

Pharmacological Cyclin-Dependent Kinase Inhibitors Inhibit Replication of Wild-Type and Drug-Resistant Strains of Herpes Simplex Virus and Human Immunodeficiency Virus Type 1 by Targeting Cellular, Not Viral, Proteins

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Pharmacological cyclin-dependent kinase (cdk) inhibitors (PCIs) block replication of several viruses, including herpes simplex virus type 1 (HSV-1) and human immunodeficiency virus type 1 (HIV-1). Yet, these antiviral effects could result from inhibition of either cellular cdks or viral enzymes. For example, in addition to cellular cdks, PCIs could inhibit any of the herpesvirus-encoded kinases, DNA replication proteins, or proteins involved in nucleotide metabolism. To address this issue, we asked whether purine-derived PCIs (P-PCIs) inhibit HSV and HIV-1 replication by targeting cellular or viral proteins. P-PCIs inhibited replication of HSV-1 and -2 and HIV-1, which require cellular cdks to replicate, but not vaccinia virus or lymphocytic choriomeningitis virus, which are not known to require cdks to replicate. P-PCIs also inhibited strains of HSV-1 and HIV-1 that are resistant to conventional antiviral drugs, which target viral proteins. In addition, the anti-HSV effects of P-PCIs and a conventional antiherpesvirus drug, acyclovir, were additive, demonstrating that the two drugs act by distinct mechanisms. Lastly, the spectrum of proteins that bound to P-PCIs in extracts of mock- and HSV-infected cells was the same. Based on these observations, we conclude that P-PCIs inhibit virus replication by targeting cellular, not viral, proteins.

To ensure specificity and avoid toxicity, most antiviral drugs are designed to target viral proteins. Such drugs, however, select for drug-resistant viral mutants. Moreover, these drugs exhibit activity against only a few closely related viruses. In contrast, antiviral drugs that target cellular proteins required for viral replication would not be constrained by these limitations. In the past several years, pharmacological cyclin-dependent kinase inhibitors (PCIs) have been shown to inhibit the replication of four clinically important viruses: human cytomegalovirus (HCMV) (6), herpes simplex virus type 1 (HSV-1) (56–58), human immunodeficiency virus type 1 (HIV-1) (9, 47, 69), and varicella-zoster virus (J. Moffat, State University of New York, Upstate Medical University, personal communication). However, it is as yet unclear whether the antiviral effects of these drugs are mediated exclusively by inhibition of their

known cellular targets, or by inhibition of yet-unknown viral targets.

Of the PCIs developed to date, the 2,6,9-trisubstituted purines (P-PCIs), such as Roscovitine (Rosco) (45) and Purvalanol (Purv) (26), are the most specific and best characterized. Rosco and Purv differ in potency (Purv is more potent than Rosco [26, 45]) but not in selectivity or mechanism of action. Both drugs inhibit cdk1, -2, and -5 and erk1 and -2 (at ≈50- to 1,000-fold higher concentrations than are needed to inhibit cdks), but they do not inhibit cdk4 or -6 or a large number of other kinases (26, 36, 45). Mechanistically, Rosco and Purv compete with ATP for binding to the ATP-binding pocket of the target cdks (16, 26, 45, 68). All known effects of Rosco and Purv on cells can be attributed to inhibition of the kinase activities of their recognized target cdks (21, 25, 44, 64). Whether the inhibitory effects of Rosco or Purv on viral replication can also be attributed to inhibition of the recognized cdk targets of P-PCIs has not been analyzed.

Replication of many DNA viruses requires cellular factors normally activated during cell cycle progression. For example, cellular cdks are known to be required for replication of several members of the *Papilloma*-, *Polyoma*-, *Adeno*-, and *Herpesviridae* families (3, 5, 7, 8, 10, 19, 24, 34, 38–41, 43, 46, 67). As expected, replication of viruses that replicate in dividing cells in which most Rosco-sensitive cdks are active, such as HCMV (6), is inhibited by Rosco. Surprisingly, Rosco also

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inhibits replication of viruses that are able to replicate in non-dividing cells where many Rosco-sensitive cdks are inactive, such as HSV-1 and HIV-1 (9, 56). Thus, for example, the inhibitory effects of Rosco on HSV-1 replication indicate that either P-PCI-sensitive cdks (such as cdk1 and -2) are required for HSV replication or that some as-yet-unidentified HSV proteins are novel targets of P-PCIs.

Mechanistically, Rosco is a global repressor of HSV-1 and HIV transcription (47, 58, 69) (but not of cellular transcription [33]), it inhibits viral DNA synthesis (HSV-1 and HCMV) (6, 57), and it blocks HSV-1 reactivation from latency (55a). Because the effects of P-PCIs, such as Rosco, may result from inhibition of either cellular cdks or viral-encoded proteins, we investigated the origin of the proteins targeted by P-PCIs (whether viral or cellular) in virus-infected cells. Here we show that P-PCIs (i) inhibit replication of wild-type strains of HSV-1 and -2 and HIV-1, but not vaccinia virus or lymphocytic choriomeningitis virus (LCMV); (ii) inhibit replication of strains of HSV-1 and HIV-1 resistant to conventional antiviral drugs that target thymidine kinase (TK) 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. Collectively, these and previous findings indicate that P-PCIs block virus replication by targeting cellular and not viral proteins.

