Antiviral Activity of CYC202 in HIV-1-infected Cells*

Emmanuel Agbottah‡, Cynthia de La Fuente‡, Sergie Nekhai§, Anna Barnett¶, Athos Gianella-Borradori¶, Anne Pumfrey‡, and Fatah Kashanchi‡**

From the ‡Department of Biochemistry and Molecular Biology, The George Washington University, School of Medicine, Washington, D. C. 20037, the §Center for Sickle Cell Disease, Howard University, Washington, D. C. 20059, ¶Cyclacel Ltd., Dundee Technopole, James Lindsay Place, Dundee DD1 5JD, Scotland, United Kingdom, and the ¶Institute for Genomic Research, Rockville, Maryland 20850.

There are currently 40 million individuals in the world infected with human immunodeficiency virus (HIV). The introduction of highly active antiretroviral therapy (HAART) has led to a significant reduction in AIDS-related morbidity and mortality. Unfortunately, up to 25% of patients discontinue their initial HAART regimen. Current HIV-1 inhibitors target the fusion of the virus to the cell and two viral proteins, reverse transcriptase and protease. Here, we examined whether other targets, such as an activated transcription factor, could be targeted to block HIV-1 replication. We specifically asked whether we could target a cellular kinase needed for HIV-1 transcription using CYC202 (R-roscovitine), a pharmacological cyclin-dependent kinase inhibitor. We targeted the cdk2-cyclin E complex in HIV-1-infected cells because both cdk2 and cyclin E are nonessential during mammalian development and are likely replaced by other kinases. We found that CYC202 effectively inhibits wild type and resistant HIV-1 mutants in T-cells, monocytes, and peripheral blood mononuclear cells at a low IC50 and sensitizes these cells to enhanced apoptosis resulting in a dramatic drop in viral titers. Interestingly, the effect of CYC202 is independent of cell cycle stage and more specific for the cdk2-cyclin E complex. Finally, we show that cdk2-cyclin E is loaded onto the HIV-1 genome in vivo and that CYC202 is able to inhibit the uploading of this cdk-cyclin complex onto HIV-1 DNA. Therefore, targeting cellular enzymes necessary for HIV-1 transcription, which are not needed for cell survival, is a compelling strategy to inhibit wild type and mutant HIV-1 strains.

As of the end of 2001, there were an estimated 40 million people living with human immunodeficiency virus type 1 (HIV-1) globally. The resurgence and development of an additional 16,000 new HIV-1 infections were calculated to take place daily, with 95% of these cases occurring in developing countries and 50% in women (1, 2). Only two viral proteins, reverse transcriptase and protease, are currently targets for HIV-1 inhibitors. However, virologic failure of antiretroviral therapy is common and primarily associated with the emergence of drug resistance (3–6). The emergence of resistance when viral loads are low during HAART or other antiviral treatments is explained by the selection for those viral variants that are less fit for replication as evidenced in the quasi-species theory, where viral populations are a mixture of distinct but related variants; the dominant wild type strain and mutants with varying degrees of fitness, i.e. mutants that are “over” and “under” fit (7). Therefore, it is widely believed that the success in HIV-1 treatment will require targeting of other HIV and/or host cellular proteins. To that end, we selected to focus on HIV-1 transcription events postintegration and its functional and physical interaction with the host cell cycle machinery.

Although HIV-1 entry, an early stage of infection, occurs regardless of cell cycle status, active high titer virion production of either M- or T-tropic viruses requires the host cell to be at the late G1 or S phase of the cell cycle (8–28). Therefore, activation (a process where cells go from G0 to the G1/S stage of the cell cycle) of latently infected cells by various stimuli leads to viral expression followed by progeny formation and cell death. Establishment of the latent infection is mainly the result of the absence of necessary host factors that are present only in induced cells, such as activated nuclear factor-kB (29–31), AP-1 (32), and CBP/p300 (33), and therefore minimal transcription from the HIV-1 long terminal repeat (LTR) occurs in G0 quiescent cells.

Tat has been shown to interact with a number of cellular factors, which may or may not be important for HIV-1-specific activated transcription (33). However, one complex, Tat-cdk9-cyclin T1 (p-TEFb), has emerged as a significant binding partner for elongation of transcription from the LTR. This complex has been shown to associate with and phosphorylate the carboxy-terminal domain (CTD) of RNA pol II, thereby enhancing elongation of transcription (34–39). In vivo treatment of peripheral blood lymphocytes and purified resting CD4+ T-lymphocytes with phorbol 12-myristate 13-acetate, phthoehaglutinin, interleukin-2, interleukin-6, or TNF-α also results in an increase in cdk9 and cyclin T1 protein levels and an increase in TAK (Tat-associated kinase) enzymatic activity (38). Furthermore, cdk9 expression during the cell cycle is periodic and peaks at the G2/S border (40).

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: George Washington University, 2300 Eye St., NW, Ross Hall, Rm. 551, Washington, D.C. 20037. Tel.: 202-994-1781; Fax: 202-994-1780; E-mail: bcmfkd@gwu.edu.

† The abbreviations used are: HIV-1, human immunodeficiency virus type 1; CBP, cAMP-response element-binding protein-binding protein; cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; CTD, carboxy-terminal domain; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; GST, glutathione S-transferase; GSK3β, glycogen synthase kinase 3β; HAART, highly active antiretroviral therapy; HCMV, human cytomegalovirus; HSV, herpes simplex virus; HTLV-1, human T-cell leukemia virus type 1; IP, immunoprecipitation; LTR, long terminal repeat; MOPS, 4-morpholinepropanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NSI, non-synergytinducing; PARP, poly(ADP-ribose) polymerase; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCI, pharmacological cyclin-depend-
Recently, a growing body of evidence has indicated the role of yet another cdk-cyclin complex, namely cdk2-cyclin E, in Tat-activated transcription. Cdk2-cyclin E is the major cdk-cyclin complex whose maximal activity is observed at the late G1/S boundary regulating the release of Rb sequestered factors, including E2F (41). We and others have shown previously that the increased kinase activity of cdk2-cyclin E complexes in HIV-1 latently infected cells is the result of the loss of p21/waf1 (42). Cdk2-cyclin E isolated from phytohemagglutinin-treated human primary T-cells phosphorylated RNA pol II CTD (43). Studies revealed that maximal activity of the kinase was during the late G1 phase of the cell cycle, which coincides with the maximal activity of the cdk2-cyclin E complex (43, 44) and that cdk2-cyclin E has been shown to be necessary for Tat-dependent transcription in vitro and in reconstituted transcription assays (45). Furthermore, it has been shown that during late G1, Tat transactivation is transactivation response-dependent, whereas at G2, Tat transactivation is transactivation response-independent (46). Here, we asked whether we could target a cellular kinase needed for HIV-1 transcription using CYC202 (R-rosocovitine), a pharmacological cyclin-dependent kinase inhibitor (PCI). We specifically decided to target the cdk2-cyclin E complex in HIV-1-infected cells because both cdk2 and cyclin E are not essential for viable mammalian development, as demonstrated by mouse knock-out models (47–50). Therefore, targeting cellular enzymes necessary for HIV-1 transcription, which are not essential for the survival of the vast majority of uninfected cells, is a compelling strategy to inhibit wild type and mutant HIV-1 strains. We found that CYC202 effectively inhibits wild type and resistant HIV-1 mutants in T-cells, monocytes, and PBMCs at a low IC50. Interestingly, the effect of CYC202 is independent of cell cycle stage and more specific for the cdk2-cyclin E complex. Finally, we show that cdk2-cyclin E is in fact loaded onto the HIV-1 genome in vivo and that CYC202 is able to inhibit the uploading of this cdk-cyclin E complex onto HIV-1 DNA.

