Purine and nonpurine pharmacological cyclin-dependent kinase inhibitors target initiation of viral transcription

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Background: Antiviral drugs that target viral proteins are very successful but have certain limitations. Consequently, cellular proteins have begun to be considered as potential targets for novel antivirals. Cyclin-dependent kinases (CDKs) are arguably the cellular proteins best studied as potential targets. Several pharmacological CDK inhibitors (PCIs) have antiviral activity against wild-type or drug-resistant strains of HIV, human cytomegalovirus, herpes simplex virus (HSV), Epstein–Barr virus, varicella-zoster virus, Kaposi's sarcoma herpesvirus, and human T-cell leukemia virus. Some PCIs such as roscovitine are apparently well tolerated and are scheduled to enter clinical trials as antivirals in 2005. However, the antiviral mechanisms of PCIs remain incompletely characterized, and PCIs with different molecular specificities may inhibit different viral functions. **Objective:** To evaluate whether PCIs with different molecular specificities target common viral functions. **Methods:** We evaluated the activities of structurally unrelated PCIs against a single model virus, HSV, in primary and immortalized fibroblasts. We tested whether roscovitine, flavopiridol, aloisine, and DRB, which preferentially inhibit different subsets of CDKs, inhibit HSV replication, transcription, or gene expression. **Results:** Aloisine was effective only in primary fibroblasts. Roscovitine, DRB, and to a lesser extent flavopiridol, inhibited HSV replication at lower concentrations in primary than in immortalized fibroblasts. These PCIs inhibited initiation of HSV transcription and accumulation of a viral protein into replication compartments. Flavopiridol also inhibited HSV transcription elongation. **Conclusions:** Structurally unrelated PCIs have common antiviral targets, although targets unique to certain PCIs also exist. We have identified two novel functional targets for antivirals, initiation of viral transcription and subcellular localization of viral proteins.

Keywords: aloisine, antivirals, DRB, flavopiridol, herpes simplex virus, protein-kinase inhibitors, roscovitine, transcription

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Conventional antiviral drugs target viral proteins, thus assuring their specificity and safety. However, these drugs tend to target only one or a few closely related viruses and quickly select for resistant mutants. Furthermore, viruses with small genomes encode only a few proteins that can be targeted by antiviral drugs and the proteins of a new pathogen must first be characterized before such drugs can be developed (for recent reviews on cellular and viral proteins as targets for antivirals, see [1–5]). In recent years, cellular proteins that are required for viral replication have begun to be considered as potential targets for novel antiviral drugs [2–4,6]. Even viruses with the smallest genomes require a large number of cellular proteins for their replication, thus increasing the number of potential targets for antiviral drugs.

Drugs that target cellular proteins required for multiple viral functions may not select for resistant strains as quickly as those that target viral proteins, as mutations in viral genes would have no effect on the targets of such drugs. Moreover, cells replicate slower than viruses, such that selection for drug-resistant cells would not occur quickly enough to affect viral replication. Finally, because the replication of many unreated viruses often requires the same cellular proteins, drugs that target cellular proteins could be used against a novel pathogen even before the proteins encoded by the novel pathogen are characterized.

Most viruses require cellular protein kinases for their replication and a considerable expertise on protein kinase inhibitors has been developed in recent years [7]. Consequently, cellular protein kinases are attractive targets for novel antiviral drugs. Perhaps the protein kinase inhibitors whose antiviral activities have been most thoroughly characterized are the pharmacological CDK inhibitors (PCIs). PCIs are a heterogeneous group of compounds that have in common their ability to preferentially inhibit CDKs. Most PCIs are small (≤600 Da), flat, heterocyclic compounds that compete with ATP for binding to the ATP-binding pocket of the target

CDKs [8–11]. PCIs establish hydrogen bonds with the target CDKs, mostly with main-chain groups or conserved residues in the ATP-binding pocket. It appears that most of the specificity of PCIs is conferred by their fitting into secondary pockets in the ATP-binding domain of the target CDKs. These pockets are not occupied by the ATP co-substrate and consequently are not so widely conserved among protein kinases.

PCIs can be classified in three groups – nonspecific, panspecific, and oligospecific [11,12]. Nonspecific PCIs inhibit CDKs and a variety of other protein kinases; panspecific PCIs inhibit most or all CDKs indiscriminately and oligospecific PCIs have preference for only a subset of CDKs. Monospecific PCIs may also exist but none have been described. Oligospecific PCIs can be further classified accordingly to whether they preferentially inhibit CDKs involved in transcription (CDK7, CDK8 and CDK9) or those involved in the regulation of the cell cycle (CDK1, CDK2, CDK4, CDK6 and CDK7) [12].

PCIs were originally developed as anticancer drugs and are proving to be apparently well tolerated in clinical trials against cancer [10,11,13–18]. Dose-limiting toxicities for oligospecific PCIs are diarrhea, which responds to conventional treatments, and vomiting [1,3,5]. Relatively high incidences of thrombosis in patients treated with a panspecific PCI (flavopiridol) appear to have been caused, at least in part, by the delivery system used – 72 h continuous intravenous (i.v.) infusion [14,19] – in that this toxicity was not observed in another Phase II clinical trial in which the same drug was administered as bolus in daily i.v. applications [20]. More recently, PCIs have also been found to inhibit replication of a variety of human pathogenic viruses including HIV-1, human cytomegalovirus (HCMV), varicella-zoster virus (VZV), Epstein–Barr virus (EBV), and herpes simplex virus (HSV)-1 and -2 [5,21–26]. Together with their limited serious adverse effects in preclinical and clinical trials, the antiviral activities of PCIs make them attractive as potential antivirals. In fact, PCIs are currently scheduled to begin clinical trials as antivirals in 2005 [101]. However, the antiviral effectiveness *in vivo* at doses that produce no major adverse effects, the molecular targets, and the antiviral mechanisms of PCIs must be characterized before PCIs can be developed as clinical antiviral drugs.

Many groups have focused their research efforts in elucidating the antiviral mechanisms of PCIs. These studies have focused mainly on the panspecific flavonoid flavopiridol (Flavo), the oligospecific purine type roscovitine (Rosco), and the oligospecific ribofuranosylbenzimidazoles TRB and DRB [23,27–31]. Flavo inhibits CDKs indiscriminately whereas Rosco preferentially inhibits CDKs involved in cell-cycle regulation, and TRB and DRB preferentially inhibit CDKs involved in transcription. Different viral functions have been identified as functional targets of PCIs in the different studies. For example, Rosco was shown to prevent initiation of the transcription of HSV-1, DNA replication of HCMV and HSV-1, and structural phosphorylations of VZV [27–30]. In contrast, Flavo, DRB and TRB were shown to inhibit elongation of HIV transcription [23,31]. It is possible that different PCIs inhibit replication of different viruses through different mechanisms. However, the studies discussed used different viruses and PCIs, which prevents a direct comparison between the different types of PCIs.