Specificity of inhibition of viral replication by P-PCIs. To assess the specificity of P-PCIs, we analyzed the inhibitory effects of Rosco and Purv on the replication of vaccinia virus and LCMV (DNA- and RNA-containing viruses, respectively, that are not known to require cdks to replicate), HSV-1 (a DNA virus that requires cdks to replicate [1, 2, 15, 56]), and HIV-1 (an RNA virus that requires cdks to replicate [9, 14, 22, 42, 48, 70]). Inhibition of HSV-1 replication by Rosco and Purv was efficient and dose dependent, whereas inhibition of LCMV and vaccinia virus was neither efficient nor dose dependent (Fig. 1A). Cells treated with doses of these drugs that are effective against HSV-1 still retained the ability to support efficient viral replication (LCMV and vaccinia virus). Thus, inhibition of HSV replication is specific in that P-PCI-treated cells are able to support replication of two other viruses (which replicate in the cytoplasm).

Because Rosco is a competitive inhibitor, we verified that the doses of Rosco used in these experiments were inhibitory for cdks at intracellular concentrations of ATP (which are approximately 100-fold higher than the standard concentrations of ATP used in kinase assays) (Fig. 1B). A 33 to 100 μ M concentration of Rosco was sufficient to inhibit cdk2 at physiological concentrations of ATP (high submillimolar to low millimolar range).

If P-PCIs act by targeting cellular proteins, they should be as active against HSV-1 mutants resistant to conventional antiviral drugs and against wild-type HSV-2 as they are against wild-type HSV-1. To test this hypothesis, we analyzed the effects of Rosco and Purv on wild-type HSV-1 (KOS), wild-type HSV-2 (strains 333 and 186), and HSV-1 mutants that are resistant to phosphonoacetic acid (PAA) and acycloguanosine (ACG) (PAA^{r5}) or only to ACG (ACG^{r5}; *dlPstTK*⁻). ACG requires activation by HSV TK, and both ACG and PAA target HSV-1 DNA polymerase (11, 12, 30). In contrast to cellular TK, HSV-TK is a rather nonspecific nucleoside kinase.

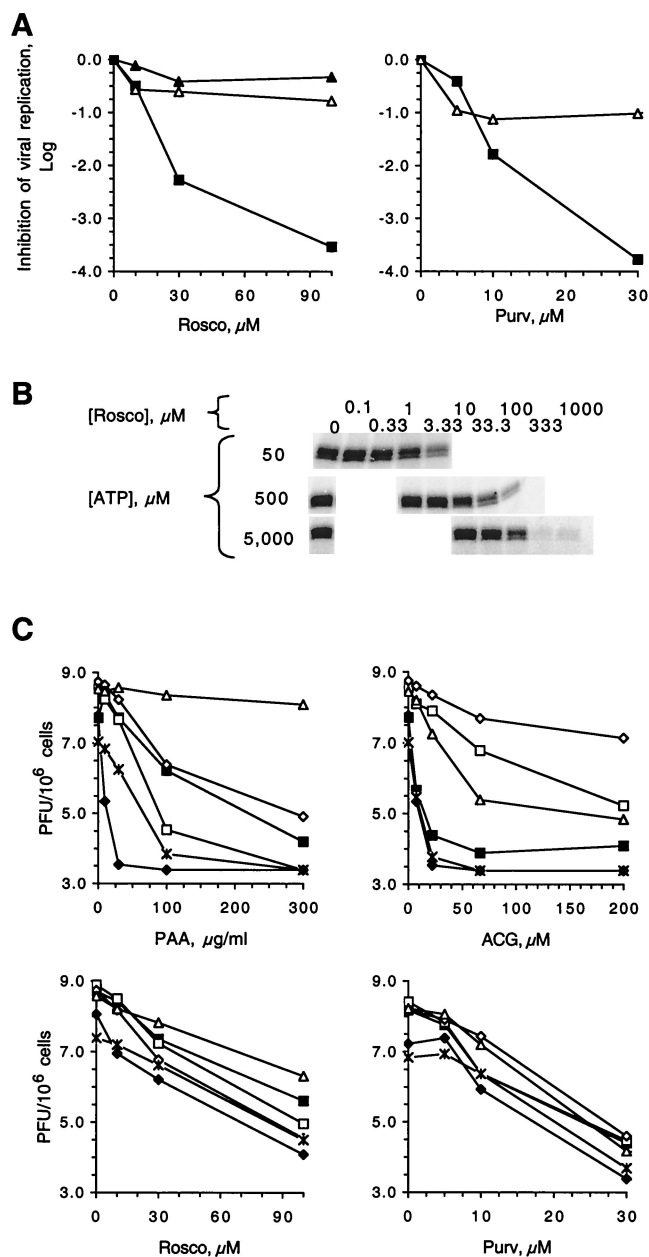


FIG. 1. Rosco and Purv inhibit wild-type and drug-resistant strains of HSV-1 and -2, but not vaccinia virus or LCMV. (A) Vero cells infected with HSV-1, vaccinia virus, or LCMV were treated with increasing concentrations of Rosco or Purv. Inhibition of viral replication at 20 h postinfection, expressed as the log, is plotted against concentration of drug. Δ , vaccinia virus; \blacktriangle , LCMV; \blacksquare , HSV-1. (B) Inhibition of cdk2/cyclinA phosphorylation of histone H1 by Rosco was evaluated in the presence of increasing concentrations of ATP, as indicated on the left of each gel. Kinase reactions were performed in vitro, phosphorylated histone H1 was then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and gels were dried and exposed on a PhosphorImager. (C) Vero cells were infected with wild-type HSV-1 (KOS), wild-type HSV-2 (strain 186 or 333), or drug-resistant HSV-1 mutants (ACG^{r5}, *dlPstTK*⁻, and PAA^{r5}). Infected cells were treated with the indicated concentrations (on x axes) of PAA, ACG, Rosco, or Purv. Viral titers at 20 h postinfection were plotted against drug concentration. \blacksquare , HSV-1 KOS; \square , HSV-1 ACG^{r5}; \diamond , HSV-1 *dlPstTK*⁻; Δ , HSV-1 PAA^{r5}; \times , HSV-2 strain 333; \blacklozenge , HSV-2 strain 186.

