Nuclear Localization Signal of HIV-1 as a Novel Target for Therapeutic Intervention

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ABSTRACT

Background: Human immunodeficiency virus type 1 (HIV-1) is a lentivirus and shares with other members of this retroviral subfamily the ability to replicate in nondividing cells, in particular, cells of the monocyte/macrophage lineage. This feature relies on the presence of a specific nuclear localization signal (NLS) within the viral matrix protein (MA p17), which to some degree can be complemented by the activity of the viral *vpr* gene product. The MA p17 NLS ensures efficient transportation of the viral preintegration complex into the nucleus of an infected macrophage and confers persistence of HIV-1 in quiescent T cells, and therefore presents an attractive target for therapeutic intervention.

Materials and Methods: Nuclear localization signals (NLS) in general and the HIV-1 MA p17 NLS in particular are characterized by a stretch of positively charged amino acids including one or more lysine residues. A series of compounds potentially capable of binding and reacting with lysine by forming Schiff base adducts was synthesized. Our special consideration was to make compounds that would preferentially bind to two closely contiguous amino functions, as opposed to isolated single lysine residues. We assumed that this approach might specifically target the compound to NLS while affecting other regions less, thus reducing nonspecific cytotoxicity. Antiviral activity was assessed in primary monocytes and in peripheral blood lymphocytes (PBL) infected with HIV-1_{ADA} strain. Viral replication was monitored by reverse transcriptase (RT) activity in the supernatant. Efficiency of nuclear importation of the viral preintegration complex was estimated by the formation of 2-LTR circle forms of HIV-1 DNA and also by in situ PCR techniques.

Results: Arylene bis(methyl ketone) compounds with a nitrogenous third substituent, especially a pyrimidinic side-chain, inhibited HIV-1 replication in human monocytes at an IC_{50} as low as 1 nM. These compounds did not block HIV-1 replication in peripheral blood lymphocyte cultures. The inhibitory effect observed in monocyte cultures appeared in the context of markedly reduced nuclear importation of viral DNA in the presence of the drug. No cytotoxic effects of the compounds was observed in vitro at concentrations as high as 10 μM. An amidinohydrazone derivative of the most active compound was about 100 times less active than the parent, indicating that carbonyl groups were instrumental in the antiviral effect.

Conclusions: These early results suggest that retroviral replication in nondividing cells is susceptible to pharmaceutical intervention targeted against the NLS activity of HIV-1 proteins in the viral preintegration complex. The compounds described efficiently block translocation of viral DNA to the nuclei of infected primary monocytes, and inhibit viral replication. This inhibition is effective only in nondividing cells and is not seen in proliferating cultures, such as activated PBLs. Thus, drugs that target HIV-1 NLS may be useful to specifically block the macrophage arm of HIV infection and could thereby be of value in treating macrophage-specific manifestations of HIV disease, such as HIV-1 dementia. In combination with other drugs, potential therapeutics exploiting this target may also help to control the progression of HIV-1 infection and disease.

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INTRODUCTION

Cells of the monocyte/macrophage lineage are believed to play an extremely important role in the pathogenesis of human immunodeficiency virus (HIV) disease and may also be important in the transmission and progression of infection. Because HIV type 1 (HIV-1) strains isolated from infected individuals during the early stages of infection are predominantly monocytotropic, non-syncytium-inducing viruses (1–4), it appears that monocytes could be the earliest target of HIV infection after introduction into a new host. Low cytopathicity of the virus towards monocytes in vitro suggests that these cells might represent a productive reservoir of HIV in the body, at least early in the course of infection (5–8). Since macrophages are antigen-presenting cells that also regulate immune responses by producing a wide variety of cytokines and other bioactive molecules, HIV infection of this population could in principle account for a significant degree of dysregulation of the immune system (reviewed in Ref. 9 and 10). HIV-infected macrophages are responsible, at least in part, for such devastating and frequent complications of acquired immunodeficiency syndrome (AIDS) as central nervous system (CNS) disease (6,11–13) and pulmonary disease (14–16). Finally, some investigators studying HIV-1-infected chimpanzees have attributed the asymptomatic course of infection in these animals to the lack of a robust macrophage component to the infection (17).

Recent studies have identified nuclear importation of the retroviral preintegration complex as a crucial step in HIV-1 replication in nondividing cells such as monocytes (18). In contrast to HIV-1 replication in proliferating T cells, where frank disruption of nuclear membrane during mitosis directly exposes cellular genomic DNA to viral preintegration complexes, viral propagation in nondividing monocytes relies on the active energy-dependent translocation of the preintegration complex through the intact nuclear membrane and into the nucleus (18–20). Further work has demonstrated that nuclear importation of the HIV-1 DNA occurs in the context of the high molecular weight complex comprising viral RNA, DNA, and several viral proteins, including matrix antigen (MA p17) and virus protein R (Vpr) (21,22). This complex can gain access to the host genome of both proliferating and growth-arrested cells (18,21–23). Subsequent analysis revealed that nuclear translocation of the HIV-1 preintegration complex in growth-arrested T cells and in cultured monocytes relies on the nuclear targeting properties of the MA p17 and Vpr proteins, and in particular on the presence of a nuclear localization signal (NLS) in the N-terminal portion of MA p17 (22,23).

The HIV-1 MA p17 NLS belongs to a well-characterized class of relatively simple nuclear localization sequences. Generally, nuclear localization signals of this type are distinguished by short stretches of amino acids that contain a high percentage of basic residues. One putative consensus signal that has been proposed is Lys-Lys/Arg-X-Lys/Arg, where X is one of a variety of amino acids (24). The MA p17 NLS (Gly-Lys-Lys-Lys-Tyr-Lys-Leu-Lys [23]) conforms very well to this consensus sequence. Mutations introduced into MA p17 NLS (e.g., Gly-Thr-Thr-Lys-Tyr-Lys-Leu-Lys) significantly attenuate viral replication in monocytes (22) and viral persistence in quiescent T cells (25). In addition, nuclear importation of the HIV-1 preintegration complex can be partially inhibited by an excess of the prototypic NLS of simian virus 40 large T antigen (26). These results prompted us to design a series of compounds potentially capable of binding to and reacting with the MA p17 NLS. In the present article, we describe the rationale behind the synthesis of such compounds and demonstrate their efficacy in blocking HIV-1 replication in primary human monocyte cultures.