EXPERIMENTAL PROCEDURES

Cells and Reagents—OM10.1, AHC2, and U1 cells are HIV-1-infected lymphocytic and monocytic cells, respectively. OM10.1 and AHC2 contain a single integrated copy of a wild type genome in parental CEM (12D7) cells, whereas U1 cells harbor two copies (one wild type and one mutant) of the viral genome in parental U973 cells (46). HL1 cells (HLV-1/Tat (51)) contain a single copy of full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene. These cells can be used to produce HIV-1 virion in the presence of wild type Tat, TNF-α, or sodium butyrate. MT-2 cells are infected with several copies of human T-cell leukemia virus type 1 (HTLV-1) and produce full-length viral particles. CEM and H9 (T-lymphocytic), and THP-1 (monocytic) cells are uninfected and have been described previously (46). All cells were cultured at 37 °C with up to 105 full-length viral particles. CEM and H9 (T-lymphocytes), HL-60 (monocytes), and PBMCs at a low IC50. Interestingly, the effect of CYC202 is independent of cell cycle stage and more specific for the cdk2-cyclin E complex. Finally, we show that cdk2-cyclin E is in fact loaded onto the HIV-1 genome in vivo and that CYC202 is able to inhibit the uploading of this cdk-cyclin complex onto HIV-1 DNA.

**Antiviral Activity of CYC202**

**Kinase Assays**—Kinase assays were performed in mammalian expression systems infected with wild type HIV-1 or with cdk2-cyclin E, -anti-cdk4-cyclin D, -anti-cdk7-cyclin H, and -anti-cdk9-cyclin T antibodies in a 20-μl reaction volume containing 50 μM ATP, 1 μCi of [γ-32P]ATP, and 100 ng of GST-CTD or 200 ng of histone H1 in TTK kinase buffer containing 50 mM HEPS (pH 7.9), 10 mM MgCl2, 6 mM EGTA, and 2.5 mM dithiorthioethanol for 30 min at 30 °C. Phosphorylated CST-GDT or histone H1 was resolved on 4–20% SDS-PAGE and subjected to autoradiography. Phosphorylation of p53 by GSK3β was carried out according to a procedure previously published (53). Phosphorylation of serine 37 of p53 by DNA-PK has been shown to create a site for GSK3β phosphorylation at serine 33 in vitro. Therefore, 3 μg of wild type GST-p53 (42) was incubated with DNA-PK (20 units, Promega Corp., Madison, WI) for 5 h at 30 °C in the following buffers (40 μl final volume): 25 mM HEPES (pH 7.5), 150 mM NaCl, 20 μM MgCl2, 0.1% Nonidet P-40, 20 μM ZnCl2, 1 mM dithiorthioethanol, 250 ng of DNA, 4.2 μg spermidine, 10 μM ATP. Samples were then incubated at 65 °C for 20 min to inactivate DNA-PK and the reaction subsequently incubated for 30 min with GSK3β in GSK3 kinase buffer (10 μg MPBS (pH 7.2), 10 mM MgCl2, 0.2 mM EDTA, 5 μM Na3 ATP, 10 μM of [γ-32P]ATP) in a final volume of 40 μl. Reactions were terminated by the addition of 20 μl of SDS-sample buffer and GSK3 phosphorylation was detected by SDS-PAGE and autoradiography.

**Peptide kinase assays**, we used our previously published procedure (54). Whole cell lysates were prepared from OM10.1 cells and PBMCs in IP buffer. Lysates (2 mg) were treated with protein A-Sepharose CL-4B (Sigma) to avoid nonspecific binding and were centrifuged. The supernatants were incubated with antibodies against cdk2 and cdk2-cyclin A, -anti-cdk4-cyclin D, -anti-cdk7-cyclin H, and -anti-cdk9-cyclin T antibod-

**Immunoblotting**—Cells were pelleted by centrifugation, washed with PBS without Ca2+ and Mg2+, and lysed with lysis buffer (55). The lysate was precipitated in ice for 15 min and centrifuged at 4 °C for 10 min. Total cellular protein was separated on 4–20% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.) overnight at 0.08 A. After transfer, the blots were washed twice with 0.1% Nonidet P-40 for 4 °C. Finally, the blots were washed twice with a 180 °C SDS-PAGE, dried, and exposed to a PhosphorImager cassette. The Rb peptide J sequence was: 516GLPTPTKTMPSHRL, and peptide G1 was 722RPPTLSPIHPHR.

**Immunoprecipitation**—Cells were pelleted by centrifugation, washed with PBS without Cu2+ and Mg2+, and lysed with lysis buffer (55). The lysate was precipitated in ice for 15 min and centrifuged at 4 °C for 10 min. Total cellular protein was separated on 4–20% Tris-glycine gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.) overnight at 0.08 A. After transfer, the blots were washed twice with 0.1% Nonidet P-40 for 4 °C. Finally, the blots were washed twice with a 180 °C SDS-PAGE, dried, and exposed to a PhosphorImager cassette. The Rb peptide J sequence was: 516GLPTPTKTMPSHRL, and peptide G1 was 722RPPTLSPIHPHR.

**Kinase Activity of CYC202**

**Kinase Assays**—Kinase assays were performed with immunoprecipitates from both infected and uninfected parental U973 cells (46) and wild type and resistant HIV-1 mutants. The immunopurified cyclins and substrates were incubated at 30 °C for 1.5 h at 37 °C and 5% CO2. The resulting formazan crystals were solubilized in 0.04 N HCl in isopropyl alcohol. The optical density of the solubilized formazan was read at 575 and 650 nm with a SpectraMax 340 (Molecular Devices, Sunnyvale, CA) plate reader. The

**Location Signal**—Cells were kept at 37 °C for 12 days, and supernatants were collected for p24 ELISA.

**Antibodies**—against cdk1, cdk2 (M-2), cdk7 (C-19), cdk8 (L-19), cyclin A (H-432), poly-ADP-ribose) polymerase PARP (N-20), caspase-3 (H-277), cyclin D1, and actin were purchased from Santa Cruz Biotechnology. The cdk inhibitor CYC202 (6-benzylamino-2-(R-1-ethyl-2-hydroxyethylamino)-9-isopropylpurine) and the kinase-inactive acid metabolite of CYC202, m-CYC202PMF (6-benzylamino-2(R)1-(carboxy[propyl]amino)-9-isopropylpurine) (Cyclacel Ltd., Dundee, Scotland, UK) were dissolved in dimethyl sulfoxide at 10 mM stock concentrations. m-CYC202 has been shown to inhibit other kinases in vitro, including cdk4-cyclin D1 (IC50 of 46 ± 5.5 μM), cdk7-cyclin H (IC50 of 24.4 ± 2.28 μM), and cdk2-cyclin A (IC50 of 78.13 ± 11.01 μM). These IC50 values are 5–100-fold greater than those observed for CYC202 (52).