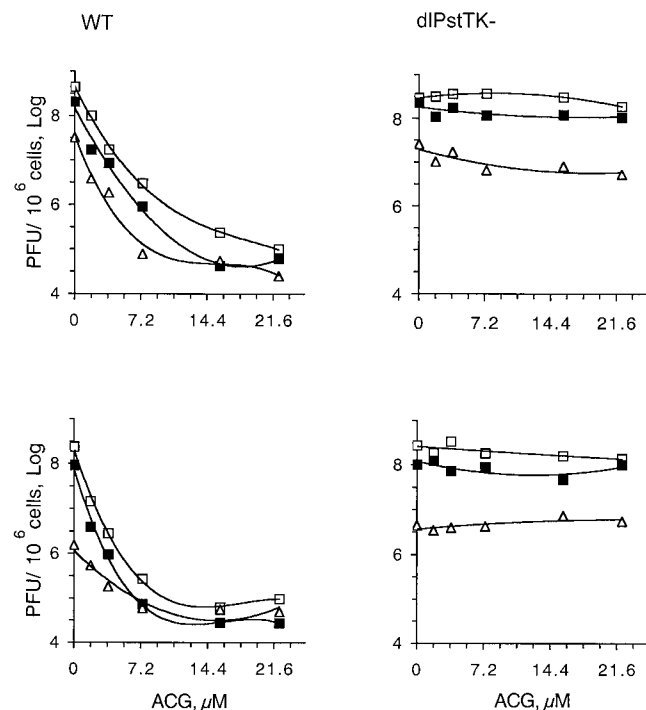


FIG. 2. The antiviral effects of P-PCIs and ACG, a conventional antiviral drug, are additive. Vero cells were infected with wild-type HSV-1 (wild type [WT]) or an ACG-resistant mutant (*dlPstTK*⁻). Infected cells were treated with the concentrations of ACG indicated on the x axes, in the presence of the concentrations of Rosco (top graphs) (□, 0 μ M; ■, 10 μ M; △, 30 μ M) or Purv (bottom graphs) (□, 0 μ M; ■, 60 μ M; △, 15 μ M). Viral titers at 20 h postinfection are plotted against concentrations of ACG.

Thus, HSV TK could, in theory, phosphorylate P-PCIs such that phosphorylated P-PCIs could target proteins other than their recognized cdk targets. In this scenario, the antiherpesvirus activity of P-PCIs could result from inhibition of these putative novel targets, and HSV TK would be required for their antiviral activity.

The susceptibility of wild-type strains of HSV-1 and -2 to conventional antiviral drugs was strain dependent (Fig. 1C), consistent with the fact that these drugs target viral proteins whose drug sensitivities vary in a strain-specific manner (27). In contrast, wild-type strains of HSV-1 and -2 exhibited similar sensitivities to both Rosco and Purv (Fig. 1C), consistent with the hypothesis that P-PCIs target exclusively cellular proteins. Also consistent with this hypothesis, all drug-resistant HSV-1 mutants were as sensitive to P-PCIs as their wild-type counterparts (Fig. 1C).

If P-PCIs inhibit viral replication by inhibiting the activities of cellular proteins, or viral proteins that are not inhibited by conventional antiviral drugs, the combined effects of P-PCIs and conventional antiviral drugs should be either additive or synergistic. To test this hypothesis, Vero cells infected with HSV-1 strain KOS or the KOS mutant *dlPstTK*⁻ were treated with increasing concentrations of ACG and P-PCIs. Partially inhibitory concentrations of both P-PCIs and ACG had additive effects (Fig. 2). In these tests, the HSV inhibitory concentrations of ACG were reduced by as much as fourfold in the

presence of partially inhibitory concentrations of Rosco or Purv, and the effects of P-PCIs against ACG-resistant *dlPstTK*⁻ were not affected by ACG (Fig. 2). These experiments show that P-PCIs target proteins other than HSV-1 TK, but they do not test the origin of these targets (whether viral or cellular).

These results demonstrate that HSV TK is not required for the antiviral activity of P-PCIs and that P-PCIs do not target the same sites on viral proteins that are targeted by other nucleoside or nucleotide analogues (i.e., the sites on HSV DNA polymerase that are targeted by PAA or phosphorylated acyclovir).

P-PCIs inhibit wild-type and drug-resistant strains of HIV.

We next tested the sensitivity to P-PCIs of HIV-1, an RNA virus that requires cdks to replicate. To this end, CEMx174 cells were infected with a high multiplicity of HIV-1 strain NL 4-3 (3 ng of p24 per 10^6 cells) and treated with Rosco. Rosco inhibited HIV-1 replication by more than 3 orders of magnitude in a dose-dependent manner (Fig. 3A). Moreover, inhibition of HIV-1 replication by Rosco prevented the loss of cell viability that normally results from HIV-1 replication (data not shown). As shown previously for HSV-1 (56), a close correlation existed between the concentrations of Rosco that inhibited HIV-1 replication and cell division (Fig. 3A and B), and inhibition of HIV-1 replication was reversible after removal of drug (data not shown).

