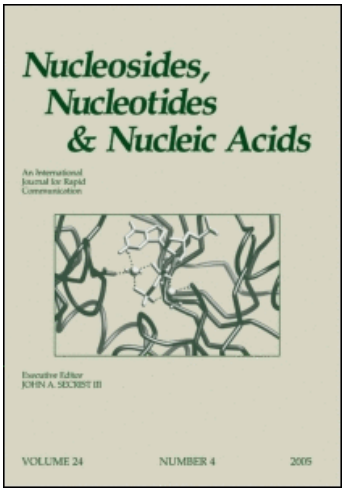

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CDK Inhibitory Nucleoside Analogs Prevent Transcription from Viral Genomes

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CDK INHIBITORY NUCLEOSIDE ANALOGS PREVENT TRANSCRIPTION FROM VIRAL GENOMES

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▫ *Targeting viral proteins has lead to many successful antivirals. Yet, such antivirals rapidly select for resistance, tend to be active against only a few related viruses, and require previous characterization of the target proteins. Alternatively, antivirals may be targeted to cellular proteins. Replication of many viruses requires cellular CDKs and pharmacological CDK inhibitors (PCIs), such as the purine-based roscovitine (Rosco), are proving safe in clinical trials against cancer. Rosco inhibits replication of wild-type or (multi-)drug resistant HIV, HCMV, EBV, VZV, and HSV-1 and 2. However, the antiviral mechanisms of purine PCIs remain unknown. Our objective is to characterize these mechanisms using HSV as a model. We have shown that Rosco prevents initiation of transcription from viral, but not cellular, genomes. This inhibition is promoter independent, but genome dependent, and requires no viral proteins. This is a novel antiviral mechanism and a previously unknown activity for purine PCIs.*

PURINE CDK INHIBITORS AS POTENTIAL ANTIVIRAL DRUGS

The requirement for CDKs in nuclear viral DNA replication is arguably the best characterized involvement of cellular protein kinases in viral replication. CDKs are a family of proline-directed serine/threonine protein kinases that complex with regulatory subunits, the cyclins.[1] The human genome encodes for 25 putative cyclins and 13 putative CDKs,[2] 10 of which have been shown to interact with cyclins. CDKs 1, 2, 3, 4, 6, and 7 are involved in regulation of the cell cycle. CDKs 7, 8, and 9 are involved in regulation of transcription, and CDKs 5 and 11 are involved in neuronal functions. CDKs 2, 5, 6, and 9 are also involved in cell

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differentiation, and CDKs 1, 2, 4, 5, 6, and 11 participate in apoptosis (recently reviewed in Knockaert et al.[3]).

Purine *P*harmacological *C*DK *I*nhibitors (PCIs)

The realization that certain CDKs are upregulated in cancer cells stimulated the search for PCIs. The first specific PCI was the 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine (olomoucine), from which the 2-(1-D,L-hydroxymethylpropylamino)-6-benzylamino-9-isopropylpurine (roscovitine, Rosco) and other second and then third generation PCIs were then developed. PCIs are chemically diverse, low molecular weight (<600 Da), flat, hydrophobic heterocycles (Figure 1). All characterized PCIs are competitive with respect to the ATP co-substrate. According to their specificities, PCIs can be classified as non-specific (inhibit with similar potencies CDKs and other protein kinases), pan-specific (preferentially inhibit CDKs but do not discriminate well among different CDKs), and oligo-specific (preferentially inhibit only a subset of CDKs). These last can be further subdivided

