis through reduction of self-primed reverse transcription [37-48] or by enhancement of the efficiency and processivity (i.e. the ability of an enzyme to repetitively continue its catalytic function without dissociating from its substrate) of RT [42,49-55] during the synthesis of viral minus- and plus-strand DNA. *In vitro* experiments using purified components to reconstruct reverse transcriptase reactions have shown that strand transfer is extremely inefficient. This is due to the occurrence of competing intramolecular self-priming reactions along the RNA template that result in the formation of secondary structures that interfere with synthesis of high fidelity DNA copies of the viral RNA genome [37-38]. *In vitro* experiments using viral RNA have shown that the presence of NC substantially decreases the extent of self-priming, and results in consistent increases in the level of transfer from ~ 3% in the absence of NC to as much as 65% in its presence [4,37-44]. A third role of the NC in reverse transcription is in the promotion of template switching in RT reactions to yield full-length DNA products [37,40,42,52,56-58].

Facilitation by the NC of various reverse transcriptase catalyzed reactions, such as annealing and strand transfer, is mediated primarily by its well documented chaperone activity [59]. Thus, although the mechanistic details associated with the NC protein's ability to catalyze the rearrangement of nucleic acids into optimal thermodynamically stable base-paired conformations are still being worked out [60], it is clear that its highly basic structure is essential for this activity. In contrast, drastic alterations, or even elimination of the zinc fingers generally cause only a somewhat limited reduction in the chaperone activity of the protein [4,26,35,54,61].

The Importance of the NC in Integration

An obligatory step in the establishment of retroviral infection is the integration of reverse transcribed viral DNA into the host cell's genome [62-63]. Integration is mediated by retroviral integrase, and sequences derived from the LTRs flanking the proviral DNA. At the completion of its synthesis, the proviral DNA is a blunt-ended linear molecule whose termini, corresponding to the boundaries of the LTRs, are specified by the primers for plus- and minus-strand DNA synthesis [24]. The LTR sequences are specifically recognized by the retroviral IN [64]. *In vivo*, the proviral DNA becomes associated with both host and viral proteins to form a large preintegration complex (PIC) in which integration reactions are catalyzed [65-68]. In HIV-1, the PIC has been observed to be comprised of MA, RT, NC, viral protein (Vpr) and the cellular protein HMG-I(Y) [69-73]. There are indications that other cellular proteins may also be present in these complexes [73-74].

Shortly after completion of viral DNA synthesis and in association with the PIC, viral IN catalyzes the cleavage of a dinucleotide (adjacent to a highly conserved CA dinucleotide) from each 3'-end of the U3 and U5 viral DNA LTRs. This results in the formation of recessed $CA_{OH's}$ on the 3'-ends, and is thus termed 3'-processing, or end processing. These recessed 3'-hydroxyl groups provide the sites of attachment of the provirus to host DNA and thus ultimately define the ends of the integrated provirus. Concomitant with the formation of the recessed 3'-ends, a two nucleotide overhang at each complementary 5'-end of the substrate is produced. The 5'-end dinucleotide guides the formation of a stable IN-DNA complex, and it has been proposed that the formation of this intermediate may prevent the "primed" substrate from being diverted into potential non-productive side reactions prior to entry of the PIC into the nucleus [73,75]. After entry into the nucleus and association with host cell DNA, IN, in the context of the PIC, catalyzes a concerted reaction in which the 3'-OH groups at the viral DNA ends are used to attack phosphodiester bonds on opposite strands of the target DNA, at positions staggered by four to six bases in the 5' direction, and therefore on the same face of the double helix [76-79]. The end result is the joining of the 3'-ends of the processed proviral DNA to the 5'-ends on opposite strands of the target DNA. This strand transfer reaction results in the formation of an intermediate that has the 5'-ends of the viral DNA strands, and the 3'-ends of the target DNA strands remaining unjoined. This results in single stranded gaps in the target DNA that flank the 5'-ends of the viral DNA, and reflects the distance between the adjoining sites [73]. The gaps must be filled in, and the two nucleotide 5' overhangs of viral DNA removed, to restore the continuity of the host DNA [73]. Thus, the target sequence on either side of the integrated viral DNA is duplicated by a length that is characteristic of the virus (5 bp in HIV-1) [73]. This "gap repair" is presumed to be mediated by host cell enzymes.

A number of model systems have been developed to study IN catalyzed reactions *in vitro* [80-82]. Most of these utilize purified recombinant IN, synthetic linear retrovirus-like DNA donors, model DNA substrates, and a divalent cation such as Mn^{2+} or Mg^{2+}. Because of the low intracellular concentration of Mn^{2+} ($<10^{-7}$ M), it is generally assumed that the more abundant Mg^{2+} (intracellular concentration of ~ 10^{-3} M) is the relevant cofactor *in vivo* [73]. Results from early *in vitro* experiments showed that IN is both necessary

and sufficient for the catalysis of strand transfer. However experiments with PICs isolated from infected cells demonstrate a more efficient reaction, implying that some components or features of the *in vivo* PIC complex may enhance or modify the activity of IN [73]. Likely candidates include the nuclear protein HMG-I(Y), and the retroviral NC [71,83-84] (see below). Even so, it was recently demonstrated that HIV-1 IN can promote efficient concerted integration *in vitro* in the absence of cellular or viral cofactors. This result was a consequence of careful avoidance of high IN concentrations during its purification after synthesis by recombinant methods, and the use of Mg^{2+} ions and polyethylene glycol at high concentrations in the integration assay [68,85].

Despite these observations, a number of recent reports amply demonstrate that the NC is a required cofactor in integration. Mutations to the NC Zn^{2+} fingers prevent proper 3'-end processing *via* viral IN, such that even subtle mutations where Zn^{2+} binding and RNA packaging are preserved are catastrophic to viral replication [86]. Mutations in either or both of its zinc fingers (NC_{H23C} and/or NC_{H44C}) result in virions with wild-type levels of packaged viral RNA, but which are replication defective. The lack of replication was shown to be a consequence of reduction in the quantity and quality of viral DNA synthesized, and defects in integration [86]. It was also demonstrated that NC plays a role in stabilizing viral DNA produced during infection, and NC with intact zinc fingers was shown to be required for efficient removal of the 3'-terminal dinucleotides by IN *in vivo* [86].

