Review

Five years of progress on cyclin-dependent kinases and other cellular proteins as potential targets for antiviral drugs

Luis M Schang*, Mireille R St. Vincent† and Jonathan J Lacasse†

Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada
†Both authors contributed equally to this manuscript

*Corresponding author: Phone: +1 780 492 6265; Fax: +1 780 492 3383; E-mail: luis.schang@ualberta.ca

In 1997–1998, the pharmacological cyclin-dependent kinase (CDK) inhibitors (PCIs) were independently discovered to inhibit replication of human cytomegalovirus, herpes simplex virus type 1 and HIV-1. The results from small clinical trials against cancer were then suggesting that PCIs could be safe enough to be used clinically. It was thus hypothesized that PCIs could have the potential to be developed as novel antivirals targeting cellular proteins. Consequently, *Antiviral Chemistry & Chemotherapy* published in 2001 the first review on the potential of CDKs, and cellular proteins in general, as potential targets for antivirals. The viral functions inhibited by PCIs, or their cellular targets, were then just starting to be characterized. The antiviral spectrum of PCIs and their effects on viral disease were still mostly untested. Even their actual specificity was not yet completely characterized. In addition, cellular proteins were not accepted as valid targets for antivirals. Significant progress has been made in the last 5 years in understanding the antiviral activities of PCIs and the potential roles of cellular proteins in general as targets for antivirals. The first clinical trials of the antiviral activities of PCIs and other inhibitors of cellular protein kinases have now been scheduled. Herein, we review the progress made since the publication of the first review on PCIs as potential antiviral drugs and on CDKs, and cellular proteins in general, as potential targets for antiviral drugs. We also highlight the major issues that still need to be addressed before PCIs or other drugs targeting cellular proteins can be developed as clinical antivirals.

Keywords: antiviral drugs, cyclin-dependent kinases, herpes simplex virus, human immunodeficiency virus, pharmacological inhibitors

Introduction

Pharmacological cyclin-dependant kinase (CDK) inhibitors (PCIs) were discovered to inhibit viral replication while performing basic virology experiments aimed at identifying the roles of cellular proteins in viral replication (Bresnahan *et al.*, 1997; Mancebo *et al.*, 1997; Schang *et al.*, 1998). At the time, PCIs were starting to be tested as potential anticancer agents, showing promise in small preclinical and clinical trials (Drees *et al.*, 1997; Pippin *et al.*, 1997; Patel *et al.*, 1998b; Senderowicz *et al.*, 1998). As a result, it was speculated that PCIs could have the potential to be developed as novel antiviral drugs (Bresnahan *et al.*, 1997; Mancebo *et al.*, 1997; Schang *et al.*, 1998; Schang, 2001). These speculations implied a major change in the commonly accepted approaches to design and develop antiviral drugs. It was widely accepted at the time that antiviral drugs must target viral proteins, and that viral resistance and the limited number of targets were the major challenges to antiviral therapy (for example, see Balzarini *et al.*, 1998; De Clercq, 2002). Viral targets ensure the specificity and safety of antiviral drugs, as viral proteins are only expressed in infected cells (Table 1). This is a time-tested and proven concept that has led to the development of the 41 antiviral drugs in clinical use (Table 2), and also to a large number of drugs under development (Table 3). Unfortunately, this approach has several limitations. Drugs that target viral proteins promptly select for resistance, for example, and the number of potential viral targets is limited for those viruses with small genomes, such as human papillomavirus. This approach is not conducive to the prompt development of drugs against newly identified viral

Table 1. Selected advantages and disadvantages of cellular and viral proteins as targets for antiviral drugs

Target	Advantages	Disadvantages
Viral protein	• Specificity (→safety)	• Selection for drug-resistant mutants • Limited number of targets • Narrow specificity (that is, one virus or one virus family) • Viral pathogen must be isolated and its proteins must be characterized
Cellular protein	• Selection for drug-resistant mutants less likely to occur • Large number of potential targets • Broad specificity (that is, all viruses that require the targeted cellular proteins) • Viral proteins need not be characterized	• Potential for toxicity

pathogens either, as the proteins encoded by such pathogens must first be characterized.