**Kinase Assays**—Kinase assays were performed with immunoprecipitates from both infected and uninfected parental U973 cells (46) and wild type and resistant HIV-1 mutants. The immunopurified cyclins and substrates were incubated at 30 °C for 1.5 h at 37 °C and 5% CO2. The resulting formazan crystals were solubilized in 0.04 N HCl in isopropyl alcohol. The optical density of the solubilized formazan was read at 575 and 650 nm with a SpectraMax 340 (Molecular Devices, Sunnyvale, CA) plate reader. The
DNA Fragmentation Assay—CYC202-treated and untreated cells were lysed with buffer containing 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.2% Triton X-100, and the fragmented DNA in the lysate was separated from the unfragmented chromosomal DNA by precipitation at 12,000 × g for 30 min. Fragmented DNA in the supernatant was then digested with 100 ng/ml ribonuclease A (Invitrogen), 20 ng/ml protease K (Invitrogen), and 1% SDS at 37 °C for 45 min, purified by phenol-chloroform extraction, and precipitated with ethanol/sodium deoxycholate. The DNA was then electrophoresed on a 1.6% agarose gel containing 0.5 µg/ml ethidium bromide.

PBMC Infection—Phytohemagglutinin-activated PBMCs were kept in culture for 2 days prior to each infection. Isolation and treatment of PBMCs were performed by following the guidelines of the Centers for Disease Control (55). Approximately 5 × 10^6 PBMCs were infected with either the T-tropic Uganda strain, subtype A envelope, or the tsWIE (CUGAAGCCGAAGGAGGCATG-3') reverse primer, 5'-GCCACTGCTAGAGATTTCCACACTG-3' and 5'-GGCAACAAAUUGGUUGUGG-3', second primer, 5'-GCCACUUGGUUCCUAAUCC, sense primer, 5'-GGAUGGAACAGGCUCCC, antisense primer, 5'-GAACAGGCACACCACGAGC-3', and then fixed with 70% ethanol. For fluorescence-activated cell sorting (FACS) analysis, cells were stained with a mixture of propidium iodide buffer (PBS with Ca^2+ and Mg^2+) and then analyzed by flow cytometry (FACSCalibur, Becton Dickinson and Company). Sorted cells were resuspended with cold PBS without Ca^2+ and Mg^2+, resuspended in 1 × binding buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2) and MgCl2, and incubated at room temperature for 15 min. Cells were acquired and analyzed using CELLQuest software (BD Biosciences).

RESULTS

Inhibition of HIV-1 in T-cells, Monocytes, and PBMCs in the Presence of CYC202—We reported previously that in latently HIV-1-infected cells, the expression of a number of cdk-cyclin complexes can be targeted to inhibit viral progeny formation (55). We chose several ATP analogs, including roscovitine (CYC202) and the acid metabolite of CYC202 known to have reduced kinase activity compared with the parent compound (m-CYC202) to further test the effect of this cdk inhibitor in HIV-1-infected cells. To test the effect of CYC202, we initially used HIV-1 latently infected OM10.1 and U1 cells. OM10.1 cells are T-cells with a single integrated copy of wild type HIV-1 genome, and U1 is a monocytic cell line containing one integrated copy of wild type and one integrated copy of a mutant HIV-1 genome. Both cell lines can be induced with various mitogens including TNF-α, interleukin-2, and phytohemagglutinin to produce HIV-1 virions. We first induced OM10.1 and U1 cells with TNF-α and p24 gag ELISA. PCRs were performed for both the promoter (5'-LTR) and Env gene (3'-most open reading frame).

Inhibition of HIV-1 in T-cells, Monocytes, and PBMCs in the Presence of CYC202—We reported previously that in latently HIV-1-infected cells, the expression of a number of cdk-cyclin complexes can be targeted to inhibit viral progeny formation (55). We chose several ATP analogs, including roscovitine (CYC202) and the acid metabolite of CYC202 known to have reduced kinase activity compared with the parent compound (m-CYC202) to further test the effect of this cdk inhibitor in HIV-1-infected cells. To test the effect of CYC202, we initially used HIV-1 latently infected OM10.1 and U1 cells. OM10.1 cells are T-cells with a single integrated copy of wild type HIV-1 genome, and U1 is a monocytic cell line containing one integrated copy of wild type and one integrated copy of a mutant HIV-1 genome. Both cell lines can be induced with various mitogens including TNF-α, interleukin-2, and phytohemagglutinin to produce HIV-1 virions. We first induced OM10.1 and U1 cells with TNF-α and p24 gag ELISA. PCRs were performed for both the promoter (5'-LTR) and Env gene (3'-most open reading frame).

Inhibition of HIV-1 in T-cells, Monocytes, and PBMCs in the Presence of CYC202—We reported previously that in latently HIV-1-infected cells, the expression of a number of cdk-cyclin complexes can be targeted to inhibit viral progeny formation (55). We chose several ATP analogs, including roscovitine (CYC202) and the acid metabolite of CYC202 known to have reduced kinase activity compared with the parent compound (m-CYC202) to further test the effect of this cdk inhibitor in HIV-1-infected cells. To test the effect of CYC202, we initially used HIV-1 latently infected OM10.1 and U1 cells. OM10.1 cells are T-cells with a single integrated copy of wild type HIV-1 genome, and U1 is a monocytic cell line containing one integrated copy of wild type and one integrated copy of a mutant HIV-1 genome. Both cell lines can be induced with various mitogens including TNF-α, interleukin-2, and phytohemagglutinin to produce HIV-1 virions. We first induced OM10.1 and U1 cells with TNF-α and p24 gag ELISA. PCRs were performed for both the promoter (5'-LTR) and Env gene (3'-most open reading frame).

Inhibition of HIV-1 in T-cells, Monocytes, and PBMCs in the Presence of CYC202—We reported previously that in latently HIV-1-infected cells, the expression of a number of cdk-cyclin complexes can be targeted to inhibit viral progeny formation (55). We chose several ATP analogs, including roscovitine (CYC202) and the acid metabolite of CYC202 known to have reduced kinase activity compared with the parent compound (m-CYC202) to further test the effect of this cdk inhibitor in HIV-1-infected cells. To test the effect of CYC202, we initially used HIV-1 latently infected OM10.1 and U1 cells. OM10.1 cells are T-cells with a single integrated copy of wild type HIV-1 genome, and U1 is a monocytic cell line containing one integrated copy of wild type and one integrated copy of a mutant HIV-1 genome. Both cell lines can be induced with various mitogens including TNF-α, interleukin-2, and phytohemagglutinin to produce HIV-1 virions. We first induced OM10.1 and U1 cells with TNF-α and p24 gag ELISA. PCRs were performed for both the promoter (5'-LTR) and Env gene (3'-most open reading frame).
that preferentially induced syncytium (SI, THA/92/00) and one that was a non-syncytium inducing (NSI, UG/92/00) virus (55). Activated PBMCs were efficiently infected with either SI or NSI strains (Fig. 1C), and CYC202 but not m-CYC202 effectively inhibited SI or NSI viral production (Fig. 1C). The kinetics of viral replication in PBMC showed maximal viral progeny at day 18. Both 1 and 10 μM concentrations of CYC202 inhibited NSI virus production, but only 10 μM concentrations of CYC202 inhibited SI virus production. No inhibition was seen with these viral strains at 0.1 μM CYC202 (data not shown). Collectively, these data indicate that CYC202 is a specific inhibitor of HIV-1 during latent and acute viral infection in cultured cell lines and in primary cells.

