Amelioration of nephropathy in mice expressing HIV-1 genes by the cyclin-dependent kinase inhibitor flavopiridol

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Received 2 October 2002; returned 20 December 2002; revised 10 January 2003; accepted 25 January 2003

Cumulative evidence suggests that human immunodeficiency virus-associated nephropathy (HIVAN), the third leading cause of end-stage renal disease in African-Americans, may respond to therapeutic strategies that interrupt HIV-1 expression in infected renal epithelium. We recently demonstrated that suppression of HIV-1 transcription in infected glomerular visceral epithelial cells by flavopiridol, a small-molecule inhibitor of the cyclin-dependent kinases required for HIV-1 promoter activity, reversed HIV-induced proliferation and dedifferentiation in vitro. To address whether flavopiridol could ameliorate HIV-induced renal disease, we utilized a well-established HIV-1 NL4-3 transgenic mouse model of HIVAN. HIV-1 proviral transgene expression in whole kidney was markedly suppressed by a 20 day treatment with flavopiridol. Following treatment, histopathological, serological and urinary indices of nephrosis were normalized in flavopiridol-treated but not in vehicle-treated transgenics. Microarray analysis showed that 82% of the dysregulated genes in HIVAN kidney were normalized to control levels by flavopiridol, whereas continued dysregulation of most of the remaining 18% was attributable to an effect from flavopiridol alone. These results demonstrate for the first time that targeting the cyclin-dependent kinases that support HIV-1 expression can ameliorate HIV-induced disease in an animal model.

Keywords: kidney, renal, therapy, AIDS, model

Introduction

Human immunodeficiency virus-associated nephropathy (HIVAN) is the third leading cause of end-stage renal disease in African-Americans,¹ and recent evidence suggests that the development of HIVAN is associated with the infection and compartmentalization of HIV-1 in kidney parenchyma.²,³ However, unlike many other manifestations of HIV-1 infection, current highly active antiretroviral therapy (HAART) has shown limited benefit in HIV-associated renal disease in large patient cohorts.⁴ This suggests that current therapies may not be interrupting pathogenic mechanisms in the kidney that occur following HIV-1 infection of renal epithelium and the establishment of a viral reservoir in kidney.²³ Indeed, other systemic illnesses in non-HIV-infected patients have also been associated with the collapsing glomerulopathy seen in HIVAN,⁵ suggesting that the development of HIVAN renal disease may become independent of active virus replication.

However, several lines of evidence suggest that the expression of HIV-1 gene products in the kidney is pathogenic even in the absence of ongoing virus replication. Transgenic mice and rats that express replication-incompetent HIV-1 proviral transgenes develop kidney disease similar to HIVAN.⁶–⁸ Reciprocal kidney transplant studies between HIV-1 transgenic and non-transgenic siblings further suggest that HIV-1 gene expression in the kidney is required, but perhaps not

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sufficient, for the development of renal disease. In addition, more recent studies have shown that the major phenotypic abnormalities in HIVAN kidneys, such as epithelial proliferation and dedifferentiation, are recapitulated in vitro by expressing HIV-1 genes in cultured renal epithelium. Together with evidence of ongoing HIV-1 transcription in infected renal epithelium from newly diagnosed HIVAN patients on HAART, these observations argue for exploring chemotherapeutic strategies that specifically interrupt HIV-1 gene expression in the kidney as opposed to other phases of HIV-1 replication.

Unlike current antiretroviral therapies, small-molecule inhibitors of cyclin-dependent kinases (CDKs) can suppress HIV-1 gene expression by inhibiting RNA polymerase II activity on the HIV-1 promoter. Moreover, as suggested by the dependence on coordinated CDK-2, -7 and -9 activities to achieve full Tat-enhanced HIV-1 transcription, HIV-1 gene expression, but not most cellular gene expression, appears to be quite sensitive to CDK inhibition. Yet, neither this therapeutic strategy nor its possible effects on cellular transcription has been explored in vivo for HIV-induced renal disease or for other HIV-related pathologies caused solely by HIV-1 gene expression, such as CD4+ T cell depletion, lymphoid interstitial pneumonitis, myocarditis and cachexia.

