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Critical role of reverse transcriptase in the inhibitory mechanism of CNI-H0294 on HIV-1 nuclear translocation

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ABSTRACT **HIV-1 replication requires the translocation of viral genome into the nucleus of a target cell. We recently reported the synthesis of an arylene bis(methyl ketone) compound (CNI-H0294) that inhibits nuclear targeting of the HIV-1 genome and thus HIV-1 replication in monocyte cultures. Here we demonstrate that CNI-H0294 inhibits nuclear targeting of HIV-1-derived preintegration complexes by inactivating the nuclear localization sequence of the HIV-1 matrix antigen in a reaction that absolutely requires reverse transcriptase. This drug/reverse transcriptase interaction defines the specificity of its antiviral effect and is most likely mediated by the pyrimidine side-chain of CNI-H0294. After binding to reverse transcriptase, the carbonyl groups of CNI-H0294 react with the nuclear localization sequence of matrix antigen and prevent its binding to karyopherin α, the cellular receptor for nuclear localization sequences that carries proteins into the nucleus. Our results provide a basis for the development of a novel class of compounds that inhibit nuclear translocation and that can, in principle, be modified to target specific infectious agents.**

Establishment of integrated HIV provirus and productive viral infection depend critically on the successful nuclear translocation of the HIV-1 genome following viral entry into a host cell (1). The HIV-1 genome is transported into the nucleus of a target cell as part of a large preintegration complex comprised of viral nucleic acids and proteins including integrase (IN), reverse transcriptase (RT), viral protein R (Vpr), and matrix antigen (MA) (2, 3). In nonproliferating cells such as terminally differentiated macrophages, nuclear translocation of this preintegration complex depends critically on two viral proteins, MA and Vpr (3–5). Within MA, there is a sequence of basic amino acids (residues 26–32) that is highly conserved among immunodeficiency viruses. This region is homologous to the nuclear localization sequence (NLS) of simian virus 40 large T antigen and functions as a nuclear targeting signal for HIV-1 preintegration complexes (4, 5). Contiguous lysine residues in the NLS are essential to confer its NLS activity (4, 6).

Import of NLS-containing proteins across the nuclear pore complex is mediated by karyopherin αβ heterodimers (also termed NLS receptor/importin) which bind NLS-containing proteins in the cytosol and target them to the nucleus (7–10). Karyopherin α binds the NLS (11) whereas karyopherin β enhances the affinity of α for the NLS (12) and mediates docking of karyopherin–NLS protein complexes to nucleoporins (a collective term for nuclear pore complex proteins) that contain FXFG peptide repeats (12, 13). The GTPase Ran (14, 15) and its interacting protein p10 (also termed NTF2) (16) impart mobility to the translocation process (14, 16) by catalyzing the disruption of karyopherin αβ heterodimers that have docked to a nucleoporin (12, 17).

Partial reactions in nuclear import can be reproduced *in vitro* using a solution binding assay and recombinant karyopherins (12). We used this assay to investigate the mechanism of action of CNI-H0294, a recently described compound designed to form Schiff base adducts with contiguous lysines in the MA NLS (18). This compound was shown to be a potent inhibitor of nuclear import of the HIV-1 genome and thus of virus replication in cultures of primary monocytes (18). We demonstrate that CNI-H0294 inhibits binding of HIV-1 preintegration complexes to karyopherin α. This effect of the compound critically depends on the presence of RT in the preintegration complex. Interaction between CNI-H0294 and RT determines the specificity of the compound and its low cytotoxicity. However, the critical event in the drug's mechanism of action is the inactivation of the MA NLS.

MATERIALS AND METHODS

Infection with Mutant HIV-1 or HIV-Like Pseudovirions. H9 cells were infected with HIV-1 or HIV-like pseudovirions (19) at a multiplicity adjusted according to p24 content (50 ng p24 per 10^6 cells). The MA NLS^- virus contains substitutions of isoleucine residues for lysines in positions 26 and 27 of MA in an NLHX backbone (20), thus inactivating the NLS (4, 5). The Vpr^- virus has the initiating ATG of the *vpr* gene changed to GTG, thus abolishing expression of this gene. The ΔMA NLS pseudovirions have leucine substituting for lysine in position 28 of MA. This mutation abrogates nuclear translocation of HIV-1 *gag* RNA in growth-arrested H9 cells infected with pseudovirions (L.D. and M.B., unpublished results). After a 1 h absorption, excess viruses or pseudovirions were washed away, and cells were incubated for an additional 2- to 3-h period at 37°C prior to analysis.