MATERIALS AND METHODS

Synthesis of Compounds

Compound H-0294 was prepared by reacting 3,5-diacetylaniline (27) with 2-amino-4-chloro-6-methylpyrimidine in water with catalytic HCl at 90–100°C for 30 min followed by base neutralization to give 2-amino-4-(3,5-diacetylphenyl)amino-6-methylpyrimidine, which was methylated by heating at reflux with a 16-fold excess of 1.75 M methyl iodide in 1:1:2 dichloromethane-acetonitrile-tetrahydrofuran for 48 hr. After an aqueous workup, 2-amino-4-(3,5-diacetylphenyl)amino - 1,6 - dimethylpyrimidinium iodide (Compound H-0294) was isolated as a dihydrate.

Compound H-2194 was prepared by reacting Compound H-0294 with 2.5 equivalents of 0.3 M aminoguanidine hydrochloride in 1:1 methanol: 2-methoxyethanol at reflux for 16 hr.

Cell Cultures and Infection with HIV-1

Primary human monocytes were obtained from peripheral blood by Ficoll-Hypaque centrifugation and adherence to plastic as described previously (7). Briefly, after Ficoll-Hypaque (Pharmacia) separation, peripheral blood mononuclear cells (PBMCs) were washed four times with Dulbecco's modified Eagle medium (DMEM) (the last wash was done at 800 rpm to remove platelets) and resuspended in monocyte culture medium (DMEM supplemented with 1 mM glutamine, 10% heat-inactivated human serum, 1% penicillin+streptomycin mixture [Sigma Chemical Co., St. Louis, Mo., U.S.A.]) at a density of 6×10^6 cells/ml. Cells were seeded in 24-well plates (1 ml per well) and incubated for 2 hr at 37°C, 5% CO_2. Following incubation, cells were washed three times with DMEM to remove nonadherent cells and incubation was continued in monocyte culture medium supplemented with 250 U/ml human M-CSF (Sigma). Cells were allowed to mature for 7 days prior to infection with the monocyte-tropic strain, HIV-1_{ADA} (28). Two hours after infection, cells were washed with medium and cultured in RPMI supplemented with 10% human serum. In experiments where polymerase chain reaction (PCR) analysis was performed, virus was pretreated with RNAse-free DNAse (Boehinger-Mannheim) for 2 hr at room temperature and then filtered through a 0.2-μm pore nitrocellulose membrane prior to infection.

PBMCs were purified by Ficoll-Hypaque centrifugation and activated by 10 μg/ml phytohemagglutin (PHA)-P (Sigma) and 20 U/ml recombinant human IL-2 (rhIL-2) in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (HyClone). After 24 hr incubation, cells were washed and inoculated with HIV-1_{ADA} in RPMI 1640 supplemented with 10% FBS. After a 2 hr adsorption, free virus was washed away and cells were cultured in RPMI 1640 supplemented with 10% FBS and 20 U/ml rhIL-2.

p24 and RT Assay

For the p24 assay, sequential 1:9 dilutions of culture supernatant were prepared and analyzed by ELISA as suggested by the manufacturer (Cellular Products, Buffalo, NY, U.S.A.). For the reverse transcriptase (RT) assay, 10 μl of culture supernatant was added to 40 μl of reaction mixture (final composition was 50 mM Tris-HCl, pH 7.8; 20 mM KCl; 5 mM $MgCl_2$; 1 mM DTT; 0.1% Triton X-100; 0.2 OD/ml polyA; 0.2 OD/ml oligo$(dT)_{12-18}$; and 40 μCi/ml ^{3}H-dTTP (76 Ci/mmol, DuPont) and incubated 2 hr at 37°C. 5 μl of the reaction mixture was then spotted onto the DE 81 (Whatman) paper. The paper was air dried and washed five times with 5% Na_2HPO_4, followed by rinsing with distilled water. After air drying, the paper was put on a Flexi Filter plate (Packard), covered with scintillation fluid, and counted in a Top Count Microplate Counter (Packard). Results are expressed as counts per minute in 1 ml of supernatant (cpm/ml).

Assay of the HIV-1 Nuclear Translocation

Efficiency of nuclear translocation was estimated by the ratio between the 2-LTR- and *pol*-specific PCR products, which reflects the portion of 2-LTR circle DNA molecules as a fraction of the entire pool of intracellular HIV-1 DNA. Viral 2-LTR circle DNA is formed exclusively within the nucleus of infected cells and thus is a convenient marker of successful nuclear translocation (18,23).

PCR ANALYSIS OF HIV-1 DNA. Total DNA was extracted from HIV-1-infected cells using the IsoQuick extraction kit (Microprobe Corp., Garden Grove, CA, U.S.A.). DNA was then analyzed by PCR using primer pairs that amplify the following sequences (18,21,29): a fragment of HIV-1 (LTR/gag) that is the last one to be synthesized during reverse transcription and therefore represents the pool of full-length viral DNA molecules; a fragment of polymerase gene (*pol*); a 2-LTR junction region found only in HIV-1 2-LTR circle DNA molecules; or a fragment of the cellular α-tubulin gene. Dilutions of 8E5 cells (containing one integrated copy of HIV-1 DNA per genome) into CEM cells were used as standards. Amplification products were transferred to nylon membrane filters and hybridized to ^{32}P-labeled oligonucleotides corresponding to internal sequences specific for each PCR amplification fragment (18,29), followed by exposure to Kodak XAR-5 film or a phosphor screen.