In the experiments reported herein, we compared the antiviral mechanisms of structurally unrelated panspecific PCIs (Flavo), nonspecific PCIs that target preferentially CDKs involved in transcription (DRB), and oligospecific PCIs that target preferentially CDKs involved in the cell cycle (Rosco, aloisine) against a single model virus, HSV-1. From these studies, we conclude that all tested PCIs prevent initiation of HSV-1 transcription with cell-type dependent potencies. Furthermore, we observed that PCIs also affect the subcellular localization of HSV-1 proteins. We are now in the process of evaluating whether PCIs also target initiation of transcription and subcellular localization of proteins of other viruses. These results further advance our understanding of the antiviral mechanisms of PCIs.

Materials & methods

Cells & viruses

Vero cells (African Green Monkey kidney fibroblasts) and human foreskin fibroblasts (HFF) were maintained in Dulbecco's modified Minimum Eagle's Medium (DMEM) supplemented with 5% fetal bovine serum, 50mU/ml penicillin and 50ng/ml streptomycin (complete medium). A low passage (p10) HSV-1 strain KOS was used throughout this study. Viral stocks were propagated and titrated on monolayers of Vero cells.

HSV-1 infection

Vero cells were infected with 2.5 or 20 plaque forming units (PFU) of HSV-1 strain KOS per cell in serum-free media, as described for each experiment. After 1 h at 37°C, inocula were

removed and cells were washed twice with cold phosphate-buffered saline (PBS – 1 mM KH_2PO_4, 154mM NaCl, 3 mM Na_2HPO_4, pH7.4) and then incubated in complete media supplemented or not with flavopiridol (Flavo), DRB, aloisine, (*R*)-roscovitine (*R*-Rosco), or (*S*)-roscovitine (*S*-Rosco). HSV-1 replicated with comparable efficiency in HFF or Vero cells in the absence of any drug with yields in the order of 10^7 to 5×10^8 PFU/10^6 cells (depending on experiment). These yields are in normal ranges and consistent with our previous experience.

Drugs

Cycloheximide (CHX) and DRB were purchased from commercial suppliers. CHX was prepared in serum-free DMEM as a 5 mg/ml stock. The stock was diluted to 50 µg/ml in complete medium and added to cells 1h prior to infection. DRB was diluted in ethanol as a 6.267 mM stock and used at concentrations from 25 to 150 µM. Flavo, aloisine, *R*- and *S*-Rosco were a generous gift from Dr L Meijer (CNRS, Station Biologique Roscoff, France). Flavo was diluted in dimethylsulfoxide (DMSO) as a 10mM stock and used at concentrations from 7.815 to 250 nM. Aloisine and *R*- and *S*-Rosco were prepared in DMSO as 10 (aloisine) or 100 mM stocks and used at concentrations from 5 to 100 µM. Equivalent amounts of DMSO were added to the medium in the non-drug-treated wells. Moreover, we consistently observe that these low concentrations of DMSO fail to inhibit HSV-1 replication.

Titrations

Cells and media were harvested at 24 h post-infection, transferred to 14 ml conical tubes and immediately frozen. Samples were further subjected to three freeze–thaw cycles and then to three cycles of low-energy sonication for 30 sec, separated by rest periods of 15 sec. Cellular debris was pelleted by centrifugation and supernatants were collected and stored at -80°C. Samples were serially diluted (1:10) and 100 µl of relevant dilutions were used to infect 1.5×10^5 Vero cells in 24 well plates, or 3.0×10^5 cells in 12 well plates. After 1 h incubation, inocula were removed and 1 ml of 37°C methylcellulose (2% w/v in complete medium) was added to each well. Infected cells were incubated at 37°C in 5% CO_2 for 3 to 4 days (until plaques were well defined and clear). Cells were fixed and stained with crystal violet in methanol (1% w/v crystal violet, 17% v/v methanol); washed, dried and individual plaques were counted.

Toxicity analyses

Vero or HFF cells were treated as for the viral replication assays with the exception that no virus was added. After removal of mock inocula, media containing the different concentrations of drugs were added and the cells were incubated for a further 23 h, as in the viral replication analyses. Cells were then harvested and the cell number and viability was analyzed by trypan blue exclusion. Cytostatic effects were defined as either increase in cell numbers in 24 h below that observed in the absence of any drug, or no increase in cell numbers. Cytotoxic effects were defined as increases of more than threefold in percentages of nonviable cells over the percentage of nonviable cells in the absence of any drug. Highly cytotoxic effects were defined as increases of more than sixfold in the percentage of nonviable cells over the percentage of nonviable cells in the absence of any drug. For Vero cells, 2.366% of cells were nonviable in the absence of any drug and therefore any treatment yielding more than 7.1% of nonviable cells was classified as cytotoxic and any treatment yielding more than 14.2% of nonviable cells was classified as highly cytotoxic. For HFF cells, 9.025% of cells were nonviable in the absence of any drug and therefore any treatment yielding more than 27.075% of nonviable cells was classified as cytotoxic. No HFF treatment classified as non-cytotoxic yielded more than 14.2% nonviable cells and no HFF treatment was highly cytotoxic. We did not use MTT or similar mitochondrial toxicity assays because all drugs used are cytostatic at most of the concentrations tested, and such assays cannot discriminate between cytostatic or cytotoxic effects.

Run-on analyses

For each treatment, two 100mm diameter dishes containing approximately 0.6×10^6 Vero cells each were treated for 1h with CHX in complete medium. Cells were then mock infected or infected with 20 PFU of HSV-1 per cell, always in the presence or absence of CHX. After 1 h adsorption at 37°C, inocula were removed and infected monolayers were washed twice with PBS containing CHX. Cells were then incubated with complete medium containing CHX for 4 h. Cells were washed with PBS and then further incubated for 5h at 37°C with complete medium containing CHX, 100 µM Rosco, 150 µM DRB, or 100 nM Flavo. Run-on assays were performed as originally described by Spencer, Rice and colleagues [32,33], with several modifications