It has recently been shown [68] that IN activation relies not only on the presence of intact NC zinc fingers, but also on its basic residues. NC lacking the zinc fingers binds DNA but only moderately stimulates strand transfer by IN, whereas the zinc finger domain binds DNA poorly and does not efficiently stimulate IN activity. However, the zinc finger domain can complement DNA binding to restore full activation of strand transfer by IN. The results imply that the basic residues and zinc fingers function together to stabilize IN and the retroviral LTRs to promote formation of a NC protein complex competent for integration. Additional elements of the supportive nature of the NC towards integration are its general activation of the process [83] and its stimulation of coupled joining *in vitro* [87], although the mechanism by which it facilitates this process remains to be defined. Again, as has been observed in reverse transcription, much of the involvement of the NC in integration is mediated by its chaperone activity [59].

Involvement of the NC in Genome Recognition and Packaging

Retroviral genome recognition occurs in the cytosol of infected cells *via* a complex process that is not yet well understood. Genome packaging is mediated primarily by interactions between the nucleocapsid domain of Gag and a ~80 – 150 [88-89] nt segment of the viral RNA known as the -site [8-11] which is located just upstream of the *gag* initiation codon [88,90-93]. Approximately 1500 – 4500 copies of Gag polyprotein assemble in the cytosol or at the inner cell membrane, and bud to form the immature virus particle [88-89]. Gag specifically incorporates two copies of the full length genomic RNA into budding virions, which are non-covalently linked near their 5'-end in mature particles. Genome selection is highly efficient, occurring in cytosols that contain at least 100-fold excess of non-viral and spliced viral RNAs [88,94].

Site directed mutagenesis experiments, chemical and enzymatic accessibility assays, and free energy minimization calculations indicate that the -site of the HIV-1 contains four stem-loops (SL1-SL4) connected by relatively short linkers, all of which are important for genome packaging [95-105]. These stem loops have independent and, in some cases, redundant or synergistic functions in genome selection [88,100,102-103,106]. Stem-loop SL1 contains the primary dimer initiation site (DIS), a GC-rich loop that is believed to promote dimerization through formation of a "kissing dimer" intermediate [88,107-109]. The NC is capable of catalyzing the conversion of the kissing form of SL1 to the more stable duplex form. Since it has yet to be determined conclusively if dimerization occurs prior or subsequent to binding by Gag, it is possible that NC catalyzed duplex formation might be important for packaging [88]. Takahashi and coworkers have demonstrated that the upper stem (and native GC-rich loop) of SL1 readily forms a duplex at 37 ^{0}C, whereas native SL1 requires incubation at 55 ^{0}C or treatment with NC to convert the initially formed kissing species (which is stable at 37 ^{0}C) to the duplex [88,110]. In general, retroviral DIS sites overlap with, or are adjacent to the -site, which may facilitate packaging of two copies of the genome during virus assembly [61,88,94]. In this regard, studies of several retroviruses indicate that dimerization and packaging functions are intimately linked [88,111-114]. Stem-loop SL2, which has been shown to bind the NC with high affinity [K_d = 110 (±50) nM] [6] contains the primary cleavage site for mRNA splicing (splice donor site, SD) and the overlap of this signal with the -site appears to provide a mechanism for the selective packaging of the unspliced genome [6,88,102,111]. Stem-loop SL3, the most highly conserved of the RNA stem-loops [115] also binds NC with high affinity [K_d = 170 (±65) nM] [88,101,115,116] and is the only stem-loop that has been demonstrated to be independently capable of promoting RNA packaging [89,115,117]. Some features of the NC-SL2 complex are similar to those observed for NC binding to SL3. For example, the zinc knuckles bind to exposed guanosine bases of the tetraloop, and the N-terminal tail forms a 3_{10} helix that packs against the N-terminal zinc knuckle. However, other features of the NC-SL2 structure differ significantly from those observed for NC-SL3, including the binding of the N-terminal zinc knuckle to the minor groove and base triple platform in NC-SL2, which contrasts with major groove binding of the N-terminal zinc knuckle and 3_{10} helix in the NC-SL3 structure. In contrast, SL4, which contains the *gag* initiation codon, binds to NC with only weak affinity [i.e. K_d = 47 ± 14 μM] [105]. NMR studies indicate that the GAGA tetraloop of SL4 adopts a classical GNRA-type fold (R = purine, N = G, C, A or U), a motif that stabilizes RNA-RNA tertiary interactions in other systems [105,118-120]. Numerous examples exist from phylogenetic, biochemical, and 3D structural studies, in which GNRA tetraloops stabilize the global RNA fold by forming hydrogen bonds

with nucleotides in helical and stem-loop RNA substructures [105,121-126]. This fact, along with the observation of weak binding between the NC and SL4, has led to the proposal that SL4 may serve a similar role in genome packaging, functioning not by binding to NC, but instead by stabilizing the tertiary structure of the -site [105]. It is therefore apparent that the HIV-1 NC can bind to various RNA targets in an adaptive manner *via* different subsets of inter- and intramolecular interactions [105]. These findings, combined with the existing molecular biological and biochemical data, have been used by Summers [105] to derive a model for the early stages of HIV-genome recognition and packaging. In this model, SL1 forms a duplex that stabilizes the dimeric form of the genome, and SL4 participates in additional RNA-RNA interactions that stabilize the tertiary structure of the -site. This folding leads to exposure of the SL2 and SL3 tetraloops for binding to Gag. In this model, the recognition complex comprises four Gag molecules bound tightly to a dimeric -site. Weaker and non-specific NC-RNA interactions involving other NC domains occur as additional Gag molecules assemble to form the budding particle [105].

THE FEASIBILITY OF TARGETING THE NC

The importance of the NC in various early and late stages of the virus infection cycle implies that inhibiting its functions would disrupt key steps at several stages of the cycle in a cascading fashion that might ultimately be expected to abrogate virus replication and infectivity. This consideration would seem to make the NC a prime target against which antiretrovirals can be developed. However, in this regard, an important concern is the perceived likelihood that compounds (particularly small molecules) that might inhibit NC mediated activities through covalent modification of the protein, will exhibit cross reactivity with numerous other indispensable host cell zinc finger proteins, thereby undermining cell viability. Thus, unlike RT and IN, for which there are no known equivalents in mammalian cells, zinc finger proteins are ubiquitous, which, in principle, makes the development of NC inhibitors that are specific for the NC more elusive. Consequently, the question of the feasibility of efforts directed towards development of NC inhibitors arises.

