Inhibition of Human Immunodeficiency Virus Type 1 Transcription by Chemical Cyclin-Dependent Kinase Inhibitors

DAI WANG,¹ CYNTHIA DE LA FUENTE,¹ LONGWEN DENG,¹ LAI WANG,¹ IRENE ZILBERMAN,¹
CAROLYN EADIE,¹ MARLENE HEALEY,¹ DANA STEIN,² THOMAS DENNY,²
LAWRENCE E. HARRISON,³ LAURENT MEIJER,⁴
AND FATAH KASHANCHI^{1*}

George Washington University School of Medicine, Washington, DC 20037¹; Department of Pathology and Pediatrics² and Department of Surgery,³ UMDNJ–New Jersey Medical School, Newark, New Jersey 07103; and Station Biologique, CNRS, 29682 Roscoff, France⁴

Received 4 December 2000/Accepted 14 May 2001

Cyclin-dependent kinases (cdk's) have recently been suggested to regulate human immunodeficiency virus type 1 (HIV-1) transcription. Previously, we have shown that expression of one cdk inhibitor, p21/Waf1, is abrogated in HIV-1 latently infected cells. Based on this result, we investigated the transcription of HIV-1 in the presence of chemical drugs that specifically inhibited cdk activity and functionally mimicked p21/Waf1 activity. HIV-1 production in virally integrated lymphocytic and monocytic cell lines, such as ACH₂, 8E5, and U1, as well as activated peripheral blood mononuclear cells infected with syncytium-inducing (SI) or non-syncytium-inducing (NSI) HIV-1 strains, were all inhibited by Roscovitine, a purine derivative that reversibly competes for the ATP binding site present in cdk's. The decrease in viral progeny in the HIV-1-infected cells was correlated with a decrease in the transcription of HIV-1 RNAs in cells treated with Roscovitine and not with the non-cdk general cell cycle inhibitors, such as hydroxyurea (G₁/S blocker) or nocodazole (M-phase blocker). Cyclin A- and E-associated histone H1 kinases, as well as cdk 7 and 9 activities, were all inhibited in the presence of Roscovitine. The 50% inhibitory concentration of Roscovitine on cdk's 9 and 7 was determined to be ~0.6 μ M. Roscovitine could selectively sensitize HIV-1-infected cells to apoptosis at concentrations that did not impede the growth and proliferation of uninfected cells. Apoptosis induced by Roscovitine was found in both latent and activated infected cells, as evident by Annexin V staining and the cleavage of the PARP protein by caspase-3. More importantly, contrary to many apoptosis-inducing agents, where the apoptosis of HIV-1-infected cells accompanies production and release of infectious HIV-1 viral particles, Roscovitine treatment selectively killed HIV-1-infected cells without virion release. Collectively, our data suggest that cdk's are required for efficient HIV-1 transcription and, therefore, we propose specific cdk inhibitors as potential antiviral agents in the treatment of AIDS.

Human immunodeficiency virus type 1 (HIV-1) is the etiologic agent of AIDS (3, 11). The HIV-1 infection life cycle can be divided into pre- and postintegration phases, and successful HIV-1 infection is closely related to the host cell cycle progression (33). HIV-1 can infect both dividing and quiescent cells; such as the nondividing T lymphocytes (42, 44), terminally differentiated macrophages (48), brain microglial cells (46, 26), and cells that are artificially arrested in the G₁/S or G₂ phases of the cell cycle (26, 43, 25, 27). However, productive viral infection of HIV-1 is restricted only to dividing cells (49, 5). The preintegration stage of HIV-1 infection can be restricted at either reverse transcription (49) or integration levels (5). The postintegration restriction of HIV-1 transcription is mainly regulated by cellular transcription factors (41) and enzymatic activities of cellular proteins, such as cdk9/cyclin T (20, 51, 47, 13, 21) and cdk7/cyclin H (8, 33, 50, 34), which play a critical role in Tat-mediated transactivation.

Reciprocally, HIV-1 has evolved various means to perturb the cell cycle to optimize the cellular conditions in favor of its own replication. Previous studies have indicated that HIV-1 encoded viral protein R (Vpr) can arrest the cell cycle at the

G₂ phase transiently by retaining the G₂/M p34cdc2 in the tyrosine phosphorylated inactive state (18, 14, 19). Blocking the cell cycle at the G₂ phase prolongs the active promoter stage, allowing optimal HIV-1 transcription (18). Our previous data have indicated that the expression of cyclin-dependent kinase (cdk) inhibitor p21/Waf1, is abrogated in latent HIV-1-infected cells (6). P21/Waf1 is known as a cdk2, -3, -4, and -6 inhibitor and, at low concentrations, selectively blocks G₁/S transition. In latently activated cells and, upon induction of stress, the lack of p21/Waf1 results in the loss of the G₁/S checkpoint, increased activity of cyclin E-cdk2 complex, increased retinoblastoma protein (Rb) phosphorylation, increased HIV-1 transcription, and viral progeny formation (6).

The lack of p21/Waf1 expression in HIV-1-infected cells indicated that the p21/Waf1-associated cdk's might play an important role in HIV-1 replication. This result and the requirement of cdk9 and -7 activities in HIV-1 transcription prompted us to ask whether HIV-1 production could specifically be inhibited by chemical drugs that function similarly to p21/Waf1 and inhibit cdk7 and -9 simultaneously. Several purine derivative drugs, including Olomoucine, Roscovitine, and Purvalanol A, have recently been described that inhibit specific types of cdk's (17, 32). At low concentrations, their inhibitory effects are highly specific for cdc2-cyclin B, cdk2-cyclin A, and cdk2-cyclin E and not other cyclin-cdk complexes or many other cellular kinases including ERKs, various forms of protein

* Corresponding author. Mailing address: George Washington University School of Medicine, 2300 Eye St., NW, Ross Hall, Rm. 552, Washington, DC 20037. Phone: (202) 994-1781. Fax: (202) 994-1780. E-mail: bcmfkk@gwu.edu.

kinase C (PKC), and casein kinase 2. None of these drugs can inhibit the cdk4 and cdk6 activities significantly, and their inhibitory effects on cdk7 and cdk9 have not previously been determined. The potential applications of these drugs have been explored in cancer (40) and viral diseases, including herpes simplex virus (HSV) (37, 38, 39) and cytomegalovirus (CMV) infection (4). Furthermore, Roscovitine targets cellular proteins, such that almost no resistant HSV type 1 strains could be isolated (37), which provided a new concept to develop antiresistant viral drugs.

Here we present evidence that cdk specific inhibitors are effective drugs that inhibit HIV-1 replication. The inhibition has been observed in HIV-1 latently infected monocytes and T cells, which is associated with the inhibition of viral transcription. Similar results were also obtained in infected activated peripheral blood mononuclear cells (PBMC) with either primary syncytium-inducing (SI) or non-syncytium-inducing (NSI) HIV-1 isolates. Roscovitine inhibited cdk2, -7, and -9 kinase activity with similar 50% inhibitory concentrations (IC_{50} s). In addition, Roscovitine could selectively induce apoptosis in HIV-1-infected cells, as made apparent by the activation of caspase-3 and the cleavage of PARP protein. Therefore, cdk specific inhibitors provide a possible alternative therapeutic target for HIV-1 infection.