[27]. Briefly, cells were resuspended in hypotonic sample buffer (RSB, 10 mM Tris pH 7.5, 10 mM NaCl, 5 mM $MgCl_2$), and lysed with 0.5% (v/v) Nonidet P-40. Nuclei were isolated, resuspended in 150 µl nuclear freezing buffer (NFB, 50 mM Tris pH 8.0, 5 mM $MgCl_2$, 40% glycerol, and 0.5mM dithiothreitol – DTT), immediately snap frozen and stored in liquid nitrogen. Afterwards, 150µl of thawed nuclei suspension was mixed with 150 µl of transcription run-on buffer (20 mM Tris pH8, 3 mM DTT, 20 mM $MgCl_2$); 0.5 mM of each ATP, CTP and UTP; and 10 µCi of (α-32P) GTP. Final buffer concentrations were, 30 mM Tris pH8.0, 1 mM DTT, 7.5 mM $MgCl_2$, 20% glycerol, 140mM KCl. Transcription reactions proceeded at 30°C for 30 min and were stopped by incubation with 50 µg (434 Worthington U) of DNAse I (Invitrogen, CA, USA) for 15 min at 30°C. Total nuclear RNA was isolated by standard methods. Membranes containing single-stranded DNA were pre-hybridized with 10ml rapid hybrid buffer (RHB, Amersham Biosciences, NJ, USA) at 60°C. Denatured RNA was added to 5 ml of RHB at 60°C and hybridized to the membranes for 48 h. Membranes were washed twice for 20 min in 300 mM NaCl, 30 mM sodium citrate (2 × SSC), 0.1% sodium dodecyl sulfate (SDS) at room temperature, and once for 10min in 75 mM NaCl, 7.5 mM sodium citrate (0.5 × SSC), 0.5% SDS at 50°C. Membranes were exposed to Kodak PhosphorImager screens. When indicated, 100 µM Rosco, 100 nM Flavo or 150 µM DRB were added to the run-on transcription reactions.

Immunofluorescence

For each treatment, approximately 4×10^5 Vero cells were seeded on coverslips in 24 well plates and infected with 20 PFU of HSV-1 per cell. Following 1h adsorption at 37°C, inocula were removed and the infected monolayers were washed twice with PBS at 4°C. Cells were then treated with complete medium supplemented with 125 µM DRB, 100 nM Flavo, 100 µM *R*-Rosco, 100 µM *S*-Rosco, or vehicle (DMSO). At 5 h post-infection, cells were fixed in methanol for 10 min at 4°C. Fixed cells were blocked with 0.5% bovine serum albumin (BSA) in PBS for 30 min. Cells were incubated with primary monoclonal anti-ICP4 antibody (clone 1101 897) diluted 1:500 in 0.5% BSA for 60 min at room temperature and then washed for 10min with 0.05% Tween in PBS. Cells were incubated with goat antimouse immunoglobulin (Ig)G Alexa 594-labeled secondary antibody (Molecular Probes, OR, USA) diluted 1:500 in 0.5% BSA for 30 min at room temperature and then washed with 0.05% Tween in PBS for 10 min. Nuclei were counterstained with Hoescht 33258 diluted 1:100 in PBS for 15 min. Coverslips were mounted onto microscope slides, visualized and documented using a fluorescence microscope with an ultraviolet (UV) light source (Leica DM IRB, Wetzlar, Germany) and camera (QIMAGING RETIGA 1300, Burnaby, Canada). ICP4 signal was quantitated using Quantity One software (BIO-RAD, Mississauga, Canada) and expressed as arbitrary units.

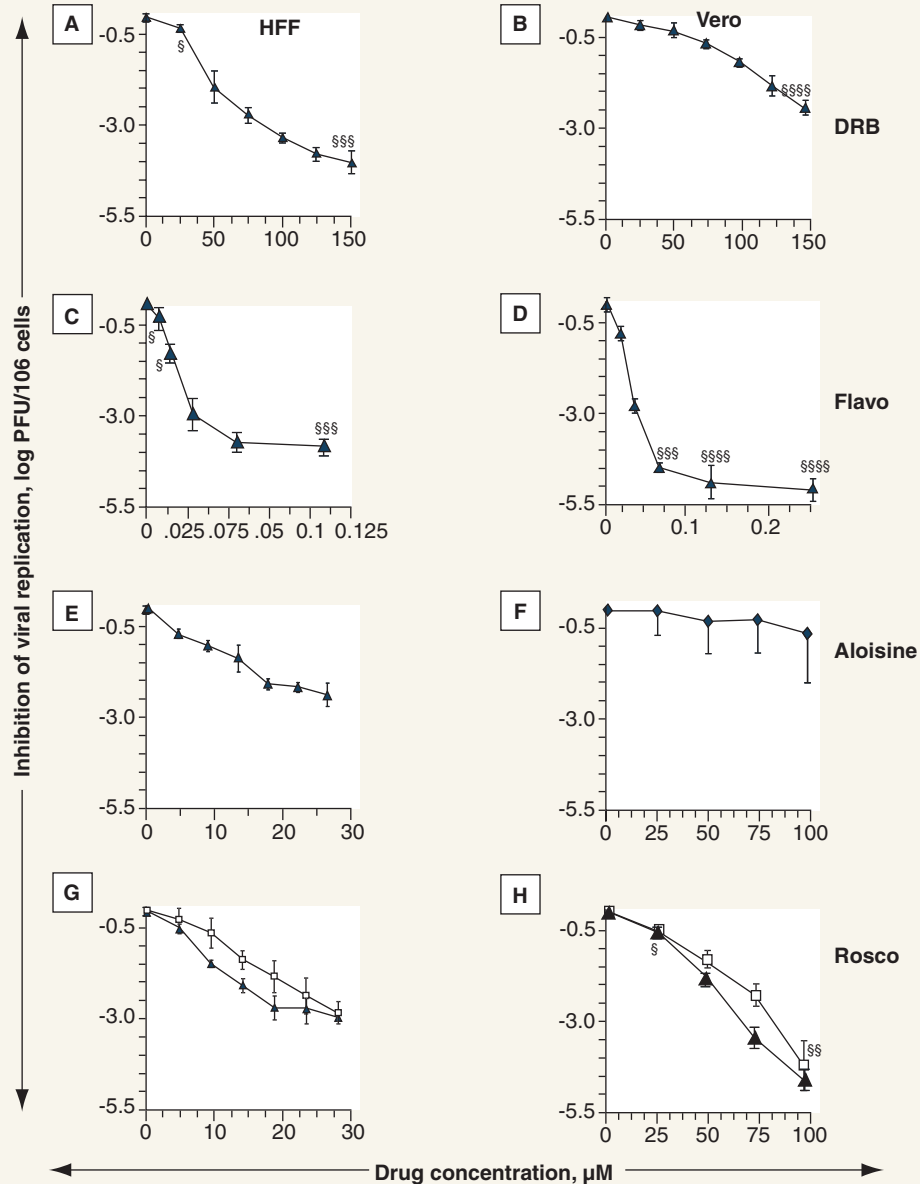
Results

The effects of PCIs on HSV-1 replication are cell-type specific

We first tested the antiviral effects of a series of structurally unrelated PCIs on the replication of a single virus, HSV-1. As the antiviral concentrations of two PCIs, Rosco and olomoucine depend on cell type [22], we further compared the PCIs in two cell lines, primary HFF and immortalized African green monkey kidney cells (Vero). HSV-1 replicates with similar efficiencies in both of these two cell lines (approximately 10^7 to 5×10^8 PFU/10^6 cells in 24 h). Dose–response analyses of the antiviral effects of DRB, Flavo, aloisine and Rosco were performed. Cells were infected with 2.5 PFU of HSV-1 per cell and treated with complete medium supplemented with the indicated concentrations of DRB, Flavo, aloisine, *R*-Rosco or *S*-Rosco (**Figure 1**). Replicated virus was harvested at 24 h post-infection and titrated to evaluate the degree of inhibition of viral replication. Due to the fact that Flavo displayed clear toxicity at high concentrations, we also evaluated cell viability in the presence of these drugs. To this end, cells were mock infected and then incubated with the different concentrations of each drug. Cell number and viability were evaluated by trypan blue exclusion at 24 h post-mock-infection. We did not use mitochondrial toxicity assays because all drugs used are cytostatic and such assays fail to discriminate between cytostatic or cytotoxic effects.