In addressing this issue, it is important to consider the variety and general characteristics of known zinc finger configurations, relative to that of the NC, and to ascertain whether any observed differences can be exploited to achieve specificity. Zinc fingers are small metal-binding peptide units that are most often observed to function in promoting specific protein-nucleic acid binding and other recognition events, and in some cases mediating protein-protein interactions as well [23,127-129]. A number of zinc binding cysteine rich motifs are known. Two of these, the common CCHH- and CCCC-type motifs, are most often observed in cellular transcription factors and steroid hormone receptor proteins [124,130-131]. A third type, the CCHC motif, is found in retroviral nucleocapsid proteins, but is otherwise relatively uncommon in eukaryotic DNA binding proteins. Systems in which it has been observed include the dynein-associated proteins that regulate embryonic development in Drosophilia, poly(ADP-ribose) polymerase [132], p53, and restin, a cytoplasmic protein observed in Reed-Stenberg cells of Hodgkin disease [133]. The zinc cores are crucial to the stability and arrangement of local protein secondary structure, and it is well established that Zn^{2+} coordination is necessary for optimal protein functioning in most cases. Although the zinc finger motif is commonly observed in a diverse set of mammalian proteins, their zinc finger defining cores are usually different from that observed in the NC.

In contrast to a variety of enzymatic zinc cores where Zn^{2+} chemically functions as a Lewis acid in catalysis [23,134-136], zinc finger arrays are usually regarded as structural elements within proteins [23]. However, these cores can be chemically active since their nucleophilic Cys thiolates are potentially vulnerable to oxidation, as has been observed for some zinc fingers containing CCHH [23,137-141], CCHC [19-23,137,140,142-143], and CCCC [137, 144,147] cores. Increasing evidence suggests that some zinc fingers are predisposed to oxidation, possibly as a means of redox or metallothionein (MT) regulation of their function, while others are relatively inert, having fixed structural functions [23,138-139,141,148-149].

Although the inherent nucleophilicity of zinc finger cores may follow predictable reactivity trends, it is clear that the actual reactivity of zinc finger arrays within proteins is modulated to a large extent by the surrounding protein environment. Results of experiments by Wilker and Lippard [150] indicate that the reactivities of “bare” zinc finger cores vary according to the ratio 1000 (CCCC):100 (CCHC):1 (CCHH). This follows a decreasing nucleophilicity trend, if one assumes net ionic charges of –2, –1 and 0 for CCCC, CCHC and CCHH zinc finger cores respectively [23], and represents the reactivity order that would be expected to hold true in the absence of other external influences. Thus, kinetic studies of model zinc finger cores, composed of mixed complexes of phenylthiolate (PhS^-) and 1-methylimidazole (MeIm), show that the rate of methylation of thiolate does in fact follow the trend $[Zn(PhS)_4]^{2-} > [Zn(PhS)_3(MeIm)]^- >> [Zn(PhS)_2(MeIm)_2]$ [150]. “Bare” cores, however, are not found in nature [23]. Accordingly, the relative reactivity of cores with the same coordination motif cannot be predicted *a priori*, and must be evaluated on an individual basis, in the context of their surrounding protein environment. Additionally, it has been postulated that structural zinc finger motifs have evolved in a manner that has promoted steric and electronic shielding of their potentially reactive core thiolates, particularly in the case of highly anionic cores [23]. Therefore, zinc fingers that utilize increasingly anionic (reactive) core motifs may exhibit a concomitant increase in the protein screening of their cores, which can be viewed as a consequence of the preservation of electroneutrality [23]. In contrast, anionic cores lacking protein screening may signify zinc fingers particularly vulnerable to oxidation, possibly because their functionality is redox or MT regulated [23].

In light of these factors, the results of a recent study of the relative chemical stability of 207 zinc finger cores representing CCCC, CCHC and CCHH arrays is of interest [23]. The study was conducted to test the hypothesis that

steric and electrostatic screening of zinc cores confers resistance to oxidation. In support of this supposition, a large increase in steric and electrostatic screening was observed to correlate with increasingly anionic (reactive) core motifs, suggesting that structural zinc fingers have evolved such that their potentially reactive zinc core thiolates are protected by the protein [23]. The predominant structural mechanism by which zinc finger cores were screened was through networks of backbone:core NH-S hydrogen bonds which, according to the results of density functional theory (DFT) and *ab initio* calculations, substantially stabilize core thiolates [23]. Also observed were an enhanced network of core NH-S hydrogen bonds associated with anionic cores, and a number of highly conserved [Arg,Lys]:[Cys_3His] interactions [23]. The results indicated that modulation of the inherent reactivity of bare cores by various steric and electrostatic interactions results in a wide diversity of core environments that may reflect differences in zinc finger functionality. Thus, for example, in a case where a CCHH core is less sterically and electrostatically screened than a CCCC core, the former would be predicted to be more labile, even though a bare CCHH core would be expected to be less reactive than a bare CCCC core. These computationally determined predictions are in alignment with experimental results. Huang *et al*. [137] have demonstrated that some electrophilic agents selectively modify HIV-1 NC protein CCHC cores, thereby disrupting HIV replication, without inhibiting cellular zinc finger proteins containing CCCC (GATA-1) or CCHH (Sp1) cores. The calculations also predicted that the steric and electrostatic screening of the N-terminal NC zinc finger would be 1.5 times greater than that of the C-terminal core [23], consistent with experiments demonstrating the N-terminal finger to be substantially less reactive than the C-terminal finger [19-21]. The CCCC core of GATA-1 was determined to be 1.5 and 2.8 times more sterically and electrostatically screened, respectively, than the HIV-1 C-terminal NC core [23]. Experimentally, GATA-1 has been observed to be much less vulnerable to electrophiles than HIV-1 NC [137], even though a bare CCCC core would be expected to be an order of magnitude more reactive than a bare CCHC core [23,137]. Compared with other zinc fingers in the study, the core of the C-terminal NC zinc finger was more exposed, particularly the Cys49 thiolate (S49). Experimentally, Cys49 is observed to be the most reactive cysteine of NC [19-20], and theoretically, S49 is predicted to be the most nucleophilic site [22,137].

The computationally and experimentally observed differences in reactivity between retroviral CCHC versus transcription factor CCHH and steroid receptor CCCC zinc finger arrays suggests that compounds that are able to attack HIV-1 NC with enhanced reactivity may possess weak activity against other cellular targets [151]. The results suggest that this reduced level of cross-reactivity could result either from steric protection of their cysteine groups, or their intrinsically lower reactivity, compared with the retroviral NC cluster environment [151]. Under such circumstances, destruction of the zinc binding sites by immediate attacks of electrophiles in particular, could dramatically affect the functionality of the HIV-1 NC. Indeed, a number of chemotypes have been identified that effectively abrogate NC mediated activities through covalent modification of the protein, without impairing the activities of other zinc finger containing proteins. These findings are discussed below.