MATERIALS AND METHODS

Cell culture, peptides, plasmids, antibodies, and drugs. ACH₂ (7, 9) and 8E5 (10) cells are both HIV-1-infected lymphocytic cells, with the integrated wild-type single-copy (ACH₂) reverse transcriptase and an integrated single-copy reverse transcriptase-defective virus (8E5) in CEM (12D7) cells (36). The CEM T cell (12D7) is the parental cell for both ACH₂ and 8E5 cells. U1 is a monocytic clone harboring two copies of the viral genome (10) from parental U937 cells. MT-2 cells are infected with several copies of human T-cell leukemia virus type 1 (HTLV-1) and produce full-length viral particles. All cells were cultured at 37°C with up to 10^5 cells per ml in RPMI 1640 media containing 10% fetal bovine serum and treated with a mixture of 1% streptomycin and penicillin antibiotics and 1% L-glutamine (Gibco-BRL).

Plasmids of HIV-LTR-CAT and pcTat were described previously (6). The Tat protein was produced in *Escherichia coli* and purified using Sephacryl S-200, followed by C₁₈ reversed-phase high-pressure liquid chromatography (24). The purified Tat protein was then dried and resuspended in phosphate-buffer saline (PBS) containing 1 mM dithiothreitol (DTT) and 0.01% bovine serum albumin. Wild-type C-terminal domain (CTD) peptide was a generous gift from M. Morange (45). Antibodies against cdk1 (C-19), cdk2 (M-2), cdk7 (C-19), cdk9 (L-19), cyclin E (M-20), cyclin A (H-432), poly(ADP-ribose) polymerase PARP (N-20), and caspase-3 (H-277) were purchased from Santa Cruz Biotechnology. The cdk inhibitors Olomoucine, Roscovitine, and Purvalanol A were synthesized in house and also purchased from Calbiochem and dissolved in dimethyl sulfoxide (DMSO) in 10 mM stock concentrations.

Lymphocyte transfection and CAT assays. Lymphocyte CEM (12D7) cells were grown to mid-log phase and were processed for protein electroporation according to a previously published procedure (24). Only one modification was introduced, in which the cells were electroporated at 230 V and plated in 10 ml of complete RPMI 1640 medium for 18 h prior to harvest and chloramphenicol acetyl transferase (CAT) assay. For CAT assays, transfected cells were harvested, washed once with PBS without Ca²⁺ and Mg²⁺, pelleted, and resuspended in 150 μ l of 0.25 M Tris (pH 7.5). Cells were freeze-thawed three times with intermittent vortexing and then incubated for 3 min at 68°C, followed by centrifugation. Supernatants were transferred to 1.5-ml Eppendorf tubes and centrifuged, and the supernatants were used for the determination of protein concentration. CAT assays were performed with 2 μ g of protein according to the method of Gorman et al. (15).

Cell extract preparation and kinase assays. Cells that were cultured to mid-log phase of growth were treated with or without tumor necrosis factor alpha (TNF- α) (10 ng/ml) for 2 h, washed with PBS without Ca²⁺ and Mg²⁺, and incubated for 48 h prior to lysis in a buffer containing 50 mM Tris-HCl (pH 7.5), 120 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.2 mM Na₃VO₄, 1 mM DTT, 0.5%

NP-40, and protease inhibitors (Protease Inhibitor Cocktail Tablets; Boehringer Mannheim [one tablet per 50 ml]). Kinases from immunoprecipitated associated complexes were then assayed by the transfer of phosphate from [γ -³²P]ATP to the substrates histone H1 or peptide representing the CTD of RNA polymerase II (Pol II) (45) in a reaction buffer consisting of 50 mM Tris (pH 7.4), 10 mM MgCl₂, 1 mM DTT, and 144 μ M ATP (40 μ Ci of [γ -³²P]ATP). Reactions were performed at 37°C for 30 min and stopped by the addition of sodium dodecyl sulfate (SDS) sample buffer. Samples were boiled for 5 min at 95°C, and the histone H1 proteins were separated on a 4 to 20% Tris-glycine gel; the CTD peptides were separated on a 20% discontinuous SDS-polyacrylamide gel electrophoresis (PAGE) gel. Gels were autoradiographed, and the bands were counted using Molecular Dynamics PhosphorImager software.

Immunoblotting. Cells were pelleted by centrifugation, washed with PBS without Ca²⁺ and Mg²⁺, and lysed with lysis buffer as described above. The lysate was incubated on ice for 15 min and microcentrifuged at 4°C for 10 min. Total cellular protein was separated on 4 to 20% Tris-glycine gels (Novex, Inc.) and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membranes; Millipore Corp.) overnight at 0.08 A. Following the transfer, the blots were blocked with 5% nonfat dry milk in 50 ml of TNE 50 (100 mM Tris-Cl [pH 8.0], 50 mM NaCl, 1 mM EDTA) plus 0.1% NP-40. Membranes were probed with a 1:200 to 1:1,000 dilution of antibodies at 4°C overnight, followed by three washes with TNE 50 plus 0.1% NP-40. The next day, the blots were incubated with 10 ml of ¹²⁵I-labeled protein G (Amersham; 50- μ l/10 ml solution) in TNE 50 plus 0.1% NP-40 for 2 h at 4°C. Finally, the blots were washed twice in TNE 50 plus 0.1% NP-40 and placed on a PhosphorImager cassette for further analysis.

Flow cytometry. For cell cycle analysis, cells treated with or without drugs were collected by low-speed centrifugation and washed with PBS without Ca²⁺ and Mg²⁺ and then fixed with 70% ethanol. For fluorescence-activated cell sorting (FACS) analysis, cells were stained with a cocktail of propidium iodide (PI) buffer (PBS with Ca²⁺ and Mg²⁺, RNase A [10 μ g/ml], NP-40 [0.1%], and PI [50 μ g/ml]) followed by cell-sorting analysis. FACS data acquired were analyzed by ModFit LT software (Verity Software House, Inc.).

Apoptosis was determined by using Annexin V and PI double staining (Annexin V-FITC; PharMingen International). Cells were washed twice with cold PBS without Ca²⁺ and Mg²⁺; resuspended in 1 $\times$ binding buffer (10 mM HEPES-NaOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl₂); 2.5 μ l of Annexin V-FITC, and 5 μ l of PI/ 10^5 cells; and incubated at room temperature for 15 min. Cells were acquired and analyzed using CELLQuest software (Becton Dickinson).

Caspase-3 assay. Cells were washed in PBS and analyzed for caspase-3 activity by using a CPP32/Caspase-3 Colorimetric Protease Assay Kit (Chemicon, Temecula, Calif.) according to the manufacturers' instructions. Briefly, cells were lysed in 150 μ l of cell lysis buffer provided in the kit. Protein concentrations of the lysates were determined by using the bicinchoninic acid assay reagent (Pierce, Rockford, Ill.). Equal amounts of lysates were incubated with the caspase-3 substrate, 200 μ M DEVD-pNA, at 37°C for 3 h. Absorbances of the samples were read every 60 min in a Spectramax 250 (Molecular Dynamics) microplate reader at 405 nm. Caspase-3 activity was proportional to the optical density at 405 nm.