Preparation of Cytoplasmic Lysates. Cytoplasmic extracts were prepared by lysing cells in cold extraction buffer [10 mM KCl/10 mM Tris·HCl, pH 7.6/0.5 mM $MgCl_2$/1 μg/ml each of leupeptin and aprotinin/1 mM phenylmethylsulfonyl fluoride (PMSF)] by 20–30 strokes of a Dounce homogenizer under the control of phase-contrast microscopy, as described (2, 21). After removal of nuclei, cytoplasmic extract was cleared by centrifugation at 15,000 × *g* for 10 min.

Analysis of Binding of Nucleoprotein Complexes to Karyopherin α. Cytoplasmic extracts prepared from HIV-1- or

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Abbreviations: IN, integrase; RT, reverse transcriptase; Vpr, viral protein R; MA, matrix antigen; NLS, nuclear localization sequence; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione *S*-transferase.

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pseudovirion-infected H9 cells were adjusted to 0.14 M NaCl and 0.1% Tween 20 and precleared with glutathione-Sepharose beads for 30 min at room temperature. Glutathione *S*-transferase (GST)–karyopherin α immobilized on Sepharose beads was then added (about 50 μg of immobilized karyopherin α per extract from 10^8 infected cells) and the mixture was incubated at room temperature for another 30 min. Beads were then pelleted by centrifugation and washed three times with PBS supplemented with 0.1% Tween 20, 1 μg/ml each of leupeptin and aprotinin, and 1 mM PMSF. HIV-1 DNA was isolated from the beads by SDS/proteinase K treatment with subsequent phenol-chloroform extraction, as described (22), while pseudovirion *gag* RNA was isolated by RNazol (Biotecx Laboratories, Houston).

Analysis of CNI-H0294 Interaction with HIV-1 Proteins in Solution. A total of 0.28 nanomol of recombinant MA (23) or RT [p66/p51 dimer (24)] were mixed with 20 nmol of [^{14}C]CNI-H0294 (specific activity, 5×10^4 cpm/nmol) and incubated 2 h at room temperature in 40 μl of binding buffer (PBS supplemented with 1% BSA/0.1% Tween 20/1 μg/ml leupeptin/1 μg/ml aprotinin/1 mM PMSF). Sheep anti-MA or rabbit anti-RT sera (both obtained from the AIDS Research and Reference Reagent Program, National Institutes of Health) or preimmune control sera were then added (at 1:100 dilution) and incubation continued for another 1 h at room temperature. Immune complexes were precipitated with protein G-agarose, washed, and then eluted with 0.1 M glycine (pH 2.8). Radioactivity of the eluate was measured in a scintillation counter.

Analysis of CNI-H0294 Interaction with HIV-1 Preintegration Complexes. Cytoplasmic lysates prepared from HIV-1-infected cells were treated with 10 μM of ^{14}C-labeled CNI-H0294 (specific activity, 5×10^4 cpm/nmol) in 1 ml extraction buffer adjusted to 0.14 M NaCl. Sodium borohydride was then added to a final concentration 10 mM and samples were incubated 1 h at room temperature prior to immunoprecipitation to reduce double bonds of Schiff bases to an irreversible secondary amine. Immunoprecipitation was performed as described above, but beads were stringently washed three times with PBS supplemented with 0.1% SDS, 1% sodium deoxycholate, 1 μg/ml each of aprotinin and leupeptin, and 1 mM PMSF.

RESULTS

CNI-H0294 was designed to react with adjacent lysines in the NLS (18), thus making it potentially capable of neutralizing NLSs on many different proteins. However, despite our expectation of high toxicity due to this compound's anticipated reactivity, CNI-H0294 exhibited remarkably low cytotoxicity in monocyte and T lymphocyte cultures *in vitro* (50% toxic dose >1 mM) and *in vivo* in mice (LD_{50} = 590 mg/kg). These results suggest that the molecular mechanism of MA NLS inactivation by CNI-H0294 is very specific. Indeed, this compound, did not block nuclear import of nucleoplasmin-coated gold particles, nor of BSA with conjugated NLS peptides that mimic the NLS of simian virus 40 large T antigen (not shown).

CNI-H0294 Inhibits Interaction Between HIV-1 Preintegration Complexes and Karyopherin α, But Does Not Affect Binding of Karyopherin α to Pseudovirion-Derived Nucleoprotein Complexes. The initial step in the process of nuclear import is binding of karyopherin α (also termed NLS-receptor/importin) to an NLS (11). Results presented in Fig. 1*A* demonstrate that wild-type HIV-1 preintegration complexes bound to GST–karyopherin α immobilized on glutathione-Sepharose beads (lane 1), in agreement with recently reported results (25). Mutant preintegration complexes that lack Vpr (MA NLS$^+$Vpr$^-$, lane 3) bound with reduced efficiency, while binding of the complexes with mutated MA NLS (MA NLS$^-$Vpr$^+$, lane 2) was even more impaired. Preintegration complexes that lack Vpr and are mutant in MA NLS (MA NLS$^-$Vpr$^-$) did not bind to karyopherin α (lane 4). These results are consistent with our analysis of MA and Vpr binding to karyopherin α which demonstrated that while Vpr can bind weakly to karyopherin α, its main role is to enhance the MA NLS-karyopherin α interaction (S.P. and M.B, unpublished data).