INHIBITION OF NC MEDIATED ACTIVITIES THROUGH COVALENT MODIFICATION OF THE PROTEIN

Susceptibility of NC zinc fingers to chemical modification was first demonstrated with C-nitroso containing antiviral agents. Specifically, 3-nitrosobenzamide and 6-nitroso-1,2-benzopyrone [NOBA and NOBP, compounds (**1**) and (**2**) respectively, Fig. (**2**)] were shown to inhibit infection of HIV-1 in human lymphocytes and also to eject zinc from isolated HIV-1 nucleocapsid zinc fingers and intact virions [142]. Both compounds were observed to react stoichiometrically with an 18-residue peptide with a sequence corresponding to the N-terminal zinc finger domain of the HIV-1 NC, with concomitant loss of zinc, although zinc ejection occured at a 10-fold faster rate for NOBA than for NOBP [142]. Importantly, the addition of NOBA or NOBP to a complex of the peptide and a synthetic 5-residue oligonucleotide with a sequence corresponding to a region of the HIV-1 -packaging signal, d(ACGCC), resulted in ejection of zinc and dissociation of the complex [142].

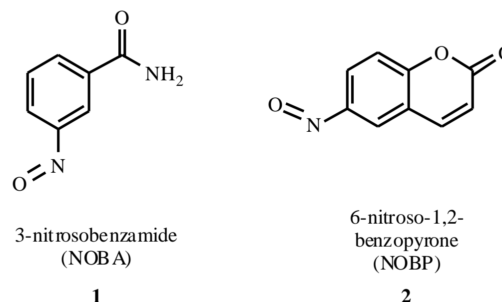


Fig. (2). The structures of C-nitroso benzamides demonstrated to inhibit HIV-1 infection in human lymphocytes, and to eject zinc from NC zinc knuckles.

Subsequent studies on the mechanism of action of NOBA revealed that its anti-HIV-1 infectivity was a consequence of inhibition of reverse transcription, albeit without direct impairment of RT [152]. The binding of HIV-1 to human lymphocytes was unaffected by NOBA, indicating that its action was not mediated by interruption of virus-host cell attachment mechanisms [152]. NOBA was also found to be unreactive towards two DNA binding enzymes, topoisomerases I and II, as well as IN and PR. Interestingly, both IN and topoisomerase I are zinc finger containing enzymes with zinc binding motifs of the classical CCHH type [152]. The failure of NOBA to react with either of these enzymes at concentrations at which zinc ejection was promoted demonstrates that NOBA specifically attacks the retroviral CCHC zinc finger configuration, and shows that some level of specificity for this motif is achievable. Although the demonstration of the specificity of NOBA towards NC zinc fingers served to prove the principle, its further development as a therapeutic agent was stymied by its inherent cellular toxicity.

Thus, armed with the knowledge that the NC zinc fingers can be selectively targeted by appropriate electrophiles, the

focus shifted to the discovery of other electron deficient species that might manifest diminished toxicity while at the same time exhibiting NC inhibitor capacity. Towards this effort, a series of electrophilic disulfide derivatives spanning a wide range of chemotypes were tested as part of the National Cancer Institute's (NCI) screening program for activity against HIV-1. The first of these that were shown to demonstrate wide-spectrum antiretroviral activity in cell culture at low micromolar and submicromolar concentrations, and to inhibit HIV-1 clinical isolates, drug resistant HIV-1 strains, and simian immunodeficiency virus (SIV), were the disulfide-substituted benzamides (DIBAs) [21]. Molecular structures of 5 of the DIBA type compounds are shown in Fig. (**3**). DIBA-1 (**3**) and DIBA-2 (**4**) are closely related congeners differing by only a single acetyl group, and DIBA-3 (**5**) is a low molecular weight derivative of DIBA-1 (i.e. compound **3**) [153]. DIBA-4 (**6**) is a congener of DIBA-1 (**3**) in which the p-amino phenylsulfonamide moiety has been replaced with a DL-isoleucine residue, and DIBA-5 (**7**) is a para-para positional isomer of DIBA-1 (**3**) in which the spatial relationship between the disulfide and benzamide has been modified [153]. The compounds were observed to inactivate cell-free virions, inhibit acute and chronic infections, and exhibit broad antiretroviral activity. Additionally, the compounds were highly synergistic with other antiviral agents, and the emergence of resistant mutants was never detected [153].

A number of studies [54-157] have shown that DIBAs act by attacking the two zinc fingers of HIV NC to eject zinc. Structurally similar DIBAs that do not inhibit HIV-1 in culture do not eject zinc, nor do analogues of the antiviral compounds where the disulfide moiety is replaced with a methylene sulfide [144]. The kinetics of NC zinc ejection by DIBAs were found to be nonsaturable and biexponential, with the rate of ejection from the C-terminal zinc finger being 7-fold faster than that from the N-terminal finger [21]. *In vitro*, antiviral DIBAs were also observed to inhibit zinc-dependent binding of NC to HIV RNA, as studied by gel shift assays, and this observation correlated well with the zinc ejection data. Other mechanistic studies of DIBA action have demonstrated that NC zinc ejection is accompanied by formation of covalent complexes between disulfide benzamide monomers and cysteine residues of the zinc depleted viral protein [154]. It was also observed that the disulfide benzamides rapidly cyclize (Fig. (**4**)) to form benzoisothiazolones under typical assay conditions (aqueous, neutral pH) and that benzoisothiazolones possess *in vitro* cellular activity similar to their disulfide benzamide precursors [154-157].

These early successes in the identification of disulfide containing compounds that mediate covalent modification of the NC with simultaneous loss of zinc and resultant loss of virus infectivity led to the initiation of a broader survey of