Cell proliferation. Cells (CEM) were initially treated with various concentrations of hydroxyurea or nocodazole and evaluated after 0, 12, 24, 48, 60, and 72 h. Subsequently, cells were incubated with 10 μ Ci of [³H]thymidine (Amersham) for 2 h prior to the end of each interval and harvested in an automatic cell harvester. The amount of radioactivity incorporated into the DNA was measured in a liquid scintillation counter (Packard) and expressed as the counts per minute (cpm). Data represented at the bottom of Fig. 3A are an average of three independent experiments.

Northern blots. Total cellular RNA was extracted using the RNeasy reagent (Gibco-BRL). Total RNA (20 μ g) was isolated 12 or 24 h posttreatment and run on a 1% formaldehyde-agarose gel overnight at 75 V, transferred onto a 0.2- μ m (pore-size) nitrocellulose membrane (Millipore, Inc.), UV cross-linked, and hybridized overnight at 42°C with ³²P-end-labeled HIV-1 full genomic RNA (Loftstrand, Gaithersburg, Md.). The next day, membranes were washed two times for 15 min each with 10 ml of 0.2% SDS-2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 37°C, exposed, and counted on a PhosphorImager cassette.

PBMC infection. Phytohemagglutinin-activated PBMC were kept in culture for 2 days prior to each infection. Isolation and treatment of PBMC were performed by following the guidelines of the Centers for Disease Control (5a). Approximately 5×10^6 PBMC were infected with either an SI (UG/92/029 Uganda strain, subtype A envelope, 5 ng of p24 gag antigen) or an NSI (THA/92/001, Thailand strain, subtype E envelope, 5 ng of p24 gag antigen) strain of HIV-1. Both viral isolates were obtained from the National Institutes of Health

FIG. 1. Inhibition of HIV-1 by various cdk inhibitors. (A) HIV-1 latently infected cells (ACH₂, 8E5, and U₁) were grown to the mid-log phase, treated with TNF-α (10 ng/ml) for 2 h, washed, and subsequently incubated with a 10 μM concentration of each cdk inhibitor. Five days later, the supernatants were collected, and the amount of newly synthesized HIV-1 was measured by p24 antigen assay. (B) Purified Tat (300 μg) was electroporated into ACH₂, 8E5, and U₁ cells and subsequently treated with various drugs for 5 days. (C) Experiment similar to that in panel A, except that both T and monocytic cell lines were treated with a combination of PMA (1 ng/ml) and PHA (1 μg/ml) for 12 h for induction of virus. (D) Experiment similar to that in panel A, except that MT-2 cells (HTLV-1 infected) were treated with TNF-α (10 ng/ml) for 2 h, washed, and subsequently treated with one of the three cdk inhibitors. Seven days later samples were collected and used for p19 gag ELISA. (E) PHA-activated PBMC (5×10^6) were infected with HIV-1 either SI (UG/92/029 Uganda strain, subtype A envelope) or NSI (THA/92/001, Thailand strain, subtype E envelope) strains. Unadsorbed viruses were washed after 6 h, and cells were cultured in fresh media with various cdk inhibitors (10 μM). Samples were collected every sixth day and used for p24 gag ELISA assay.

(NIH) AIDS Research and Reference Reagent Program (catalog numbers 1650 for strain UG/92/029 and 1651 for strain THA/92/001). After 8 h of infection, cells were washed, and fresh media were added. Drug treatment was performed (only once) immediately after the addition of fresh media. Samples were collected every sixth day and stored at -20°C for p24 gag enzyme-linked immunosorbent assay (ELISA).

HIV-1 p24 and HTLV-1 p19 ELISA. Media from HIV-1 infected cell lines were centrifuged to pellet the cells, and supernatants were collected and diluted to 1:100 to 1:1,000 in RPMI 1640 prior to ELISA. Supernatants from the infected PBMC were collected and used directly for the p24 antigen assay. The p24 gag antigen level was analyzed by using the HIVAG-1 Monoclonal Antibody Kit (Abbott Laboratories, Diagnostics Division). The HTLV-1 p19 core antigen ELISA kit was from Retro-Tek (Cellular Products).

RESULTS

Inhibition of HIV-1 transcription in the presence of cdk inhibitors. We have previously reported that in latently HIV-1-infected cells, the expression of G₁/S cdk inhibitor p21/Waf1 was impaired, indicating that the p21/Waf1 associated cdk's, as well as cdk9-cyclin T and cdk7-cyclin H complexes, play an important role in HIV-1 transcription. We therefore reasoned that cdk chemical inhibitors, which are functionally similar to p21/Waf1, could potentially inhibit viral replication in infected cells. We initially tested 14 different cdk inhibitors on both infected and uninfected T cells, among which three purine analogues, Olomoucine, Roscovitine, and Purvalanol A, were further selected primarily due to the lack of toxicity and the reversibility of inhibition on cdk's in uninfected T and monocytic cells (data not shown).

We choose HIV-1 stably integrated cells, lymphocytic ACH₂

and 8E5 cell lines, as well as monocytic U₁ cells (and their uninfected parental counterparts CEM and U937, respectively) for our initial studies, since viral transcription could be activated and scored through signals, such as Tat, TNF-α, or phorbol myristate acetate (PMA) and PHA. We first activated viruses in these cells with TNF-α (10 ng/ml) at 37°C for 2 h, followed by washing, and then we incubated cells with 10 μM concentrations of each drug. The p24 concentration in the medium was determined by ELISA assay, and the results of such an experiment are shown in Fig. 1A. All drugs inhibited HIV-1 replication to various degrees, with Olomoucine (due to its highest IC₅₀ value) being the least effective and Roscovitine and Purvalanol A being the most effective of the three drugs. A somewhat similar pattern of drug inhibition was also seen when ACH₂, 8E5, and U₁ cells were treated with either Tat or a combination of PMA and PHA to induce full-length viral transcripts. Tat-treated cells (Fig. 1B) were best inhibited in the presence of Roscovitine, whereas PHA- and PMA-treated cells were inhibited well with all three drugs (Fig. 1C). Interestingly, compared to other retrovirally infected cells such as MT-2 (infected with HTLV-1), only Purvalanol A inhibited HTLV-1 replication in these cells (Fig. 1D). We next examined the effect of all three drugs on primary HIV-1 field isolates of SI and NSI strains. Activated PBMC were infected with two independent HIV-1 viral strains of SI (UG/92/029, subtype A envelope) and NSI (THA/92/001, subtype E envelope). Cells were treated with 10 μM concentrations of each drug postinfection, and the p24 gag antigen level was determined by

FIG. 1—Continued.

ELISA. The results of such an experiment are shown in Fig. 1E, in which Roscovitine (for both isolates) and Purvalanol A (only for NSI strain) effectively blocked viral replication.