**Titration of CYC202 and Determination of IC_{50} in Infected Cells**—Results presented in the previous section demonstrated that CYC202 inhibited various HIV-1 strains. To determine accurately the IC_{50} of CYC202, we performed a closer titration curve for CYC202 in activated OM10.1 and U1 cells. Cells were grown to mid-log phase of growth and treated with 10 μg/ml TNF-α for 2 h to induce HIV-1 expression. TNF-α was then removed, and cells were subsequently treated with 0.1, 1, or 10 μM CYC202 or 0.1, 1, or 10 μM m-CYC202 in complete medium. Five days later, supernatants were collected and processed for p24 HIV-1 gag ELISA. Lanes 1–8 are from OM10.1 cells, and lanes 9–16 are from U1 cells. B, three different viral strains were used to infect either CEM or THP cells. T-tropic virus (HIV-1 MN, lanes 1–8), monoclonal virus (HIV-1 BAL, lanes 9–16), and dual-tropic virus (HIV-1 89.6, which can infect either T- or monocytes, lanes 17–25) were used. Similar concentrations of CYC202 and m-CYC202 were used as in A. Gag p24 levels are in ng/ml. C, PBMCs were infected with two field isolates and subsequently treated with CYC202. Phytohemagglutinin-activated PBMC (5 × 10^6) were infected with either SI (THA/92/00, top panel) or NSI (UG/92/029, bottom panel) strains. After 8 h, unadsorbed virus was washed away, and cells were treated with 0.1, 1, or 10 μM CYC202 or m-CYC202. Supernatants were collected every 6 days for p24 ELISA. Three of the samples for day 6 from SI-infected cells were not processed because of a sample handling error. Gag p24 levels are in pg/ml.
CYC202 Inhibits HIV-1 Transcription in Cells Undergoing Apoptosis—We next examined the effect of CYC202 on apoptosis of both HIV-1-infected and uninfected cells. We designed two sets of experiments, one with OM10.1 cells induced with TNF-α and subsequently treated with CYC202 at an IC50 of 0.36 μM, and the other with PBMCs (from donor 1, Fig. 2C) infected with the NSI strain and treated with CYC202 at an IC50 of 1.0 μM. In both systems, cells were carried out for an extended period of time. Results are shown in Fig. 3. When OM10.1 cells were treated with TNF-α to induce HIV-1 transcription, increased levels of HIV-1 Env RNA were seen 3 days postinduction (Fig. 3A, lane 2, RT/PCR/Env). At the same time low levels of caspase-3 were also observed. By day 6 there was a high level of PARP degradation (indicator of apoptosis), and no change in actin protein or RNA was observed. These results indicate that HIV-1 alone causes apoptosis in infected cells after reactivation. We next examined the effect of CYC202 in TNF-α-treated OM10.1 cells. As expected, we saw a dramatic decrease in the level of HIV-1 RNA in these cells after CYC202 treatment (Fig. 3A). There was no change in actin RNA or protein levels. As expected, there was activation of caspase-3 and PARP cleavage in these cells. Therefore, the resulting apoptosis in these cells was the result of activation of the virus and not treatment with CYC202. Finally, we performed the same experiment in CEM uninfected cells treated with TNF-α and carried the experiment up to 6 days using a similar CYC202 concentration (IC50 of 0.36 μM). We observed no PARP cleavage, caspase activation, or actin degradation in these cells. These results imply that CEM cells treated with TNF-α and CYC202 (at IC50 of 0.36 μM) do not cause any apparent apoptosis.

We next performed a similar experiment in PBMC cells. Donor 1 (Fig. 2C) was used for infection with the THA/92/00 NSI strain (similar to Fig. 1C), subsequently treated with CYC202 (IC50 of 1 μM), and samples were taken at 6 and 12 days. Similar to OM10.1 results, PBMC cells infected with the NSI strain showed Env expression and PARP degradation by day 6 (Fig. 3B), indicating that HIV-1 infection caused apoptosis in these cells. When infected PBMCs were treated with CYC202, there was a complete drop in Env expression by day 6 and an 80% drop by day 12. We then performed the control experiment with CYC202 using the same PBMCs. As expected we observed no toxicity in these cells when using 1 μM CYC202. To determine further the level of apoptosis in both OM10.1 and...
FIG. 3. Effect of CYC202 on transcription inhibition of HIV-1 in cells undergoing apoptosis. Two sets of experiments were designed: one with OM10.1 cells induced with TNF-α and subsequently treated with CYC202 at an IC₅₀ of 0.36 μM (A), and the other with PBMCs (from donor 1, Fig. 2C) infected with the NSI strain and treated with CYC202 at an IC₅₀ of 1.0 μM (B). A, OM10.1 cells treated with TNF-α were carried out to day 6. Samples were collected for days 0, 3, and 6 and processed for Western blots (100 μg of total protein extract) or reverse transcription-PCR.
Antiviral Activity of CYC202

PBMCs, we performed a number of assays including FACS and DNA laddering in both infected and uninfected cells. Fig. 3C shows results of all treatments at day 6 for OM10.1 and CEM as well as day 12 for PBMC infected and uninfected cells. OM10.1 cells induced with TNF-α showed an apoptotic population (16.03%), which was slightly elevated (27.19%) in CYC202-treated cells. No change in apoptotic cell populations was observed in CEM cells. Similarly, PBMCs infected with the NSI strain of HIV showed an enhanced apoptotic cell population (29.81%) compared with uninfected cells (3.08%). CYC202 treatment showed similar results in infected cells and no change in uninfected cells. Finally, we extracted nuclear DNA from these cells and ran it on a 1.6% agarose gel. As seen in Fig. 3D, OM10.1, OM10.1 + CYC202, as well as infected and drug-treated PBMCs showed the typical laddering pattern indicative of apoptosis. However, no DNA laddering was observed in control, uninfected cells. Collectively, these results indicate that CYC202 treatment at anywhere from 0.3 to 1.0 μM doses inhibit HIV-1 transcription but has no effect in uninfected cell lines or PBMCs.

Effect of CYC202 in Cells Infected with Resistant HIV-1 Viruses—HIV-1 rapidly mutates in vivo and during culturing in vitro. Selection of mutant strains usually occurs either as a result of immune mediated positive selection or treatment with various HIV-1 inhibitors. To date, there are many forms of escape mutants related to all of the HIV-1 open reading frames; however, much attention has been given to clinically relevant strains harboring mutations in reverse transcriptase and protease. We therefore decided to test the effect of CYC202 on mutants resistant to nucleoside (AZT, 3TC) and nonnucleoside (TIBO) inhibitors as well as a protease-resistant virus. The resistant viral strains used in this study were initially grown in the presence of appropriate inhibitors (i.e. AZT, 3TC), and their resistance status has already been determined (3, 58–61). Activated PBMCs were infected with TIBO, 3TC, or protease-resistant viruses, and MT-2 cells were infected with an AZT-resistant virus. After infection, cells were treated with either CYC202 or m-CYC202 (0.1, 0.5, 1, 5 and 10 μM). Infected PBMCs were cultured for up to 12 days and MT-2 infected cells were cultured for up to 5 days. MT-2 cells (HTLV-1-infected) were cultured for a shorter period because they also contained the Tax transactivator, which induces nuclear factor-κB and CBP/p300, and thus additionally enhances HIV-1 transcription. Treatment with CYC202, but not m-CYC202, consistently inhibited all mutant viruses tested (Fig. 4, A–D). The IC₅₀ values were estimated to be 0.5 μM (protease-resistant strain; Fig. 4D), 1.0 μM (TIBO- and 3TC-resistant strains, Fig. 4, B and C, respectively), and 5 μM (MT-2 cells with the AZT-resistant virus, Fig. 4A). The higher IC₅₀ observed in MT-2 cells (HTLV-1-positive) could be a result of Tax-mediated induction of NF-κB and CBP/p300, which allows a higher titer of virus (ng/ml versus pg/ml p24 levels, Fig. 4A), and thus more drug is needed to inhibit the transcription of HIV-1. This result also indicates that other viral coinfections may require higher levels of CYC202 to treat HIV-1 infection. Collectively, these data suggest that CYC202 is an effective inhibitor of HIV-1 mutant viruses regardless of whether the mutations are in reverse transcriptase or protease.