Because P-PCIs could inhibit proteins, such as DNA or RNA polymerases, which utilize other purine-derived substrates, we evaluated the sensitivity to Rosco of HIV-1 strains RT_{MDR}/MT-2 (which is resistant to several nucleoside and nonnucleoside reverse transcriptase inhibitors [RTIs] [35]) and L10R/M46I/L63P/V82T/I84V (which is resistant to several structurally unrelated protease inhibitors [PIs] [13]). Both RTI- and PI-resistant strains were as sensitive to Rosco as wild-type HIV-1 (Fig. 3C).

Molecular specificity of P-PCIs in HSV-infected Vero and HEL cells. Among P-PCIs, Purvs bind to their protein targets with the highest affinity and, consequently, they are the most potent inhibitors of the activities of these proteins (26). An N6-methylated derivative of Purv, methyl-Purvalanol (Me-Purv), does not bind to or inhibit cdks (26, 32). Consistent with the differences in their potencies against cdks, Purv inhibited HSV-1 replication more efficiently than Rosco, whereas Me-Purv did not inhibit HSV-1 replication (Fig. 4A).

In extracts of a variety of noninfected cells, Purv binds to all proteins known to be inhibited by this drug (32), whereas Me-Purv binds to none of them. Therefore, if Purv inhibits HSV-encoded proteins, as well as cellular proteins, it should bind to additional (viral) proteins in HSV-infected cells relative to uninfected cells. To test this possibility, we performed Purv-affinity binding assays (32) using extracts of HSV-1 and mock-infected Vero and HEL cells. The specificities, dilutions, and sources of antibodies used in this study were as follows: anti-erk2 (erk2; 1:3,000; a generous gift from Stéphane Flament, University of Nancy); anti-mitogen-activated protein kinase (erk1, erk2; 1:3,000; Sigma, St. Louis, Mo.); B0114 (HSV; 1:500; DAKO Corporation, Carpinteria, Calif.); 17 (cdk1; 1:100), M2 (cdk2; 1:500), C-22 (cdk4; 1:200), C-8 (cdk5; 1:500), H96 (cdk6; 1:200), C-19 (cdk7; 1:500), and H-169 (cdk9; 1:200) (all from Santa Cruz Biotechnologies, Santa Cruz, Calif.). The

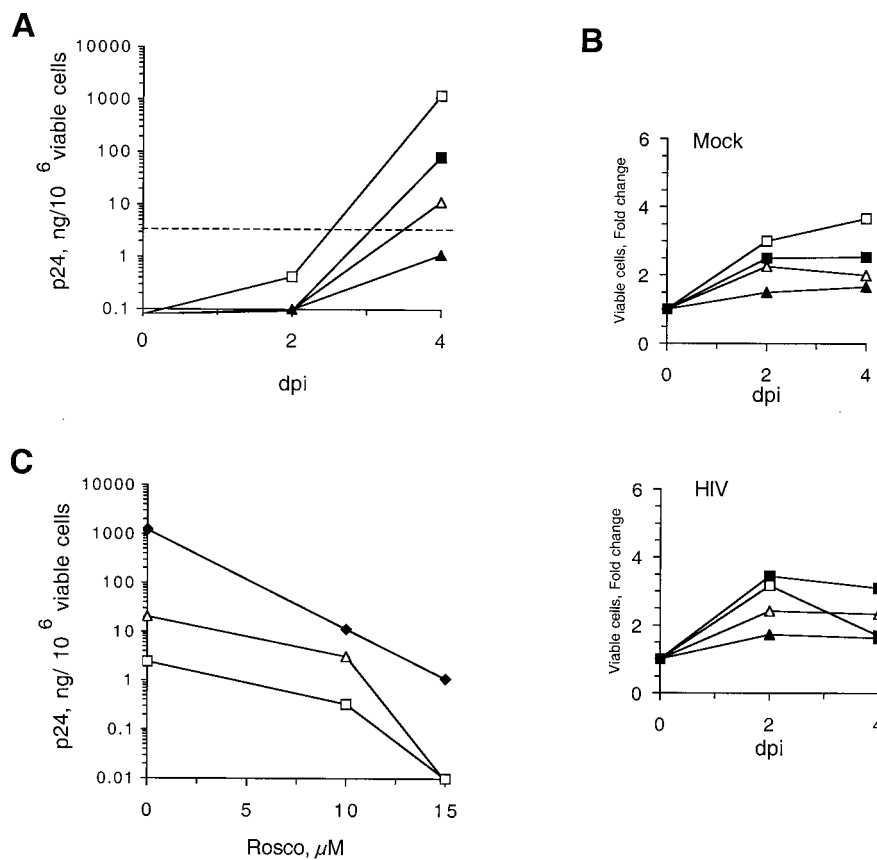


FIG. 3. Rosco inhibits replication of wild-type and drug-resistant strains of HIV-1 and enhances survival of cells in infected cultures. (A) CEMx174 cells were infected with HIV-1 and treated with Rosco. Levels of p24 at 2 and 4 days postinfection (dpi), expressed as picograms of p24 per 10^6 viable cells, are plotted against drug concentration. The level of inoculum is indicated by the dashed line. Symbols for Rosco concentrations: □, 0 μM ; ■, 5 μM ; △, 10 μM ; ▲, 15 μM . (B) Mock- or HIV-1-infected cells were treated with Rosco and counted at 2 and 4 dpi. The fold change in the number of viable cells is plotted against days postinfection for each concentration of drug. Symbols for Rosco concentration are as described for panel A. (C) CEMx174 cells were infected with PI- or RTI-resistant HIV-1 strain L10R/M46I/L63P/V82T/I84V (13) or RT_{MDR}/MT-2 (35), respectively. Infected cells were treated with Rosco and levels of p24 per 10^6 viable cells on day 4 pi are plotted against Rosco concentrations. ◆, wild type; □, PI^r; △, RTI^r. The levels of p24 produced by wild-type HIV-1 on day 4 pi in the presence of Rosco (see Fig. 4A) are included for comparison.

same spectrum of proteins bound to Purv in extracts of both HSV- and mock-infected Vero cells (Fig. 4B). Although many proteins in HEL cell extracts bound nonspecifically to immobilized Purv or Me-Purv, several proteins bound only to immobilized Purv (Fig. 4B). No novel Purv-binding proteins were detected in extracts of HSV-infected HEL cells relative to extracts of mock-infected HEL cells. Notably, the Purv-binding proteins in Vero and HEL cells had molecular masses in the range of the known targets of P-PCIs (16, 25, 26, 36, 44, 45, 64, 68).