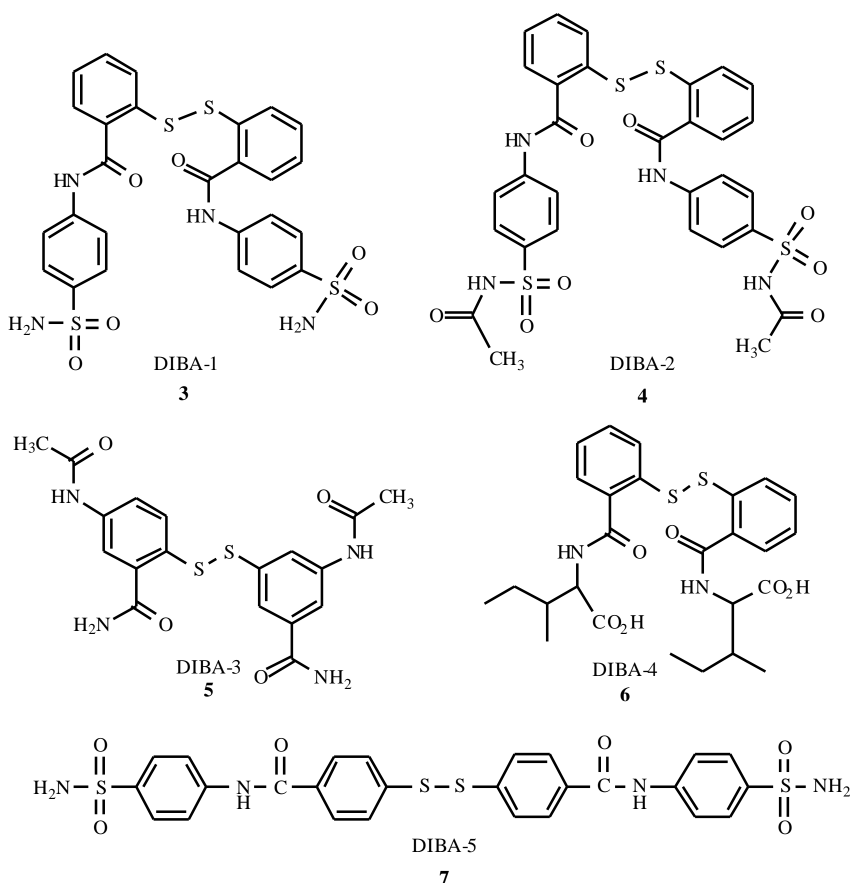


Fig. (3). The structures of disulfide substituted benzamides shown to exhibit wide-spectrum antiretroviral activity.

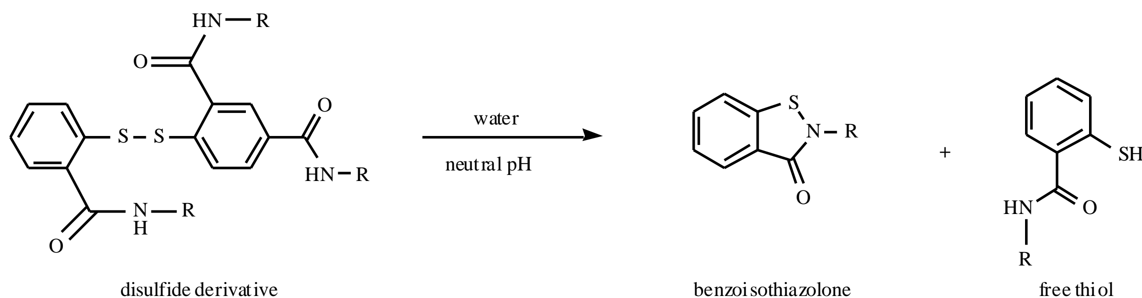


Fig. (4). Cyclization of o-substituted disulfide benzamides to benzoisothiazolones in aqueous solution at neutral pH.

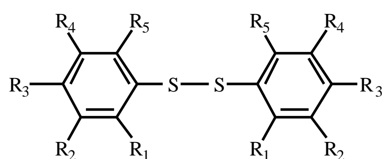
other chemotypes possessive of similar characteristics. Among the disulfide compounds surveyed were various aminobenzene derivatized aromatic disulfides, aliphatic disulfides, non-symmetrical thiurams, aromatic disulfoxides, aromatic disulfones, aromatic thiosulfonates and nonaromatic thiosulfonates (Fig. (**5**)). Because many disulfide compounds are toxic to proliferating cells, the experimental compounds were initially evaluated for anti-HIV-1 activity with a cell-based viral replication assay that made use of the proliferating CEM-SS T-cell line [158]. This screening yielded 24 candidate compounds. These compounds were then tested for anti-HIV-1 activity in non-proliferating fresh human peripheral blood monocyte/macrophage cultures, and for their ability to react with purified HIV-1 NC to effect zinc release. Those compounds scoring active by all 3 criteria were then evaluated against various HIV-1 molecular targets to ensure target specificity. Compounds found not to inhibit HIV-1 replication in the monocyte/macrophage model of HIV-1 infection were considered to be inactive. Compounds demonstrating anti-HIV-1 activity in both models but having relatively low therapeutic indices in either model were considered as moderately active. Inactive and moderately active compounds were not pursued further due to insufficient antiviral activity, even though many of these compounds reacted rapidly with NC zinc fingers [158].

These efforts resulted in the discovery of two aromatic disulfides (Fig. (**6**)) that demonstrated concentration dependent *in vitro* zinc ejection from purified NC, but did not inhibit any of the other classical anti-HIV-1 targets tested. Additionally, molecular modeling studies identified two high affinity binding sites (distal and proximal) on the N-terminal finger of the NC for both molecules [158]. A significant observation in these studies was that moderate antiviral activity correlated with the presence of electron withdrawing groups (EWG). For example, it was observed that simple unsubstituted aminobenzene disulfides were inactive, whether or not the amine group was located in the ortho or para position. However, the presence of an EWG such as CO_2H, SO_2NH, or NO_2 on the aminobenzene backbone yielded compounds with anti-HIV activity. This result is not surprising, given that the presence of an EWG would be expected to increase the electrophilicity at the reacting sulfur atom.

In the study cited immediately above [158], it was observed that overall, the aliphatic and asymmetric disulfides were inactive. The thiuram disulfides were observed to be too toxic to the proliferating T-cell cultures for there to be an observable antiviral effect. Additionally, the thiurams were inactive against HIV-1 in non-proliferating monocyte/ macro-cultures, even though a number of them reacted *in vitro* with the zinc fingers of purified NC. Of the oxygenated organosulfur derivatives tested, (disulfoxides, disulfones and thiosulfones), a number of thiosulfones demonstrated moderate anti-HIV activity in the T-cell assay [158].