Effect of CYC202 Is Independent of Cell Cycle Stage—We have shown previously that TAR-specific activated transcription initially takes place at the G₁/S border (46) and that cells blocked at G₁/S with hydroxyurea have virtually undetectable titers compared with cells that have passed the checkpoint and have reached the G₂/M phase (62). Therefore, we asked whether CYC202 could inhibit HIV-1 production in cells prior to, or after the restriction point in the mid-G₁ phase, where Rb.
protein is hyperphosphorylated. To do this, we used HLM-1 cells, which contain a single integrated copy of HIV-1 with a mutation in the Tat gene (triple termination codon). The advantage of using these cells is that they can easily be blocked at any phase of the cell cycle, released, and virus could be produced by introduction of the Tat gene
in trans. Therefore, to test the effect of CYC202 at various phases of the cell cycle, we designed two sets of experiments. First HLM-1 cells were serum starved for 3 days to induce cell stasis at the G0 phase and subsequently treated with 1 μg of purified Tat protein. To introduce inhibitors in the mid-G1 phase or past the G1/S checkpoint, cells were either immediately treated with CYC202, DNA-PK inhibitor, wild type Tat peptide, or a mutant dominant negative Tat peptide (Fig. 5A) or treated 16 h postinduction with Tat (Fig. 5C). Treated cells were cultured for 12 days, and p24 ELISAs were then performed on the supernatants. As expected, wild type Tat protein but not a mutant form (Tat, K41A) induced viral production in HLM-1 cells (Fig. 5, B and D, lanes 2 and 3). The addition of CYC202, but not m-
CYC202, inhibited viral production when added during the mid-G1 phase (Fig. 5B, lanes 4 and 5). Two different DNA-PK inhibitors, as well as the dominant negative Tat peptide, reduced viral production only when added during the mid-G1 phase (Fig. 5B, lanes 6, 7, and 9). When cells were allowed to pass the G1/S border (16 h postrelease), neither the DNA-PK inhibitors nor the Tat peptide inhibitors affected viral production (Fig. 5D, lanes 6, 7, and 9).

To ensure that cells were in fact at the G0 phase of the cell cycle and subsequently entered the G1/S after the addition of serum, we performed two independent experiments. To verify that the majority of cells were arrested in G0, transcription factor binding to the cyclin A promoter was analyzed. Takahashi et al. (63), utilizing ChIP, examined the cyclin A promoter at various stages of the cell cycle and demonstrated that at G0 early G1, the cyclin A gene was repressed by having two repressor factors, namely E2F4 and p130, bound to the promoter. We have also previously utilized a similar approach for detecting G0 cells in HTLV-1-infected cells (64). In contrast, cells that are at late G1

Fig. 4. Effect of CYC202 in cells infected with resistant HIV-1 viruses. Effects of CYC202 and m-CYC202 were tested in activated PBMCs infected with nucleoside (AZT, 3TC), nonnucleoside (TIBO), and protease-resistant mutant viruses. A–D, approximately 5 × 10^6 PBMCs were infected with HIV-1 mutant viruses (50 ng of p24 gag antigen/virus). All viral isolates were obtained from the NIH AIDS Research and Reference Reagent Program. After 8 h of infection, cells were washed, and fresh media were added. Activated PBMCs were infected with TIBO, 3TC, or protease-resistant viruses, and MT-2 cells (HTLV-1- and Tax-negative) were infected with an AZT-resistant virus. Treatments with CYC202 or m-CYC202 (0.1, 0.5, 1, 5 and 10 μM) were performed immediately after removal of the unadsorbed virus and the addition of fresh media. Infected PBMCs were cultured for 12 days and infected MT-2 cells were cultured for 5 days. Note that the p24 results for the AZT mutant in MT-2 cells are in ng/ml as opposed to pg/ml for the other viral strains.
and S phases do not have E2F4 or p130 present at the cyclin A promoter. Therefore, ChIP assays were performed as a control to verify that the majority of the cells had been arrested in the G0 phase (Fig. 5E). Chromatin from HLM-1 cells at both G0 and G1/S were incubated with control IgG, anti-E2F4, anti-p130, and anti-p300 antibodies, and primers for the cyclin A promoter (marker...
for late G1/S transcription) were used for PCR. We observed the presence of E2F4 and p130 (G0 phase markers) at the cyclin A promoter in HLM-1 cells serum starved for 3 days (Fig. 5E, lanes 3 and 4). In contrast, an activator of transcription, p300, was not detected at the cyclin A promoter in the G0 phase. HLM-1 cells in G1/S, in contrast, had no p130, and a decreased amount of E2F4 at the cyclin A promoter was observed. In addition, p300 was recruited to the cyclin A promoter at the G1/S boundary. These results indicate that HLM-1 cells were properly arrested at the G0 phase and subsequently released into G1/S by the addition of complete medium. Finally, as a further proof that serum-starved cells were indeed at the G0 phase, we performed an immunoprecipitation with anti-cyclin D1 antibody from both G0 and G1/S cells followed by a kinase reaction using GST-Rb as a substrate (64). Results are presented in Fig. 5F, where IP with cyclin D1 antibodies showed no phosphorylation of Rb from G0 cells (lane 1) but only from G1/S cells released after 16 h in complete medium (lane 2). Control cdk4-cyclin D1 purified protein complex showed abundant phosphorylation of GST-Rb (lane 3). Collectively, these cell cycle results indicate that in addition to inhibition of Tat-activated transcription, CYC202 may also affect post-transcriptional events including RNA processing, translation, packaging, or virion release. These results also indicate that inhibition of HIV-1 by CYC202 was not cell cycle phase-specific but more likely related to cdk inhibition.

Effect of CYC202 on Cdk2-Cyclin E in HIV-1-infected Cells—It has been shown previously that roscovitine exhibited the highest potency and selectivity for cdk2-cyclin E with an apparent IC50 of 0.1 μM, followed by cdk7-cyclin H with an IC50 of 0.49 μM (55). The potency of CYC202 appeared to be 7-fold greater for cdk2-cyclin E than reported previously for roscovitine, even in the presence of a 100 μM excess of free ATP. The improved potency of CYC202 was attributed to the higher potency of the R form of the enantiomer compared with the mixture of the R and S forms present in roscovitine (52). We therefore asked which of the various cdk-cyclin complexes in HIV-1-infected cells were most sensitive to CYC202. A typical kinase assay from OM10.1 cells is shown in Fig. 6. Induced OM10.1 cells were used for immunoprecipitation with various antibodies, washed, and increasing concentrations of CYC202 (0.0, 0.5, 1, 5, and 25 μM; i.e. lanes 1–5, respectively) were added to kinase reactions containing histone H1 (A) or 1 μg of GST-Rb (B) as substrates. As seen in Fig. 6A, 0.5 μM CYC202 completely inhibited the cdk2 activity on histone H1 (lane 2), whereas 5 μM CYC202 inhibited the cdk1 activity (lane 9). We also performed similar experiments for cdk4 activity and observed a complete inhibition at 25 μM CYC202 (Fig. 6B, lane 5) using GST-Rb as a substrate.

