Suppression of HIV-1 Expression by Inhibitors of Cyclin-Dependent Kinases Promotes Differentiation of Infected Podocytes

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Abstract. The glomerular lesions of HIV-associated nephropathy (HIVAN) are associated with the expression of HIV-1 in podocytes. Infected podocytes proliferate and lose several differentiation markers in vivo and in vitro, which suggests that HIV-1 gene expression induces these changes. Flavopiridol and roscovitine, newly identified inhibitors of cyclin-dependent kinase-9, markedly decrease HIV-1 promoter activity in cell lines of various lineages. In this study, the inhibitors were used to determine whether suppression of HIV-1 transcription in infected podocytes correlated with an inhibition of proliferation and a return to the differentiated phenotype. Dose-response analysis showed that both flavopiridol and roscovitine reversibly suppressed HIV-1 transcription in podocytes in vitro at an IC50 of 25 nM and 3 μM, respectively. Despite equivalent suppression of HIV-1 transcription, roscovitine was a more effective inhibitor of podocyte proliferation than flavopiridol. Suppression of HIV-1 transcription by flavopiridol or roscovitine was marked by re-expression of the podocyte differentiation markers, synaptopodin and podocalyxin. These results suggest that inhibition of HIV-1 transcription decreases podocyte proliferation and permits the reexpression of differentiation markers. Thus, suppression of HIV-1 transcription by selective cyclin-dependent kinase-9 inhibitors may be a useful therapeutic strategy for the treatment of HIVAN.

Effective treatment of HIV-associated nephropathy (HIVAN) will likely require interrupting the viral life cycle of HIV-1 in renal epithelium (1). Animal models of HIVAN suggest that suppressing HIV-1 gene expression in renal epithelium may be a particularly effective strategy (2–7). Recently, flavopiridol and roscovitine, small molecules that inactivate specific cell-cycle cyclin-dependent kinases (CDK), were found to inhibit CDK-9, markedly decreasing HIV-1 promoter activity in cell lines of various lineages (8,9). This raised the possibility that HIV-1 gene expression in renal epithelium may also be critically dependent on CDK that control RNA polymerase II activity on the HIV-1 promoter (10).

Clinical trials that have evaluated flavopiridol as an antineoplastic and use of roscovitine in animal models of acute glomerulonephritis suggest that these small molecules are not toxic to quiescent renal cells at concentrations that inhibit proliferation in vivo (11,12). As a result, they appear to be attractive therapeutic compounds. Here, we use flavopiridol or roscovitine to suppress HIV-1 gene expression in podocytes. The purpose was to determine whether a reduction in HIV-1 transcripts by these CDK inhibitors correlated with an inhibition of proliferation and a return to the differentiated phenotype of infected podocytes.

Materials and Methods

Podocyte Cell Culture

Murine podocytes immortalized with interferon-inducible temperature-sensitive SV-40 T antigen were isolated as described elsewhere (13). Podocytes were used before passage 20 and maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum, 1X PenStrep (Life Technologies), and 20 U/ml of recombinant interferon-gamma (Life Technologies) at 33°C.

Virus Production and Infection

Pleotropic VSV-G pseudotyped HIV-1 virus was prepared as follows: GFP-expressing HIV proviral plasmid, pNL4-3:d-EGFP, was created by cloning the enhanced green fluorescent protein (EGFP) coding region from pCMS-EGFP (Clontech, CA) into the gag/pol deletion site of pNL4-3:d1443 (6). 293T cells were simultaneously transfected with pNL4-3:d-EGFP, pCMV R8.91 (which provides gag/pol) (14), and pMD.G (expressing the VSV-G glycoprotein) (14). Supernatants that contained recombinant virus were collected 2 d posttransfection, passed through a 0.45-μm filter and used to infect podocytes at two to five transducing units/ml (the reciprocal of the lowest dilution of virus showing GFP expression after infection of HeLa/tat cells). Seventy percent to 80% of podocytes showed GFP expression 4 d postinfection (data not shown). All subsequent studies were started 1 d after switching podocytes from 33°C to 37°C in media without recombinant interferon-gamma to inactivate temperature-sensitive SV-40 T antigen.
**Dose Response**