Thus, through a variety of intensive drug discovery programs, other compounds, such as the thiosulfonate 1,2-dithiolane-4,5-diol-1,1-dioxide [compound (**8**)] [159-160] and azodicarboxamide [ADA; compound (**9**)] [161-162] (Fig. (**7**)) have been identified as having antiretroviral characteristics mediated through interaction with NC zinc fingers. Collectively, the results of these studies implied that drugs that are in current clinical use that contain the electrophilic disulfide moiety might, in fact, demonstrate anti-HIV-1 infectivity characteristics mediated through oxidative modification of NC zinc fingers. These include disulfiram **10** (also known as antabuse or tetraethyl thiuram disulfide, an FDA approved drug that is used to treat alcoholism), thiamine disulfide **12**, and cystamine **11** (Fig. (**7**)) [163]. Indeed, a number of compounds possessing electrophilic functional groups that might react with the metal coordinating sulfur atoms in the retroviral zinc fingers to cause zinc ejection have been shown to inhibit HIV-1 replication by ill defined mechanisms [163]. Subsequent ^{1}H NMR studies revealed that these compounds readily eject zinc from synthetic peptides with sequences corresponding to the HIV-1 NC zinc fingers, as well as from intact HIV-1 NC [163]. In contrast, the reduced forms of **10** and **11** were found to be ineffective at zinc ejection [163]. Additional studies with HIV-1 infected human T-cells in monocyte/macrophage cultures revealed these compounds to possess significant antiviral properties at non-toxic concentrations. Furthermore, mechanistic studies have revealed that the compounds inhibited neither attachment of HIV-1 to host target cells, nor the enzymatic activities of HIV-1 RT or PR [155].

A rationale for the hypothesis that anti-HIV agents can selectively target the NC zinc fingers to the exclusion of other cellular zinc finger containing proteins, based on the results of biochemical and computational experiments, has been advanced. Four compounds representing different electrophilic chemotypes (i.e. NOBA [compound (**1**)] as a representative of C-nitroso compounds, DIBA-1 [compound (**3**)] as a representative of disulfide benzamides, thiosulfonate **8** as a representative of dithioheterocyclic compounds and

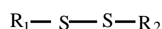


symmetrical and asymmetrical substituted phenyl disulfides

where R_1 through R_5 represent varying substituents including -H, $-NH_2$, $-NHSO_2R$, -NHC(O) R, $-NHC(NH_2)=NH$, -NCH(O)R, -NCH-R, -OH, -OMe, -Me, $-CH_2Ph$, -*t*-Bu, $-CH_2CO_2$, -OC(O)NHMe, -Cl, -Br, $-CO_2Me$, $-CO_2H$, $-SO_2Me$, $-SO_2NH_2$, $-CF_3$, $-NO_2$, -C(O)Ph, $-C(O)NHNH_2$, among other substituents

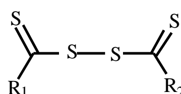
symmetrical substituted aryl disulfides

where aryl represents naphthyl, quinoyl, thiazole, benzothiazole, benzimidazoles, benzonitriles, pyridines and pyrimidine substituents.



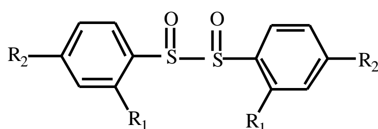
symmetrical and asymmetrical disulfides

where R_1 and R_2 represent -Me, -Pr, -t-Bu, $-CH_2Ph$, $-(CH_2)_5Me$, -C(O)Ph, $-CH_2C(O)NH_2$, $-CH_2C(O)Ph$, 2-pyridinyl, -Ph, among other substitutents



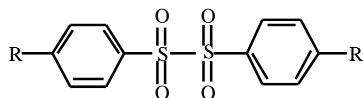
symmetrical and asymmetrical thiuram disulfides

where R_1 and R_2 represent -OMe, -OEt, -OBu, -O-i-Pr, -OBu, -OPh, $-N(Et)_2$, $-N(Me)_2$, -NHEtOH, $-NHNH_2$, $-N(CH_2Ph)_2$, among other substituents



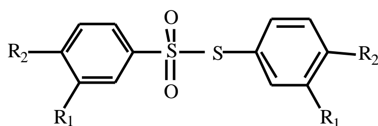
symmetrical aromatic disulfoxides

where R_1 and R_2 represent -H, -Me, -OH, or -Cl.



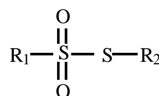
symmetrical aromatic disulfones

where R represents -H, -Me, -OH, or -Cl.



symmetrical aromatic thiosulfonates

where R_1 and R_2 represent -H, -Me, -*t*-Bu, or $-CO_2H$.



symmetrical and unsymmetrical thiosulfonates

where R_1 and R_2 represent -Ph, $-(CH_2)_2NHMe$, $-(CH_2)_2NHAc$, $-(CH_2)_2SO_2S(CH_2)_2NHAc$, or 2-imidazolinyl substitutents

Fig. (5). General structural formulas of electrophilic organosulfur compounds screened for their ability to effect zinc release from NC type zinc fingers.

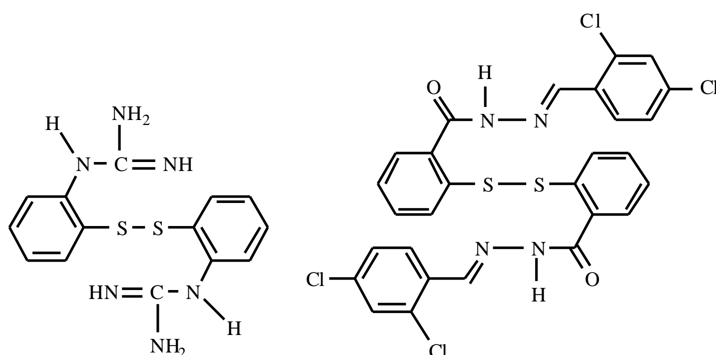


Fig. (6). Aromatic disulfides discovered through high-through-put screening, that demonstrate concentration dependent *in vitro* zinc ejection from purified NC.

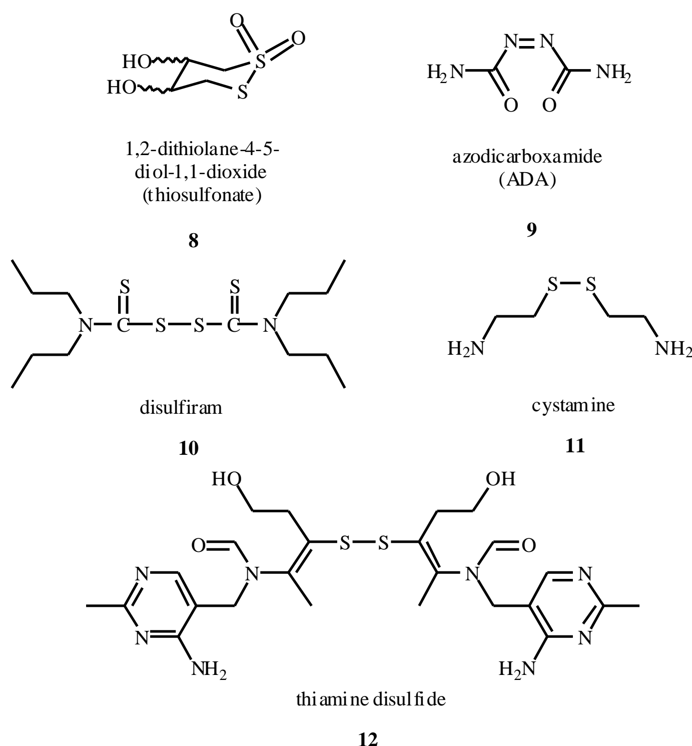


Fig. (7). Electrophilic compounds of various chemotypes that exhibit antiretroviral activity.