We demonstrated recently that flavopiridol, which shows inhibitory activity against CDK-2, -4, -6, -7 and -9, marked suppression of HIV-1 transcription in infected podocytes, the glomerular visceral epithelial cell in the kidney, without toxic effects in vitro. This correlated with the reversal of HIV-induced podocyte proliferation, apoptosis and dedifferentiation, phenotypes observed in HIVAN specimens, suggesting that a similar response might be elicited by treatment in vivo. Here, we utilize a well-established HIV-1 transgenic mouse model of HIVAN to show that flavopiridol suppresses HIV-1 proviral expression in the kidney and ameliorates murine HIVAN without significant toxicity or global alterations in renal gene expression patterns. These results indicate that targeting CDKs that support HIV-1 transcription may be a useful therapeutic strategy for HIVAN and other HIV-induced diseases.

Materials and methods

Mice

All animal protocols were carried out under IACUC-approved protocols. The Tg26 mouse model, which develops murine HIVAN secondary to renal expression of full-length and spliced HIV-1 mRNAs from the HIV-1 NL4-3 Δgag-pol proviral transgene, was used for this study. To study acute changes in renal transgene expression before and after drug treatment in a single animal, eight 30-day-old heterozygous transgenic and nine 30-day-old non-transgenic siblings each underwent a rapid left nephrectomy followed by administration of drug (described below), and then a right nephrectomy 24 h later. To study changes in renal transgene expression, renal gene expression patterns and renal function after chronic drug treatment, sibling groups consisting of two randomly selected 21-day-old heterozygous transgenic and two randomly selected 21-day-old non-transgenic siblings were enrolled in the 20 day drug treatment protocol (described below). Twenty-four sets of these sibling groups entered and completed the 20 day drug treatment protocol. Confirmation of heterozygosity was carried out by probing 10 µg of slot-blotted chromosomal DNA with an HIV-1 nef probe.

Drug treatment protocols

To study acute changes in renal transgene expression, 2.5 mg/kg flavopiridol (Aventis Pharmaceuticals, Inc., Bridgewater, NJ, USA) or an equivalent volume of vehicle (70:30 v/v PBS/DMSO) was administered by intraperitoneal injection immediately and then 12 h after the left nephrectomy. For sibling groups in the 20 day drug treatment protocol, one transgenic and one non-transgenic from each group received 2.5 mg/kg flavopiridol or an equivalent volume of vehicle, respectively, by intraperitoneal injection every 12 h for 20 days, thereby creating four separate treatment cohorts. This dose maintained the 24 h serum trough concentration above the IC50 of 25 nM for suppression of HIV-1 gene expression in infected podocytes. Single dose: peak serum concentration = 1120 nM, trough serum concentration = 34 nM; chronic dosing: peak serum concentration = 1160 nM, trough serum concentration = 44 nM, mean serum concentration over 24 h = 190 nM; values are calculated from the known pharmacology of flavopiridol in mice.

Collection of specimens

From day 17 through day 20 of the 20 day drug treatment protocol, urine was collected and pooled for each mouse. Three to five hours after the final drug dose in the 20 day drug treatment protocol, whole blood, serum and kidneys were collected from each mouse as follows: whole blood and serum were collected by heart puncture; one kidney was homogenized in Trizol reagent (Life Technologies, Gaithersburg, MD, USA) or an equivalent volume of RNA and DNA extraction; the contralateral kidney was fixed in 10% buffered formalin for histopathology. The kidneys from mice used to study acute changes in renal transgene expression were homogenized in Trizol reagent.

Analysis of blood, serum and urine

For each mouse in the 20 day drug treatment protocol, a complete blood count with differential, serum albumin, serum blood urea nitrogen, serum cholesterol, serum triglycerides,
random urine protein and random urine creatinine was determined by the Clinical Pathology Laboratory of the Center for Comparative Medicine and Surgery at the Mount Sinai School of Medicine.

**Quantitative histopathology**

The severity of renal disease in each mouse was quantified by determining the percentage of all nephrons across an entire full-length coronal kidney section that displayed any one of the following features of HIVAN: glomerulosclerosis, podocyte hyperplasia or hypertrophy, acute tubular injury, tubular microcysts and tubular atrophy with interstitial fibrosis. The mean of these percentages gave a final histopathological grade of HIVAN in each mouse that ranged from 0% to 100% disease. Samples were blinded to the pathologist before histopathological analysis.