We next examined the cellular effects of these drugs on the cell cycle progression of HIV-1-infected and uninfected cells. Cells were treated with TNF- α , washed, and grown in the presence of Olomoucine, Roscovitine, or Purvalanol A, and their cell cycle distributions were assessed 48 h after treatment. In uninfected CEM cells, at concentrations of 10 μ M Olomoucine and Roscovitine did not change the cell cycle progression significantly, whereas Purvalanol A showed an increase in the G₂/M population and induced apoptosis (Fig. 2A). We next performed similar experiments on HIV-1-infected cells and observed increased apoptosis in the Purvalanol A- and Roscovitine-treated cells (Fig. 2B). Interestingly, Roscovitine selectively induced cell death in the HIV-1-infected cells (30.21% apoptosis in ACH₂ versus 4.9% in CEM cells). Therefore, the selective killing mechanism by this particular drug might partially be responsible for the decrease in HIV-1 titers observed in the infected cells. We also reasoned that Purvalanol A may be toxic to uninfected induced cells by increasing the G₂/M population and the increase of apoptosis, as is evident in Fig. 2A. Similar results were also observed in a set of promonocytic cell lines. U937 is the uninfected monocytic

parental cell line, and U1 is the U937 cell line infected with two copies of integrated HIV-1, only one of which is wild type for viral progeny formation. Again, Roscovitine treatment of the uninfected parental cells showed no apparent apoptosis, whereas U1 cells showed an abundance of the apoptotic population (Fig. 2C). Collectively, these data imply that among all three drugs tested Roscovitine may be the best choice of an inhibitor for an induced HIV-1-infected cell. Roscovitine was able to selectively kill HIV-1 infected cells and inhibit viral production. This is in contrast to many apoptosis-inducing agents, such as TNF- α , DNA-damaging agents (gamma irradiation, mitomycin C [6]), or sodium butyrate, in which cases the apoptosis of HIV-1-infected cell accompanies massive production and the release of infectious HIV-1 virions.

Inhibition of the basal and activated transcription by cdk inhibitors. We next sought to determine whether the inhibition of HIV-1 transcription, observed above, could be mediated specifically by cdk inhibitors or by general cell cycle inhibitors. To distinguish between the two possibilities, we performed transfection experiments with all three cdk inhibitors tested above and two other well-established cell cycle inhibitors, namely, hydroxyurea and nocodazole. Hydroxyurea blocks DNA replication by inhibiting ribonucleotide reductase and thus arrests cell cycle progression at the G₁/S checkpoint, while

FIG. 2. Cell cycle analysis of infected and uninfected cells following cdk inhibitor treatment. (A) CEM (uninfected) cells were treated with TNF- α (10 ng/ml) for 2 h and then grown in the presence of a 10 μ M concentration of each cdk inhibitor. Forty-eight hours later, cells were collected, fixed with 70% ethanol, PI stained, and analyzed by FACS. (B and C) Experiments similar to the experiment in panel A were performed in ACH₂ (B) or U1 (C) cells (both latently HIV-1 infected) plus TNF- α , followed by ethanol fixation, PI staining, and analysis by FACS. Apoptosis (Apop) represents a mixture of cells from either G₁, S, or G₂/M with characteristics of cell death.

nocodazole blocks at G₂/M by promoting tubulin depolymerization. When performing transfections in CEM cells, we observed a dramatic inhibition caused by Olomoucine, Roscovitine, and Purvalanol A and not by hydroxyurea or nocodazole at ca. 10 μ M (Fig. 3A). As a control, the effects of both hydroxyurea and nocodazole were tested in CEM cells, where at ca. 10 μ M no inhibition of DNA synthesis was observed (Fig. 3A, bottom). Therefore, the basal transcription of HIV-1 was inhibited by purine analogs and not general cell cycle inhibitors.

Activated transcription by Tat was also examined in CEM cells using the LTR-CAT reporter and pcTat (CMV-driven Tat). Consistent with basal transcription results, Tat transactivation was impaired by the cdk-specific drugs Olomoucine, Roscovitine, and Purvalanol A and not by hydroxyurea or nocodazole (Fig. 3B). Interestingly, Roscovitine inhibited activated transcription better than the other two analogs. To exclude the possibility that the reduction was mediated by inhibiting the CMV promoter driving the Tat gene, we performed a similar experiment with purified Tat protein and observed a similar inhibition of CAT activity by Roscovitine (Fig. 3C). Collectively, these results suggested that the cell cycle-dependent HIV-1 transcription requires cdk activities

and, perhaps more importantly, general cell cycle inhibitors that do not target cdk do not inhibit HIV-1 postintegrative events.

Time course analysis of p24 antigen release in Roscovitine-treated cells. Based on the results obtained above, we decided to determine whether there was a time dependency in viral inhibition in ACH₂ cells treated with Roscovitine. The results of such an experiment is shown in Fig. 4A, where at a low concentration of 1 μ M no obvious decrease of p24 was apparent; however, at 10 μ M concentrations only low levels of viral p24 antigens were detected in the supernatant. Similar results were obtained in both U1 and 8E5 cells when treatment was continued for up to 21 days at either a 5 or a 10 μ M concentration; in addition, neither hydroxyurea nor nocodazole was able to inhibit HIV-1 replication at these concentrations (data not shown).

To ensure that the inhibition was at the level of viral transcription and not at any other subsequent step, we performed Northern blot analysis using whole genomic HIV-1 probes. Viral transcripts of genomic (9.5-kb), structural (4.5-kb), and regulatory (2.2-kb) RNAs were all examined using a whole HIV-1 probe. The 9.5-kb genomic RNA is the precursor for the structural and regulatory mRNA and can be packaged into

ACH₂ + TNF- α

FIG. 2—Continued.

FIG. 3. Inhibition of HIV-1 basal and activated transcription by cdk inhibitors. (A) CEM (12D7) cells were transfected with CAT reporter gene driven by HIV-1 LTR and then incubated for 20 h with a 10 μ M concentration of either hydroxyurea, nocodazole, Olomoucine, Roscovitine, or Purvalanol A. CAT assays were performed using 2 μ g of cellular extract from each sample. The graphs at the bottom of the figure represent the effect of various concentrations of general cell cycle inhibitors (hydroxyurea and nocodazole) on CEM cells. (B) Inhibition of activated transcription in CEM cells was examined using transfected HIV-1 LTR-CAT, together with pcTat plasmid. (C) Experiment similar experiment to that in panel B, except that pcTat plasmid was replaced with *E. coli*-expressed and purified Tat protein.

virions. As shown in Fig. 4B, when ACH₂ cells were treated with TNF- α and subsequently with increasing concentrations of Roscovitine, all genomic, structural, and regulatory RNAs were dramatically decreased, suggesting that the inhibition was indeed at the level of gene expression. A comprehensive count of all three classes of the RNAs showed downregulation of HIV-1 basal (doubly spliced regulatory RNAs) and activated (singly spliced structural RNAs, unspliced genomic RNA) transcription (data not shown).