ADA [compound (**9**)] as a representative of -carbonyl azoic compounds) were chosen for study [137]. Whereas all the compounds had been shown to effect zinc release from the NC, DIBA-1 [compound (**3**)] [153,158], thiosulfonate **8** [159] and ADA **9** [161-162] were also demonstrated to inhibit replication of HIV-1 and other retroviruses at concentrations that did not exert toxic effects, thus indicating a degree of selectivity of the agents for the CCHC zinc finger target in the viruses. To assess the level of discrimination displayed by these compounds towards other potential zinc finger protein targets, their propensity towards reaction with the zinc finger proteins poly (ADP-ribose) polymerase (PARP), Sp1 and GATA-1 was determined [137].

Whereas NOBA **1** was observed to be relatively promiscuous in its reactivity towards all the zinc finger proteins tested, DIBA-1 [compound (**3**)], thiosulfonate **8** and ADA **9** promoted *in vitro* zinc ejection from the NC at concentrations that did not impact the functions of PARP, Sp1 or GATA-1. Nor did these compounds inhibit HeLa nuclear extract mediated transcription. Selectivity of the interactions of these agents with the NC was supported by molecular modeling analysis which 1) identified a common saddle-shaped nucleophilic region on the surfaces of both NC zinc knuckles; 2) indicated a strong correspondence between computationally docked positions for the agents tested, and overlap of frontier orbitals within the nucleophilic loci of the NC zinc fingers; and 3) revealed selective steric exclusion of the agents from the core of the GATA-1 zinc finger [137]. Modeling analysis further demonstrated that the Cys49 thiolate of the C-terminal zinc finger is the site most susceptible to electrophilic attack [137].

Despite the increasing number of available compounds that target the NC zinc fingers and their demonstrated NC zinc finger specificity, their further development as antiretrovirals has been hampered by the observance of poor therapeutic indices *in vitro*, and/or susceptibility to *in vivo* reduction. Indeed, DIBAs and other compounds containing the disulfide moiety can be rendered non-electrophilic and hence, unreactive towards the NC due to facile degradation to the corresponding free thiols after uptake into the reducing intracellular environment.

To resolve these issues, the reduction susceptible disulfide moiety was replaced with the less reactive thioester bridge [-S(C=O)]. Additionally, haloalkanoyl derivatives were linked to the benzamide ring through the amide or thioester bridge. This yielded a series of pyridinioalkanoyl thiolesters (PATEs) [143]. Screening of a panel of these compounds led to the discovery of two PATEs [compounds (**13**) and (**14**), Fig. (**8**)] that possess virucidal activity, resistance to glutathione reduction, appreciable aqueous solubility, and the ability to inhibit HIV-1 replication in both acutely and latently infected HIV-1 cells [164]. The compounds were also shown to preferentially target the NC zinc finger when tested against other molecular targets. Generally, analogous compounds in which the disulfide moiety was replaced with an amide linkage lacked antiviral activity. Interestingly, although both lead compounds exhibited antiviral activity in cell-based assays, appreciable zinc ejection from the NC under conditions in which previously described NC active disulfides readily eject zinc was not detected [1646]. However, it was observed that in the presence of a metal activator such as Ag^{2+}, both compounds covalently modified the protein with pyridinioalkanoyl groups [164]. The PATEs reacted with NC, ejecting zinc from both fingers. The reaction has been shown to proceed *via* a two-step mechanism in which zinc is ejected from the C-terminal zinc finger faster than from the N-terminal finger [164]. However, the necessity of the presence of a Lewis acid activator such as Ag^{2+} as a precondition for the effectiveness of PATE mediated zinc ejection is clearly unacceptable for *in vivo* applications. In order to increase their therapeutic indices and identify potentially new pharmacophores, further structural refinements of the PATE chemotype have been made. Lengthening of the alkanoyl chain, and replacement of the thiol component with N-2-mercaptobenzoyl amino acid derivatives have led to a subclass of PATEs with improved antiviral potency [165]. Thus, whereas the previous PATEs incorporated the phenylsulfonyl-based benzamide substituents, the new PATEs were derived from amino acid primary amides [165]. Selective compounds were shown to be active on chronically infected CEM/SK-1, TNF -induced U1 and ACH-2 cells, and virucidal on cell-free virus, latently infected U1 cells and acutely infected primary peripheral blood mononuclear cells [165]. These characteristics imply that the new PATEs may provide chemotherapeutic elimination of viral reservoirs [165]. Another class of designed compounds that exhibit a broad range of antiviral activity with potencies equivalent to or better than those of the previously identified PATEs are benzamide-based thiolcarbamates [166-167]. It should be noted that mechanistic studies conducted alongside the antiretroviral activity experiments cited above have correlated the observed antiretroviral activity with interception of NC mediated activities through covalent modification of the NC or NC in the context of the Gag precursor.

FUTURE DIRECTIONS

Even though much has been learned in the process of designing NC-specific pharmacophores, the development of NC inhibitors as antiretrovirals is still in its infancy. Of the compounds that have been determined to arrest virus infectivity through targeting of NC zinc fingers, two compounds, a benzoisothiazolone derivative of DIBA-4 [compound (**6**)], also known as CI-1012, and ADA (**9**), have undergone further testing in clinical trials. Whereas the results of animal testing of CI-1012 were disappointing, recently completed phase I/II trials with ADA (**9**) yielded more encouraging results. The ADA trial [168] represents the first report on the safety, tolerability and preliminary efficacy of a NC inhibitor in HIV infection. ADA (**9**) was administered in escalating doses concomitant with combined antiretroviral therapy during a 3-month open-label period in