QUANTITATION OF PCR REACTIONS. Bands of correct size revealed after hybridization were quantitated with a PhosphorImager (Molecular Dynamics) by measuring the total density (integrated volume) of rectangles enclosing the corresponding product band. Efficiency of nuclear translocation of HIV-1 DNA was estimated by measurement of the amount of 2-LTR circle

DNA ($N_{2\text{-LTR}}$) relative to total viral DNA (N_{tot}) in each culture, indexed to the same ratio of appropriate control cultures

$$\frac{C_{2\text{-LTR}}}{C_{tot}}.$$

The nuclear translocation index was then defined as

$$\left(\frac{\frac{N_{2\text{-LTR}}}{N_{tot}}}{\frac{C_{2\text{-LTR}}}{C_{tot}}}\right) \times 100\%.$$

PCR in Situ Hybridization

Detection of PCR-amplified HIV-1 DNA in fixed tissue culture samples was accomplished using a previously published protocol (30–33). Briefly, after protease digestion (with 2 mg/ml pepsin at room temperature for 30 min), PCR was performed using a solution which contained PCR buffer (GeneAmp kit, Perkin Elmer Corporation, Norwalk, Conn., U.S.A.), 4.5 mM $MgCl_2$, 200 μM dNTPs, 1.0 μM of the primers (SK38/SK39 or SK145/SK431 [30]), 1 mg/ml of bovine serum albumin and 2.5 Units/25 μl of Taq DNA polymerase. The "hot start modification", whereby Taq DNA polymerase is added to the amplifying solution at 55°C increases the sensitivity of the assay to one copy with a single primer pair (32). After an initial denaturation step of 94°C for 3 min, 30 cycles were completed using the following protocol: annealing/extension at 55°C for 2 min, denaturation at 94°C for 1 min. After PCR, the probe (SK19 for SK38/39 and SK102 for SK145/431 [30]) at 50 ng/ml in a solution of 10% formamide, 5% dextran sulfate, and 300 mM NaCl, and amplified DNA are simultaneously denatured by heating to 100°C for 5 min. After the hybridization (2 hr at 37°C) and wash steps (10 min in 1 × SSC and 0.2% bovine serum albumin at 50°C) the slides are incubated in anti-digoxigenin-alkaline phosphatase conjugate (1:100 dilution, Boehinger Mannheim, Indianapolis, Ind., U.S.A.) at 37°C for 30 min, and the probe/target complex detected by incubation with chomagen, NBT/BCIP (ONCOR Corporation, Gaithersburg, Md., U.S.A.). A hybridization signal is evident as a dark blue precipitate, whereas the counterstain, Nuclear Fast Red, stains nuclei and cytoplasm pale pink. Negative controls were done with a separate cytospin on the same slide and included either mock-amplification in the absence of the Taq polymerase or amplification with primers specific for human papilloma virus (31) in place of the HIV-1 specific primers; each control consistently gave negative results.

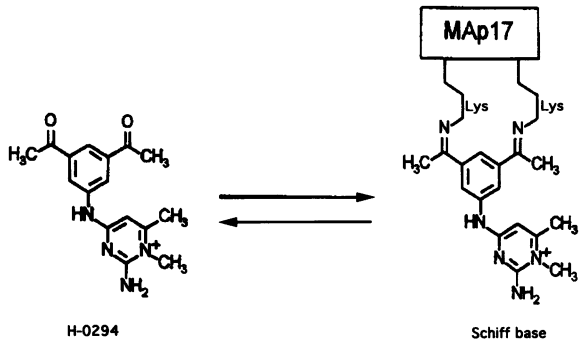

FIG. 1. Presumed interaction of anti-NLS compounds with HIV-1 MA p17 NLS

One of the anti-NLS compounds, H-0294, is shown. Reversible reaction of a carbonyl group with an amino group of any lysine (including those within the MA p17 NLS) results in the formation of a Schiff base. Formation of two Schiff bases after reaction with the adjacent lysines characteristic of nuclear localization signals would stabilize the bimolecular complex, shifting the equilibrium toward product formation.

RESULTS

Arylene Bis(methyl ketone) Compounds Block HIV-1 Replication in Primary Human Monocytes

Nuclear localization signals (NLS) are generally characterized by a stretch of basic amino acids, comprised mostly of lysine residues (reviewed in Ref. 34). We reasoned that by designing divalent carbonyl-containing compounds that potentially could interact with closely adjacent amino groups to form Schiff bases (Fig. 1), we could preferentially target the typical lysine-rich NLS sequences, while avoiding highly stable binding to other protein segments. Schiff base formation is a reversible chemical reaction, and we surmised that creation of a double Schiff-base structure with two nearby lysine residues in the NLS would result in a more stable adduct than a single Schiff base formed on an isolated lysine or other amino group (Fig. 1). Also, ε-amino groups of lysine residues within the context of the other