To facilitate analysis of HIV-1 nuclear translocation and of the mechanism of drug effects on this process, we used a simplified model of the HIV-1 preintegration complex comprising a minimal number of non-essential proteins. This model employs *gag–env* pseudovirions which exhibit an HIV-

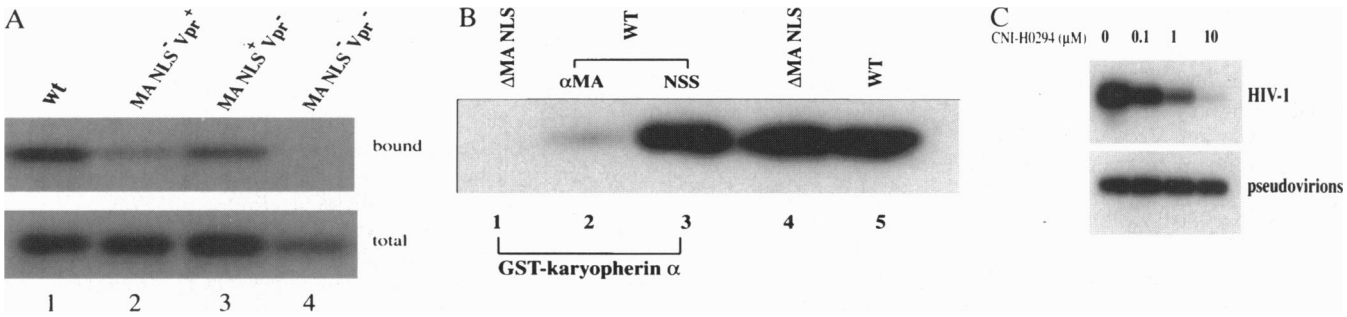
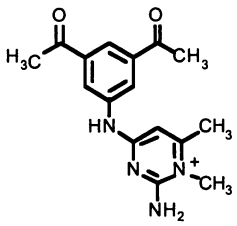
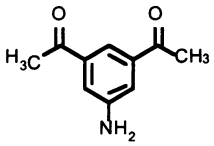
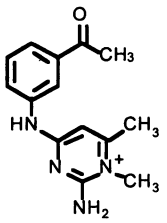
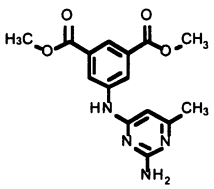


FIG. 1. Binding of HIV-1 nucleoprotein complexes to karyopherin α. (*A*) Binding of HIV-1 to karyopherin α is mediated by both MA and Vpr. Cytoplasmic extracts prepared 4 h after infection of H9 cells with equivalent amounts (100 ng of p24 per 10^6 cells) of wild-type HIV-1_{NLHX} or variants carrying inactivating mutations in MA NLS or/and Vpr were divided in two aliquots. DNA was extracted from one aliquot and quantified by PCR using primers specific for the HIV-1 *pol* gene (*Lower*). The obtained signal represented the total amount of the HIV-1 DNA in the cytoplasm. The second aliquot was incubated with GST–karyopherin α immobilized on Sepharose beads. HIV-1 DNA was extracted from the beads and analyzed by PCR using *pol*-specific primers. The obtained signal represented the amount of HIV-1 preintegration complexes bound to karyopherin α. (*B*) Binding of HIV-1 pseudovirion nucleoprotein complexes to karyopherin α is mediated by MA NLS. H9 cells were inoculated with HIV-like *gag–env* pseudovirions that contain *gag* RNA (19); equal amounts of wild-type (wt) and mutant pseudovirions that carry amino acid substitutions in the MA NLS (ΔMA NLS) were used. Cytoplasmic extracts prepared from infected cells were incubated with polyclonal anti-MA serum (αMA, lane 2), preimmune serum (NSS, lane 3), or nothing (lanes 1, 4, and 5). Samples were then mixed with GST–karyopherin α immobilized on glutathione Sepharose beads for 30 min at 25°C. Nucleic acids were extracted from Sepharose beads and quantified by RT–PCR using primers specific for HIV-1 *gag* gene (26). To control for possible differences in cell entry of wild-type versus mutant agents, HIV-specific nucleic acids were extracted directly from cytoplasmic extracts and assayed by RT–PCR (lanes 4 and 5). (*C*) CNI-H0294 inhibits interactions of karyopherin α with HIV-1 preintegration complexes, but not with pseudovirion-derived nucleoprotein complexes. Cytoplasmic lysates of H9 cells infected with HIV-1_{RF} (*Upper*) or HIV-like pseudovirions (*Lower*) were treated for 2 h with various concentrations of CNI-H0294, and were then mixed with GST–karyopherin α immobilized on glutathione Sepharose beads. HIV-1 DNA or RNA that coprecipitated with karyopherin α was quantified as in *A* and *B*.