We next performed a more comprehensive set of IP/kinase assays using various cdk-cyclin complexes and other more classical enzymes as controls. Kinase assays were performed using immobiloprecipitates of cdk1, 2, 4, 7, 9, and GSK3β, followed by a wash, and incubation with various substrates. Kinase reactions were performed with recombinant RNA pol II CTD (GST-CTD) for cdk2, cdk7, and cdk9, GST-Rb(773–928) for cdk4-cyclin D1, and purified GST-p53 for GSK3β. We have shown previously that cdk2-cyclin E can phosphorylate pol II CTD (69). PKC and PKA were also assayed by using commercial assay kits (Amer sham Biosciences Biotrak PKC and Upstate PKA assay kits). Both infected lysates from induced latent (OM10.1 and ACH2) and uninfected (CEM and HL-60) cell lysates were used for immunoprecipitation followed by kinase assays in vitro in the presence of various concentrations of CYC202 (0.01, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 μM). Results in Table I indicate that the cdk2-cyclin E complex was more sensitive to CYC202 (IC50 of 0.13–0.15 μM) compared with cdk4-cyclin D1 (IC50 of 0.63–0.79 μM), cdk9-cyclin T1 (IC50 of 1.29–1.43 μM), or other controls including cdk4-cyclin D1 (IC50 of 10.70–11.30 μM). Similar levels of cdk-cyclin complexes were immunoprecipitated using these four cdk antibodies as observed in IP/Western blots (data not shown). Finally, to validate further the effect of CYC202 and its specificity on the cdk2-cyclin E complex, we performed an additional experiment with infected OM10.1 and PBMCs. A similar experiment has been described in Fig. 3, A and B, where OM10.1 cells were treated with TNF-α and subsequently with 0.36 μM CYC202, and PBMCs were infected with the NS1 strain and treated with CYC202 at an IC50 of 1.0 μM. Samples were collected postinduction of infection, and lysates were processed for immunoprecipitations with anti-cdk2 and anti-cdk4 antibodies. To avoid nonspecific binding, whole cell lysates were treated with protein A-Sepharose CL-4B and subsequently used for immunoprecipitations. The immunopurified cyclins and substrates were incubated at 30 °C for 30 min in R buffer that contained [γ-32P]ATP and 5 μg of Rb peptides (J and G1), and separated on an 18% SDS-PAGE, dried, and exposed to PhosphorImager cassette. The Rb peptide J sequence was KGLPTPTKMTPRSRIL, and peptide G1 was ITPTPTTLPPIHPTR.

![Fig. 6. Effect of CYC202 on cdk1, 2, and 4 in HIV-1-infected cells.](http://www.jbc.org/)

Fig. 6. Effect of CYC202 on cdk1, 2, and 4 in HIV-1-infected cells. A, induced OM10.1 cells were used for immunoprecipitation with antibodies against cdk1, 2, and 4, washed, and increasing concentrations of CYC202 (0.0, 0.5, 1, 5, and 25 μM; i.e. lanes 1–5, respectively) were added to the in vitro kinase reactions containing 3 μg of histone H1 (A) or 1 μg of GST-Rb (B) as substrates. B, similar to A, except cdk4 was immunoprecipitated for a kinase reaction, and cdk4 activity was inhibited at 25 μM CYC202 (lane 5). C, two sets of experiments similar to Fig. 3, A and B, were performed with OM10.1 cells induced with TNF-α and subsequently treated with CYC202 at an IC50 of 0.36 μM or PBMCs (from donor 1) were infected with the NS1 strain and treated with CYC202 at an IC50 of 1.0 μM. Samples were collected postinduction of infection, and lysates were processed for immunoprecipitations with anti-cdk2 and anti-cdk4 antibodies. To avoid nonspecific binding, whole cell lysates were treated with protein A-Sepharose CL-4B and subsequently used for immunoprecipitations. The immunopurified cyclins and substrates were incubated at 30 °C for 30 min in R buffer that contained [γ-32P]ATP and 5 μg of Rb peptides (J and G1), and separated on an 18% SDS-PAGE, dried, and exposed to PhosphorImager cassette. The Rb peptide J sequence was KGLPTPTKMTPRSRIL, and peptide G1 was ITPTPTTLPPIHPTR.)
no reduction in activity is seen for cdk4 in CYC202-treated infected cells. Collectively, these results clearly indicate that CYC202 preferentially inhibits the cdk2-cyclin E complex with an IC_{50} of at least 4-fold lower than the IC_{50} for cdk7-cyclin H and 10-fold lower than the IC_{50} for cdk9-cyclin T1, all of which are required for HIV-1 transcription.

**CYC202 Prevents the Recruitment of the Cdk2-Cyclin E Complex to the HIV-1 Genome—**The kinase experiments presented above indicate that CYC202 preferentially inhibits cdk2-cyclin E. To address whether cdk2-cyclin E directly participates in HIV-1 transcription, we determined whether cdk2-cyclin E was recruited to the HIV-1 DNA in vivo using ChIP. We also selectively inhibited expression of various cdk-cyclin complexes by siRNA interference followed by ChIP to measure the binding of various cdk-cyclin complexes to the HIV-1 DNA in vivo. These experiments served as a proof of principle to determine whether cdk2-cyclin complexes could indeed be found on the HIV-1 promoter and possibly on the downstream sequences such as the Env gene. OM10.1 cells were induced by TNF-α and subsequently transfected with siRNAs against cdk2, 4, 7, or 9. 48 h post-transfection, cells were cross-linked and processed for ChIP assay. PCRs of immunoprecipitated cdk2 were performed for the promoter (5′-LTR) and the Env gene (3′-most open reading frame). Treatment with the corresponding siRNA reduced the level of cdk2, cdk4, and cdk7 and cdk9 but not actin (Fig. 7B) and prevented recruitment of these kinases to the HIV-1 DNA (Fig. 7A, lanes 4, 8, and 12, LTR panel). RNAi-mediated knock-down of cdk2 and cdk9 correspondingly reduced the presence of these enzymes at the Env region (Fig. 7A, lanes 4 and 12, Env panel). No cdk7 or cdk4 was found on the Env region in the controls, in the absence of RNAi treatment (Fig. 7A, lanes 6 and 14, Env panel), indicating that these two kinases were not participating in HIV-1 transcription elongation.

We next asked whether CYC202 could inhibit recruitment of cdk2 or cdk9 to the HIV-1 LTR or Env region. Treatment with a low concentration of CYC202 had a slight effect on cdk2 loading onto the LTR (30% drop), but CYC202 dramatically reduced the presence of cdk2 on the 3′-Env region (Fig. 7C, lane 2). At the same time, a low concentration of CYC202 had no effect of cdk9 on the LTR and partially reduced (60%) its presence in the Env region (Fig. 7C, lane 6). In contrast, higher concentrations of CYC202 inhibited loading of both cdk2 and cdk9 onto the LTR and Env regions (Fig. 7C, lanes 10 and 14). These results demonstrate that inhibition of cyclin activity by a specific inhibitor eliminates its presence on the HIV-1 DNA. Moreover, cdk2 recruitment is more sensitive to inhibition by CYC202 as the RNA pol II travels downstream toward the 3′-end of the HIV-1 genome. These results further argue that both cdk2 and cdk9 are present on the HIV-1 genome (throughout the entire 9.5 kb) and that the recruitment of both is targeted by CYC202.