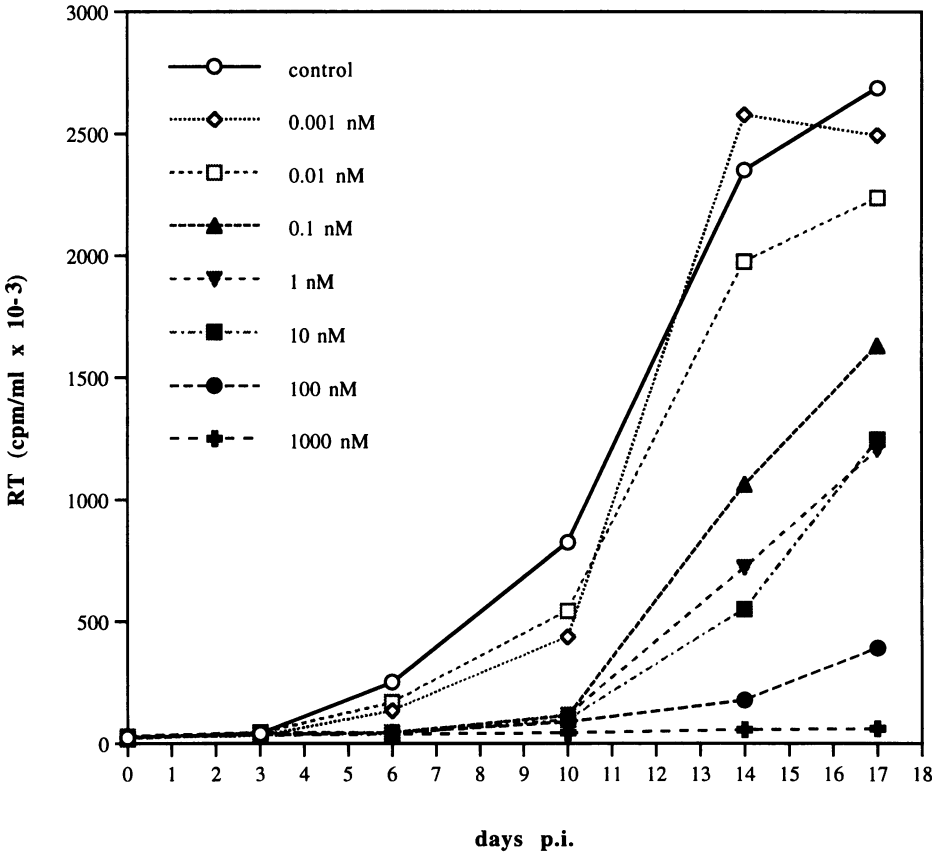

FIG. 2. Dose response curves of H-0294 in human monocytes infected with HIV-1_{ADA}

Monocyte cultures were infected with HIV-1_{ADA} with MOI 1 ng p24/10^6 cells in the presence of indicated concentrations of H-0294. After an overnight incubation, free virus was washed away, medium was changed, and incubation was continued in the presence of drug. Half the volume of culture medium was changed every 3–4 days, and samples were withdrawn for RT analysis at the same time. A representative experiment out of three performed with cells from different donors is shown. Each concentration of the drug was assayed in triplicate and presented data are means.

basic amino acids comprising the NLS should have a higher probability of being neutral rather than positively charged (due to reciprocal repulsion of positive charges), and might therefore be more susceptible to reaction with carbonyls to produce Schiff base adducts. With these considerations, we synthesized a series of mono- and divalent ketoaryl compounds with different modifications in the side groups (P. Ulrich, L. Dubrovsky, A. Cerami, and M. Bukrinsky, manuscript in preparation). Several of these compounds demonstrated pronounced anti-HIV effects in primary human monocyte cultures. In this report, we describe the inhibitory effect of one of the compounds, H-0294, on HIV-1 replication in monocytes and provide an initial functional characterization of this inhibition.

Compound H-0294 Inhibits HIV-1 Replication in Primary Monocytes with $IC_{50} \approx 1$ nM

The potential cytotoxicity of H-0294 was tested in monocyte cultures by trypan blue exclusion assay or lactate dehydrogenase (LDH) release. By either assay, no cytotoxic effect was observed at concentrations of the compound up to 10 μM (data not shown).

Results presented in Fig. 2 show the effect of various concentrations of H-0294 on HIV-1 replication in monocytes. From this experiment, we estimated that the IC_{50} for this compound fell between 1 and 10 nM. At 1 μM, H-0294 inhibited HIV-1 replication in monocytes by more than 95%. In the presence of inhibitory concentrations of H-0294, we observed not only a delay

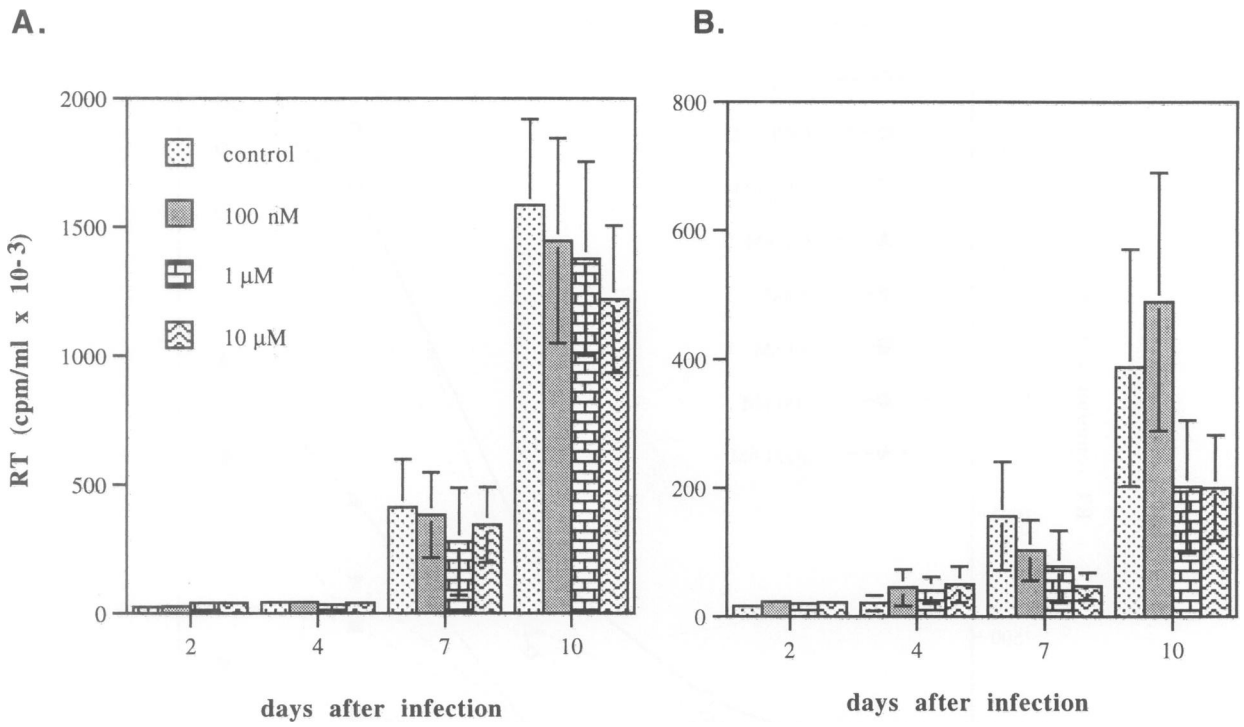

FIG. 3. Effect of H-0294 on HIV-1 replication in activated PBLs

RT activity in culture supernatants was assessed at the indicated days postinfection as described in Materials and Methods. Cultures were infected and maintained under concentrations of H-0294 as indicated (key indicating concentrations in Panel A applies to Panel B as well). (A) Cultures were infected with HIV-1_{ADA} at MOI of 10 ng p24/10^6 cells. (B) Cultures were infected with HIV-1_{ADA} at MOI of 1 ng p24/10^6 cells. Note difference in *y*-axis scales between Panels A and B.

in the development of infection but also a decrease in total RT activity at the peak of infection, thus indicating an irreversible inactivation of the virus by the compound. The compound was also tested on activated PBL cultures and, in accordance with our prediction, the antiviral effect was much less evident in these actively dividing cell populations (Fig. 3). In fact, no antiviral effect was detected if cultures of replicating PBLs were infected at the multiplicity of 10 ng p24/10^6 cells (Fig. 3A), the same MOI as used to infect monocyte cultures, although some inhibition was observed when dividing PBL cultures were infected at a 10-fold lower MOI (Fig. 3B).