like core but are composed exclusively of Gag (MA, CA, NC, and p6) and Env (gp41 and gp120) proteins (19). These pseudovirions package HIV-1 *gag* RNA and, as we have shown recently (26), translocate this RNA into the nucleus of an infected cell in a manner similar to the behavior of HIV-1 preintegration complexes. Results presented in Fig. 1*B* demonstrate that karyopherin α binds nucleoprotein complexes formed in pseudovirion-infected CD4$^+$ T cells (lane 3). Binding required a functional MA NLS as mutation of the NLS (Fig. 1*A*, lane 1) or pretreatment of nucleoprotein complexes with polyclonal anti-MA antibodies (lane 2) greatly diminished binding to karyopherin α. Thus, we conclude that pseudovirion-derived nucleoprotein complexes interact with karyopherin α in a manner similar to HIV-1 preintegration complexes.

We next examined the effect of CNI-H0294 on the interaction between karyopherin α and HIV-1 versus pseudovirion nucleoprotein complexes and found that CNI-H0294 inhibited in a dose-dependent manner binding of karyopherin α to HIV-1 preintegration complexes (Fig. 1*C Upper*). Quantitation on a Phosphorimager demonstrated that 0.1 μM and 1 μM of CNI-H0294 reduced karyopherin α/HIV-1 binding 8- and 25-fold, respectively. These results explain the inhibition of HIV-1 nuclear import by the compound and correlate well with the dose-response curve obtained when HIV-1-infected monocyte cultures were treated with CNI-H0294 (18). Surprisingly, CNI-H0294 did not inhibit binding of karyopherin α to pseudovirion-derived nucleoprotein complexes (Fig. 1*C Lower*) or to purified recombinant MA (results not shown). These results suggest that the mechanism of CNI-0294 inhibition requires a factor(s) present in the HIV-1 preintegration complex but absent from pseudovirion-derived complexes.

Table 1. Structure-function analysis of anti-HIV activity of CNI compounds

Compound	Structure	IC_{50}
CNI-H0294		50 nM
CNI-H1894		>10 μM
CNI-H1494		1 μM
CNI-H3094		>10 μM

CNI compounds were added at various concentrations (10 pM to 10 nM) to cultures of primary human monocytes together with HIV-1_{ADA} and were present throughout the entire experiment. A 50% inhibitory concentration (IC_{50}) was determined at day 9 after infection. Some compounds did not achieve 50% inhibition at maximal concentration yet exhibited anti-HIV activity; in these cases the results are presented as >10 μM.

Structure-Activity Relationships Within the CNI-H Group of Compounds. To further investigate the mechanism of action of CNI-H0294, we examined the structure-activity relationships within compounds of the CNI-H series (Table 1). Absence of the reactive carbonyl groups (compounds CNI-H1494 and CNI-H3094) or the pyrimidine side chain (compound CNI-H1894) resulted in a dramatic decrease of the drug's potency. As the carbonyl groups were designed to react with lysine residues within MA NLS (18), it was not surprising that their absence decreased the drug's activity. In contrast, a role for the pyrimidine side chain was unexpected, and suggested that this side group may be involved in binding CNI-H0294 to the preintegration complex. Interestingly. a similar pyrimidine ring is present in a group of nonnucleoside inhibitors of RT (27), suggesting that RT could provide a binding site for CNI-H0294.

CNI-H0294 Binds to RT. Binding of CNI-H0294 to RT or MA was tested *in vitro* using ^{14}C-labeled CNI-H0294 and recombinant RT and MA proteins (Fig. 2). Specific immunoprecipitation was used to quantify the amount of bound CNI-H0294. Preliminary experiments showed that both anti-RT and anti-MA reagents specifically recognized and immunoprecipitated RT and MA, respectively (not shown). As shown in Fig. 2, about 17,000 cpm, or 0.34 nmol of CNI-H0294 (specific activity, 50,000 cpm/nmol), were immunoprecipitable from incubations of drug with 0.28 nmol RT, suggesting that CNI-H0294 binds to RT in a 1:1 molar ratio. The specificity of this interaction was further confirmed by immunoprecipitation experiments using cold CNI-H0294 to compete out precipitable counts associated with labeled drug (Fig. 2). No binding was observed with recombinant MA, in agreement with our earlier results, and no radioactivity was precipitated by immune sera if the recombinant protein was omitted from the reaction mixture (not shown). In similar experiments, we did not detect binding of CNI-H0294 to Vpr nor to IN, two other proteins known to be present within the HIV-1 preintegration complex (results not shown). These experiments established that CNI-H0294 bound directly to RT, but not to other proteins of the HIV-1 preintegration complex. Of interest, CNI-H0294 did not significantly inhibit reverse transcription of HIV-1 in infected cells (18) nor did it block in