**Real-time RT–PCR**

cDNA from 2 μg of whole kidney RNA was prepared using Omniscript RT Kit (Qiagen, Valencia, CA, USA) and used for real-time RT–PCR with SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA, USA) on an iCycler (Bio-Rad Laboratories, Hercules, CA, USA) to determine the relative expression of HIV-1 NL4-3 env (forward primer: 5′-TGTCCAAAGGTATCCTTGGAGCCATTCC-3′; reverse primer: 5′-AGTAGAATTTCCCTCCACAAAT-3′; GenBank accession no. AF324493) versus glycer-aldehyde-3-phosphate dehydrogenase (forward primer: 5′-ACCACAGTCCATGCCCCATCAC-3′; reverse primer: 5′-TCACCCACCTGGTGTGA-3′; GenBank accession no. NM008084), an endogenous gene not affected by the concentrations of flavopiridol used in this study.10 RNA from HIV-1 NL4-3-infected podocytes, collected before and 24 h after suppression of HIV-1 gene expression with 50 nM flavopiridol, served as a control. Three separate analyses were carried out on each kidney RNA sample.

**Microarray gene analysis**

Comparisons of renal gene expression patterns between mice in the 20 day drug treatment protocol were analysed at the end of the treatment period by hybridizing whole kidney cRNA, prepared as per Affymetrix GeneChip Target Preparation Protocols (Affymetrix, Santa Clara, CA, USA), to Murine Genome U74Av2 Arrays (Affymetrix) by the Mount Sinai School of Medicine Shared Microarray Facility. One sibling treatment group was studied, and the histopathological grade of HIVAN in each mouse that ranged from 0% to 100% disease. Samples were blinded to the pathologist before histopathological analysis.

### Results

**Suppression of HIV-1 proviral expression in kidney by flavopiridol**

Murine models of HIVAN have shown that HIV-1 gene expression in infected renal epithelium results in renal disease similar to human HIVAN.6–9 Given that current therapies are not directed against viral gene expression, we investigated whether flavopiridol, a small molecule CDK inhibitor of HIV-1 transcription in both lymphoid and non-lymphoid cell types,10,12 could ameliorate murine HIVAN. Thirty-day-old non-transgenic and heterozygous Tg26 HIV-1 transgenic siblings (Figure 1a) underwent a left unilateral nephrectomy followed by a right nephrectomy 24 h later. Flavopiridol (2.5 mg/kg) or an equivalent volume of vehicle was dosed immediately after the first nephrectomy and then 12 h later to maintain the 24 h trough serum concentration above the minimum required to suppress HIV-1 transcription by 50% in vitro.10,12,26 By collecting one kidney before and then after administration of flavopiridol or vehicle, the direct effect of flavopiridol or vehicle on kidney transgene expression in each mouse could be determined. Despite variability in HIV-1 transcript levels between the pre-treatment transgenic kidneys, HIV-1 transcript levels in the post-treatment transgenic kidneys were significantly suppressed by flavopiridol (Figure 1b) but not by vehicle (Figure 1c). To determine whether this acute suppression by flavopiridol could be maintained chronically,
Kidney transgene expression levels were compared between 24 transgenic sibling pairs (without prior nephrectomies), with one sibling receiving 20 days of flavopiridol and the other sibling receiving 20 days of vehicle. This analysis showed that HIV-1 transcript levels remained suppressed in the kidneys of transgenics treated with flavopiridol (2.5 mg/kg every 12 h) for 20 days when compared with vehicle-treated transgenics (Figure 1d). This difference was still apparent despite the known decrease in HIV-1 transcript levels in kidneys of vehicle-treated transgenics with progressive renal disease (Figure 1e). Thus, flavopiridol caused sustained suppression of HIV-1 transcription in whole mouse kidney.