Since we hypothesized that the activity of H-0294 depended on its two carbonyl groups, we tested a derivative of this drug that had amidinohydrazone groups substituting both carbonyls (Compound H-2194). In agreement with our prediction, H-2194 was significantly less active than H-0294 in monocyte cultures (Fig. 4). The estimated IC_{50} for H-2194 is about 100 times higher (approximately 100 nM) than for the H-0294. Plausible explanations for the residual antiviral activity of H-2194 are the presence of small amounts of the unreacted parent compound (H-0294), and/or the hydrolysis of H-2194 guanylhydrazones to reconstitute active carbonyl groups.

Compound H-0294 Blocks Nuclear Importation of HIV-1 DNA

The effect of H-0294 appeared to reproduce the effect of NLS mutations (22,23). To further substantiate this possible molecular mechanism of action, we analyzed the effect of this arylene bis(methyl ketone) compound on synthesis and nuclear importation of the viral genome following infection of monocytes. Results presented in Fig. 5 A, B demonstrate that the drug did not significantly affect viral reverse transcription in infected cultures but dramatically reduced the fraction of total HIV-1 DNA represented by 2-LTR circle forms. Quantitative evaluation by

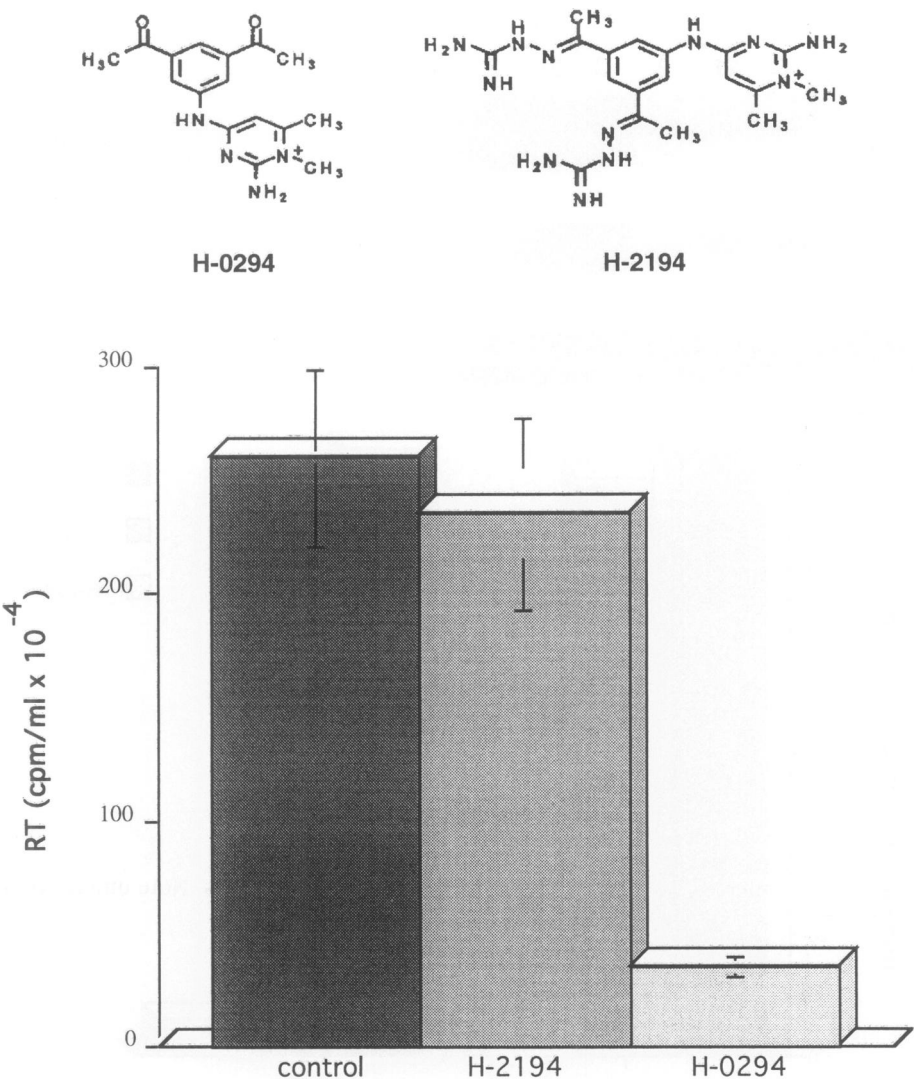

FIG. 4. Inhibition of HIV-1 nuclear translocation by H-0294 relies on the presence of carbonyl groups
Primary human monocytes were infected with HIV-1_{ADA} in the presence of 10 nM concentration of H-0294 or H-2194, or without drugs (control). Half the medium was changed every 3 days, and drugs were present throughout the whole experiment. Results of the RT analysis done on the 10th day after infection are presented. Each sample was assayed in quadriplicate and data are means ± SD.

Phosphorimager analysis showed 2-LTR circle DNA to be 95% lower at 48 hr and 97% lower at 96 hr after infection in treated cultures (Fig. 5B). Interestingly, similar analysis for H-2194 demonstrated only 5–8% reduction in 2-LTR circles, corroborating the difference in potency noted for these compounds in the antiviral test (Fig. 4).