Infected and wild-type podocytes were treated with flavopiridol (Developmental Therapeutics Program, National Cancer Institute, MD) or roscovitine (Calbiochem, CA) at twofold increments ranging from 0.78 to 200 nM and 0.19 to 50 μM, respectively, for 12 h, followed by RNA extraction with TRIZOL reagent (Life Technologies). Each sample (10 μg RNA) was analyzed by Northern blot analysis that used a probe for nef (1) and glyceraldehyde-3-phosphate dehydrogenase. Signal intensity after autoradiography was calculated by use of the UN-SCAN-IT gel v4.3 (Silk Scientific, Orem, UT).

**Proliferation**

Six-well plates seeded with 2.2 × 10⁴ infected podocytes were treated with media that contained 50 nM flavopiridol or 12.5 μM roscovitine for 3 d (media changed every 12 h), followed by a 2-d washout period in which media without inhibitors was used. Cells were counted at days 0, 1, 2, 3, and 5. Parallel cultures were treated identically and extracted for RNA at day 0, 1, 2, 3, and 4. Each sample (10 μg RNA) was analyzed by Northern blot by use of a probe for nef and glyceraldehyde-3-phosphate dehydrogenase.

**Apoptosis**

Infected podocytes plated on coverslips were treated with flavopiridol or roscovitine as described above for 36 h. DMSO-treated wild-type and infected podocytes served as controls. Every 12 h, cells from each condition were fixed in 60% acetone/3.7% formaldehyde at −20°C for 15 min, then placed in 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Oncogene Research Products, MA) for 1 min. The apoptotic index was calculated as the ratio of DAPI-enhanced late apoptotic nuclei per 100 normal nuclei in 10 randomly selected 40× fields per coverslip (15).

**Differentiation**

Infected podocytes plated on type I collagen were treated with flavopiridol or roscovitine as described above for 9 d after the loss of SV-40 T antigen activity. Differentiation of wild-type podocytes after the loss of SV-40 T antigen activity served as a comparison (13). Twenty micrograms of RNA per sample, extracted at day 0, 3, 6, and 9, was analyzed by Northern blot and probed for synaptopodin and podocalyxin transcripts (cDNA gifts from Masaaki Sunamoto, generated by reverse transcription–PCR from murine podocytes and verified by sequence analysis).

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**Figure 1.** Dose-dependent suppression of HIV-1 transcription in podocytes by flavopiridol. (A) Northern blot detection of HIV-1 and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) transcripts from HIV-1–infected (lanes 1 through 10) or wild-type (lanes 11 through 20) podocytes after a 12-h treatment with increasing concentrations of flavopiridol. Suppression of full-length (F), singly spliced (S), and multiply spliced (M) HIV-1 transcripts is evident. (B) Dose-response curve based on the abundance of multiply spliced transcripts.

**Figure 2.** Dose-dependent suppression of HIV-1 transcription in podocytes by roscovitine. (A) Northern blot detection of HIV-1 and G3PDH transcripts from HIV-1–infected (lanes 1 through 10) or wild-type (lanes 11 through 20) podocytes after a 12-h treatment with increasing concentrations of roscovitine. Suppression of F, S, and M HIV-1 transcripts is evident. (B) Dose-response curve based on the abundance of multiply spliced transcripts.
Results
Dose-Dependent Suppression of HIV-1 Genes

Recent observations suggest that flavopiridol suppresses HIV-1 transcription by inhibiting CDK-9 at 10- to 20-fold lower concentrations than is needed to inhibit cell-cycle CDK (8). Thus, flavopiridol may suppress HIV-1 expression without rapidly affecting cell-cycle progression. In contrast, roscovitine inhibits HIV-1 expression at concentrations that also inhibit cell-cycle CDK (9). Similar to observations in other cell types, flavopiridol suppressed HIV-1 transcription in podocytes at an IC$_{50}$ of 25 nM (Figure 1). Roscovitine, on the other hand, suppressed HIV-1 transcription in podocytes at concentrations expected to inhibit cell-cycle CDK, with an IC$_{50}$ of 3 μM (Figure 2). For both inhibitors, suppression of HIV-1 transcription appeared to reach a maximum of 85% to 90%. Glyceraldehyde-3-phosphate dehydrogenase expression, a marker of endogenous transcriptional activity, was not significantly altered in both infected and wild-type podocytes.