Figure 1. Suppression of HIV-1 gene expression in mouse kidney by flavopiridol. (a) Confirmation by slot blot of the presence or absence of integrated HIV-1 NL4-3 provirus in DNA from transgenic (Tg) and non-transgenic (Non-Tg) siblings treated with flavopiridol or vehicle after a unilateral nephrectomy. (b and c) HIV-1 transcript levels in the pre-treatment (filled rectangles) versus the post-treatment transgenic kidneys after administration of flavopiridol (white bars) or vehicle (hatched bars). HIV-1 transcripts are significantly suppressed in the cohort of four flavopiridol-treated mice (pre-nephrectomy: 0.742 ± 0.0788, post-nephrectomy: 0.149 ± 0.148, *P < 0.01) but not in the cohort of four vehicle-treated mice (pre-nephrectomy: 0.832 ± 0.258, post-nephrectomy: 0.779 ± 0.594, P > 0.05). This recapitulates the suppression of HIV-1 transcription in infected mouse podocytes by flavopiridol in vitro (inset in b), where HIV-1 transcript levels are 2–3 logarithms greater than in mouse kidney. (d) Box-and-whiskers graph of HIV-1 transcript levels in kidneys from flavopiridol-treated transgenics shows suppressed expression compared with vehicle-treated transgenics after 20 days of treatment. As previously described, however, the inability of the latter group’s kidneys to support proviral expression with progressive renal disease decreases the significance of the difference (P < 0.1). (e) Correlation of HIV-1 transcript levels with urine [protein/creatinine] from transgenic kidneys after 20 days of vehicle treatment depicts this loss of proviral expression with progressive renal disease.
Amelioration of nephropathy by flavopiridol

The development of murine HIVAN in Tg26 mice, which shows many of the clinical and pathological hallmarks of human HIVAN, is associated with HIV-1 proviral expression in the kidney and not with HIV-induced circulating factors. Thus, we examined the effects of flavopiridol on several clinical parameters of nephrosis. Analysis of urine protein versus creatinine levels at the end of the 20 day treatment period revealed normal ratios in transgenics treated with flavopiridol, but not vehicle, when compared with non-transgenics treated with flavopiridol or vehicle (Figure 2a).

Quantification of the degree of histopathological renal disease in each treatment cohort (Figure 2b) confirmed that flavopiridol had ameliorated the phenotypic abnormalities in murine HIVAN (Figure 2c versus d). Four serological indices of nephrosis—albumin, blood urea nitrogen, cholesterol and triglycerides—were also normalized in flavopiridol-treated transgenics (Table 1). Interestingly, the unexpected decrease in lipid levels in non-transgenics suggests that flavopiridol is altering normal lipid metabolism. In total, these data indicate that flavopiridol ameliorated murine HIVAN in this animal model of HIV-induced renal disease.

Figure 2. Amelioration of murine HIVAN by flavopiridol. (a) Urine [protein/creatinine] in mice (Non-Tg = non-transgenic; Tg = transgenic) after 20 days of treatment with flavopiridol or vehicle shows that none (0/24) of flavopiridol-treated transgenics versus 79% (19/24) of the vehicle-treated transgenics had ratios that exceeded those in the vehicle-treated non-transgenics (P < 0.0001). (b) The histopathological grade of HIVAN in the same mice shows that 4% (1/24) of flavopiridol-treated transgenics versus 71% (17/24) of the vehicle-treated transgenics had values that exceeded those in the vehicle-treated non-transgenics (P < 0.0001). (c) Periodic-acid Schiff-stained kidney section of a vehicle-treated transgenic shows focal glomerulosclerosis with podocyte hyperplasia and numerous tubular microcysts (×250). (d) Periodic-acid Schiff-stained kidney section of the flavopiridol-treated transgenic sibling of (c) shows no histological abnormalities of the glomerular or tubulo-interstitial compartments (×250).
other groups with different cell types and 82% (112/137) of these genes was normalized to the levels kidney. With flavopiridol treatment, the expression level of two-fold or greater in the vehicle-treated HIVAN kidney when compared with the vehicle-treated non-transgenic groups. cRNA probes were produced from kidneys after the 20 day regimen of flavopiridol or vehicle, and then hybridized to Affymetrix array chips containing 12000 mouse genes. This analysis showed that 137 genes were dysregulated by alterations in renal gene expression patterns with non-selective therapeutic or toxic effects, we examined the gene table 1. Therapeutic versus toxic effects of chronic flavopiridol treatment