2-LTR circle DNA represents a useful marker for successful nuclear translocation of HIV-1 DNA. This index was introduced several years ago (18) and has been shown since then to correlate well with the actual amount of viral DNA within the nuclear compartment (18,20,22,23, 26). In addition, we sought an alternative direct demonstration that the potential mechanism of antiviral action of H-0294 is its interference with nuclear translocation of the viral genome. This was accomplished by exploiting an in situ PCR technique (30–32). Results presented in Fig. 6A demonstrated that HIV-1 DNA-specific signal (blue color) remained localized in the cytoplasm of monocytes infected in the presence of 100 nM of H-0294. Control infections show normal nuclear localization of the HIV-1 DNA-specific signal (Fig. 6B). Control amplifications performed without Taq polymerase or with HIV-unrelated

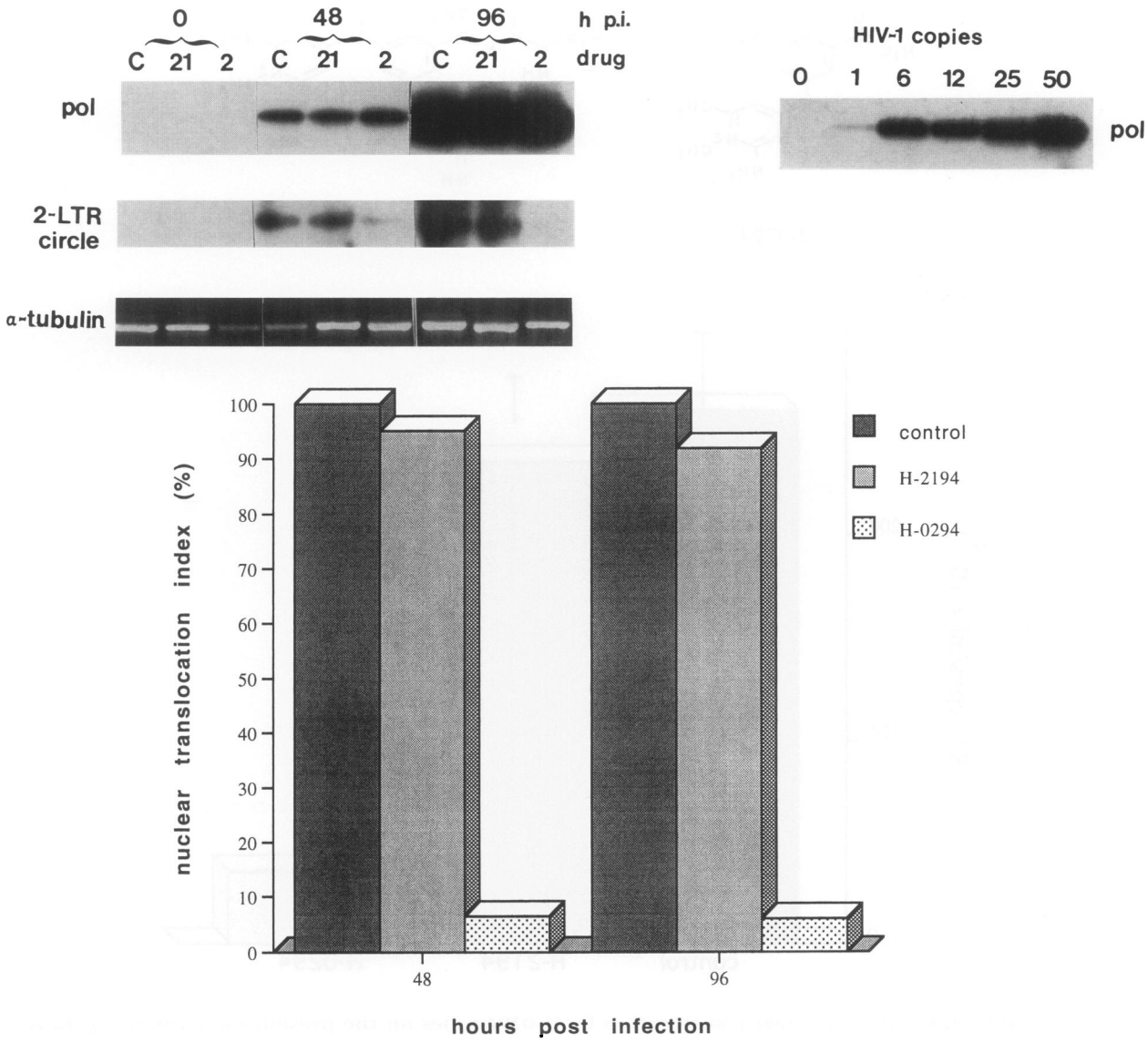

FIG. 5. PCR-based analysis of nuclear translocation of HIV-1 genome

(A) Results of the PCR analysis with primers specific for HIV-1 polymerase gene (*pol*) and 2-LTR circle junction (2-LTR circle) or for the cellular α-tubulin gene. A million monocytes were infected with HIV-1_{ADA} in the presence of H-0294 (2) or H-2194 (21). Total cellular DNA was extracted immediately after a 2-hr adsorption period (0 hr p.i.), 48 hr p.i. and 96 hr p.i. HIV-1-specific products were revealed after Southern hybridization to ^{32}P-labeled probe, while the tubulin-specific fragment was revealed by staining the gel with ethidium-bromide. Control infection (C) was performed without drugs. Results of the PCR reactions with serial dilutions of 8E5 cells, each containing one integrated copy of HIV-1 genome (53), using *pol*-specific primers are shown on the right. The number of HIV-1 copies in each dilution is shown above the corresponding lane. (B) Results of the experiment described in Fig. 4A were analyzed on a phosphorimager. Nuclear translocation index is expressed as

$$\left(\frac{\frac{N_{2\text{-LTR}}}{N_{tot}}}{\frac{C_{2\text{-LTR}}}{C_{tot}}} \right) \times 100\%.$$