To enhance our ability to detect viral proteins that bind to Purv, Western blot analyses of cell extracts and proteins eluted from Purv-binding beads were performed by using a polyclonal antibody directed against HSV-1-infected cells. Even under low-stringency conditions, no HSV proteins that bound to Purv were identified (Fig. 4C and data not shown).

Cellular targets of P-PCIs that may be required for viral replication. Based on the results of the experiments just described, we concluded that the antiviral effects of P-PCIs result from their inhibition of cellular and not viral proteins. We

therefore initiated preliminary tests to characterize the cellular targets of P-PCIs that might mediate their antiviral effects. We first determined whether the proteins that bind to Purv in extracts of mock- and HSV-infected cells are the same proteins whose activities are known to be inhibited by P-PCIs (including Purv) in vitro (26, 45, 68). P-PCI-sensitive cdk2 and -5 and erk2 all bound to Purv efficiently in extracts of mock- or HSV-infected cells, whereas cdk4 and -6, which are not inhibited by P-PCIs (26, 32, 45, 68), did not (Fig. 4D and E). P-PCI-sensitive cdk1 did not bind to Purv as expected since (i) only active cdk1 (i.e., cdk1 complexed with cyclin B1 and properly phosphorylated) binds to Purv (32); (ii) cdk1 is activated exclusively during mitosis; and (iii) only a small proportion of cells in our cultures were undergoing mitosis at harvest (data not shown). Among the cdks whose sensitivities to Purv or Rosco are unknown (cdk7, -8, and -9), cdk7, but not cdk9, bound to Purv efficiently in extracts of mock- and HSV-infected cells (Fig. 4D and E). Thus, the specificity of P-PCIs is not affected by the presence of viral proteins, and a novel target of P-PCIs, cdk7, was identified.