Antivirals targeting cellular proteins have several potential benefits (Table 1). Inhibition of cellular proteins required for multiple viral functions could minimize the selection for resistance, for example, and a number of cellular proteins are required for replication of the viruses with the smallest genomes. Furthermore, many cellular proteins are required for replication of even distantly related viruses. Drugs targeting cellular proteins could thus be used against a novel pathogen even before its proteins are fully characterized. On the negative side, targeting cellular proteins could also lead to unwanted negative side effects, as such targets are also commonly expressed in many uninfected cells. It should be considered, however, that a large body of expertise already exists on targeting cellular proteins without major toxicities. All clinical drugs other than anti-infective agents target cellular proteins. Some of these drugs are used against lethal diseases, circumstances in which riskier drugs still have a positive risk-benefit profile. Others, however, are used against minor diseases, against which only very safe drugs have positive risk-benefit profiles.

Herein, we will review the progresses made in the last 5 years on the characterization of the antiviral activities of PCIs, and on the potential of cellular proteins in general as targets for antivirals. Although we will focus mostly on PCIs and other protein kinase inhibitors, we will also review a few selected potential cellular targets other than protein kinases, so to bring attention to the many different kinds of potential cellular targets for antiviral therapy.

CDKs

CDKs are a family of protein kinases that phosphorylate a serine or threonine followed by a proline. CDKs share sequence homology and activation by regulatory subunits, most commonly cyclins (Meyerson *et al*., 1992). Nine CDKs and nine other kinases with restricted sequence homology to CDKs were known 5 years ago (reviewed in Schang, 2001). The human genome has since been sequenced (Lander *et al*., 2001; Venter *et al*., 2001), and there are now 13 proteins classified as CDKs, as well as four other related (but dual-specificity) protein kinases classified as CDC2 (CDK1)-like kinases (CLKs), and five PCTAIRE-motif protein kinases (PMPK; Manning *et al*., 2002). All CDKs, CLKs and PMPK are classified together with the mitogen-activated protein kinases, GSK, DYRK, and others in the CMGC family of protein kinases (which includes a total of 61 proteins; Manning *et al*., 2002).

CDKs regulate progression through the cell-cycle, transcription, the neuronal cytoskeleton, apoptosis, and other cellular functions. CDK1, CDK2, CDK3, CDK4, CDK6, and CDK7 regulate the cell-cycle; CDK7, CDK8 and CDK9 regulate transcription and CDK5 phosphorylates cytoskeleton proteins in neurons (reviewed in Schang, 2001). Far less is yet known about CDK10, which may regulate certain transcription factors and progression through the G2 phase of the cell-cycle (Li *et al*., 1995; Bagella *et al*., 2006), or CDK11, which participates in cell-cycle regulation, apoptosis and splicing (Hu *et al*., 2003; Li *et al*., 2004; Petretti *et al*., 2006).

CDKs are activated by specific regulatory subunits (most commonly cyclins), by phosphorylation at specific residues and by de-phosphorylation at others. CDK activities are further regulated by inhibitory proteins, by the expression and degradation of cyclins, inhibitory proteins and CDKs themselves or by their subcellular localization. CDK1 is activated by cyclins A and B, whereas CDK2 is activated by cyclins A and E. CDK4 and CDK6 are activated by cyclins D1, D2 and D3. CDK7 is activated by cyclin H and a third subunit, Mat1. CDK8 is activated by cyclin C; CDK9, by cyclins T and K. CDK5 is activated by a non-cyclin like subunit, p35. The cyclins activating

Table 2. Clinical antiviral drugs (drug combinations omitted)