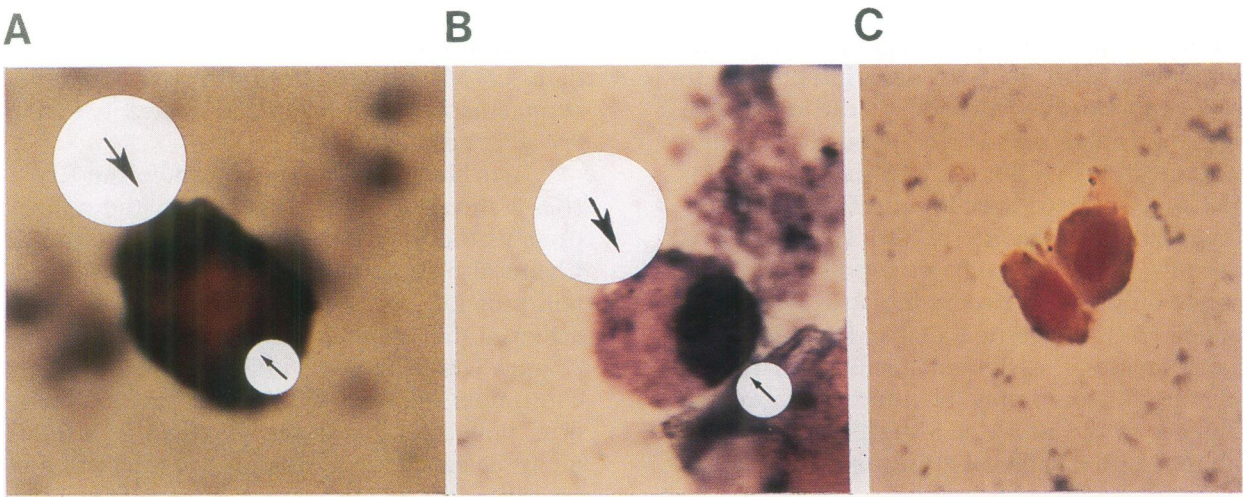

FIG. 6. PCR in situ hybridization analysis of HIV-1-infected monocytes

Monocytes were inoculated with 10 ng p24/10^6 cells of HIV-1_{ADA}, washed after 2 hr absorption and incubated for additional 48 hr. One hundred nanomolar concentration of H-0294 was present in experimental samples at all steps of the procedure. At the end of a 48-hr incubation, culture medium was removed, cells were washed three times with PBS and fixed with 10% buffered formalin for 24 hr. Monocytes were scraped from the plate with rubber policemen and analyzed by in situ PCR as described in Materials and Methods. (A) Monocytes treated with H-0294; (B) untreated (control) infected monocytes; (C) negative control (without Taq polymerase). Large arrows point to the cytoplasm, and small arrows to nuclei.

primers do not show HIV-specific DNA staining (Fig. 6C).

DISCUSSION

Since the discovery of HIV as the causative and pathogenic agent of AIDS, a number of potentially useful strategies for antiviral therapy have emerged (reviewed in Ref. 35 and 36). These strategies have targeted various steps of HIV replication, from binding of the virus to its cellular receptor as the initial step of infection, to the assembly of mature HIV virions and their budding from the cell membrane during the final steps. Unfortunately, relatively few drug candidates developed as a result of these endeavors have emerged as useful and efficient therapeutics in clinical practice (reviewed in Ref. 37). The rapid evolution of escape mutants owing to the extreme genetic lability of the virus, combined with the very nature of the retroviral life cycle, where the infectious agent integrates into the host cell genome and replicates in the same manner as normal cellular genes, have prominently mitigated against successful drug discovery efforts. Given the difficulty of interfering with expression of the virus once provirus has become established in the cellular genome, pharmaceutical approaches that target early, preintegration steps of HIV replication remain attractive strategies.

One of the early obligate steps in retroviral replication within an infected cell is the translocation of the viral genetic material into the cell nucleus (19). This requirement was previously unappreciated as a potential target for antiretroviral therapy because most retroviruses rely on the natural disruption of the nuclear membrane during mitosis to gain access to the nuclear compartment, therefore depending on cell division for productive infection (20,38). HIV-1 and other members of the lentivirus subfamily of retroviruses have in addition a different mechanism to achieve nuclear access. This mechanism is employed for HIV-1 replication in nondividing cells (39,40) and depends on the nuclear targeting properties of two viral proteins, MA p17 and Vpr (18–20,22,25). Recent studies have identified a specific nuclear localization signal (NLS) within MA p17 which is responsible for targeting the preintegration complex of HIV-1 into the nucleus of a nondividing cell (23,25). The HIV-1 NLS conforms closely to other well-characterized nuclear localization sequences (34), comprising a stretch of basic amino acids, predominantly lysine residues (23). The NLS-dependent nuclear translocation of the HIV-1 genome represents an

appealing target for anti-HIV therapy because: (i) it functions prior to viral integration, such that interference with this step has the potential to prevent productive infection of the cell; (ii) it relies on a characteristic well-defined set of molecular features within the NLS that are in principal amenable to specific chemical modifications; (iii) the activity of the HIV-1 NLS is critically sensitive to base substitutions that change only one or two amino acids within the peptide (34), suggesting that a few molecular changes within the NLS can inactivate its nuclear targeting function; and (iv) the well conserved sequence of the HIV-1 NLS suggests that escape mutants still capable of using this mechanism may evolve only infrequently.

Recognizing this potential vulnerability, a set of compounds potentially capable of binding and reacting with the free ε-amino groups of lysine residues was synthesized and tested. Some of the compounds (i.e., H-0294) proved to be extremely potent inhibitors of HIV-1 replication in monocytes, with an IC_{50} in the low nM range. For maximal effect, these drugs had to be added to static monocyte cultures during the early stages of infection (within 24 hrs after addition of the virus to cells). This result correlates well with the kinetic of HIV-1 replication in monocytes, which shows accumulation of HIV-1 DNA within the nucleus by 24–48 hr after inoculation (M. Bukrinsky, unpublished results). No effect of the drugs on the expression of integrated provirus by activated U1 (monocytic cell line) or ACH2 (T cell line) cells was observed (data not shown). Likewise, the antiviral effect of the compounds on HIV-1 infection in activated PBL cultures was limited, and revealed only at low multiplicity of infection (MOI). At the standard MOI employed in macrophage screening experiments, no antiviral activity of the compounds was detected in dividing PBL cultures. Taken together, these results support the hypothesis that compounds designed to modify the HIV-1 NLS can be effective antiviral agents in nondividing cells when administered prior to integration of the viral cDNA into the host genome.