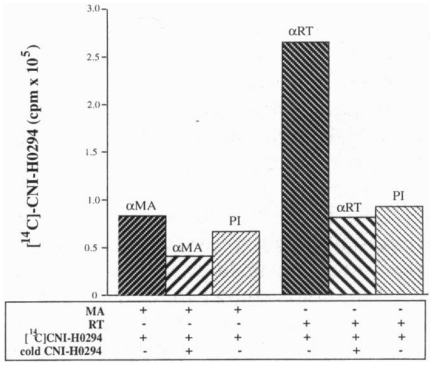


FIG. 2. CNI-H0294 binds to recombinant RT in solution. [^{14}C]CNI-H0294 (20 nmol) was mixed with 0.28 nmol of recombinant MA or RT (a p51/p66 heterodimer) in 40 μl of binding buffer. Samples were incubated for 2 h at 37°C in the presence or absence of 200 nmol of unlabeled CNI-H0294. MA and RT proteins were immunoprecipitated by using protein G-agarose and sheep polyclonal anti-MA (αMA) or rabbit anti-RT (αRT) sera, respectively. Preimmune sera (PI) was used as control. Bound material was eluted from protein G using 0.1 M glycine buffer (pH 2.8) and the radioactivity in the eluate was quantified in a scintillation counter.

concentrations up to 50 μM the enzymatic activity of HIV-1 RT *in vitro* (results not shown), suggesting that an effect on RT activity cannot account for the antiviral action of the compound.

Binding to RT Is Critical for the Anti-HIV Activity of CNI-H0294. The role of CNI-H0294/RT interaction in the drug's activity was analyzed in experiments with compound CNI-H3094. As CNI-H3094 does not have reactive carbonyl groups but contains the active pyrimidine side chain (see Table 1), it could effectively compete with CNI-H0294 for binding to the same site on the HIV-1 preintegration complex, albeit it did not inhibit nuclear import of HIV-1. Fig. 3*A* shows that unlabeled CNI-H3094 inhibits binding of ^{14}C-labeled CNI-H0294 to RT in a dose-dependent manner. Likewise, CNI-H3094 restored binding of HIV-1 preintegration complexes to karyopherin α in the presence CNI-H0294 (Fig. 3*B*). A 5-fold excess of CNI-H3094 (Fig. 3*B*, lane 4) reduced significantly the inhibitory effect of CNI-H0294 on binding of HIV-1 preintegration complexes to karyopherin α, and a 10-fold excess (lane 5) completely eliminated the inhibitory effect. In a control experiment, CNI-H3094 did not inhibit binding of HIV-1 preintegration complexes to karyopherin α (Fig. 3*B*, lane 1); this correlates with the compound's lack of anti-HIV activity (Table 1). Finally, CNI-H3094 eliminated the inhibitory effect of CNI-H0294 on HIV-1 replication in monocyte cultures (Fig. 3*C*). These results confirm the critical role of CNI-H0294/RT interaction in the drug's mechanism of action and also show a direct correlation between the drug's binding to RT, inhibition of HIV-1/karyopherin α interaction, and repression of viral replication.

CNI-H0294 Inactivates the NLS of MA Without Disrupting MA Association With the HIV-1 Genome. The results presented above indicate a direct role for RT in the anti-HIV effect of CNI-H0294 and provide a molecular explanation for the high specificity of the compound. However, these results do not explain how CNI-H0294 prevents binding of HIV-1 preintegration complexes to karyopherin α, as RT does not bind to directly to karyopherin α. One possibility was that binding of CNI-H0294 to RT disrupts the preintegration complex and causes dissociation of MA from HIV-1 cDNA. To test this hypothesis, cytoplasmic lysates of HIV-1-infected H9 cells were treated with CNI-H0294 and mixed with immobilized karyopherin α or subjected to immunoprecipitation with antibodies that bind MA. Although CNI-H0294 blocked the interaction of the preintegration complexes with karyopherin α (Fig. 4*A*, lane 2), it did not prevent immunoprecipitation of viral DNA with anti-MA serum (Fig. 4*A*, lane 3); thus MA was still associated with HIV-1 cDNA but lost its ability to bind karyopherin α. As binding of preintegration complexes to karyopherin α is controlled mainly by the MA NLS (Fig. 1 *A* and *B*), these results indicate that CNI-H0294 neutralizes the NLS activity of MA, either directly (through chemical modification) or indirectly (e.g., by steric hindrance). To discriminate between these two possibilities, we treated cytoplasmic extracts of HIV-1-infected cells with [^{14}C]-CNI-H0294 and then with sodium borohydride to reduce the reversible Schiff bases hypothesized to form between the compound and the lysines of MA NLS and convert the attached drug molecules to irreversible adducts (see *Materials and Methods*). MA was then immunoprecipitated with specific serum in a buffer containing of 0.1% SDS and 1% sodium deoxycholate which disrupts weak protein–drug and protein–protein interactions in the preintegration complex (2) without disrupting covalent bonds. Under these conditions, a significant amount of radioactivity was immunoprecipitated by anti-MA serum (Fig. 4*B*), in contrast to results obtained with recombinant MA (Fig. 2). These results corroborated requirement for RT for the drug's effect and suggested that the CNI-H0294 had been covalently linked to MA by borohydride treatment. Without borohydride treatment, no radioactivity was immunoprecipitated with MA