Drug	Target	Virus
Nucleoside and nucleotide analogues		
Cyclic nucleoside analogues		
Brivudin	Viral DNA polymerase	HSV-1, VZV
Entecavir	Viral DNA polymerase (RT)	HBV
Idoxuridine	Viral DNA polymerase	HSV-1, HSV-2
Trifluridine	Viral DNA polymerase	HSV-1, HSV-2
Ribavirin (plus pegylated interferon-2α)	Viral DNA polymerase (RT)	RSV (HCV)
Acyclic nucleoside analogues		
Acyclovir	Viral DNA polymerase	HSV-1. HSV-2, VZV
Valaciclovir	Viral DNA polymerase	HSV-1, HSV-2, VZV, EBV, HCMV
Penciclovir	Viral DNA polymerase	HSV-1, HSV-2
Famciclovir	Viral DNA polymerase	HSV-1, HSV-2, VZV
Ganciclovir	Viral DNA polymerase	HCMV
Valganciclovir	Viral DNA polymerase	HCMV
2′,3′-Dideoxynucleosides		
Zidovudine	Viral DNA polymerase (RT)	HIV-1
Didanosine	Viral DNA polymerase (RT)	HIV-1
Zalcitabine	Viral DNA polymerase (RT)	HIV-1
Stavudine	Viral DNA polymerase (RT)	HIV-1
Lamivudine	Viral DNA polymerase (RT)	HIV-1, HBV
Abacavir	Viral DNA polymerase (RT)	HIV-1
Emtricitabine	Viral DNA polymerase (RT)	HIV-1
Nucleotide phosphonates		
Tenofovir disoproxil fumarate	Viral DNA polymerase (RT)	HIV-1
Adefovir dipivoxil	Viral DNA polymerase (RT)	HBV
Cidofovir	Viral DNA polymerase	HCMV (in AIDS patients)
Oligonucleotides		
Fomivirsen	Viral IE mRNA	HCMV (in AIDS patients)
Peptidomimetics		
Saquinavir	Viral protease	HIV-1
Ritonavir	Viral protease	HIV-1
Indinavir	Viral protease	HIV-1
Nelfinavir	Viral protease	HIV-1
Amprenavir	Viral protease	HIV-1
Darunavir	Viral protease	HIV-1
Fosamprenavir	Viral protease	HIV-1
Lopinavir	Viral protease	HIV-1
Atazanavir	Viral protease	HIV-1
Enfuvirtide	Viral fusion glycoprotein	HIV-1
Sialic acid analogues		
Oseltamivir	Viral neuraminidase	Influenza A and B
Zanamivir	Viral neuraminidase	Influenza A and B
Cyclic amines		
Amantadine	Viral matrix protein	Influenza A
Rimantadine	Viral matrix protein	Influenza A
Others		
Foscarnet	Viral DNA polymerase	HCMV, HSV-1, HSV-2
Nevirapine	Viral DNA polymerase (RT)	HIV-1
Delavirdine	Viral DNA polymerase (RT)	HIV-1
Efavirenz	Viral DNA polymerase (RT)	HIV-1
Tripanavir	Viral protease	HIV-1, HIV-2

AIDS, acquired immune deficiency syndrome; HCMV, human cytomegalovirus; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HSV, herpes simplex virus; IE, immediate-early; RT, reverse transcriptase; RSV, respiratory synticial virus; VZV, varicella-zoster virus.

Table 3. Selected potential antiviral drugs under development targeting viral proteins (natural products and immunomodulatory agents omitted)

Compound	Target	Virus	Status	Reference
Telbivudine	Viral DNA polymerase (RT)	HBV	Phase III	Bryant *et al*., 2001
Dextran-2-sulphate	Viral envelope glycoprotein	HIV	Phase III	Callahan *et al*., 1991
Maribavir	Viral phosphotransferase	HCMV, EBV	Phase II	Zacny *et al*., 1999
Rilpivirine	Viral DNA polymerase (RT)	HIV	Phase II	Jansenn *et al*., 2005
S-1360	Viral integrase	HIV	Phase II	Yoshinaga *et al*., 2002
BMS-488043	Viral envelope glycoprotein	HIV	Phase IIa	Lin *et al*., 2003
Valopicitabine	Viral polymerase (RdRp)	HBV, HCV genotype 1	Phase IIb	Lim *et al*., 2002
PA-457	Viral maturation	HIV	Phase IIb	Li *et al*., 2003
VX-950	Viral serine protease	HCV genotype 1	Phase Ib	Perni *et al*., 2003
ST-246	Putative viral phospholipase	VV	Phase I	Yang *et al*., 2005
WM5	Viral transcription transactivator	HIV	Experimental	Cecchetti *et al*., 2000
Bay 57-1293	Viral helicase-primase complex	HSV	Experimental	Kleymann *et al*., 2002
cf 1743	Viral DNA polymerase	VZV	Experimental	McGuigan *et al*., 2000
HDP-CDF*	Viral DNA polymerase	HSV, HCMV	Experimental	Beadle *et al*., 2002