Reduction in Apoptosis

To rule out that the reduction in HIV-1 transcripts is not simply a reflection of cell death by enhanced apoptosis, we determined the apoptotic index of infected podocytes up to 36 h after the addition of 50 nM flavopiridol or 12.5 μM roscovitine, the first doses to achieve at least a 70% reduction in HIV-1 transcripts, respectively (Figures 1 and 2). At baseline, HIV-1-infected podocytes displayed a threefold greater apoptotic index than wild-type podocytes (Figure 3). This HIV-induced cytopathicity was reduced at 12 h and further at 24 and 36 h, after the addition of flavopiridol or roscovitine, which indicates that these CDK inhibitors selectively decrease apoptosis of HIV-1-infected podocytes. This was further confirmed by the reexpression of HIV-1 genes to pretreatment levels after washout of the inhibitors (see below).

Proliferation and Reversible Suppression of HIV-1

Markers of podocyte proliferation appear in vivo in HIVAN (16–18). Roscovitine (12.5 μM) significantly inhibited proliferation of HIV-1-infected podocytes during 3 d of treatment (Figure 4A). This roscovitine effect is predicted on the basis of previous data that have shown inhibition of cell-cycle CDK at this drug concentration (9). In contrast, 50 nM flavopiridol nonsignificantly inhibited proliferation during 3 d of treatment despite equivalent suppression of HIV-1 transcription. After washout of the inhibitors, HIV-1 gene expression and proliferation resumed in both flavopiridol- and roscovitine-treated podocytes (Figure 4, A and B).

Podocyte Differentiation

Loss of differentiation of HIV-1–infected podocytes, characterized by the down-regulation of several mature podocyte
markers, is a consistent feature of the glomerular dysfunction in HIVAN (16–18). We examined the possibility that suppression of HIV-1 genes by these CDK inhibitors after the loss of SV-40 T antigen activity would permit the reexpression of maturity markers in infected podocytes. Reexpression of maturity markers in uninfected podocytes during the transition from dedifferentiated to differentiated cells after the loss of SV-40 T antigen activity served as a comparison. After switching to the nonpermissive temperature for SV-40 T antigen activity, wild-type podocytes gradually reexpress synaptopodin and podocalyxin during the subsequent 9 d (Figure 5). Treatment of HIV-1–infected podocytes with 50 nM flavopiridol or 12.5 μM roscovitine was similarly marked by the gradual reexpression of these maturity markers. In contrast, infected podocytes treated with vehicle alone failed to reexpress synaptopodin or podocalyxin.

**Discussion**

One of the characteristic features of HIVAN is focal segmental glomerulosclerosis, often of the collapsing variant (16–18). Podocyte dysfunction appears to be a crucial step in the development of this glomerular lesion. After HIV-1 infection, podocytes lose markers of differentiation and begin to proliferate *in vivo*, a process that is recapitulated by infecting podocytes with HIV-1 *in vitro*. In this study, we demonstrate that the CDK inhibitors, flavopiridol and roscovitine, markedly suppress HIV-1 transcription in podocytes and promote the reexpression of these maturity markers. In contrast, infected podocytes treated with vehicle alone failed to reexpress synaptopodin or podocalyxin.

**Suppression of HIV-1 gene expression may not be the only mechanism whereby these small molecule CDK inhibitors affect the proliferation, differentiation, or apoptosis of infected podocytes.**

**References**


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**Figure 5.** Reexpression of podocalyxin and synaptopodin during treatment with flavopiridol or roscovitine. Northern blot detection of reexpressed podocalyxin and synaptopodin in wild-type podocytes treated with vehicle alone (lanes 1 through 4) and in infected podocytes treated with 50 nM flavopiridol (lanes 9 through 11) or 12.5 μM roscovitine (lanes 12 through 14) during 9 d of differentiation. Podocalyxin and synaptopodin remain down-regulated in infected podocytes treated with vehicle alone (lanes 5 through 8).