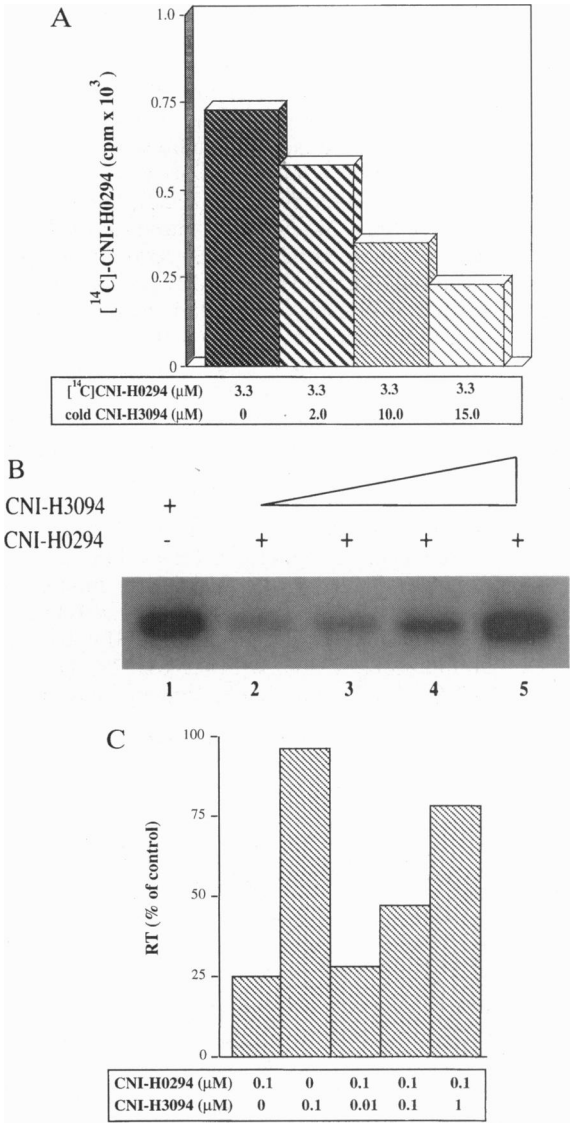


FIG. 3. CNI-H0294 interacts with RT to produce the anti-HIV effect. (*A*) CNI-H3094 competes with CNI-H0294 for binding to RT. Recombinant RT (0.2 μM) was incubated with 3.3 μM of ^{14}C-labeled CNI-H0294 and increasing concentrations of unlabeled (cold) CNI-3094. The amount of [^{14}C]CNI-H0294 that bound to RT was measured as in Fig. 2. (*B*) CNI-H3094 reduces CNI-H0294-mediated inhibition of HIV–karyopherin α interaction. Cytoplasmic lysates prepared from H9 cells infected with HIV-1_{RF} were treated with 10 μM CNI-H3094 (lane 1) or with 1 μM CNI-H0294 and 10 μM (lane 5), 5 μM (lane 4), 1 μM (lane 3), or no CNI-H3094 (lane 2). The amount of preintegration complexes available for interaction with karyopherin α was quantified as in Fig. 1*A*. (*C*) CNI-H3094 inhibits anti-HIV activity of CNI-H0294 in monocyte cultures. Monocytes infected with HIV-1_{ADA} were cultured in the presence of CNI-H0294 and CNI-H3094 in various concentrations. Nine days after infection, RT activity in culture supernatants was quantified. The results are presented as percent of total RT activity in untreated cultures (control). Three independent samples were assayed for each drug concentration, and the standard deviation was less than 15%.

(not shown). In control experiments, no radioactivity was precipitated by anti-IN serum, or by anti-MA serum from the cytoplasmic extract of cells infected with pseudovirions (Fig. 4*B*) which lack RT and thus do not bind CNI-H0294 (Fig. 2).