*Hexadecyloxypropyl-cidofovir (HDP-CDF) is being tested against a variety of other viruses as well, including adenovirus and vaccinia virus (VV).
HCMV, human cytomegalovirus; EBV, Epstein–Barr virus; HBV, hepatitis B virus; HCV, hepatitis C Virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; RdRp, RNA-dependent DNA polymerase; RT, reverse transcriptase; VZV, varicella-zoster virus.

CDK10 or 11, if any, remain unknown. Twelve cyclins were known in 2001, whereas 25 have now been identified by sequence analyses of the human genome (Murray & Marks, 2001). However, it is unclear whether all putative cyclins regulate CDKs. Cellular CDKs are also activated by viral-encoded cyclins, such as the Kaposi's sarcoma-associated herpesvirus (KSHV) vCyc (Godden-Kent *et al*., 1997; Li *et al*., 1997).

PCIs

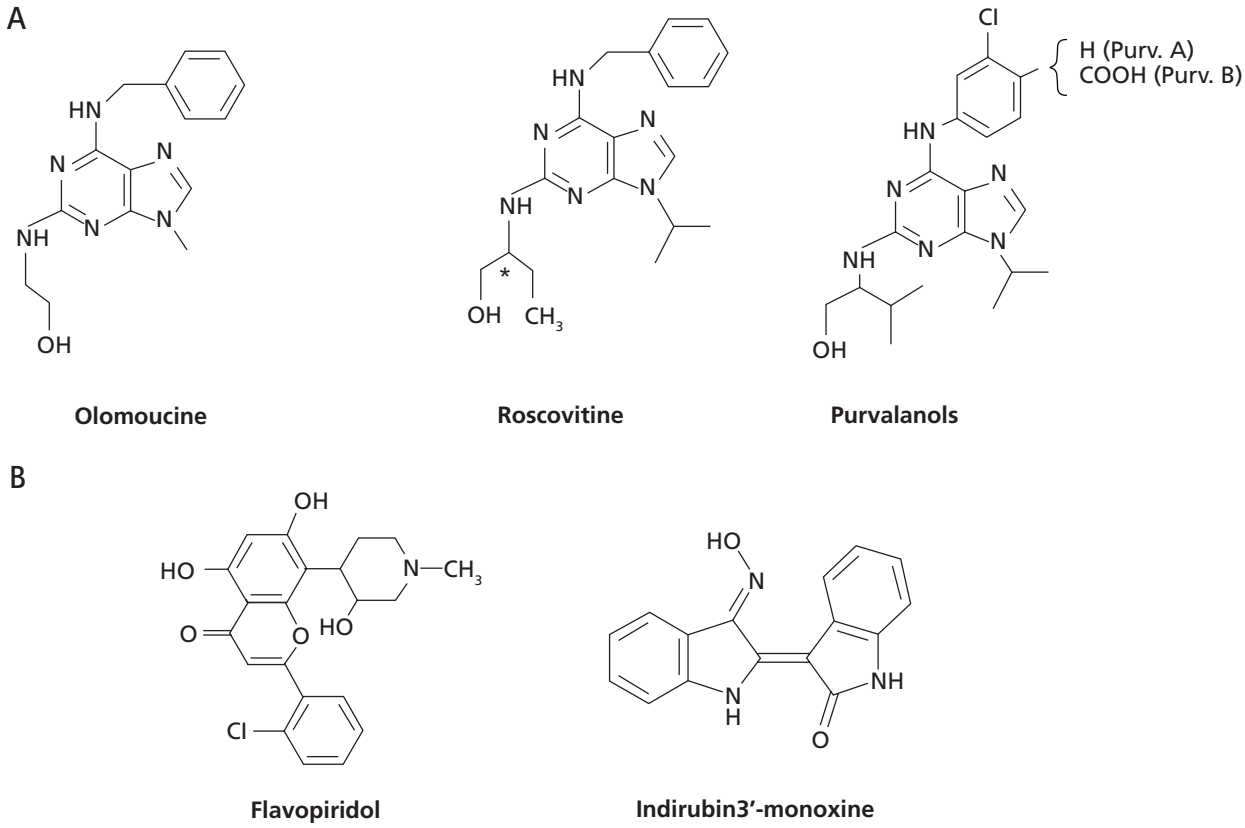
The PCIs are a heterogeneous family of small molecule inhibitors of CDKs (reviewed in Meijer, 1995, 1996; Meijer & Kim, 1997; Schang, 2001; Schang, 2002; Meijer *et al*., 2003; Schang, 2005a). They were among the first highly specific protein kinase inhibitors discovered and, consequently, are among the most characterized (Meijer, 1995, 1996; Meijer & Kim, 1997; Bach *et al*., 2005). Although most PCIs are flat heterocycles, their heterocycles are highly diverse, including substituted purines, pyrimidines, flavonoids, and bis-indoles, among many others (Figure 1). Most have molecular masses <600 Da and some, <300 Da. They bind to the ATP-binding pocket of their target CDKs and inhibit them most commonly by competing with the ATP co-substrate.