Roscovitine inhibits cyclin E- and cyclin A-associated histone H1 kinase, as well as cdk7 and cdk9 kinase activities in HIV-1-infected cells. Roscovitine has been reported to inhibit cdk1, -2, and -5, but not cdk4 or -6, and the activity of this drug to date has not been reported on cdk3, -7, -8, or -9 (31). cdk9-cyclin T complex is a critical complex in the control of the Tat protein function (20, 51, 47), and cdk7 and -2 have also been shown to associate with the Tat complex (33). We therefore examined the effects of Roscovitine on cdk2, -7, and -9

B)

pC Tat	-	+	+	+	+	+	+	+	+
Hydroxyurea	-	-	+	-	-	-	-	-	-
Nocodazole	-	-	-	+	-	-	-	-	-
Olomoucine	-	-	-	-	-	+	-	-	-
Roscovitine	-	-	-	-	-	-	+	-	-
Purvalanol A	-	-	-	-	-	-	-	+	-
DMSO	-	-	-	-	-	-	-	-	+
HIV-LTR-CAT	+	+	+	+	+	+	+	+	+

%Conversion	5.7%	93.4%	98.7%	98.9%	82.8%	57.2%	27.6%	53.9%	91.0%
-------------	------	-------	-------	-------	-------	-------	-------	-------	-------

C)

Tat Protein	-	▲		▲	
Roscovitine	-	-	-	+	+
HIV-LTR-CAT	+	+	+	+	+

% Conversion	25.3%	97.7%	97.8%	24.5%	15.5%
--------------	-------	-------	-------	-------	-------

FIG. 3—Continued.

activity using histone H1 and RNA Pol II CTD peptides as in vitro substrates. Cyclin A or E immunoprecipitates were obtained from uninfected and infected cells that were treated with Roscovitine and used in kinase assays. The results of such an experiment are shown in Fig. 5A, where cyclin A or cyclin E immunoprecipitate showed an average of twofold induction from ACH₂ cells. Importantly, the addition of Roscovitine decreased the histone H1 kinase activity from both cyclin immunoprecipitates in ACH₂ cells and not in control CEM cells. Similar reductions of cyclin A, cyclin E, cdk1, and cdk2 protein levels were also detected by Western blot analysis. Lower levels of these proteins in Roscovitine-treated cells might be due to the induced apoptosis or to the accelerated digestion of activated cyclin-cdk complexes in the infected cells. It is interesting to note that the transcription of cyclin A depends on the presence of an active cyclin E complex in the cell. The data in Fig. 5A indicate that the kinase activity of the cyclin E complex is lowered by more than eightfold (ACH₂ + TNF- α versus ACH₂ + TNF- α + Roscovitine) in HIV-1-infected and -induced cells. This may explain the observed lower cyclin A kinase activity and points to Roscovitine's primary effect on the cyclin E-associated complex. Alternatively, the levels of cdk 1 and 2 protein also show a ~5-fold drop in the same extracts,

FIG. 4. Time course study of HIV-1 progeny formation in the presence of Roscovitine. (A) ACH₂ (HIV-1 latently infected) cells were induced and grown in the presence of 1, 10, or 50 μ M Roscovitine. At days 2, 4, and 6, the supernatants were collected, and the released HIV-1 particles were measured by using a p24 antigen ELISA assay. (B) Twenty micrograms of total RNA was separated on an RNA-formaldehyde-agarose gel, transferred, and probed with full-length labeled HIV-1 genome RNA. The hybridized genomic RNA, singly spliced (structural), and doubly spliced (regulatory) RNAs were exposed and counted by using Molecular Dynamic PhosphorImager software. All samples were initially treated with TNF- α , and lanes 1 to 4 contained either DMSO or 1, 10, or 50 μ M Roscovitine, respectively. Both actin probe hybridization and ethidium bromide staining of the gel are shown below.

which may indicate that Roscovitine selectively targets the cyclin-cdk complex in infected rather than in uninfected cells. The mechanism of this downregulation on cdk1 and 2 in infected cells remains to be determined; however, preliminary Northern blot data indicate that transcription of these cdk's is not affected by Roscovitine (data not shown).

We examined the effect of Roscovitine on cdk9-cyclin T complex from both infected and uninfected cells. cdk9-cyclin T immunoprecipitates were washed and mixed with an in vitro-synthesized CTD peptide for kinase assays. The result of such an experiment is shown in Fig. 5B, where the cdk9 activity in

ACH₂ cells is ca. twofold higher than in CEM cells and, again, Roscovitine treatment led to a decrease of cdk9 levels and its activity on phosphorylation of the RNA Pol II CTD. We next performed an in vitro kinase assay on the cdk9 immunoprecipitates in the presence of various concentrations (0.1, 0.25, 0.5, 1, 1.5, 2, 5, or 10 μ M) of Roscovitine. The substrate used in this experiment was the CTD peptide of RNA Pol II. As shown in Fig. 5C, the in vitro IC₅₀ value of Roscovitine on cdk9 was calculated to be $\sim$ 0.6 μ M. This value was similar to the IC₅₀s that have been reported with cdk2-cyclin E (0.7 μ M), cdk2-cyclin A (0.7 μ M), and cdk1-cyclin B (0.65 μ M) (31). Finally, we determined the IC₅₀ of Roscovitine inhibition on cdk7 kinase activity to phosphorylate the CTD peptide of RNA Pol II. As calculated from Fig. 5D, the in vitro IC₅₀ value was also $\sim$ 0.6 μ M. Taken together, our data implies that all cdk's that might be involved in the transcriptional regulation of HIV-1 (cdk2, -7, and -9) were equally sensitive to Roscovitine treatment.

Roscovitine selectively sensitizes HIV-1-infected cells to apoptosis. Apoptosis and necrosis are two pathways that lead to cell death. Apoptosis is characterized by a series of morphological features, including cell shrinkage, plasma membrane blebbing, phosphatidylserine translocation to the outer leaflet of the plasma membrane, nuclear condensation, and DNA fragmentation (1). To distinguish apoptotic from necrotic cells, we performed a flow cytometry experiment using Annexin V and PI double staining. Annexin V is a sensitive probe for phosphatidylserine, and PI was used to detect membrane loss, since membrane loss leads to the accessibility of PI staining to DNA. Exposure of HIV-1-infected ACH₂ cells to TNF- α alone did not result in an increase of Annexin V-stained cells; however, Roscovitine-treated cells, especially in presence of the TNF- α , resulted in a remarkable increase in the number of apoptotic cells. At 48 h after drug treatment, most of the apoptotic cells were found at the late stage of apoptosis and not in the necrotic population (Fig. 6A). We next investigated whether the apoptotic cascade through the caspase-3 pathway was activated under drug treatment. Caspase-3, a cysteine protease, is present in cells as an inactive procaspase-3 form and, in many cases, this enzyme is activated at the onset of apoptosis. We reasoned that cdk inhibitor treatment could result in an increase of the cleaved of procaspase-3 in HIV-1-infected cells, thus increasing the caspase-3 activity on substrates such as PARP. PARP is a 112-kDa nuclear protein, which specifically has been shown to be cleaved by caspase-3. PARP is a protein necessary for the ribosylation of a number of critical substrates in DNA damage checkpoint, including p53, DNA-PK, PCNA, DNA polymerase alpha and beta, topoisomerase I and II, and RNA Pol I and II, as well as histones and lamins (2). In immunoblotting experiments, we observed that PARP was almost completely cleaved in induced ACH₂ cells when exposed to Roscovitine, implying that caspase-3 is active in drug-treated cells (Fig. 6B). Finally, to further control for the caspase-3 activity, we utilized treated lysates with the caspase-3 substrate, DEVD-pNA. Briefly, cells were lysed, and equal amounts of lysates were incubated with the caspase-3 substrate, DEVD-pNA, at 37°C for 3 h. Absorbances of the samples were read every 60 min in a Spectramax 250 microplate reader at 405 nm. The result of such an experiment is shown in Fig. 6C, in which