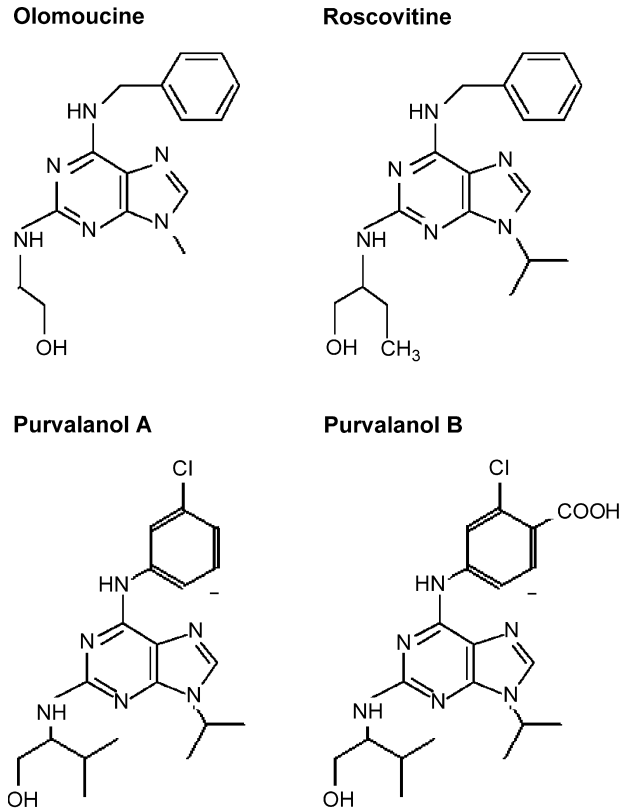

FIGURE 1 Chemical structures of four purine PCIs, 6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine (olomoucine), 2-(1-D,L-hydroxymethylpropylamino)-6-benzylamino-9-isopropylpurine (roscovitine), 6-[(3-Chloro) anilino]-2(1R)-(isopropyl-2-hydroxyethylamino)-9-isopropylpurines (purvalanol A), and 2*R*)-2-[[6-[(3-Chloro-4-carboxyphenyl)amino]-9-(1-methylethyl)-9 *H*-purin-2-yl]amino]-3-methyl-1-butanol (purvalanol B).

into "transcription specific" (preferentially inhibit CDKs involved in transcription), and "cell cycle specific" (preferentially inhibit CDKs involved in cell cycle regulation).[4–6] Mono-specific PCIs may also exist but none has been discovered. Purine PCIs such as Rosco are oligo-specific, inhibiting only CDKs 1, 2, 5, and 7, but not CDKs 4, 6, or 8.[7–10] At concentrations approximately 50- to 1,000-fold higher than those that inhibit CDKs, purine PCIs inhibit ERK1, ERK2, and DYRK1a, but not other protein kinases, phosphatases, DNA polymerases, ATPases, or topoisomerases (for a total of 63 enzymes).[8–11] Purine PCIs also inhibit CDK1 and CDK2 in vivo,[12,13] which results in inhibition of cell cycle progression at the G1/S and G2/M transitions.[9,12–16] They also inhibit expression of selected cell cycle regulated genes, cellular DNA synthesis,[9,10,17–19] and phosphorylation of selected CDK substrates.[9,17] As expected from their competitive nature and the high intracellular concentrations of ATP, all in vivo effects require high concentrations of PCIs. For example, 10 to 180 μM Rosco is required to observe the biological responses expected from inhibition of its target CDKs in different cell types.[20–27] Lower concentrations are usually required in non-dividing or primary cells than in transformed or rapidly dividing cells.

Toxicity of Purine PCIs in Preclinical and Clinical Trials

Although CDKs are commonly considered essential for mammalian cells, recent knockout experiments indicate that CDK2 or 4 are surprisingly nonessential for mice.[28,29] Furthermore, several PCIs under test in preclinical and clinical trials are proving to be surprisingly safe for animals and humans.[30–52] For example, Rosco decreased cell proliferation in rats without major toxicity at doses of 2.8 mg/kg per day (*ip*) for 5 days. Diarrhea, as a major toxicity, developed at doses of 3.0 mg/kg per day. Rosco is also nontoxic for mice, up to 20.0 mg/kg *i.v.*, 2,000 mg/kg *p.o.*, or 200 (3 times a day for 10 days), or 500 (3 times daily for 4 days) mg/kg *i.p.*[52] These last two treatments inhibited tumor cell growth, indicating that Rosco is safe in vivo at concentrations that have anti-proliferative effects. In vitro, such concentrations also inhibit viral replication.[53] In phase I clinical trials, Rosco was well tolerated orally up to 1,600 mg/day,[45,48] or 2,500 mg/day for 5 days every 3 weeks. The maximum tolerated dose was 3,200 mg/day with vomiting as limiting toxicity.[45] Plasma concentrations higher than those that fully inhibit HIV replication in vitro were reached in humans without toxicity.[45] Rosco is currently in phase II trials for glomerulonephritis, breast cancer, and small cell lung cancer (www.cyclacel.com). The low toxicity of PCIs in animal experiments and human clinical trials against cancer suggests that these drugs may be useful as antivirals in humans at nontoxic concentrations.

Antiviral Activity of Purine PCIs

HIV, HTLV, KSHV, HCMV, VZV, HSV-1, HSV-2, EBV, adeno-, and others viruses, require CDKs for their replication, and, as expected, their replication is inhibited by PCIs.[7,16,54–61] Because PCIs display these antiviral activities in vitro at

concentrations that are proving safe in vivo in clinical trials against cancer, PCIs are considered as potential clinical antivirals. However, the targets and antiviral mechanisms of PCIs must be properly characterized before they can be developed as antivirals. Several groups, including ours, are performing such characterization. The current status of progresses will be discussed below.