According to their specificities, PCIs can be classified as non-specific, pan-specific, oligo-specific or mono-specific (Schang, 2001; Schang, 2002; de la Fuente *et al*., 2003; Fischer & Gianella-Borradori, 2003; Schang, 2005a). The oligo-specific PCIs can be further subclassified as specific for CDKs involved mostly in transcription or in cell-cycle regulation (Schang, 2001; Schang, 2002; Schang, 2005a).

The non-specific PCIs, such as staurosporine, inhibit not only CDKs, but also many other unrelated protein kinases and consequently are not likely to be of great pharmacological use. These drugs will not be discussed any further. The pan-specific PCIs such as flavopiridol inhibit CDKs preferentially over other protein kinases, but do not discriminate well among different CDKs (Table 4). The oligo-specific PCIs, such as roscovitine, preferentially inhibit only a subset of CDKs (Table 4), usually those that are most closely related by sequence homology. No mono-specific PCI was described when the original review was prepared (Schang, 2001), but several potentially mono-specific PCIs have since been reported (for examples, see Soni *et al*., 2000; Toogood, 2001; Schoepfer *et al*., 2002; Misra *et al*., 2004; Voigt *et al*., 2005; Chassagnole *et al*., 2006). However, there is yet no experimental evidence publically available that any of them has been tested against all CDKs. This classification is not absolute, and the number of potential targets analysed for some PCIs is rather limited.

Several articles have reviewed the structure, specificities, and biochemical, cellular and antitumoural activities of several PCIs, as well as their toxicities (for example, see Knockaert *et al*., 2002; Senderowicz, 2002; Fischer & Gianella-Borradori, 2003; Fisher *et al*., 2003; Meijer *et al*., 2003). In this article, we will focus on their antiviral activities, highlighting their specificities and other activities or toxicities when necessary. We will focus on those PCIs that have been shown to inhibit viral replication or specific viral functions.

Figure 1. Structures of five selected pharmacological cyclin-dependent kinase inhibitors



The structure of three oligo-specific pharmacological cyclin-dependent kinase (CDK) inhibitors (PCIs) with preference for CDKs mainly involved in the cell-cycle **(A)**; and two pan-specific PCIs **(B)** are presented. The asterisk in roscovitine indicates the anomeric carbon that determines the *R* and *S* isomers (adapted from Schang, 2001)

Oligo-specific PCI with preference for CDKs involved in the cell-cycle

This group can be further sub-classified into PCIs with preference for CDK1, CDK2, CDK5 and CDK7, and those with preference for CDK4 and CDK6. The former includes the purine-containing PCIs, such as olomoucine [6-benzylamino-2-(2-hydroxyethylamino)-9-methylpurine], roscovitine [2-(1-D,L-hydroxymethylpropylamino)-6-benzylamino-9-isopropylpurine], purvalanol [6-[(3-chloro)anilino]-2(1*R*)-(isopropyl-2-hydroxyethylamino)-9 -isopropylpurine] (Figure 1; Table 4), as well as the phenylaminopyrimidines, the O^6-cyclohexylmethylguanines and the recently discovered thiazole ureas (Vesely *et al*., 1994; Meijer *et al*., 1997; Binarova *et al*., 1998; Gray *et al*., 1998; Davies *et al*., 2002; Ruetz *et al*., 2003; Payton *et al*., 2006). The latter includes the indolopyrrolocarbazoles, fascaplycin and tri-amino pyrimidine derivatives, amongst others (Soni *et al*., 2000; Soni *et al*., 2001; Sanchez-Martinez *et al*., 2003).