The antiviral effects of the tested PCIs were cell-type dependent, as expected, in that the concentrations required to reach maximal inhibition of HSV-1 replication were lower in primary HFF than in immortalized Vero cells. Likewise, toxicity was also cell-type dependent. For example, 150 µM DRB resulted in a decrease in viral titer

Figure 1. Responses of HSV-1 replication in Vero and HFF cells to Flavo, DRB, aloisine, *R*-Rosco or *S*-Rosco.



Reductions in viral replication per 106 cells are plotted as log against drug concentration. HFF or Vero cells (Vero) were infected with 2.5 PFU of HSV1-KOS per cell. After 1 h inocula were removed, cells were washed twice with 1 ml PBS, and drug solutions in complete medium were added. Cells were treated with either **(A,B)** 0, 25, 50, 75, 100, 125, or 150 µM DRB, **(C)** 0, 7.8125, 15.625, 31.75, 62.5, or 125 nM Flavo, **(D)** 0, 15.625, 31.75, 62.5, 125, or 250 nM Flavo, **(E)** 0, 5, 10, 15, 20, 25, or 30 µM aloisine, **(F)** 0, 25, 50, 75, or 100µM aloisine, **(G)** 0, 5, 10, 15, 20, 25, or 30µM R- (open squares) or S- (black triangles) Rosco, or **(H)** 0, 25, 50, 75, 100 µM *R*- (open squares) or *S*- (black triangles) Rosco. At 24 h post infection, virus was harvested and titrated by standard plaque assays. Note the different scales in the x-axes. As previously reported, antiviral concentrations of these drugs are cytostatic. HFF: Human foreskin fibroblast; HSV: Herpes simplex virus; PBS: Phosphate-buffered saline; PFU: Plaque forming unit.

§: Non cytostatic concentrations; §§: Probably cytotoxic concentration (percentage of non-viable cells increased approximately threefold above the percentage of non-viable cells in the absence of any drug);

§§§: Cytotoxic concentration (percentage of non-viable cells increased more than threefold above the percentage of non-viable cells in the absence of any drug); §§§§: Highly cytotoxic concentration (percentage of nonviable cells increased more than sixfold above the percentage of non-viable cells in the absence of any drug).

Table 1. Comparison of IC_{50} and maximum antiviral effects of different PCIs in two cell lines.

PCI	HFF		Vero	
	IC_{50}, µM	*Maximum inhibition, %§*	*IC_{50}, µM*	*Maximum inhibition, %§*
DRB	48	99.97	77	96.31
Flavo	0.020	99.96	0.024	99.90
Aloisine	15	99.72	N/A	69.10
R-Rosco	17.5	99.83	72	99.992
S-Rosco	10	99.87	50	99.997

N/A: Not available (inhibition of viral replication was not significant enough to calculate IC_{50}).

§: Maximum inhibition of HSV-1 replication attained by non-cytotoxic concentrations of the drug, expressed as a % of viral replication in the absence of any drug for the respective cell type.

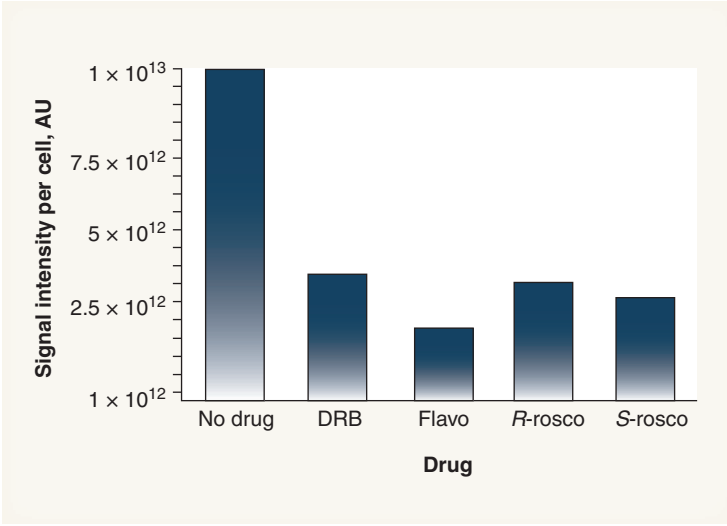
of 4.01 orders of magnitude in HFF cells, but of only 2.31 orders of magnitude in Vero cells (**Figure 1A & B**). However, DRB was cytotoxic for Vero and HFF cells at 150 µM. The IC_{50} of non-cytotoxic concentrations of DRB in HFF cells was approximately half of its IC_{50} in Vero cells (48 and 77 µM, respectively) (**Table 1**). Flavo also demonstrated some degree of cell type specificity, albeit to a minor extent. Noncytotoxic concentrations of Flavo inhibited HSV replication by 3.7 orders of magnitude in HFF cells, with an IC_{50} of 20 nM, but by only 3.0 orders of magnitude in Vero cells with an IC_{50} of 24 nM, (**Figure 1C & D**) (**Table 1**). Although high concentrations of Flavo apparently inhibited HSV-1 replication to a higher extent in Vero than in HFF cells (by 5.13 and 3.84 orders of magnitude, respectively) (**Figure 1**), inhibition at these concentrations was actually mediated in part by cytotoxicity (**Figure 1**) [27,34,35]. We have nonetheless included these concentrations in order to compare our results with the results of experiments previously reported using such concentrations [23,36–38]. Aloisine, a novel phenyl-pyrrolo-pyrazine [39], demonstrated complete cell-type selectivity. Thus, 30 µM aloisine reduced viral replication by 2.56 orders of magnitude in HFF cells whereas 100 µM had only a marginal effect on viral replication in Vero cells (**Figure 1E & F**) (**Table 1**). As a result of these marginal effects of aloisine on HSV-1 replication, we decided not to pursue this drug any further and therefore we did not perform toxicity analyses of aloisine. Rosco inhibits HSV-1 replication more potently in fetal human primary lung fibroblasts (HEL) than in immortalized Vero cells [40]. Consistently, Rosco inhibited HSV-1 replication in neonate HFF cells more potently than in immortalized Vero cells. In these experiments, we further compared the antiviral activity of the purified *R*- and *S*-isomers of Rosco. Even though both drugs inhibited HSV-1 replication to approximately the same extent, *S*-Rosco was more potent than *R*-Rosco. Thus, the IC_{50} of *S*-Rosco was 1.5- to twofold lower than that of *R*-Rosco in HFF (10µM and 17.5µM, respectively) or Vero cells (50µM and 72µM, respectively) (**Figure 1G & H**) (**Table 1**). Some loss of Vero cell viability (average of 9.5% nonviable cells) was observed at 100µM *R*-Rosco in two of four experiments. No cytotoxicity was observed at any concentration of *S*-Rosco.