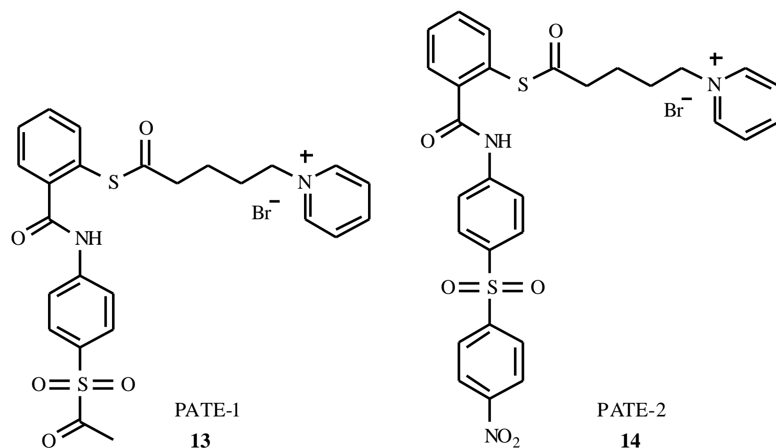


Fig. (8). Structures of two antiretroviral pyridinioalkanoyl thioesters that were developed to circumvent problems associated with first generation NC inhibitors, such as susceptibility to *in vivo* reduction, poor solubility, and unimpressive toxicity profiles.

patients with advanced AIDS and documented phenotypic resistance. Although tolerance to ADA (**9**) was dose dependent, and ADA exhibited some cytotoxicity at higher doses, 27% of patients had consistent viral load reductions compared with baseline, and 45% showed a CD4 cell recovery from baseline. Importantly, no phenotypic resistance to ADA (**9**) was observed. Though the overall effects of ADA (**9**) on viral load appeared rather modest, it should be noted that the methods used in the study to measure viral load did not discriminate between infectious and non-infectious particles. Since ADA (**9**) targets the zinc fingers of the NC to produce non-infectious virus particles, a reduction in viral load may not be immediately observed since current test methods measure total genetic load and not infectious load.

An important aspect of the study was that ADA (**9**) administration represented quasi-monotherapy, since the study participants presented with proven resistance to the combination antiretroviral therapy to which ADA (**9**) was added [168]. Additionally, there was no evidence of the emergence of resistance to ADA (**9**) during the course of the trial. The study demonstrates that zinc finger inhibitors potentially represent an important contribution to combination therapy in AIDS because they are unlikely to foster the development of escape mutants. It remains to be seen whether these observations can be confirmed in larger studies of longer duration. Additionally, it is important for ADA (**9**) to be evaluated for potential synergism with other clinical antivirals [168].

In a third recently reported *in vivo* study [169], 2-mercaptobenzamide PATE zinc knuckle inhibitors were observed to inhibit HIV-1 in a transgenic murine system. In this model, the infectious HIV-1 is induced from an integrated provirus. Inhibition of transgenic spleen cell p24 expression was observed, with potencies comparable to results observed in experiments using human peripheral blood lympocytes. Transgenic mice treated *in vivo* with 2-mercaptobenzamide thioesters expressed significantly lower plasma p24, and splenocytes from these animals produced fewer infectious virions. The results imply that these thioesters may provide an effective means for inhibiting the expression of HIV from integrated viral reservoirs [169].

The hypothesis that retroviral zinc fingers can be targeted was conceived with the discovery that some C-nitroso compounds inhibited PARP mediated activities through covalent modification of its CCHC motif [170-171] Accordingly, the bulk of the research devoted to targeting retroviral zinc fingers has focused on development of electrophilic agents that covalently modify the zinc fingers. However, there are in principle other avenues of approach to disruption of NC protein mediated activities that warrant consideration. One would be development of zinc chelators that can strip zinc away from solvent accessible CCHC arrays, thereby compromising the structural integrity of the protein and arresting the progress of CCHC zinc finger protein mediated activities. For example, it has been reported that a number of rationally designed zinc binding heterocycles exhibit inhibitory effects on the binding of HIV enhancer binding protein (HIV-EP1) to DNA *via* sequestration of zinc from the HIV-EP1 zinc finger [172]. A second approach would be the generation of RNA aptamers that bind to the NC with high affinity, and intercept its binding to viral RNA. Recently, RNA aptamers that bind to mature NC protein were isolated from a RNA library [173]. The aptamers were shown to specifically bind to the NC with high affinity and compete for RNA binding to the NC protein [173]. Both approaches warrant further investigation.

CONCLUSIONS

The hypothesis that the NC can be targeted has met with resistance due to the ubiquity of mammalian zinc finger proteins, and the perception that the similarities between NC protein zinc finger arrays and those of cellular zinc finger containing proteins, precludes the possibility of developing NC-specific chemotherapeutics. While such concerns are understandable, the fact is that differential susceptibility to chemical species by zinc fingers is now well documented. Although studies aimed at understanding the basis of these differences are ongoing, it is increasingly apparent that the nuances that define zinc finger reactivity and susceptibility to various compounds cannot be predicted *a priori*, and are modulated to varying extents by a variety of factors including the steric and electrostatic shielding by the surrounding protein environment, the chemical potentials of the sulfur atoms, and hydrophilic/hydrophobic characteristics. Nevertheless, the feasibility of selectively targeting zinc fingers has been demonstrated, and efforts must be undertaken to further exploit the differences in zinc finger reactivity for therapeutic benefit [164]. Ongoing molecular modeling studies [22,151] of the interactions of inhibitors with the retroviral zinc finger verses other zinc finger motifs should help in defining the factors contributing to differential reactivity toward different types of zinc fingers, and aid in the design and refinement of the selectivity of various compounds towards reaction with the retroviral zinc finger [159].

ACKNOWLEDGEMENTS

I would like to thank Peter Kutchukian and Seokwon Kim for helpful discussions and critical reading of the manuscript.

ABBREVIATIONS

ADA	=	Azodicarboxamide
CA	=	Capsid protein
DFT	=	Density functional theory
DIBA	=	Disulfide-substituted benzamide
EWG	=	Electron withdrawing group
HIV	=	Human immunodeficiency virus
IN	=	Integrase
LTR	=	Long terminal repeat
MA	=	Matrix protein
MeIm	=	1-methylimidazole
MT	=	Metallothionein

NC = Nucleocapsid protein

NCI = National cancer institute

NOBA = Nitrosobenzamide

NOBP = Nitrosobenzopyrone

nt = Nucleotide

PARP = Poly (ADP-ribose) polymerase

PATE = Pyridinoalkanoyl thioester

PBS = Primary binding site

PhS^- = Phenylthiolate

PIC = Preintegration complex

PPT = Polyurine tract

PR = Protease

R = Repeated sequences

RT = Reverse transcriptase

SD = Major splice-donor site

SIV = Simian immunodeficiency virus

SL1-4 = Stem-loops 1-4

-sssDNA = Minus-strand strong stop DNA

$_+$sssDNA = Plus-strand strong stop DNA

Vpr = Viral protein

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