The different purine-containing PCIs differ in potency and fine specificity, but not in mechanism of action or general specificity (Table 4). They bind to the ATP-binding pocket of CDK2 (and presumably of the other target CDKs). However, their purine rings are rotated to different degrees with respect to that in ATP. Consequently, the purine-type PCIs make several contacts with CDK residues that make no contacts with ATP. Most of these interactions are hydrophobic. They make only a limited number of hydrogen bonds, the most common ones with the backbone carbonyl and NH groups of Leu83.

When the first review was published it was still questioned to some extent whether drugs binding to the fairly well conserved ATP-binding pocket could be specific for only one or a few kinases. The large number of small molecule inhibitors of an also large number of protein kinases that have since been developed, with ever increasing specificity, has largely addressed such concerns. It is now widely accepted that the ATP-binding pocket can confer specificity for a small number of protein kinases and there is indeed a large number of drugs under development that target the ATP-binding pockets of different protein kinases. However, the high degree of conservation of the ATP-binding pockets among CDK1, CDK2 and CDK5

Table 4. Specificity profile of selected oligo- or pan-specific PCIs

		Oligo-specific							Pan-specific		
		Rosco			Olo	Purv A		Purv B	Flavo		Ind
Group	Kinase	IC_{50}, μM	Inhib. at 10 μM, %	K_d, μM t	IC_{50}, μM	IC_{50}, μM	Inhib. at 10 μM, %	IC_{50}, μM	IC_{50}, μM	K_d, μM t	IC_{50}, μM
AGC	AKT1,2,3	>100 e	<2 r	*n/t*	*n/t*	*n/t*	<20 (AKT1)	*n/t*	*n/t*	*n/t*	*n/t*
	ROCK1,2;CRIK	*n/t*	<20 f,r	*n/t*	*n/t*	*n/t*	43 (ROCK2)	*n/t*	*n/t*	*n/t*	*n/t*
	GRK2,3,4,5,6,7	*n/t*	<14 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	NDR2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PKA	>50 l,y	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PKACαβγ	>1,000 c	<20 f	*n/t*	>2,000 c,d	9 b	<20	33.8 j	*n/t*	*n/t*	*n/t*
	PKACα	*n/t*	7 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	145 o, 122 z	>10	6.3 ai
	PRKX	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PDK1	*n/t*	1-<20 f,n,r	*n/t*	*n/t*	*n/t*	<20	*n/t*	*n/t*	*n/t*	*n/t*
	PKCα	>100 c,l	<20 f,n,r	*n/t*	>1,000 c,d	>10 b	<20	>100 b,h	6 o	*n/t*	27 ai
	PKCβ1	>100 c,l	<20 n,r	*n/t*	>1,000 c,d	>10 b	*n/t*	>100 b,h	*n/t*	*n/t*	***4 ai***
	PKCβ2	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	20 ai
	PKCγ	100 c,l	15-<20 n,r	*n/t*	800 c,d	>10 b	*n/t*	>100 b,h	*n/t*	*n/t*	8.4 ai
	PKCδ,ζ	>1,000 c	<3 r	*n/t*	>1,000 c,d	>100 b	*n/t*	>100 b,h	*n/t*	*n/t*	>100 ai
	PKCε,η	>100 c	0 r	*n/t*	>930 c,d	>100 b	*n/t*	>100 b,h	*n/t*	*n/t*	20-52 ai
	PKCθ,ι	*n/t*	<11 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PKG1,2	>1,000 c	9 r	*n/t*	>2,000 c,d	>10 b	*n/t*	>100 b,h	*n/t*	*n/t*	9 ai
	PKN1,2	*n/t*	<20 n,r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MSK1,2	*n/t*	<20 f,r	>10	*n/t*	*n/t*	<20	*n/t*	*n/t*	2	*n/t*
	p70S6K	130 h	<20 f,n,r	*n/t*	>1,000 d	*n/t*	21	15 h	*n/t*	*n/t*	*n/t*
	RSK1	*n/t*	22-31 f,r	*n/t*	*n/t*	*n/t*	***85***	*n/t*	*n/t*	*n/t*	*n/t*
	RSK2	*n/t*	14 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	RSK3	*n/t*	24 r	3.2	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	1.6	*n/t*
	SGK1,2,3	*n/t*	3-<20 f,r	*n/t*	*n/t*	*n/t*	<20	*n/t*	*n/t*	*n/t*	*n/t*
CAMK	CaMK1a,g	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	CaMK4	*n/t*	4 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CaMK1d	*n/t*	1 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	CaMK2α	32 h, >10 r	2 r	>10	>1,000 d	*n/t*	*n/t*	13h	*n/t*	1.3	*n/t*