The results presented in **Figure 1** and **Table 1** confirm that the antiviral effects and toxicity of PCIs are cell-type specific, as expected for drugs that target cellular proteins. These results also indicated that it was more difficult for any tested PCIs to inhibit HSV-1 replication in Vero than in HFF cell, which is consistent with previously published experiments [22]. We used Vero cells for all subsequent experiments, on the assumption that any effect that is detectable in Vero cells will likely be even more pronounced in HFF cells.

PCIs inhibit accumulation of ICP4 in HSV-1 replication compartments

Our previous results have shown that inhibition of HSV-1 replication by Rosco results from prevention of initiation of HSV-1 transcription [27,41] and inhibition of HSV-1 DNA replication [28]. To evaluate whether the effects on viral transcription are common to PCIs or unique to Rosco, we tested the effects of PCIs on HSV-1 gene expression, using the HSV-1 immediate–early protein ICP4 as an indicator. Vero cells were infected with 20PFU of HSV-1 per cell and treated with vehicle, 125 µM DRB, 100 nM Flavo, 100 µM *R*-Rosco, or 100 µM *S*-Rosco. Although this concentration of Flavo showed some cytotoxic effects at 24h, we analyzed in these and the following experiments doses of the different drugs that had similar effects on HSV-1 replication. Since these and the following experiments were terminated at 10h or earlier, no cytotoxicity was observed (**Figure 3**). Cells were fixed in 4°C methanol at 5h post-infection, ICP4 was detected by immunofluorescence and ICP4 signal was quantitated using Quantity One software. Quantitation by immunofluorescence was used in these experiments because we are in the process of comparing levels and accumulation of ICP4 into replication compartments in a cell-by-cell basis, a comparison that requires quantitation of ICP4 levels in individual cells. We have

Figure 2. Effects of DRB, Flavo, *R*-Rosco or *S*-Rosco on accumulation of ICP4 to replication compartments.



Vero cells were infected with 20PFU of HSV-1 strain KOS per cell. After 1h, inocula were removed, cells were washed twice with cold PBS and complete medium containing the different drugs was added. Cells were treated with no drug, 125 µM DRB, 100 nM Flavo, 100 µM *R*-Rosco, or 100µM *S*-Rosco. At 5 h post-infection, cells were fixed in 4°C methanol. Immediate–early protein ICP4 was visualized by immunofluorescence with anti-ICP4 primary antibody and Alexa 594 conjugated secondary antibody. ICP4 signal was quantitated using Quantity One software and plotted as arbitrary units (AU). X-axis crosses the Y-axis at the background level (8.5×10^{11}). The results presented are from one of two qualitatively identical experiments. HSV: Herpes simplex virus; PBS: Phosphate-buffered saline; PFU: Plaque-forming unit.

shown before that PCIs inhibit ICP4 expression as evaluated by metabolic labeling, arguably the most quantitative technique [41].

In two independent experiments, ICP4 was expressed at high levels in the absence of PCIs, as expected, but at much lower levels in cells infected in the presence of any PCI. As measured by immunofluorescence, ICP4 levels at 5h post-infection were approximately two thirds to three quarters lower in the presence of DRB, Flavo, *R*-, or *S*-Rosco than in nontreated cells (**Figure 2**). Since the ICP4 levels in nontreated cells were saturating for the quantitation system, the degree of inhibition of ICP4 quantitated in these experiments is most likely an underestimate of the actual degree of inhibition. These results indicate that three structurally diverse PCIs prevent immediate-early viral gene expression to similar extents.

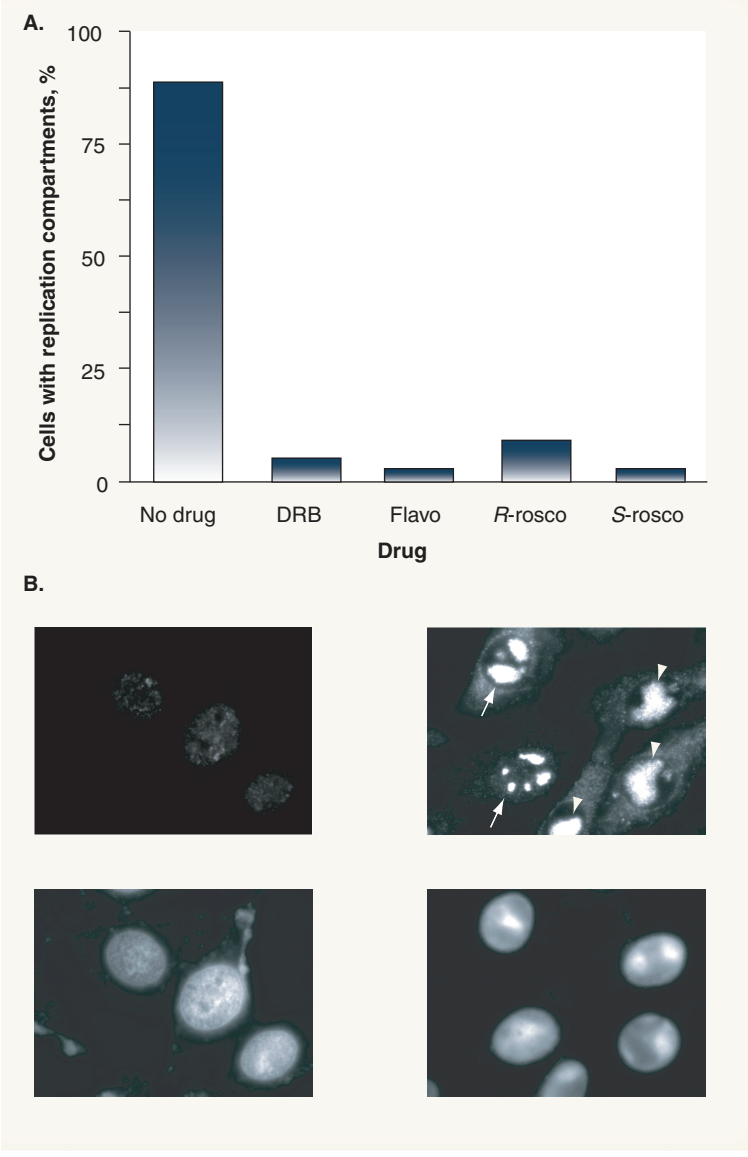
During the analyses of these experiments, we also noted that PCIs appear to affect the subcellular localization of ICP4. HSV-1 genomes and viral proteins form intranuclear domains, referred to as viral replication compartments [42], in which HSV-1 genes are expressed and HSV-1 genomes are replicated. Since ICP4 is among the proteins that localize to the replication compartments, we further examined the formation of these compartments in the presence of PCIs [43]. Replication compartments were identified by the globular accumulation of ICP4 in the nucleus (**Figure 3**). As expected, ICP4 localized to replication compartments in most cells infected in the absence of PCIs (approximately 90% of cells). In contrast, ICP4 failed to localize to replication compartments in cells infected in the presence of any PCI (**Figure 3**). For example, approximately 1% of cells showed ICP4 accumulation into replication compartments in the presence of Flavo or *S*-Rosco. *R*-Rosco and DRB, which lead to 6.8 and 2.7% of cells showing accumulation of ICP4 in replication compartments, respectively, were less potent than *S*-Rosco and Flavo in these experiments (**Figure 3**).