FIG. 5. Inhibition of kinase activities by Roscovitine in HIV-1-infected and uninfected cells. ACH₂ (HIV-1-infected) or CEM (uninfected) cells treated with TNF- α (10 ng/ml) for 2 h were washed and incubated with 10 μ M concentrations of each of the cdk inhibitors. Twenty-four hours later, the cells were harvested and lysed in lysis buffer (see Materials and Methods). The protein concentration was quantified by use of the Bio-Rad protein concentration kit and also by Coomassie blue staining after on 4 to 20% Tris-glycine PAGE. (A) A total of 1 mg of cellular extract was used for immunoprecipitation with 5 μ g of cyclin A or E antibody and washed twice with TNE 150 plus 0.1% NP-40 and twice with the kinase buffer. The cyclin A- and E-associated cdk2 kinase activity was determined, using histone H1 as a substrate. The cdk1, cdk2, cyclin A, and cyclin E levels were analyzed on a PVDF membrane probed with specific antibodies and detected with ¹²⁵I-labeled protein G. The counts represent quantitation of the autoradiographic bands using the PhosphorImager software program. (B) Immunoprecipitation experiments similar to those shown in panel A were performed, followed by assay for cdk9 kinase activity using the CTD dipeptide from RNA Pol II. (C) In vitro titration of cdk9 inhibition by Roscovitine. Cdk9 protein complex was immunoprecipitated from CEM cells, and the enzymatic activity was assayed in the presence of increasing concentrations of Roscovitine. (D) Active cdk7 protein complex was immunoprecipitated from CEM cells, and the IC₅₀ value was determined as in panel C.

induced ACH₂ cells showed an average of ~5-fold-higher activity when treated with Roscovitine.

DISCUSSION

In this study, we have demonstrated that HIV-1 transcription requires cellular cdk's, namely, cdk2, -7, and -9. cdk's may function in at least two ways: to control HIV-1 transcription and to keep HIV-1-infected cells alive. Exposure of HIV-1-

infected cells to cdk-specific inhibitors, such as Roscovitine, resulted in the loss of HIV-1 transcription and the induction of apoptosis in HIV-1-infected cells. More importantly, the apoptosis was not seen in uninfected control cells. These two mechanisms may be responsible for the inhibitory effects exerted by Roscovitine in HIV-1 progeny formation. When examining the effects of Roscovitine on primary HIV-1 SI and NSI field isolates, we found that activated PBMC infected with either of these two viruses did not support viral replication. It

FIG. 6. Roscovitine treatment induces apoptosis in HIV-1-infected cells. (A) ACH₂ (HIV-1 latently infected) cells were treated with TNF- α (10 ng/ml) for 2 h and then grown in the presence of 10 μ M Roscovitine for 48 h. Cells were collected, stained with PI and FITC-Annexin V, and analyzed by FACS. The lower left, upper left, lower right, and upper right panels represent the normal, necrotic, early-apoptotic, and late-apoptotic cells, respectively. (B) Cellular extracts from Roscovitine-treated and untreated cells were resolved on a 4 to 20% Tris-glycine gel and transferred to a PVDF membrane. Membranes were then probed with rabbit polyclonal anti-caspase-3 or anti-PARP antibody (Santa Cruz). The antigen-antibody complexes were detected using ¹²⁵I-labeled protein G. (C) Cells were washed in PBS and analyzed for caspase-3 activity using a colorimetric protease assay kit (Chemicon). Lysates were incubated with the caspase-3 substrate, 200 μ M DEVD-pNA, at 37°C for 3 h. Absorbances of samples were read every 60 min in a microplate reader at 405 nm. Symbols: ●, CEM + TNF- α ; ■, CEM + TNF- α + Roscovitine; ▲, ACH₂ + TNF- α ; ◆, ACH₂ + TNF- α + Roscovitine.

C)

FIG. 6—Continued.

is also interesting to note that, similar to HSV-1 infection (37), both HIV-1 strains have not shown the emergence of resistant viruses after 6 months of initial treatment (data not shown).

In eukaryotic cells, 10 cdk's have thus far been identified. Aside from the function of regulating cell cycle progression, they are involved in a broad range of biological processes, such as transcription, DNA repair, differentiation, and apoptosis (22). Recent studies have shown that Tat may target cdk9 (20, 51, 47), cdk7 (8, 33, 50, 34), and cdk2 (33) to transactivate the HIV-1 promoter. The target of cdk9 is known to be the CTD of RNA Pol II. In the absence of Tat, cdk9 phosphorylates serine 2 of the CTD, and cdk7 phosphorylates serine 5. However, in the presence of Tat, cdk9 can phosphorylate both serines 2 and 5 (50). The functional association of Tat with cdk2 is still unclear. cdk2 might be the substrate for Tat-associated kinases, including cdk9 and/or cdk7. cdk activities are also required for the survival of HIV-1-infected cells in either the latent or activated stages, an idea that is supported by our observation that the HIV-1-infected cells undergo apoptosis when the cdk activities are blocked by Roscovitine. This could serve as an antiapoptosis mechanism developed by HIV-1 during latent infection.

The ATP binding pocket in the catalytic subunit of cdk's (i.e., cdc2 and/or cdk2) is a major domain for interaction with inhibitors. It is located in a pocket between the small and large lobes of the kinase and contains two acceptor amino acids (Thr-14 and Tyr-15). Competitive inhibition with chemical cdk inhibitors may therefore modulate the activity of various cdk's in cells. Potentially, modulation of cdk activity through phosphorylation is an intriguing concept, since recent independent reports from the laboratories of Q. Zhou and K. A. Jones have shown that the phosphorylation of cdk9 modulates Tat transcriptional activity.

A number of studies have demonstrated that hydroxyurea can inhibit HIV-1 replication by reducing the intracellular pool of deoxynucleotides, which is essential for successful reverse transcription of HIV-1 RNA in both activated and resting PBMC (12, 28, 29, 30). However, in this study we examined the postintegrative events related to HIV-1 transcription and the

subsequent steps prior to progeny formation. The postintegrative HIV-1 progeny formation remained essentially the same in the presence of hydroxyurea and was slightly higher in the G₂/M cells when blocked with nocodazole (data not shown). Since the treatment of cells with hydroxyurea or nocodazole (at low concentrations) did not decrease the HIV-1 replication and since the requirement of efficient HIV-1 transcription in G₁ or G₂ phase is limited to the availability of cdk's, cell cycle blockers that do not specifically target cdk's would not inhibit HIV-1 postintegrative events.

It is important to note that our current study does not address issues related to the initiator and effector caspases involved in the apoptosis of infected cells in sufficient detail. Also, events related to the apoptosis unfold too rapidly to determine their temporal sequence in HIV-1-infected cells. However, some of our preliminary studies suggest that caspase-3, -7, and -8 are activated in infected cells and may be responsible for the apparent apoptosis in both treated ACH₂ and U1 cells. The activation is further evident from the release of cytochrome c, from Western blots of the activated caspase-3, -7, and -8, from the presence of the Smac-DIABLO complex with the mXIAP (inhibitor of caspase activity) as detected by immunoprecipitations followed by Western blotting, and from the inhibition of caspase activity seen with z-VAD-fmk peptide. Furthermore, substrates that have tested positive for caspase-3 (the executioner) activity in both ACH₂ and U1 cells were Rb, SREBP-1, heteroribonuclear protein C1, PKC, DNA-PK_{cs}, U1-70, and PARP (L. Deng and F. Kashanchi, unpublished results). Therefore, the apparent apoptosis of the infected cells following the addition of the cell cycle inhibitor, Roscovitine, may ultimately be linked to mitochondrial dysfunction, but the exact sequence of events leading to apoptosis awaits further detailed analysis and experimentation.