	CaMK2β	*n/t*	9 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	2.6	*n/t*
	CaMK2γ	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	CaMK2δ	*n/t*	9 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	AMPKα1	*n/t*	<20 f,n	>10	225 d	*n/t*	50	*n/t*	*n/t*	6.6	*n/t*
	AMPKα2	*n/t*	<20 f,n	*n/t*	226 d	*n/t*	50	*n/t*	*n/t*	*n/t*	*n/t*
	CHK1	*n/t*	5-<20 f,n,r	*n/t*	*n/t*	*n/t*	22	*n/t*	*n/t*	*n/t*	*n/t*
	MARK2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PASK	*n/t*	9 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	DAPK2; DRAK1	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	DRAK2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	5.1	*n/t*
	DAPK3	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	MAPKAPK2,5	*n/t*	2-<20 f,r	*n/t*	*n/t*	*n/t*	<24	*n/t*	*n/t*	*n/t*	*n/t*
	MAPKAPK3	*n/t*	1 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MNK2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	1.9	*n/t*
	smMLCK	90 c	*n/t*	*n/t*	>1,000 c,d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	skMLCK	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PHKg1	*n/t*	11-<20 f,r	>10	*n/t*	*n/t*	64	*n/t*	*n/t*	>10	*n/t*
	PHKg2	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	2	*n/t*
	PIM1,2	*n/t*	3-25 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	***0.52-0.65***	*n/t*
	PKD2,3	*n/t*	0-17 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CHK2	*n/t*	13-40 n,r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	TSSK1,2	*n/t*	<4 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
CK1	RLCK1	100 v,x (*R*); 120 v (*S*)	*n/t*	*n/t*	*n/t*	>3.33 b	*n/t*	3.33 b,j	*n/t*	*n/t*	9 ai
	CK1α	*n/t*	18 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CK1δ	17 f	47-50 f,n,r	*n/t*	*n/t*	*n/t*	25	>3.333 h	*n/t*	*n/t*	*n/t*
	CK1ε	*n/t*	44 r	0.16	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	CK1γ1,2	*n/t*	23-57 r	1.4-3.3	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	CK1γ3	*n/t*	36 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
CMGC	CDK1-cyclin A	*n/t*	*n/t*	***50 d***	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK1-cyclin B	0.45-0.65 c,g,h,u, 23 e, 2.69 l, 3.62 y; 0.35-0.45 u,v,x (*R*); 0.95 u, 0.55 v (*S*)	>95 n, 82 r	*n/t*	7 c,d,g,u	0.004 b,g	*n/t*	0.006 b,h,j	0.20-0.485 g,m,o	*n/t*	0.180 g,ai
	CDK1-cyclin E	*n/t*	*n/t*	*n/t*	10 d		*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK2-cyclin A	0.7 c,h,l, 0.25 f; 1.2 e (*R*); 1.8 e (*S*)	93n, 86r	*n/t*	7 c,d	0.07 b, 0.1 f	98	0.006 b,h,j	*n/t*	*n/t*	0.44 ai
	CDK2-cyclin E	0.7 c, 0.17 y; 0.95 e, 0.1 l, 0.40 x (*R*); 1.4e, 0.24 l (*S*)	*n/t*	*n/t*	7 c,d	0.035 b	*n/t*	0.009 b,j	1.34 m	*n/t*	0.5 ab, 0.25 ai
	CDK2	*n/t*	*n/t*	2.9	*n/t*		*n/t*	*n/t*	0.1	*n/t*	*n/t*
	CDK3-cyclin E	1.4 e (*R*); 1.5 e (*S*)	*n/t*	*n/t*	*n/t*		*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK4-cyclin D1	>100 c, 75 e, 14 l,y	*n/t*	*n/t*	>1,000 c,d	0.85 b	*n/t*	>10 b,j	0.1-0.155 m,q	*n/t*	***3.33 ai***
	CDK5-p35	0.16 c,g*,h*, 0.20 v,x (*R*); 0.35 v (*S*)	98 r	*n/t*	3 c,d,g*	0.075 g*	*n/t*	0.006 b,h*,j	0.17 g*	*n/t*	0.1 g*,ai
	CDK5	*n/t*	*n/t*	2	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	0.043	*n/t*
	CDK6-cyclin D1	51 e	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK6-cyclin D2	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	***0.663 m***	*n/t*	*n/t*
	CDK6-cyclin D3	>100 c	*n/t*	*n/t*	>250 c,d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK7-cyclin H	0.45-0.76 l,k,l,y, <5 p	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	0.2 ab,ac	*n/t*	***>4 ab***