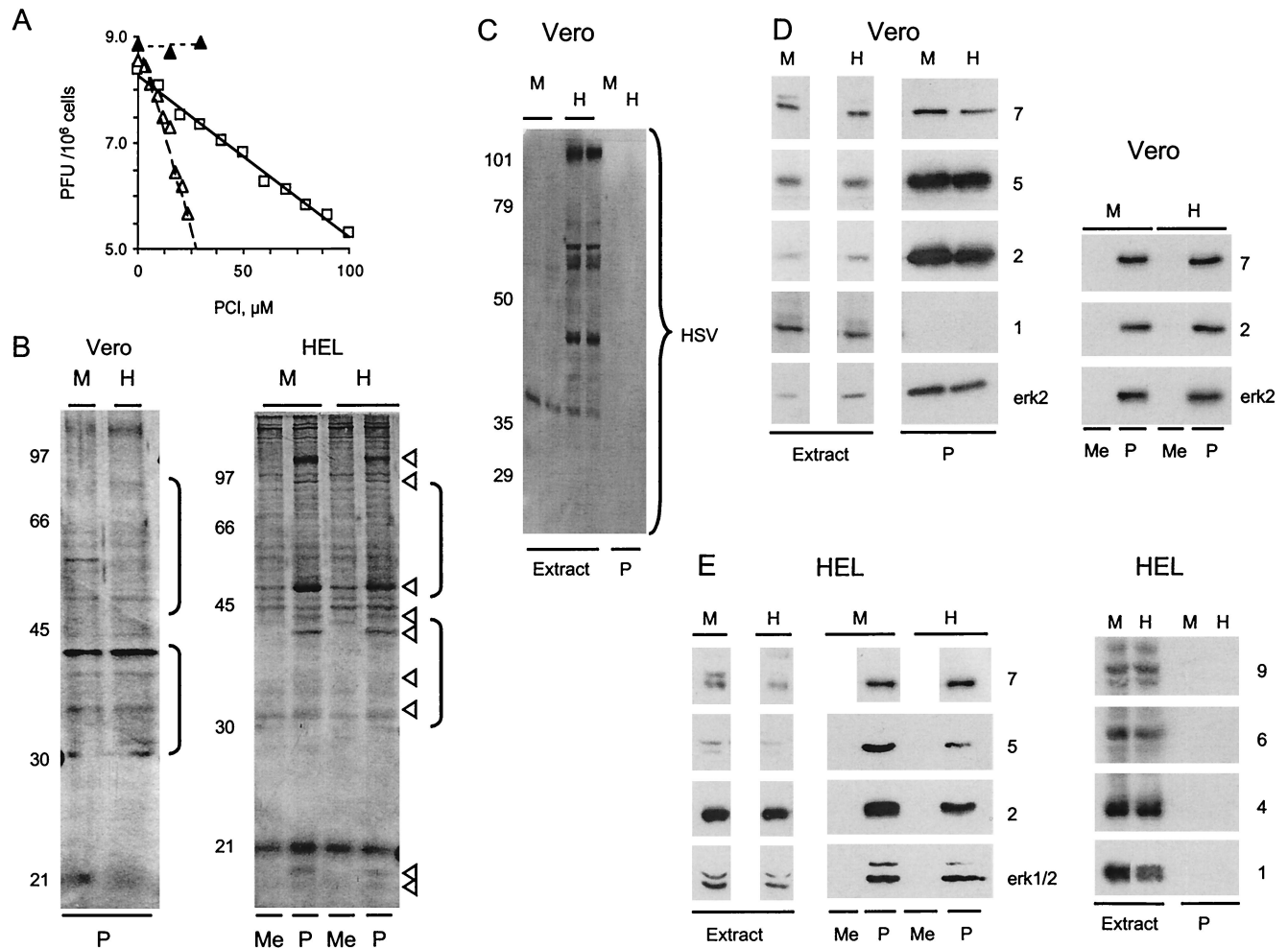


FIG. 4. Proteins that bind to Purv in mock- or HSV-infected Vero or HEL cells. Protein extracts of mock- (M) or HSV-infected (H) Vero and HEL cells were incubated with Purv (P) or Me-Purv (Me) covalently linked to agarose beads. Purv-binding proteins were eluted, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and silver stained or immunoblotted with specific antibodies (see text for descriptions of antibodies used). The antibodies specific for cdk4, -6, and -9 did not recognize simian cdk4, -6, or -9 with high affinity and, thus, could not be used in Vero cells. (A) Vero cells were infected with wild-type HSV-1 and treated with the concentrations of drug indicated on the *x* axis. Viral titers at 20 h postinfection are plotted against concentrations of Me-Purv (▲), Purv (△), or Rosco (□). (B) Silver-stained Purv-binding proteins in extracts of mock- (M) or HSV (H)-infected Vero and HEL cells. Molecular masses are indicated on the left in kilodaltons, and the ranges of molecular masses of the cdk's targeted by Purv *in vitro* and their corresponding cyclins are indicated by the brackets on the right. No Vero cell protein bound to Me-Purv was detected (data not shown). Proteins in HEL cell extracts that bound to Purv, and not to Me-Purv, are indicated by white arrowheads on the right side of the gel. (C) Western blots of Purv-binding proteins in mock- (M) or HSV (H)-infected Vero cell extracts. Purv-binding proteins (P) or total proteins in cell extracts (Extract) were resolved by SDS-PAGE, transferred, and blotted with antibodies specific for HSV-1 proteins. Molecular masses are indicated on the left in kilodaltons. (D) Western blots of Purv- (P) or Me-Purv (Me)-binding proteins in mock- (M) or HSV (H)-infected Vero cell extracts. Purv- or Me-Purv-binding proteins or total proteins in cell extracts (Extract) were resolved by SDS-PAGE, transferred, and blotted with antibodies specific for cdk7, -5, -2, and -1 or erk2 as indicated on the right of each blot. Only the relevant sections of the blots are shown. Exposure times differed for each antibody. (E) Western blots of Purv- (P) or Me-Purv (Me)-binding proteins in mock- (M) or HSV (H)-infected HEL cell extracts. Purv- or Me-Purv-binding proteins or total proteins in cell extracts were resolved by SDS-PAGE, transferred, and blotted with antibodies specific for cdk7, -5, and -2, erk1 and -2, and cdk9, -6, -4, or -1 as indicated on the right side of each blot.

In a final series of experiments, we asked whether inhibition of HSV-1 replication by P-PCIs might be a consequence of inhibition of erks, which are partially sensitive to these drugs *in vitro* (26, 45) and bind to Purv *in vivo* (Fig. 4). Vero cells were serum starved for 4 days in the presence of PD98059, a specific inhibitor of erk activation (18). Cells were then either infected with HSV-1 in the presence of a low serum concentration, or restimulated with fresh serum and left uninfected, always in the presence of PD98059. PD98059 had no effect on HSV-1

replication, even though it inhibited activation of erks, as shown by the block in reentry of arrested cells into the cell cycle (Fig. 5A).

Given that (i) Rosco inhibits transcription of HSV-1 genes, (ii) cdk7 is involved in cellular transcription, (iii) at least one P-PCI (Purv) binds to cdk7 (Fig. 1), and (iv) another P-PCI (Olomoucine) inhibits cdk7 activity (52), we determined whether cdk7 activity is sensitive to Rosco. *In vitro*, Rosco competitively inhibited cdk7 (Fig. 5B). The 50% inhibitory