CNI-H0294 Inhibits MA NLS-, But Not Vpr-Mediated Binding of HIV-1 Preintegration Complexes to Karyopherin α. The reported role for MA NLS and Vpr in HIV-1 nuclear import (3–5) prompted us to investigate the effects of CNI-H0294 on

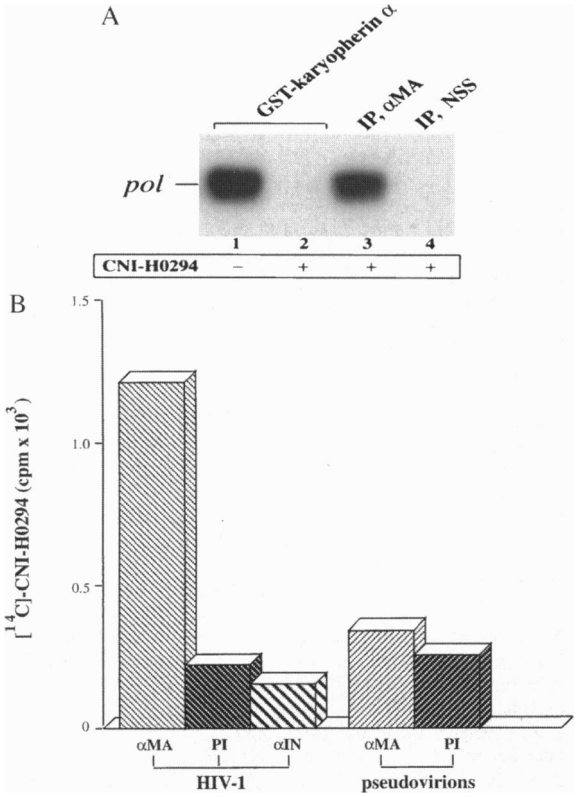


FIG. 4. Analysis of CNI-H0294 interactions with the HIV-1 preintegration complex. (*A*) CNI-H0294 does not disrupt the interaction between MA and HIV-1 cDNA. Cytoplasmic extracts prepared from H9 cells infected with HIV-1_{RF} were treated with 10 μM CNI-H0294 (lanes 2, 3, and 4) or left untreated (lane 1). Samples were divided into two aliquots. One aliquot was mixed with GST–karyopherin α immobilized on Sepharose beads and the HIV-1 DNA that bound was quantified by PCR. The second aliquot was immunoprecipitated (IP) with anti-MA serum (αMA, lane 3) or with preimmune serum (NSS, lane 4) as control. (*B*) CNI-H0294 reacts with MA, but only when MA is associated with the HIV-1 preintegration complex. Cytoplasmic extracts from HIV-1_{RF}-infected H9 cells were treated with [^{14}C]CNI-H0294 (10 μM). After borohydride reduction the extracts were immunoprecipitated with anti-MA (αMA), anti-IN (αIN), or preimmune serum (PI). As control, similar reactions were performed using lysates of pseudovirion-infected cells which lack RT and thus do not bind CNI-H0294 (see text). Immunoprecipitated radioactivity was quantified in a scintillation counter.

the interaction between karyopherin α and preintegration complexes derived from viruses that carry mutations in Vpr (Vpr$^-$), MA NLS (MA NLS$^-$), or both (Fig. 5). These viruses (except for MA NLS$^-$Vpr$^-$ double mutant which was slightly attenuated) entered target cells and reverse transcribed their genome with similar efficiencies (Fig. 5 *Upper*). The presence of CNI-H0294 diminished binding of karyopherin α to wild-type (Fig. 5 *Lower*, lanes 1 and 2) and Vpr$^-$ complexes (lanes 3 and 4) by 95% but had no effect on binding to MA NLS$^-$ complexes (lanes 5 and 6) which is only 1–5% of that observed with MA NLS$^+$ complexes. This result correlates well with our previous study which showed a 95% inhibition of HIV-1 replication in monocyte cultures treated with 1 μM CNI-H0294 (18). The inability of CNI-H0294 to block binding of MA NLS$^-$ complexes to karyopherin α can be explained by the lack of a consensus NLS in Vpr and our recent demonstration that Vpr binds to karyopherin α in an NLS-independent manner (S.P. and M. B., unpublished data).