Currently, clinical treatment of AIDS patients with a combination of anti-HIV-1 drugs has been successful in reducing the viral load in the bloodstream. However, eradication of the long-lived chronically and latently infected cells cannot be achieved by highly active antiretroviral therapy (HAART) (33). In addition, reverse transcriptase and protease inhibitors do not block virus particle production in latently infected cells; rather, they act by preventing de novo infection. In this study, our data suggest that purine-derived cdk inhibitors have the potential for novel anti-HIV-1 therapy. Our assumption is based on the following rationales. (i) The transcription of newly synthesized HIV-1 RNAs in activated cells could be inhibited by cdk inhibitors and, for the first time, we show that cdk9-cyclin T and cdk7-cyclin H, which are required for HIV-1 transcription, can effectively be inhibited by Roscovitine. Functionally, Roscovitine inhibits HIV-1 transcription because the LTR requires and utilizes more than one cdk for its robust activated transcription, a scenario that may be unique to viral and not so much to cellular promoters. (ii) Roscovitine was able to induce apoptosis selectively in the HIV-1-infected cells and not in uninfected cells. (iii) Targeting cellular proteins and selective killing of HIV-1-infected host cells may be an effective method to prevent development of resistant viruses, which is a novel approach to eradicate latently and chronically infected cells. Finally, we recently have tested similar cdk inhibitors on other human and primate retroviruses (including Simian immunodeficiency virus, HIV-2, and HTLV-1), as well as

on HHV-8, and found that these viruses were sensitive only to a select set of cdk inhibitors (M. Healey, D. Wang, and F. Kashanchi, unpublished results). Therefore, we predict that most viruses that have cell cycle stimulatory functions and require active cdk's for their survival may be targets for these drugs. Future experiments will determine whether cdk inhibitors are effective blockers in Simian/human immunodeficiency virus animal models.

ACKNOWLEDGMENTS

We thank John Stephens (Department of Pediatrics, UMDNJ) for assistance with the HIV-1 p24 assay. We are also grateful to Ron Rhone for assistance in preparing the manuscript.

This work was supported by NIH grants AI44357, AI43894, and 13969 to F.K. and in part by NIH grant RR14753 to T.D. and F.K. L. Wang and L. Deng were supported by a grant from the Alexandrine and Alexander Sinsheimer Foundation.

REFERENCES

- Allen, R. T., W. J. Hunter III, and D. K. Agrawal. 1997. Morphological and biochemical characterization and analysis of apoptosis. *J. Pharmacol. Toxicol. Methods* **37**:215–228.
- Alvarez-Gonzalez, R., T. A. Watkins, P. K. Gill, J. L. Reed, and H. Mendoza-Alvarez. 1999. Regulatory mechanisms of poly(ADP-ribose) polymerase. *Mol. Cell. Biochem.* **193**:19–22.
- Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dautet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**:868–871.
- Bresnahan, W. A., I. Boldogh, P. Chi, E. A. Thompson, and T. Albrecht. 1997. Inhibition of cellular Cdk2 activity blocks human cytomegalovirus replication. *Virology* **231**:239–247.
- Bukrinsky, M. I., N. Sharova, M. P. Dempsey, T. L. Stanwick, A. G. Bukrinskaya, S. Haggerty, and M. Stevenson. 1992. Active nuclear import of human immunodeficiency virus type 1 preintegration complexes. *Proc. Natl. Acad. Sci. USA* **89**:6580–6584.
- Centers for Disease Control. 1991. Isolation, culture, and identification of HIV: procedural guide. Centers for Disease Control, Atlanta, Ga.
- Clark, E., F. Santiago, L. Deng, S. Chong, C. de La Fuente, L. Wang, P. Fu, D. Stein, T. Denny, V. Lanka, F. Mozafari, T. Okamoto, and F. Kashanchi. 2000. Loss of G₁/S checkpoint in human immunodeficiency virus type 1-infected cells is associated with a lack of cyclin-dependent kinase inhibitor p21/Waf1. *J. Virol.* **74**:5040–5052.
- Clouse, K. A., D. Powell, I. Washington, G. Poli, K. Strebel, W. Farrar, P. Barstad, J. Kovacs, A. S. Fauci, and T. M. Folks. 1989. Monokine regulation of human immunodeficiency virus-1 expression in a chronically infected human T cell clone. *J. Immunol.* **142**:431–438.
- Cujec, T. P., H. Okamoto, K. Fujinaga, J. Meyer, H. Chamberlin, D. O. Morgan, and B. M. Peterlin. 1997. The HIV transactivator Tat binds to the CDK-activating kinase and activates the phosphorylation of the carboxy-terminal domain of RNA polymerase II. *Genes Dev.* **11**:2645–2657.
- Folks, T. M., K. A. Clouse, J. Justement, A. Rabson, E. Duh, J. H. Kehrl, and A. S. Fauci. 1989. Tumor necrosis factor alpha induces expression of human immunodeficiency virus in a chronically infected T-cell clone. *Proc. Natl. Acad. Sci. USA* **86**:2365–2368.
- Folks, T. M., J. Justement, A. Kinter, C. A. Dinarello, and A. S. Fauci. 1987. Cytokine-induced expression of HIV-1 in a chronically infected promonocyte cell line. *Science* **238**:800–802.
- Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, et al. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. *Science* **224**:500–503.
- Gao, W. Y., A. Cara, R. C. Gallo, and F. Lori. 1993. Low levels of deoxynucleotides in peripheral blood lymphocytes: a strategy to inhibit human immunodeficiency virus type 1 replication. *Proc. Natl. Acad. Sci. USA* **90**:8925–8928.
- Garriga, J., J. Peng, M. Parreno, D. H. Price, E. E. Henderson, and X. Grana. 1998. Upregulation of cyclin T1/CDK9 complexes during T cell activation. *Oncogene* **17**:3093–3102.
- Goh, W. C., M. E. Rogel, C. M. Kinsey, S. F. Michael, P. N. Fultz, M. A. Nowak, B. H. Hahn, and M. Emerman. 1998. HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat. Med.* **4**:65–71.
- Gorman, C. M., L. F. Moffat, and B. H. Howard. 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* **2**:1044–1051.
- Gray, N. S., L. Wodicka, A. M. Thunnissen, T. C. Norman, S. Kwon, F. H. Espinoza, D. O. Morgan, G. Barnes, S. LeClerc, L. Meijer, S. H. Kim, D. J. Lockhart, and P. G. Schultz. 1998. Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. *Science* **281**:533–538.
- Gray, N., L. Detivaud, C. Doerig, and L. Meijer. 1999. ATP-site directed inhibitors of cyclin-dependent kinases. *Curr. Med. Chem.* **6**:859–875.
- Gummuluru, S., and M. Emerman. 1999. Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. *J. Virol.* **73**:5422–5430.
- He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, and N. R. Landau. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G₂ phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.* **69**:6705–6711.
- Herrmann, C. H., and A. P. Rice. 1993. Specific interaction of the human immunodeficiency virus Tat proteins with a cellular protein kinase. *Virology* **197**:601–608.
- Herrmann, C. H., R. G. Carroll, P. Wei, K. A. Jones, and A. P. Rice. 1998. Tat-associated kinase, TAK, activity is regulated by distinct mechanisms in peripheral blood lymphocytes and promonocytic cell lines. *J. Virol.* **72**:9881–9888.
- Johnson, D. G., and C. L. Walker. 1999. Cyclins and cell cycle checkpoints. *Annu. Rev. Pharmacol. Toxicol.* **39**:295–312.
- Kashanchi, F., E. T. Agbottah, C. A. Pise-Masison, R. Mahieux, J. Duvall, A. Kumar, and J. N. Brady. 2000. Cell cycle-regulated transcription by the human immunodeficiency virus type 1 Tat transactivator. *J. Virol.* **74**:652–660.
- Kashanchi, F., J. F. Duvall, and J. N. Brady. 1992. Electroporation of viral transactivator proteins into lymphocyte suspension cells. *Nucleic Acids Res.* **20**:4673–4674.
- Lewis, P. F., and M. Emerman. 1994. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J. Virol.* **68**:510–516.
- Lewis, P., M. Hensel, and M. Emerman. 1992. Human immunodeficiency virus infection of cells arrested in the cell cycle. *EMBO J.* **11**:3053–3058.
- Li, G., M. Simm, M. J. Potash, and D. J. Volsky. 1993. Human immunodeficiency virus type 1 DNA synthesis, integration, and efficient viral replication in growth-arrested T cells. *J. Virol.* **67**:3969–3977.
- Lori, F., A. Malykh, A. Cara, D. Sun, J. N. Weinstein, J. Lisiewicz, and R. C. Gallo. 1994. Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. *Science* **266**:801–805.
- Malley, S. D., J. M. Grange, F. Hamed-Sangsari, and J. R. Vila. 1994. Synergistic anti-human immunodeficiency virus type 1 effect of hydroxamate compounds with 2',3'-dideoxyinosine in infected resting human lymphocytes. *Proc. Natl. Acad. Sci. USA* **91**:11017–11021.
- Malley, S. D., J. M. Grange, F. Hamed-Sangsari, and J. R. Vila. 1994. Suppression of HIV production in resting lymphocytes by combining didanosine and hydroxamate compounds. *Lancet* **343**:1292.
- Meijer, L., A. Borgne, O. Mulner, J. P. Chong, J. J. Blow, N. Inagaki, M. Inagaki, J. G. Delcrois, and J. P. Moulinoux. 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. *Eur. J. Biochem.* **243**:527–536.
- Meijer, L., S. Leclerc, and M. Leost. 1999. Properties and potential applications of chemical inhibitors of cyclin-dependent kinases. *Pharmacol. Ther.* **82**:279–284.
- Nekhai, S., R. R. Shukla, and A. Kumar. 1997. A human primary T-lymphocyte-derived human immunodeficiency virus type 1 Tat-associated kinase phosphorylates the C-terminal domain of RNA polymerase II and induces CAK activity. *J. Virol.* **71**:7436–7441.
- Okamoto, H., T. P. Cujec, B. M. Peterlin, and T. Okamoto. 2000. HIV-1 replication is inhibited by a pseudo-substrate peptide that blocks Tat transactivation. *Virology* **270**:337–344.
- Pierson, T., J. McArthur, and R. F. Siliciano. 2000. Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Annu. Rev. Immunol.* **18**:665–708.
- Ross, E. K., A. J. Buckler-White, A. B. Rabson, G. Englund, and M. A. Martin. 1991. Contribution of NF-kappa B and Sp1 binding motifs to the replicative capacity of human immunodeficiency virus type 1: distinct patterns of viral growth are determined by T-cell types. *J. Virol.* **65**:4350–4358.
- Schang, L. M., J. Phillips, and P. A. Schaffer. 1998. Requirement for cellular cyclin-dependent kinases in herpes simplex virus replication and transcription. *J. Virol.* **72**:5626–5637.
- Schang, L. M., A. Rosenberg, and P. A. Schaffer. 1999. Transcription of herpes simplex virus immediate-early and early genes is inhibited by Roscovitine, an inhibitor specific for cellular cyclin-dependent kinases. *J. Virol.* **73**:2161–2172.
- Schang, L. M., A. Rosenberg, and P. A. Schaffer. 2000. Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins. *J. Virol.* **74**:2107–2120.
- Senderowicz, A. M., and E. A. Sausville. 2000. Roscovitine, a specific inhibitor of cellular cyclin-dependent kinases, inhibits herpes simplex virus DNA synthesis in the presence of viral early proteins. *J. Natl. Cancer. Inst.* **92**:376–387.