<table>
<thead>
<tr>
<th>Therapy</th>
<th>Vehicle (wild-type)</th>
<th>Vehicle (transgenic)</th>
<th>Flavopiridol (wild-type)</th>
<th>Flavopiridol (transgenic)</th>
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<tr>
<td>blood urea nitrogen (mg/dL)</td>
<td>18 ± 4</td>
<td>22 ± 4</td>
<td>17 ± 5</td>
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<td>albumin (g/dL)</td>
<td>2.9 ± 0.3</td>
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<td>cholesterol (mg/dL)</td>
<td>123 ± 17</td>
<td>183 ± 70</td>
<td>101 ± 12</td>
<td>95 ± 14</td>
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<tr>
<td>triglycerides (mg/dL)</td>
<td>108 ± 54</td>
<td>140 ± 82</td>
<td>64 ± 38</td>
<td>60 ± 30</td>
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<tr>
<td>Toxicity</td>
<td></td>
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<tr>
<td>rate of growth (g/day)</td>
<td>0.45 ± 0.11</td>
<td>0.44 ± 0.07</td>
<td>0.42 ± 0.06</td>
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<td>complete blood count</td>
<td></td>
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<td>white blood cells (10^3/µL)</td>
<td>3.10 ± 1.16</td>
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<td>neutrophils</td>
<td>0.48 ± 0.33</td>
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<td>lymphocytes</td>
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<td>monocytes</td>
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<td>0.11 ± 0.06</td>
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</tr>
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<td>eosinophils</td>
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<td>0.09 ± 0.13</td>
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<td>basophils</td>
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<td>0.03 ± 0.03</td>
<td>0.05 ± 0.08</td>
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<td>haemoglobin (g/dL)</td>
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<td>haematocrit (%)</td>
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<td>platelets (10^3/µL)</td>
<td>1119 ± 352</td>
<td>1116 ± 382</td>
<td>1336 ± 267</td>
<td>1184 ± 461</td>
</tr>
</tbody>
</table>

\*n = 24; \*P < 0.001; \*P < 0.02; \*P > 0.05.

Flavopiridol toxicity

Our prior in vitro study with podocytes, and analyses by other groups with different cell types and in vitro transcription assays suggest that the selectivity of flavopiridol for the CDK control of HIV-1 transcription over cellular transcription and cell-cycle progression may be abrogated at high doses. Thus, given the transient supra-therapeutic serum concentrations from bolus intraperitoneal administration, we first examined the mice for dose-limiting toxicities described by others during pre-clinical and Phase I testing of flavopiridol. Aside from a trend towards a decrease in leucocyte counts (P < 0.05), neither the transgenic nor the non-transgenic adolescent mice displayed any significant abnormalities in growth, gastrointestinal or haematological indices with a 20 day course of continuous flavopiridol treatment (Table 1).

Because higher doses of flavopiridol may induce global alterations in renal gene expression patterns with non-selective therapeutic or toxic effects, we examined the gene expression patterns in kidneys from our four treatment groups. cRNA probes were produced from kidneys after the 20 day regimen of flavopiridol or vehicle, and then hybridized to Affymetrix array chips containing 12000 mouse genes. This analysis showed that 137 genes were dysregulated by two-fold or greater in the vehicle-treated HIVAN kidney when compared with the vehicle-treated non-transgenic kidney. With flavopiridol treatment, the expression level of 82% (112/137) of these genes was normalized to the levels found in the vehicle-treated non-transgenic kidney. Within this group of 112 genes, many are known to be involved in renal fibrosis, proliferation, apoptosis, inflammation and loss of microvasculature (examples in Table 2). In contrast, only 2% (127/5471) of the genes expressed in the flavopiridol-treated non-transgenic kidney were altered by two-fold or greater when compared with the vehicle-treated non-transgenic kidney. None of these overlapped the normalized genes from the HIVAN kidney, arguing against, but not disproving, a non-selective benefit from flavopiridol on the dysregulated renal gene expression pattern in HIVAN. Interestingly, though, of the remaining 25 genes in HIVAN kidney that did not correct with flavopiridol treatment, 17 of these were also dysregulated in the flavopiridol-treated non-transgenic kidney (examples in Table 2), indicating an effect from flavopiridol alone on these genes.