PCIs inhibit HSV-1 transcription

The results presented in **Figure 2** demonstrate that several PCIs inhibit accumulation of a HSV-1 protein, ICP4, in agreement with previous results which demonstrated that Rosco inhibits transcription of HSV-1 genes [27]. Previous results also indicate that Rosco inhibits HSV-1 gene transcription in the presence or absence of immediate–early proteins, such as ICP4 [5,41]. Therefore, we further evaluated the effects of DRB and Flavo on HSV-1 transcription in the presence or absence of these proteins, using a previously described CHX-release experimental design [27,41]. Vero cells were infected in the presence of CHX with 20PFU of HSV-1 per cell. After 5h, cells were transferred to complete medium containing vehicle (DMSO), 50 µg/ml CHX, 100 µM Rosco, 150 µM DRB, or 100 nM Flavo. Members of our group have shown previously that high levels of all HSV-1 immediate–early proteins are synthesized after removal of CHX following this procedure (from the transcripts that over accumulated during the 5 h incubation in the presence of CHX) [41]. Nuclei were isolated at 10h post-infection, and run-on assays were performed as described [27]. We analyzed promoter specific (i.e., sense) and non-promoter specific (i.e., antisense) transcription, by probing with single stranded DNA sense or antisense to selected HSV genes.

Run-on transcription assays performed with nuclei of mock-infected cells (negative controls) resulted in only background levels of hybridization to viral genes. Run-on

Figure 3. Effects of DRB, Flavo, *R*-Rosco, or *S*-Rosco on accumulation of ICP4 to replication compartments.



A. Vero cells were infected with 20 PFU of HSV-1 per cell and fixed as described in Figure 2. Percentages of cells containing ICP4 accumulated in either small or large replication compartments are plotted against drugs. The results presented are from one of two qualitatively identical experiments. **B.** Representative pictures of nuclear diffuse ICP4 accumulation (top left panel), taken from *S*-Rosco treated infections, and small and large replication compartments (arrows and arrowheads, respectively), taken from non-treated infections (top right panel). The bottom panels are the same fields counterstained with Hoescht 33258, showing the nuclei present in the respective fields.
HSV: Herpes simplex virus; PFU: Plaque-forming unit.

transcription assays performed with the nuclei of cells infected with HSV-1 in the absence of any drug (positive controls) resulted in abundant transcription of all HSV-1 genes, as expected (**Figure 4**). Most transcription was from the sense strand (promoter-specific) but a small fraction was from the antisense strand (non-promoter-specific), which is characteristic of HSV-1 transcription [27,32,33].

Run-on assays performed with the nuclei of cells infected with HSV-1 and maintained in CHX resulted in abundant transcription of the immediate–early gene *ICP4* (**Figure 4**). Another IE gene, *ICP27*, was transcribed under these conditions less efficiently than *ICP4*, to levels similar to those in the absence of any drug. Since the concentrations of CHX required to efficiently reverse the effect of the drug are not sufficient to completely inhibit protein synthesis, some transcription of early and late genes, to lower levels than those in the absence of any drug, are also observed under these conditions. Run-on assays performed with the nuclei of cells infected with HSV-1 for 5 h in the presence of CHX and then further incubated for 5 h with 100µM Rosco, 150 µM DRB or 100 nM Flavo resulted in almost complete inhibition of transcription of all tested HSV-1 genes (**Figure 4**). All PCIs prevented promoter-specific and nonpromoter-specific transcription. Flavo, which inhibits transcription elongation, also moderately inhibited transcription when it was present during the run-on transcription (**Figure 4**). In contrast, DRB, which acts primarily on transcription initiation, or Rosco, which acts primarily at or before transcription initiation [27], did not inhibit HSV-1 transcription when either were present during the run-on transcription (**Figure 4**). These findings are consistent with the lack of effects of DRB on cellular run-on transcription, and of Rosco on HSV-1 run-on transcription under similar circumstances [27]. Therefore, the inhibition of initiation of HSV-1 gene transcription in the presence of immediate–early proteins is common to at least three different PCIs.