41. Siekevitz, M., S. F. Josephs, M. Dukovich, N. Pfeffer, F. Wong-Staal, and W. C. Greene. 1987. Activation of the HIV-1 LTR by T cell mitogens and the trans-activator protein of HTLV-I. *Science* **238**:1575–1578.
42. Spina, C. A., J. C. Guatelli, and D. D. Richman. 1995. Establishment of a stable, inducible form of human immunodeficiency virus type 1 DNA in quiescent CD4 lymphocytes in vitro. *J. Virol.* **69**:2977–2988.
43. Springett, G. M., R. C. Moen, S. Anderson, R. M. Blaese, and W. F. Anderson. 1989. Infection efficiency of T lymphocytes with amphotropic retroviral vectors is cell cycle dependent. *J. Virol.* **63**:3865–3869.
44. Tang, S., B. Patterson, and J. A. Levy. 1995. Highly purified quiescent human peripheral blood CD4⁺ T cells are infectible by human immunodeficiency virus but do not release virus after activation. *J. Virol.* **69**:5659–5665.
45. Trigon, S., H. Serizawa, J. W. Conaway, R. C. Conaway, S. P. Jackson, and M. Morange. 1998. Characterization of the residues phosphorylated in vitro by different C-terminal domain kinases. *J. Biol. Chem.* **273**:6769–6775.
46. Watkins, B. A., H. H. Dorn, W. B. Kelly, R. C. Armstrong, B. J. Potts, F. Michaels, C. V. Kufta, and M. Dubois-Dalcq. 1990. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science* **249**:549–553.
47. Wei, P., M. E. Garber, S. M. Fang, W. H. Fischer, and K. A. Jones. 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92**:451–462.
48. Weinberg, J. B., T. J. Matthews, B. R. Cullen, and M. H. Malim. 1991. Productive human immunodeficiency virus type 1 (HIV-1) infection of non-proliferating human monocytes. *J. Exp. Med.* **174**:1477–1482.
49. Zack, J. A., S. J. Arrigo, S. R. Weitsman, A. S. Go, A. Haislip, and I. S. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **61**:213–222.
50. Zhou, M., M. A. Halanski, M. F. Radonovich, F. Kashanchi, J. Peng, D. H. Price, and J. N. Brady. 2000. Tat modifies the activity of CDK9 to phosphorylate serine 5 of the RNA polymerase II carboxyl-terminal domain during human immunodeficiency virus type 1 transcription. *Mol. Cell. Biol.* **20**:5077–5086.
51. Zhu, Y., T. Pe'ery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M. B. Mathews, and D. H. Price. 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation in vitro. *Genes Dev.* **11**:2622–2632.