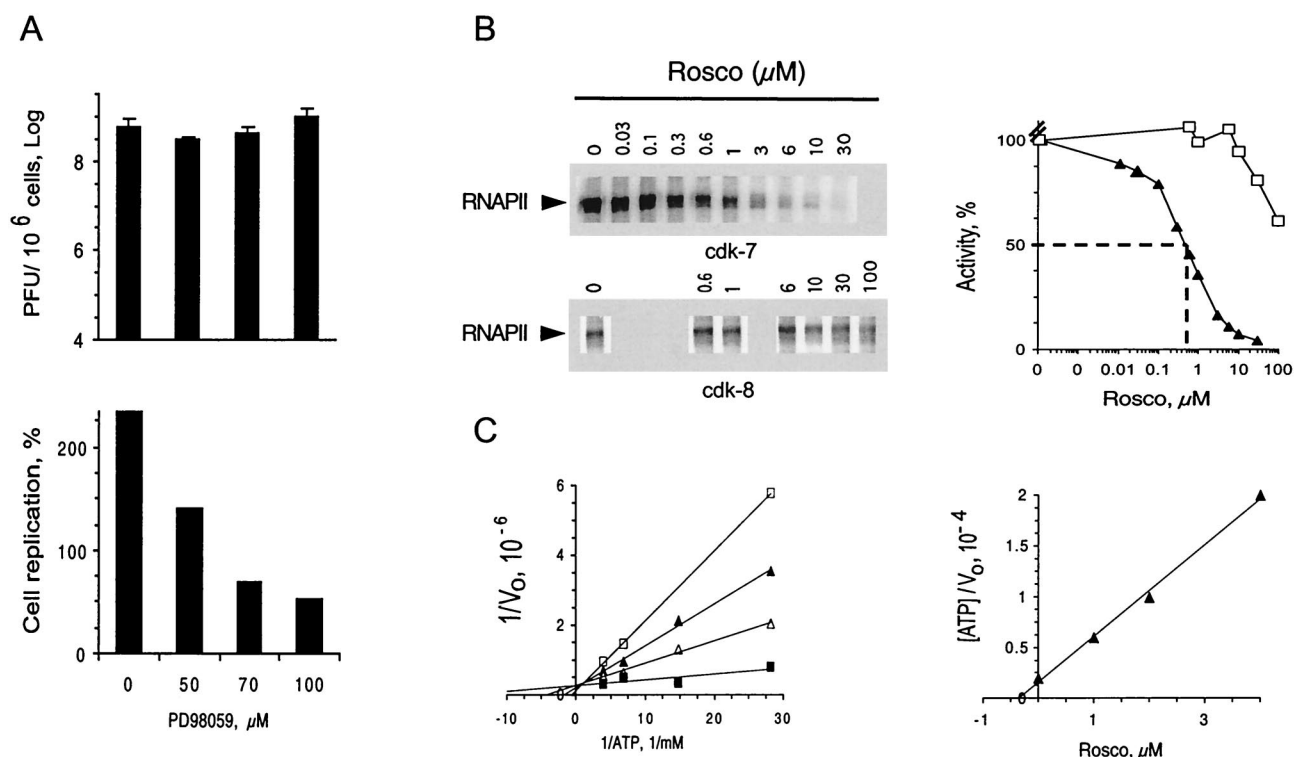


FIG. 5. *cdk7*, but not *cdk8* or *erks*, may mediate the anti-HSV effects of Rosco. (A) Vero cells were incubated in a low serum concentration and PD98059 for 4 days. Cells were infected with HSV-1, treated with PD98059 for 20 h, and harvested, and viral titers were determined (top panel). Another set of cells was stimulated with fresh serum and treated with PD98059, and the percentage of cell replication was determined (bottom panel). (B) RNAP II hypo- and hyperphosphorylated *in vitro* by *cdk7* (\blacktriangle) or *cdk8* (\square) was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were dried and exposed on a PhosphorImager. Only the relevant sections of gels are shown (left panel). Phosphorylation levels were quantitated using ImageQuant software and are plotted against Rosco concentration (right panel). The IC_{50} is indicated by the dashed line. The double lines on the y axes indicate a discontinuity on the scale. (C) RNAP II was phosphorylated by *cdk7* in the presence of increasing concentrations of ATP and Rosco: \blacksquare , 0 μM ; \triangle , 1 μM ; \blacktriangle , 2 μM ; \square , 4 μM . A Lineweaver-Burk plot is presented; inhibition was shown to be competitive (left panel). The slopes of the regression functions presented in this graph were plotted against the concentration of Rosco to calculate the K_i (right panel).

concentration (IC_{50}) (0.45 μM) and K_i (0.35 μM) of Rosco for *cdk7* were similar to the IC_{50} and K_i values for Rosco for other sensitive *cdks* (45) (Fig. 5C). In contrast to its effects on *cdk7*, Rosco did not inhibit the much weaker *in vitro* kinase activity of *cdk8* (Fig. 5B).

In this paper, we have shown that P-PCIs (i) inhibit replication of viruses whose proteins share little if any sequence homology (HSV and HIV-1) and are not sensitive to the same antiviral drugs, (ii) are equally potent against viral strains that are not equally sensitive to conventional antiviral drugs, (iii) inhibit replication of viral mutants that are resistant to conventional antiviral drugs, (iv) act by a mechanism that differs from conventional antiviral drugs, and (v) bind to cellular but not viral proteins in infected cells. Thus, our data indicate that P-PCIs inhibit viral replication by targeting cellular, not viral, proteins.

Efforts are currently under way to identify the specific cellular targets of P-PCIs that mediate the inhibition of viral replication. In one series of experiments described above, we demonstrated that *cdk7* is a previously unrecognized target of both Rosco and Purv (Fig. 4 and 5). In contrast, *cdk1* and -9 were not detected among Purv-binding proteins (Fig. 4), *cdk8* was shown to be insensitive to Rosco (Fig. 5), and HSV-1

replication was unimpaired when erk activation was inhibited (Fig. 5). Thus, in these preliminary tests we have identified a novel potential target of the antiviral effects of P-PCIs and *cdk7*, and excluded four other potential targets for the anti-herpesvirus activities of PCIs, erk1, erk2, *cdk8*, and *cdk9*. As a subunit of TFIIF, *cdk7* is required for global cellular transcription (20, 53, 61, 63), a function not inhibited by Rosco (33). Moreover, the RNA polymerase II (RNAP II) complexes engaged in transcription of HSV genomes are depleted in TFIIF (which contains *cdk7* as the kinase subunit) and TFIIE (which activates the kinase activity of TFIIF) (29). Lastly, *cdk7* is not known to be involved in DNA replication. Thus, inhibition of *cdk7* by P-PCIs does not account for the majority of the inhibitory effects of P-PCIs on HSV replication *in vivo*. Consequently, inhibition of *cdk2* and/or -5 is most likely the basis for inhibition of HSV replication by P-PCIs. With regard to HIV-1, we can only conclude that cell-cycle-inhibitory concentrations of at least one P-PCI (Rosco) are required to inhibit HIV-1 replication. Thus, *cdk1* and -2, which are sensitive to P-PCIs and regulate cell cycle progression, or *cdk5* or -7, which are approximately as sensitive to P-PCIs as *cdk1* and -2, appear to be the most likely candidate targets of Rosco-mediated inhibition of HIV-1 replication.