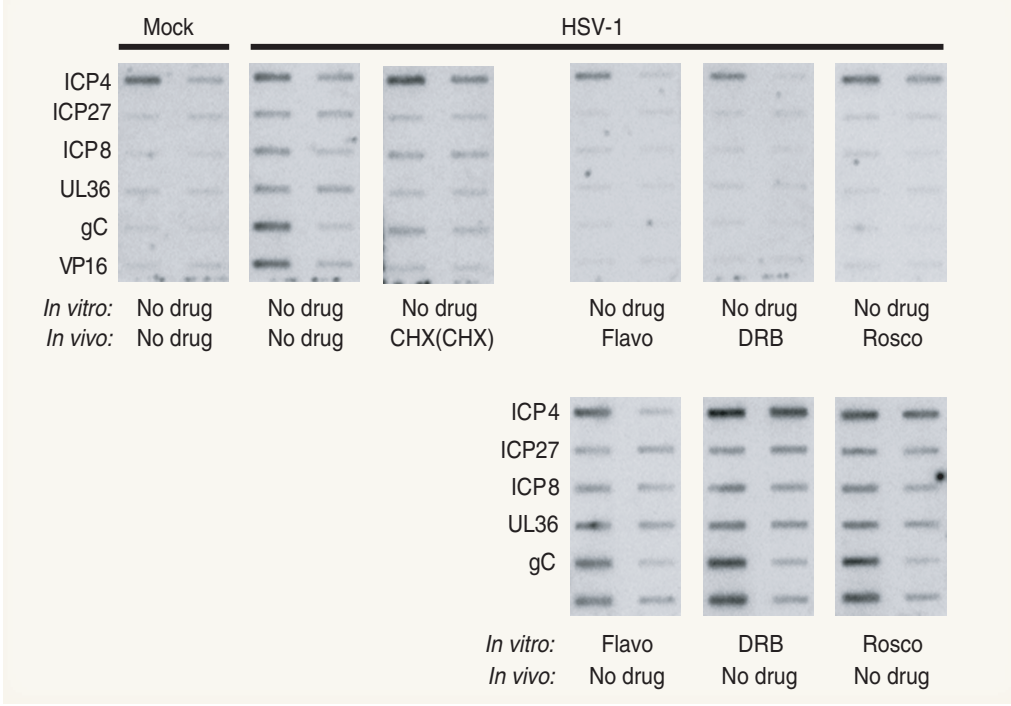
Discussion

Herein, we have shown that three structurally unrelated PCIs, which preferentially inhibit different subsets of CDKs, all inhibit viral gene expression. We further show that the three PCIs affect the subcellular localization of viral proteins and that both oligo- and polyspecific PCIs, such as Rosco and Flavo, respectively, inhibit initiation of viral transcription, a function that is not targeted by any available antiviral drug. Lastly, we showed that *S*-Rosco inhibits HSV-1 replication with a IC_{50} approximately 1.5 to twofold lower than those of *R*-Rosco.

PCIs inhibit replication of a number of human pathogenic viruses, including HIV-1, HCMV, VZV, EBV, HSV-1 and HSV-2 [22–26,29,44,46] and are showing only limited serious adverse effects in clinical trials against cancer [10,11,13–18]. Consequently, PCIs have been repeatedly proposed as potential antiviral drugs, and Rosco and Flavo have already been tested in an animal model of HIV-induced nephropathy [2,4–6,11,22,23,26,29,47,51], and are now scheduled to enter Phase I clinical trials as antivirals in 2005. However, the antiviral mechanisms of PCIs remain only partially characterized.

One outstanding question is whether different PCIs inhibit viral replication through a common mechanism. Using HSV-1, HCMV and VZV as models, Rosco has been shown to prevent initiation of viral transcription, DNA replication and phosphorylation of viral proteins [25,27,29,52]. Using HIV, Flavo and DRB have been shown to inhibit elongation of transcription [23,31,53], and using other retroviruses, Rosco and other related PCIs have also been shown to inhibit expression of cellular proteins required to activate specific viral promoters [36]. One of the major difficulties in analyzing these results together is that different viruses and cells were used to study the different antiviral effects. Therefore, we tested the effects of structurally unrelated PCIs, which also have different specificities, on a single virus. We included two PCIs that preferentially target CDKs involved in transcription (DRB and Flavo) and two PCIs that preferentially target CDKs involved in cell-cycle progression (aloisine and Rosco). From these experiments, we concluded that structurally unrelated PCIs, which primarily target CDKs required for cell-cycle progression or transcription, all inhibit initiation

Figure 4. PCIs inhibit initiation of HSV-1 transcription.



Nine membranes slot-blotted with single-stranded DNA same sense as, or complementary to, six HSV-1 genes and probed with RNA isolated from run-on transcription reactions. Cells were (Mock), or infected with HSV-1 (HSV-1) in the presence of CHX. Five hours later, cells were transferred to medium containing (*in vivo*) vehicle (No Drug), CHX (CHX), Flavo (Flavo), DRB (DRB), or Rosco (Rosco). Nuclei were isolated at 10 h post-infection, and run-on transcription reactions were performed in the presence of (*in vitro*) vehicle (no drug), Flavo (Flavo), DRB (DRB), or Rosco (Rosco). RNA was purified and probed with membranes containing single-stranded DNA complementary to (+), or same sense as (-), two immediate-early (ICP4, ICP27), two early (ICP8, UL36) and two late (gC, VP16) HSV-1 genes. The higher background in the ICP4 sense probe is consistently observed after CHX-treatments, and is most likely due to cross-hybridization with CHX-inducible cellular RNAs.
CHX: Cyclohexamide; HSV: Herpes simplex virus.

of viral transcription. However, we have not tested whether these PCIs inhibit viral replication only as a result of inhibition of viral transcription. Moreover, we have used only one virus as a model, HSV-1. It is possible, and even likely, that PCIs may inhibit different functions of different viruses – a question that we are currently addressing. From results published by our group and others, we hypothesize that PCIs most likely inhibit several viral functions.

We have previously shown that the antiviral potencies of Rosco and olomoucine are cell-type dependent [22]. Herein, we show that three other PCIs – DRB, Flavo and aloisine – also inhibit HSV-1 replication with cell-type dependent potencies (**Figure 1**). Interestingly, the extent of cell-specificity was dependent on the specific drug. For example, aloisine had almost no antiviral effect on Vero cells, whereas Flavo was only marginally more potent in HFF than in Vero cells. Although cell-specific potency was expected because PCIs act on cellular targets, the specific mechanisms of these differences are still unknown. Immortalized or transformed cells could internalize PCIs less efficiently than primary cells, or they could metabolize or export PCIs more efficiently. Transformed cells, for example, commonly overexpress drug-exporting pumps. Overexpression of the multidrug resistance protein (MRP)1 confers partial resistance to Flavo [54] and might indiscriminately confer resistance to other PCIs. However, such genes may or may not be overexpressed in immortalized cells and their actual levels in Vero cells have not, to the best of our knowledge, been analyzed. The difference of HSV-1 sensitivity to PCIs in different cells may also reflect the differences in intracellular concentrations of ATP or on levels of other CDK activities. ATP levels and cell-cycle related CDK activities, such as CDK1 and 2, are commonly lower in primary than in immortalized or transformed cells. These different levels are consistent with the higher antiviral potencies of PCIs in primary than in immortalized or transformed cells. In contrast, levels of CDK9 or CDK7 activity have not, to the best of our knowledge, been reported to vary among fibroblast cell lines (HFF and Vero cells are both fibroblasts). High concentrations of Rosco or Flavo, but not of DRB or aloisine, could overcome the resistance of Vero cells to their antiviral activities. In the case of Flavo, however, this apparent antiviral activity was mediated, at least in part, by cytotoxicity (**Figure 1**) [27,34,35]. High concentrations of Flavo inhibit transcription by RNA polymerase II and consequently, expression of most cellular genes [34,35]. In contrast, the concentrations of Rosco used in these experiments have no major effects on global cellular gene expression, or even on expression of a viral gene recombined in the cellular genome [27,35].

The antiviral concentrations of all tested PCIs were found to be lower in primary cells, in which viruses commonly replicate *in vivo*, than in immortalized or transformed cells, which are the target of the antitumoral activities of PCIs. Therefore, the antiviral concentrations of PCIs *in vivo* could be expected to be as low as those required to inhibit viral replication in primary cells *in vitro*, and below the concentrations used with limited undesirable major side effects in the ongoing clinical trials against cancer [10,11,13–18].