Discussion

Models of HIVAN pathogenesis suggest that inhibiting HIV-1 gene expression in infected renal epithelium may be an effective therapeutic strategy. Here, we show a direct relationship between suppression of HIV-1 transcription in mouse kidney and amelioration of murine HIVAN through evaluation of both renal function and renal gene expression patterns in Tg26 HIV-1 transgenic mice. We find that a 20 day administration of flavopiridol suppressed HIV-1 proviral
expression and protected HIV-1 transgensics from developing HIV-induced renal disease without global alterations in cellular transcription or normal renal function. By targeting HIV-1 gene expression in this non-lymphoid reservoir with a small-molecule CDK inhibitor, we build on existing strategies that focus on halting HIV-induced immunodeficiency related to ongoing or latent activation of HIV-1 expression in lymphoid tissues. Furthermore, similar to their activity in infected lymphocytes, small-molecule CDK inhibitors of HIV-1 transcription may also attenuate the HIV-1 replication in infected renal epithelium implicated in a recent study. Despite many remarkable similarities between the murine HIVAN of Tg26 mice and human HIVAN, clinical observations indicate that differences in renal disease pathogenesis may exist in humans that are not addressed in this therapeutic animal model. For example, heterozygous Tg26 transgenics have HIV-1 proviral DNA and broadly express HIV-1 genes in all renal epithelial cell types, but exhibit very low levels of HIV-1 expression in secondary lymphoid tissues and tubulo-interstitial mononuclear cells when compared with renal parenchyma. In contrast, in situ detection of both HIV-1 DNA and mRNA in the renal epithelium of human HIVAN biopsies is quite focal, and there is qualitatively less HIV-1 expression in infected renal epithelium than in adjacent infected, tubulointerstitial mononuclear cells. Secondly, marked, diffuse mononuclear tubulointerstitial infiltrates in excess of glomerular damage are present in up to two-thirds of human cases of HIV-induced collapsing glomerulopathy, which typically develops in at-risk patients when HIV-induced lymphoid disease is poorly controlled. This is not characteristic of heterozygous Tg26 transgenics, which do not have readily apparent HIV-induced lymphoid disease or marked tubulointerstitial infiltrates in diseased kidneys. Interestingly, in anecdotal, well-described cases where HIV-induced collapsing glomerulopathy reversed following the initiation of antiretroviral therapy in HAART-naive patients, the reversal of renal disease correlated with the loss of tubulointerstitial infiltrates, not with the loss of HIV-1 expression in infected renal epithelium. Together, these observations suggest that HIV-induced perturbations in immunity and inflammation in humans may impact the progression of renal disease, a possibility that is not explored in the current therapeutic animal study.

Based on the known side-effects of HAART, often due to previously unrecognized cellular targets, it is reasonable to assume that small-molecule CDK inhibitors may alter cellular transcription and possibly other cellular processes. Yet, flavopiridol has been administered chronically to both animals and humans at doses that are expected to inhibit a broad range of CDKs in vivo without significant toxicity. Our analysis of the renal gene expression patterns after chronic drug treatment in one set of siblings suggests that flavopiridol and possibly other small-molecule CDK inhibitors may have a large therapeutic window. Importantly, though, we do not demonstrate here whether the therapeutic benefit is mediated through the inhibition of basal, Tat-independent HIV-1 transcription or cell-cycle-controlled, Tat-enhanced HIV-1 transcription. Likewise, although we demonstrate a direct correlation between the suppression of HIV-1 transcription and protection from the development of murine HIVAN, we cannot exclude that flavopiridol’s ability to target cell-cycle progression via CDK inhibition also contributes to the amelioration of murine HIVAN, as HIV-1 expression can cause aberrant cell-cycle progression of infected renal epithelium. Indeed, during the 12 h dosing regimen used in this study, serum concentrations of flavopiridol are expected to peak near 1100 nM, above the IC50 range of 100–400 nM for inhibition of cell-cycle CDKs by flavopiridol in vivo. It will be important to investigate these questions and whether flavopiridol can attenuate or reverse pre-existing HIV-induced renal disease. Our results support further evaluation of small-molecule CDK inhibitors as a potential therapeutic strategy for HIVAN and other HIV-induced diseases.
**Acknowledgements**

We thank Luis Schang and Mary Klorman for critical reading of the manuscript, and Paul Klorman for the use of Tg26 mice (colony funded by the NIH grant DK 56492-02). This work was supported by a National Kidney Foundation Research Award (P.J.N.).

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