During the preparation of the manuscript, two other groups also showed that Rosco and Purv inhibit replication of HIV-1 at concentrations that are nontoxic for uninfected cells (47, 69). In these reports, Rosco was found to inhibit HIV-1 transcription (both basal and Tat-activated) but not cellular transcription, consistent with our previous findings that Rosco (and Olomoucine) inhibit transcription of HSV-1 but not transcription of two cellular genes or expression of a large number of cellular proteins (56–58). Kashanchi and collaborators attempted unsuccessfully to select for Rosco-resistant mutants of HIV-1 (69). Similarly, we reported extensive efforts to isolate Rosco-resistant mutants of HSV-1, also to no avail (56), under conditions in which we easily selected for HSV-1 mutants resistant to PAA (which targets the viral DNA polymerase [56]). Moreover, no HCMV mutants resistant to PCIs have been reported. Although it is not possible to conclude that viral mutants resistant to P-PCIs cannot be selected, it is clearly far more difficult to select for viral mutants resistant to P-PCIs than to select for viral mutants resistant to conventional antiviral drugs.

All inhibitory drugs must bind to their respective target proteins in order to inhibit the activities of these proteins. Yet, binding of some drugs to proteins is too labile to be detected in pull-down assays. As noted above, however, all known targets of Purv inhibition have been detected among Purv-binding proteins (32). Therefore, we conclude that the failure to detect HSV proteins in the Purv-binding fraction indicates that HSV proteins are not targeted by Purv with high affinity. Theoretically, P-PCIs could be recognized by HSV DNA polymerase and incorporated into nascent DNA, where they could act as chain terminators. In this scenario, P-PCIs could interact with HSV proteins with low affinity, yet they would still inhibit HSV replication. Contradicting this hypothesis, we observed no cross-resistance between Rosco or Purv and two drugs that act on different sites of the HSV DNA Pol (ACG, a chain terminator, and PAA) (Fig. 1 and 2).

It is important to emphasize that inhibition of HSV-1 and HIV-1 replication by P-PCIs is not secondary to inhibition of cell cycle progression but rather a direct consequence of inhibition of cdk activity. Thus, both HSV and HIV-1 can replicate in nondividing cells (23, 37, 56, 62, 71) and P-PCIs inhibit HSV-1 transcription and DNA replication in less than 2 h (31, 56, 58), whereas they inhibit cell cycle progression only after 12 to 18 h. Yet, PCIs inhibit viral replication and cell cycle progression at the same concentrations in several cell lines, suggesting that both biological effects (inhibition of viral replication and inhibition of cell cycle progression) are consequences of inhibition of the same cdks.

Based on their broad antiviral activities and the novel proteins which they target, a logical question is whether PCIs will prove useful as clinically effective antivirals. Pharmacological inhibition of cdk activities appears to result in surprisingly few and rather minor toxic effects in animal experiments and human clinical trials. In preclinical animal trials, cell-cycle-inhibitory doses of the non-purine-derived PCI flavopiridol, which inhibits cdk1, -2, -4, and -9 and several other enzymes, had no major toxic effects (4, 17, 49). Moreover, concentrations of flavopiridol in plasma above cell-cycle-inhibitory and antiviral concentrations in cell culture had no major toxic effects in extended clinical trials in humans (28, 54, 55, 59, 60, 65, 66).

The dose-limiting toxicity in these trials was secretory diarrhea, which responded to standard treatments (28, 60). The second most important toxicity was fatigue, and other less prominent toxicities included fever, asthenia, and anorexia but not immunosuppression (59, 60, 65). Significantly, some patients have been treated with flavopiridol for more than 4 years without suffering from significant toxic effects (60). Little data on the toxicity of Rosco or Purv for humans are available as yet. To date, Rosco has proven to be nontoxic in animal models (50, 51), and the first phase I human clinical trial of Rosco against cancer has been completed recently. This clinical trial demonstrated that Rosco has no acute toxicity for humans. Additional data on the potential chronic effects of Rosco on humans should be available soon, as a larger-scale phase I/II clinical trial of Rosco against cancer is currently underway.

In sum, P-PCIs (i) inhibit replication of two unrelated viruses that require cdks, but not two viruses not likely to require cdks, (ii) inhibit replication of viruses able to replicate in nondividing cells, (iii) inhibit replication of drug-resistant mutants of HSV-1 and HIV-1, (iv) act additively with drugs that target viral proteins, and (v) target the same cellular proteins in mock- and HSV-1-infected cells. Thus, P-PCIs exhibit significant antiviral activity and act by a mechanism different from that of several conventional antiviral drugs. Notably, the use of PCIs as antivirals in a clinical setting would not be constrained by three intrinsic limitations of conventional antiviral drugs: selection for drug resistance, narrow antiviral specificity, and a limited number of potential molecular targets. Should the lack of toxicity of PCIs be confirmed in ongoing and future clinical trials, PCIs may well constitute novel and powerful tools against a spectrum of clinically significant viral infections.

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