**DISCUSSION**

Anticancer drugs such as cdkI, topoisomerase 1 enzyme (top 1) inhibitors, nonnucleoside antimetabolites, and estrogen receptor ligands have recently been shown to be promising candidates for viral inhibition studies. At high doses, these drugs are used for cancer therapy; however, at lower concentrations they exhibit antiviral activities in cultured cells and promote infected cells to apoptosis (66).

During the past few years, two laboratories have contributed significantly to our understanding and scope of cdk inhibitors in virally infected cells. The Schang and Schaffer laboratories have demonstrated the effect of roscovitine in viral inhibition and have defined the mechanism of inhibition. Initially, their results suggested the involvement of a cellular cdk(s) in the replication of a DNA virus. They observed the involvement of cellular cdk in the transcription of viral genes. When infected cells were treated with ATP analogs, viral replication was inhibited, and no resistant mutants were observed after extensive passage (67). Roscovitine inhibited HSV replication, transcription of IE and E genes, and viral DNA synthesis when added postinfection or after release from a cycloheximide block (68). Other related experiments showed that roscovitine inhibited HSV replication after release from a block in viral DNA synthesis induced by phosphonoacetic acid or following a shift down of cells infected with HSV-1 DNA ts mutants from the nonpermissive to the permissive temperature (68). To show further the range of specificity, it was shown that PCIs (i) inhibit replication of wild type strains of HSV-1 and -2 and HIV-1, but not vaccinia virus or lymphocytic choriomeningitis virus; (ii) inhibit replication of strains of HSV-1 and HIV-1 resistant to conventional antiviral drugs that target thymidine kinase or DNA polymerase (HSV-1), or reverse transcriptase or protease (HIV-1); and (iii) bind to the same subset of proteins in mock- and HSV-infected cells (69, 70). Along these lines, HSV-1 has been shown to encode a protein, DNA polymerase processivity factor encoded by the UL42 gene, which behaves much like a cyclin and binds to cdk1. This new complex is active and can be inhibited by roscovitine (71). Finally, readers are referred to a publication with a comprehensive list of all RNA and DNA viruses that utilize cellular kinases for their latency, transcription and replication (72).

Studies related to cdk5 and HCMV, Epstein-Barr virus, and VZV inhibition are also worth noting here. In HCMV-infected cells cdk2-cyclin E activity is induced and can be inhibited with roscovitine at an IC_{50} of 1.2 μM, with no apparent toxicity to uninfected cells (up to 96 h at 15 μM) (73). Furthermore, HCMV infection and IE protein expression caused a dramatic increase in the abundance of cdk2 in the nuclei, and roscovitine was used to investigate the role of cdk2 expression in the inhibition of etoposide-induced apoptosis by HCMV IE1. Etoposide-induced apoptosis occurred in cells treated with 1–10 μM roscovitine (74). In the lytic stage of Epstein-Barr virus infection,
Antiviral Activity of CYC202

A) Presence of cdk2-cyclin E, cdk7-cyclin H, and cdk9-cyclin T complexes on the HIV-1 genome and inhibition by CYC202. A), presence of various cdk-cyclin complexes on the HIV-1 genome using RNAi followed by ChIP and PCR. OM10.1 cells were treated with TNF-α for 2 h, washed, and subsequently electroporated with siRNA against cdk2, 4, 7, and 9 (10 μg/ml each). 48 h later cells were cross-linked and processed for ChIP. Supernatants were also processed for p24 ELISA after 48 h (untreated, 8.3 ng/ml; cdk2 RNAi, 1.6 ng/ml; cdk4 RNAi, 12.8 ng/ml; cdk7 RNAi, 3.1 ng/ml; and cdk9 RNAi, 1.9 ng/ml). Specific DNA sequences in the immunoprecipitates were detected by PCR by using primers specific for the HIV-1 LTR (forward primer, 5'-ACCTTTCTCGGGAAGGGCGGATC-3'; reverse primer, 5'-GCAACTAGTGGATTCTCCACACTG-3') or Env region (forward primer, 5'-CTTGCTAGCCAAAGGCACTC-3'; reverse primer, 5'-TAAACATGCTTCCTCCTGTC-3'). siRNA sequences were designed using the Oligoengine Work station (www.oligoengine.com). (RNAi indicates no RNA added. I indicates input DNA isolated without immunoprecipitation (for RNAi sequences, see “Experimental Procedures”). B), Western blot of RNAi-transfected cells in A. Whole cell lysates (100 μg) were processed after transfection (A, 48 h) and Western blotted against cdk2, 4, 7, 9, and actin antibodies. C), inhibition of loading of cdk2 and cdk9 on the HIV-1 genome using CYC202. After induction of OM10.1 with TNF-α, cells were treated with two concentrations of CYC202 (1.5 and 12 μM) or m-CYC202. 48 h later, samples were collected and treated for ChIP analysis and PCR using LTR and Env primers as described in A.

cdk activity is also involved in the progression from G1 to the S phase, and S phase cdk activity appears to be essential for the expression of viral immediate early and early lytic proteins and hence for lytic viral replication. Further, it was observed that both purvalanol A and roscovitine block Epstein-Barr virus lytic replication, whereas well characterized inhibitors of other protein kinases, such as mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and PKC, did not. The effects of purvalanol A was shown to be the result of inhibition of expression of viral immediate-early and early proteins, including the replication proteins encoded by the BALF2, BALF3, BMRF1, and BBLF2/3 genes (75). Finally, the effect of roscovitine has been tested in VZV infection. Roscovitine prevented VZV replication at levels that were not cytotoxic, did not induce apoptosis in the host cells, and were lower than that needed to block HSV-1 (76). Control cells treated with 25 μM roscovitine did not appear to undergo apoptosis; however, infected cells with VZV treated with 25 μM roscovitine or 10 μM purvalanol A caused a 10-fold reduction in VZV yield after 24 h (77).

Retroviral infections have also been shown to utilize cellular cdk activity and hence may be a good target of inhibition by PCIs. For instance, we have shown previously that purvalanol A can inhibit HTLV-1-activated transcription at an IC50 of 0.035 μM. The target of inhibition was shown to be cdk2-cyclin E in HTLV-1-infected cells, and RNA pol II CTD phosphorylation was inhibited in drug-treated cells (78). The effect of PCIs had also been investigated on other retroviral infections including HIV-1. Roscovitine inhibited HIV-1-activated transcription by inhibiting cdk2, 7, and 9 (62, 78, 79). Furthermore, dose-response analysis had shown that both flavopiridol and roscovitine reversibly suppressed HIV-1 transcription in podocytes at an IC50 of 25 and 3 μM, respectively. Interestingly, despite equivalent suppression of HIV-1 transcription, roscovitine was a more effective inhibitor of podocyte proliferation than flavopiridol, and suppression of HIV-1 transcription by flavopiridol or roscovitine was marked by reexpression of the podocyte differentiation markers (80). Finally, the effect of roscovitine has also been shown on drug-resistant strains of HIV-1 (81). Collectively, these studies imply that cdk inhibitors may be a new class of drugs that could either be used alone or in combination with HAART in HIV-1-infected patients.

Although HAART has been shown to be effective for drug-naive patients, as a long term solution, HAART might ultimately result in the development of multi-drug-resistant viruses. Alternative therapies, including topical microbicides (82) and vaccines (83), for prevention of HIV infection are currently being explored; yet many challenges still remain. We believe that because of the emergence of HIV-1 drug-resistant viruses, the current lack of HIV-1 Tat transcription inhibitors on the market, and perhaps most importantly, the fact that multiple transcription factors or kinases are used for the optimal function of the HIV-1 LTR promoter, the use of cdk inhibitors (PCI)
to inhibit HIV-1 transcription may be a viable form of treatment. CYC202 inhibits cellular kinases that are not essential for cell survival (i.e., cdk2-cyclin E) (52) but are important for HIV-1 progeny formation. Consistent with this notion, several laboratories have recently shown that targeting cellular proteins (using siRNA) can reduce HIV-1 infection and progeny formation (84–93).