Complete inhibition of viral replication by H-0294 was achieved at relatively low MOI, 10 ng p24 per 10^6 cells (about 200 HIV-1 particles per cell [41]) under our experimental conditions. Experimental infection at higher MOI resulted in low but measurable levels of viral production even in the presence of relatively high concentrations (1 μM) of the drug. This result may in part be attributable to the small number of dividing cells in monocyte cultures (42), which could support low levels of HIV-1 replication independent of MA p17 NLS function, and to the complementation of MA p17 NLS activity by the *vpr* gene product (22).

To achieve nuclear translocation, the HIV-1 NLS is thought to be recognized by the cellular proteins that mediate nuclear importation (43). Chemical disruption of these cellular/viral protein/protein interactions may have been achieved by elimination of required positive charges within the NLS and/or through sterical constraints imposed by the presence of bulky drug molecules at the recognition site. Experiments designed to test directly the interaction of potential anti-NLS drugs with molecular features of the HIV-1 nuclear localization signal and to define the nature, stability, and kinetics of this interaction are now in progress. Several lines of evidence already available, however, tend to corroborate the intended direct interaction between anti-NLS compounds and MA p17 as the actual mechanism of action of anti-NLS compounds: (1) H-0294 efficiently blocks nuclear translocation of the HIV-1 genome (Fig. 5 and 6); (2) chemical derivatives of H-0294 lacking active carbonyl groups (e.g., H-2194), the functional groups intended to interact with the retroviral NLS, demonstrate greatly decreased antiviral activity (Fig. 4); and (3) the high specific activity of anti-NLS compounds for HIV-1 replication in nondividing cells versus a markedly lower benefit in actively dividing cultures correlates with the suggested mechanism.

Perspectives for the Use of Anti-NLS Compounds as Anti-HIV Drugs

Only future animal studies and human clinical trials can directly assess whether anti-NLS compounds can contribute to the pharmaceutical arsenal against HIV disease. It is perhaps encouraging, however, that the intended target populations of this new class of potential therapeutics are nondividing cells. Maturation of macrophages in vivo requires only few, if any, cell divisions (reviewed in Ref. 44), and at which stage in its life cycle the macrophage is infected by HIV-1 remains controversial (40,42). Although under some in vitro conditions, HIV-1 replication in monocytes appears to depend on cell proliferation (42,45), there is convincing evidence that cell division is not absolutely required for HIV-1 infection of macrophages (40),

and certain macrophage populations highly susceptible to HIV-1 infection (e.g., microglial cells) do not divide at all under in vitro culture conditions, as evidenced by ^{3}H-thymidine incorporation and BrdU staining (46) (M. Dubois-Dalcq, personal communication). Thus the potential for anti-NLS compounds to block HIV-1 infection in macrophage populations in vivo appears well founded, and at a minimum this class of inhibitors may be useful in establishing the distinct roles of macrophage and microglial infections in the clinical manifestations of HIV disease.

The utility of anti-NLS compounds may also extend, albeit to a lesser degree, to other cell types susceptible to HIV infection. Infection of quiescent T cells by HIV-1 has been shown to take place in vitro (47,48), and probably constitutes an important pathway for the spread of infection in vivo at various stages of the disease (29). Although HIV-1 does not establish productive replication in quiescent T cells, the extrachromosomal retroviral DNA can persist in the cytoplasm of such cells for a considerable period of time, and initiate replication upon activation of the host cell (25,47,49,50). A recent report suggests that the duration of viral persistence in the quiescent T cell depends on the presence of a functional NLS (25), thus widening the potential HIV infection-limiting role of the anti-NLS class of drug candidates. In this regard, it was encouraging that antiviral effects of H-0294 were apparent in activated PBL cultures, even if only at low MOI. This potentially indicates that a functional NLS also enhances viral replication in proliferating cell populations, supporting the use of anti-NLS compounds in this context as well.

For maximal effect, anti-NLS drugs should be present at the earliest possible time after exposure of the host to infective virus. During early stages of the infection, macrophages and cells of the macrophage lineage (i.e., dendritic cells) may be the primary reservoir of HIV-1 in the body, supporting infection of T cells by antigen presentation activities (51) as well as via the release of free virus. Direct cell-to-cell transmission of the virus may constitute the major route by which infection spreads during the early stages of the disease, after resolution of the initial viremia. The molecular mechanisms of this mode of transmission are still obscure, but to the extent that nondividing cells are involved, functional NLS activity to provide access to the host cell genome will also be required and a corresponding therapeutic opportunity may therefore exist despite the absence of free virus in the blood. The realization of this possibility will depend on the design of anti-NLS compounds that are sufficiently membrane permeable to penetrate to intracellular compartments. Our preliminary experiments indicate that H-0294, for instance, can inhibit HIV-1 infection in vitro when only the viral inoculum is pre-exposed to drug and the culture remains subsequently untreated (L. Dubrovsky and M. Bukrinsky, unpublished results). Since the HIV envelope is budded from the host membrane, this suggests that anti-NLS drug candidates can be designed to diffuse through the cell membrane.

Although the potential benefits of early initiation of anti-NLS therapy seem the most promising in principle, these compounds may be helpful even if administered later in the disease. It is well established that macrophages, through the abnormal production of cytokines and other biologically active molecules, may mediate significantly the immune dysregulation observed in AIDS (reviewed in Ref. 52). Therefore, reduction in the number of infected macrophages could potentially reduce the disruption of normal immune functions in HIV-infected individuals.

After identifying the HIV-1 nuclear localization signal on MA p17 as a potentially vulnerable retroviral target and experimentally demonstrating its critical function for viral replication in nondividing cells, we were able to design a set of candidate drugs specifically to interfere with this retroviral target. As a class, anti-NLS compounds may be useful not only against HIV, but also against other viruses that replicate in nondividing cells and therefore require nuclear transport for replication, for instance, the measles virus, which replicates in macrophages or the herpes virus that infects neurons. As the development of such anti-NLS compounds progresses, they are likely to be of particular value in combination therapy with other therapeutics that target the T cell arm of HIV-1 infection to alleviate the progression of HIV disease.

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