Several purine PCIs, including Rosco, inhibit HIV transcription and reactivation from latency, HCMV DNA replication and late gene expression,[62] activation of a structural protein and transcription of VZV,[63,64] transcription, DNA replication, and reactivation from latency of HSV-1 and HSV-2,[16,65,66] and lytic DNA replication and gene expression of EBV.[67] It is currently accepted that the most important antiviral activity of purine PCIs is on viral transcription. However, the precise mechanisms whereby purine PCIs inhibit viral, but not cellular, transcription remain incompletely characterized. Earlier work centered on flavopiridol and other non-purine PCIs that inhibit CDK9 at least as efficiently as they inhibit CDK1 or 2. These "CDK9-specific" PCIs were shown to inhibit HIV transcription elongation, mostly by targeting CDK9.[55,68,69]

More recent work has focused on purine PCIs such as Rosco, which inhibit preferentially CDK1, 2, 5, and 7. Since these PCIs inhibit accumulation of viral transcripts as efficiently as flavopiridol, they were hypothesized to also act primarily on CDK9.[70] Although Rosco inhibited immunoprecipitated CDK9 of non-assessed purity,[70] it failed to inhibit highly purified recombinant CDK9 at 0.5 μM,[71] concentration at which it significantly inhibits highly purified recombinant or native CDK7.[57,71] A closely related purine PCI, purvalanol, also inhibited viral transcription, but did not bind to CDK9 in the same cell extracts in which it bound CDK2, 5, and 7.[57] Furthermore, flavopiridol and other "CDK9-specific" PCIs inhibit global cellular transcription, which requires CDK9, whereas purine PCIs such as Rosco do not.[72,73]

Because CDK9 is required for transcription elongation, but not for transcription initiation,[74] we tested whether Rosco inhibited initiation or elongation of HSV-1 transcription. Using "run-on" transcription assays, we have recently shown that Rosco prevents initiation, but does not affect elongation, of HSV-1 transcription.[72] Therefore, the biological effects of Rosco on viral transcription occur at a step at which CDK9 is not known to play a limiting role. We thus conclude that Rosco does not inhibit viral transcription as a consequence of inhibition of CDK9. Consistent with this conclusion, Rosco also inhibits transcription of HIV mutants in the tat gene or TAR sequences,[7] which consequently cannot be activated by CDK9, and transcription of HTLV, which is not known to require CDK9.[58] Furthermore, whereas CDK9-specific PCIs inhibit viral and cellular transcription with similar efficiency, purine PCIs such as Rosco inhibit specifically viral transcription, while having no major effects on cellular transcription.[16,65,72,73]

The "run on" transcription assays also indicated that Rosco inhibits with similar efficiency transcription driven by a number of HSV-1 promoters, which are activated by different transcription factors.[72] This led us to the hypothesis that the effects of Rosco on viral transcription are genome specific, not promoter specific.

To test this hypothesis, we recombined copies of an HSV-1 promoter into the cellular genome, and then infected these cells with HSV-1. In agreement with our hypothesis, Rosco inhibited transcription driven by the HSV-1 promoters in the viral genome, but not by the copies of the same promoter recombined into the cellular genome.[72]

FUTURE DIRECTIONS AND CONCLUSIONS

We and others are currently working on addressing the mechanisms whereby purine PCIs prevent initiation of transcription in a genome-dependent manner, including the identification of the intracellular targets of PCIs that are required for viral replication. Our current work indicates that HSV transcription requires a kinase that is known to be susceptible to inhibition by purine PCIs, and that inhibition of HSV-1 transcription does not require specific sequences in the viral genome.

Regardless of the specifics of the mechanisms, genome-specific inhibition of viral transcription may be a major advantage for antiviral drugs. First, such a mechanism indicates that the same drug may be useful against a variety of viruses, as its activity would depend only on having genomes that are recognized as "non-self" by infected cells. Second, such mechanisms would be independent of viral gene and genome sequences. It could thus be expected that viral resistance against a drug that prevents initiation of transcription of extra-chromosomal genomes would be difficult to select for. Indeed, no PCI-resistant strains of any virus has been reported, although extensive efforts to select for such a mutant of HIV, HSV, or HCMV have been attempted.[7,16] Moreover, drugs that prevent initiation of transcription of extra-chromosomal genomes should be active against viral strains that are resistant to conventional antiviral drugs, which target viral proteins. Indeed, PCIs are fully active against HIV and HSV-1 strains that are already resistant to current antiviral drugs.[16,53]

In sum, CDK inhibitory purine derivatives have shown good potential as antiviral drugs. Among other advantages, purine PCIs are active against many unrelated viruses, including strains resistant to current antiviral drugs, and resistance against purine PCIs in not easily selected for. In the immediate future, the antiviral mechanisms of purine PCIs can be expected to be fully characterized. In the more distant future, the antiviral activity of purine PCIs in vivo at nontoxic doses must be evaluated in clinical trials. We can expect that the full antiviral potential of purine PCIs will be characterized in the coming years.

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