Although DRB, Rosco and Flavo all appear to inhibit ICP4 expression to similar extents in Vero cells, HSV-1 replication was inhibited by 3.0 to 4.5 orders of magnitude by non-cytotoxic concentrations of Rosco or Flavo, but only by 2 orders of magnitude by non-cytotoxic concentrations of DRB (**Figure 1**). However, the experiments presented in **Figures 2 & 4** were each performed at a single time point (5 and 10h post-infection, respectively), and DRB appears to delay rather than inhibit expression of HSV-1 genes (data not shown). In contrast, Rosco inhibits rather than delaying expression of HSV-1 genes [22,27,28,41]. Experiments in progress are aimed at identifying whether DRB inhibits or delays expression of HSV-1 genes.

PCIs also inhibited localization of ICP4 to viral replication compartments, an antiviral effect that has not been described previously. PCIs may directly inhibit the formation of replication compartments by preventing phosphorylation of cellular or viral proteins. This hypothesis is supported by previous reports in which Rosco inhibited phosphorylation of ICP4 and phosphorylation or other post-translational modifications of ICP0 [52,55]. ICP0 is another HSV immediate–early protein, which interacts and co-localizes with ICP4. The effects of Rosco on ICP4 phosphorylation, however, have been evaluated only at late times post-infection [52]. Therefore, more experiments are needed before it can be concluded that the effects of PCIs on ICP4 phosphorylation and subnuclear localization are related. Inhibition of ICP4 localization to replication compartments would in turn lead to the previously observed inhibition of HSV-1

DNA replication [28]. Alternatively, direct inhibition of HSV-1 DNA replication may result in indirect inhibition of the formation of the replication compartments [28]. PCIs other than Rosco may also delay immediate–early protein synthesis, such that at 5h post-infection there is insufficient ICP4 to visibly localize to replication compartments. Experiments in progress are aimed at characterizing the mechanisms of inhibition of formation of replication compartments by PCIs, a potentially novel antiviral mechanism.

In these studies, Flavo was the only PCI that inhibited HSV-1 transcription elongation, although not as efficiently as it inhibited initiation of HSV-1 transcription (**Figure 4**). The effect of Flavo on transcription elongation likely results from inhibition of CDK9, which is directly involved in this process. However, DRB, which also inhibits CDK9, failed to inhibit elongation of HSV-1 transcription under our conditions. The concentrations of ATP used in the run-on transcription reactions may be too high for a competitive inhibitor (DRB), while having no effect on a noncompetitive one (Flavo).

Since it has one chiral carbon, Rosco has two optical isomers, (*R*) and (*S*). *R*-Rosco inhibited purified CDK1 *in vitro* with IC_{50} approximately twofold lower than that of the *S* isomer [56]. *R*-Rosco was also found to co-crystallize preferentially with CDK2, even though both isomers inhibited this kinase with very similar IC_{50} values [56]. In another study, the IC_{50} of both isomers toward CDK2 were dependent on the activating cyclin, A or E. Based on these results, it was hypothesized that *R*-Rosco may be biologically the most active isomer [57]. Surprisingly, we found that *S*-Rosco inhibits HSV-1 replication with IC_{50} approximately 1.5- to twofold lower than the *R* isomer (**Figure 1**)(**Table 1**). According to results from the group of De Azevedo and colleagues [56], *R*-Rosco may well be more potent but less specific than *S*-Rosco. Under this scenario, *R*-Rosco would bind to more (irrelevant for HSV-1 replication) cellular proteins than *S*-Rosco. Thus, at sub-saturating concentrations, more *S*- than *R*-Rosco would be available to inhibit the kinases required for HSV-1 replication. In contrast, both isomers should be capable of inhibiting HSV-1 replication with similar efficiencies at saturating concentrations, which is consistent with the dose-responses observed in the experiments presented in **Figure 1**.

Expert opinion

PCIs have been postulated to inhibit viral gene expression by inhibiting the accessibility of transcription factors to viral promoters [41,45], by inhibiting elongation of viral transcription [23], or by inducing downregulation of expression of cellular genes required for viral transcription [36]. Our previous results indicate that the effects of Rosco on HSV-1 replication are not mediated by downregulation of cellular factors in that Rosco inhibits the accumulation of HSV-1 transcripts even when protein synthesis is inhibited by CHX [27]. The experiments presented herein, together with previously published results, further indicate that Rosco, Flavo, and DRB inhibit elongation of run-on HSV-1 transcription only moderately (Flavo) or inefficiently (Rosco and DRB) [27]. Therefore, we conclude that the major antiviral mechanism of PCIs against HSV-1 is the prevention of initiation of viral transcription. This is a novel antiviral mechanism not used by any current antiviral drug. We have recently shown that this inhibition is genome-specific but independent of promoter-specific factors [27]. Such a mechanism requires no specific viral proteins or sequences and would, therefore, lead to the observed ability of PCIs to inhibit replication of a variety of unrelated viruses and to their observed inability to promptly select for drug-resistant strains.

Outlook

Although much progress has been made in the seven years since PCIs were first shown to have antiviral properties, several major questions still remain unanswered. Several groups are currently working to address these questions, including ours, and PCIs will enter clinical trials as antivirals in 2005. Consequently, we can expect that the antiviral activities of PCIs *in vivo* will be fully addressed in the coming years. We can also expect that the antiviral mechanisms of PCIs will soon be fully characterized, and that the relevant kinases will be identified. Such characterizations may then allow for the development of novel compounds, which would inhibit viral replication by the same major mechanisms used by PCIs. Even if PCIs were eventually proven not to be useful as clinical antivirals, the study of these drugs has raised the awareness of cellular proteins as possible targets for antiviral drugs. PCIs have already been useful to further uncover the uniqueness of the regulation of viral gene expression, and they may help identifying novel targets for antiviral drugs in the near future.

Highlights

- Pharmacological cyclin-dependant kinase (CDK) inhibitors (PCIs) such as roscovitine and flavopiridol are considered potential antivirals due to the fact that they have potent antiviral activity *in vitro* and are showing minimal serious adverse effects for humans in early clinical trials against cancer.
- Other PCIs had been shown to target different functions of different viruses in various other cell types.
- However, it had not been evaluated whether PCIs also target a common viral function.
- Three structurally unrelated PCIs, which also inhibit different subsets of CDKs, targeted the same viral function.
- The *S*-isomer of roscovitine was more potent as antiviral than the *R*-isomer.
- PCIs were more potent antivirals in human primary cells in which viruses replicate *in vivo* than in transformed cells, which are the target of the antitumoral activities of PCIs.
- PCIs inhibited a viral function that is not targeted by current antiviral drugs, initiation of transcription.
- PCIs also inhibited the proper subcellular localization of viral proteins, another function that is not targeted by any current antiviral drug.
- The antiviral activities of PCIs appear to require no specific viral proteins or sequences and would therefore lead to the ability of PCIs to inhibit replication of a variety of unrelated viruses and their inability to promptly select for drug-resistant strains.

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101 Website of the biopharmaceutical company, Cyclacel. www.cyclacel.com Accessed November 2004.

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