DISCUSSION

Results presented in this paper reveal the molecular mechanism of action of CNI-H0294, the first compound to specifically target nuclear import of HIV-1. A model by which CNI-H0294 can inactivate the MA NLS and thus prevent nuclear import of HIV-1 is depicted in Fig. 6. We suggest that the compound first binds to HIV-1 preintegration complexes via RT and then forms reversible Schiff bases with contiguous lysines in an adjacent MA NLS. This interaction prevents binding of karyopherin α to the MA NLS and significantly inhibits nuclear translocation of the HIV-1 preintegration complex. This model has important implications for the future design of drugs aimed at disrupting nuclear import. First, our results with pseudovirion-derived nucleoprotein complexes

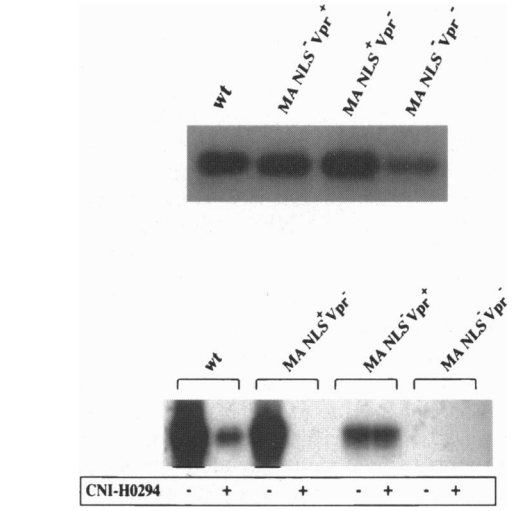


FIG. 5. CNI-H0294 inhibits MA-, but not Vpr-mediated binding of HIV-1 preintegration complexes to karyopherin α. H9 cells were infected with equal amounts of wild-type (wt) HIV-1_{NLHX} or with mutant HIV-1_{NLHX} that lack Vpr (Vpr$^-$) or carry a mutation that inactivates the MA NLS (MA NLS$^-$). Infected cells were washed and incubated for 4 h. An aliquot of each sample was used to quantify the total HIV-1 DNA (*Upper*) and the rest was used to prepare cytoplasmic extracts. Extracts were incubated with 1 μM CNI-H0294 (*Lower*, lanes 2, 4, 6, and 8) or were left untreated (lanes 1, 3, 5, and 7). The amount of preintegration complexes available for binding to karyopherin α was determined as in Fig. 1*A*.

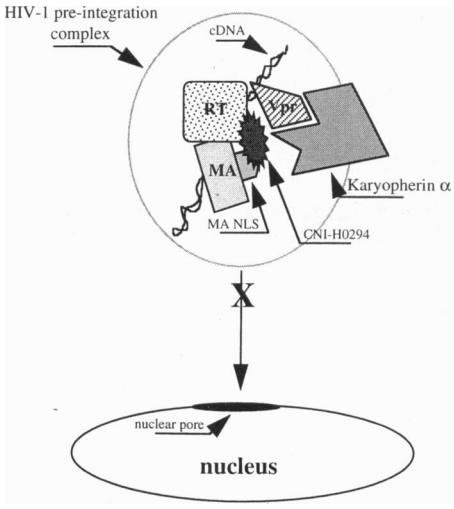


FIG. 6. Proposed mechanism of CNI-H0294 action. CNI-H0294 binds to HIV-1 preintegration complexes via RT, and then reacts with an adjacent MA NLS. This interaction prevents binding of karyopherin α to MA NLS; this nearly abolishes nuclear import of the preintegration complexes. Note that Vpr binds to karyopherin α even in the presence of CNI-H0294; this explains the low level of import detected in the presence of the drug.

indicate that formation of the functional complex capable of binding karyopherin α and translocating into the nucleus resides entirely within the HIV-1 Gag proteins. Although other proteins present in the HIV-1 preintegration complex (e.g., Vpr, IN, and RT) may enhance nuclear translocation, they are not necessary for this process. Our results suggest that RT and MA NLS are located in close proximity within the HIV-1 preintegration complex as CNI-H0294 is very small yet seems to bind RT and the MA NLS simultaneously. As MA is made from the Gag precursor and RT is made from the Gag–Pol precursor, the ratio of RT to MA in the virion is expected to be 1:50 because the translational frameshift that leads to synthesis of the Gag–Pol precursor (rather than the Gag precursor) occurs about 2% of the time (28). Interestingly, about 1–2% of the virion MA protein is phosphorylated and only these molecules are incorporated into the HIV-1 preintegration complex (29, 30). This leads to an important conclusion that there is roughly an equivalent number of RT and MA molecules per HIV-1 preintegration complex. Given an efficient inactivation of MA NLS by CNI-H0294, one can speculate that most if not all RT and MA molecules are in close proximity in the preintegration complex.

CNI-H0294 is targeted to HIV-1 by binding to RT, most likely via its pyrimidine side chain. This targeting is critical for the compound's activity and specificity. Modifications of the side chain may lead to more effective compounds—e.g., those that inhibit RT activity as well as nuclear translocation. Alterations of the side groups may also serve to target the inhibitor to other nucleoprotein complexes, provided that we know their protein composition. This could be a potential strategy for designing drugs against viruses that replicate in nondividing cells and critically depend on nuclear targeting, such as measles or herpes viruses.

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