We determined previously that viral replication is cell cycle-dependent and requires cellular cdk-cyclin complexes and that Tat-activated transcription predominantly occurs at the G/S phase of the cell cycle (46). We confirmed these results in vivo by transfecting Tat into HLM1 cells (HIV-1/Tat−), which contain a single full-length HIV-1 provirus with a triple termination codon at the first AUG of the Tat gene, and chemically blocking the cells with either hydroxyurea (a general G/S blocker) or nocodazole (a general M phase blocker). HIV-1 attained peak viral replication in cells blocked with nocodazole, whereas G/S blockage by hydroxyurea resulted in the dramatic inhibition of virion production (62). These results were consistent with the notion that G/S kinases, such as cdk2-cyclin E, could be targeted to inhibit HIV-1 replication.

In 2003, the Barbacid laboratory first reported that embryonic fibroblasts lacking cdk2 proliferate normally and become immortal after continuous passage in culture. Additionally, cdk2−/− mice were viable and survived for up to 2 years, indicating that cdk2 is also dispensable for the proliferation and survival of most cell types (50). Others have reported similar findings demonstrating that the E type cyclins were also largely dispensable for mouse development. Cyclin E-deficient cells proliferated actively under conditions of continuous cell cycling but were unable to reenter the cell cycle from the quiescent G0 state (47–49). Therefore, cdk2 and cyclin E are not essential for the growth of normal, noncancerous cells, and use of inhibitors against the cdk2-cyclin E complex may pose a viable option to inhibit HIV-1 in infected cells, perhaps without compromising uninfected cells of the host.

The HIV-1 Tat protein activates viral gene expression by promoting transcriptional elongation via phosphorylation of the RNA pol II CTD. To date, three cdk-cyclin complexes, cdk7-cyclin H (subunits of transcription factor TFIH), cdk9-cyclin T, and cdk2-cyclin E, have been shown to phosphorylate the CTD on the HIV-1 promoter. We have recently reported that cdk2-cyclin E could phosphorylate the CTD (78), was associated with RNA pol II, and was found in elongation complexes assembled on the promoter (44, 94). Recombinant cdk2-cyclin E stimulated Tat-dependent HIV-1 transcription in a reconstituted transcription assay, and immunodepletion of cdk2-cyclin E blocked Tat-dependent transcription.

Here, we examined the effect of the cdk inhibitor CYC202 (R-roscovitine, an ATP analog) on HIV-1 latent and infected PBMCs. CYC202 was an effective inhibitor of latently infected cells with an IC50 between 0.36 and 1.8 μM. CYC202 also inhibited both SI and NSI strains effectively in PBMCs. Observing a range of IC50, rather than absolute numbers in infected cells, is expected because of drastic variations in ATP levels in cells, variability of cell type, and differences in the activity of a specific kinase in question, the number of copies of the viruses, the stage of the host cell cycle, or the presence of mixed infections. This is in contrast to in vitro kinase assays where the levels of ATP and other components are carefully controlled (Table I). Therefore, in vivo results can be considered as more reliable in assessing the efficacy of a drug against HIV-1. CYC202 also selectively induced apoptosis in HIV-1-infected cells without virion release (Fig. 3B), which agrees with previous data using a mixture of the two isomeric forms of roscovitine (55, 62, 69, 72). This is in contrast to the killing of HIV-1-infected cells with agents such as DNA-damaging agents (γ-irradiation), high concentrations of TNF-α, or sodium butyrate, where apoptosis is followed by massive virion release (42). We also consistently observed a dose-dependent response with four different resistant viral strains (Fig. 4). This was also expected because the initial mutant viruses were developed against reverse transcriptase or protease and not against the LTR or Tat. To date, the wild type virus in OM10.1 cells has not shown any resistant escape mutants (in the LTR, gag, or Env; data not shown) after ongoing culture in the presence of CYC202 for up to a year. Therefore, treatment with cdk inhibitors may avoid the generation of escape mutants because the inhibitory target is a cellular protein and not a viral protein.

An interesting and provocative finding in our studies was observed after CYC202 treatment of infected cells prior to or after the G1 checkpoint (Fig. 5). To our surprise, only CYC202, among several inhibitors tested, was able to inhibit HIV-1 production regardless of whether cells were treated before or after the G/S checkpoint. This indicates that CYC202 is able to inhibit Tat-activated transcription through a specific complex such as cdk2-cyclin E (Table I) and is consistent with previously published results demonstrating that CYC202 inhibits the cdk2-cyclin E complex (52). We are currently trying to define the mechanism of this inhibition and are studying the effect of cdk2-cyclin E in transcriptional elongation, capping of HIV-1 RNA, the presence of either singly spliced or genomic RNA after CYC202 treatment, and possible involvement in transport of RNA from the nucleus to the cytoplasm, translation, or packaging of virions. Our preliminary data indicate that CYC202 can inhibit transport of singly spliced and genomic RNA.

Our in vitro kinase assays clearly indicate that the mode of action of CYC202 is inhibition of cdk2-cyclin E (Table I). To determine whether cdk2-cyclin E is loaded onto the HIV-1 genome, we performed ChIP assays and demonstrated that CYC202 treatment inhibited loading of cdk2 onto the HIV-1 genome (Fig. 7C). This is an interesting result and indicates that inhibition of cdk activity eliminates the presence of cdk2 from the HIV-1 DNA and that cdk2 physically becomes more sensitive to CYC202 during the transcriptional elongation process. This may suggest that after CYC202 treatment, cdk2 is either free in the nucleus or cytoplasm without its cyclin partner or simply has a shorter half-life and gets degraded. Along these lines, we have observed previously that the Tat peptide inhibitor (41/44), which targets the cdk-cyclin complex, physically removes the cdk from the complex and leaves behind the cyclin partner on the HIV-1 DNA. Studies are in progress to address whether CYC202 has similar functions in infected cells.

PCIs have emerged as the most promising antiviral agents within the past few years. We have demonstrated that PCIs are potent in inhibiting cdk-cyclin complexes, which are important for viral activation, without the emergence of resistant viruses, making PCIs ideal candidates as broad range antiviral drugs. Further studies dealing with the timing involved in the initiation of treatment, possible combination with current HAARTs, and the effectiveness of these drugs in coinfection, will allow a more complete assessment of the therapeutic promise of PCIs in the treatment of HIV-infected individuals.

REFERENCES
1. Joint United Nations Programme on HIV/AIDS, World Health Organization (2000) UNAIDS, Geneva, Switzerland
2. Joint United Nations Programme on HIV/AIDS, World Health Organization

3. F. Kashanchi and C. Zeng, unpublished results.
Antiviral Activity of CYC202 in HIV-1-infected Cells
Emmanuel Agbottah, Cynthia de La Fuente, Sergie Nekhai, Anna Barnett, Athos
Gianella-Borradori, Anne Pumfery and Fatah Kashanchi

J. Biol. Chem. 2005, 280:3029-3042.
doi: 10.1074/jbc.M406435200 originally published online November 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406435200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 94 references, 50 of which can be accessed free at
http://www.jbc.org/content/280/4/3029.full.html#ref-list-1