Table 4 (continued)

		Oligo-specific						Pan-specific		
		Rosco			Olo	Purv A		Purv B	Flavo		Ind
Group	Kinase	IC_{50}, μM	Inhib. at 10 μM, %	K_d, μM t	IC_{50}, μM	IC_{50}, μM	Inhib. at 10 μM, %	IC_{50}, μM	IC_{50}, μM	K_d, μM t	IC_{50}, μM
	CDK8	>100 i, >50 p	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CDK9-cyclin T1	0.6 k, <5 p, 1.70 y	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	0.001-0.011 w,ab,ac	*n/t*	0.05 ab
	PCTAIRE1	*n/t*	*n/t*	0.99	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	0.18	*n/t*
	CLK1	*n/t*	35 r	2.1	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	2.2	*n/t*
	CLK2	*n/t*	***90r***	0.47	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	1.4	*n/t*
	CLK3	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	***1***	*n/t*
	CLK4	*n/t*	*n/t*	***4.5***	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	DYRK1A	***3.1f; 11-18 v,x (R); 4.2 v (S)***	15f	*n/t*	*n/t*	0.3 f	94	*n/t*	*n/t*	*n/t*	*n/t*
	DYRK3,4	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	plant GSK-3A	220 c	*n/t*	*n/t*	130 c,d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	GSK3A	*n/t*	24 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	GSK3B	130 g, 32.2 y	7-<20 f,n,r	*n/t*	100 g	>10 b, 13 g	<20	>10 b,j	0.45 g	*n/t*	0.22 g
	p44mpk	*n/t*	*n/t*	*n/t*	25 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	GST-erk1	30 c	*n/t*	*n/t*	30 c,d,u	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	His-tagged erk1	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>100 ai
	Erk1	34 c,h; 25 v (*R*); 16 v (*S*)	14 r	*n/t*	***50 c***	9 b	*n/t*	3.33 b,h,j	*n/t*	*n/t*	*n/t*
	Erk2	14 c,h, 1.17 l; 20 v,x (*R*); 13v (*S*)	<20-45 f,n,r	*n/t*	***40 c***	*n/t*	74	1 h,j	*n/t*	*n/t*	>100 ai
	JNK1	*n/t*	<20 f,n	>10	*n/t*	>1 b	<20	1.2 b, >10 j	*n/t*	>10	0.8 aj
	JNK2	*n/t*	<20 n	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	1.4 aj
	JNK3	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	1.0 aj
	p38α,β,γ	*n/t*	<20 f,n,r	>10	*n/t*	*n/t*	<20	*n/t*	*n/t*	>10	*n/t*
	p38δ	*n/t*	2-<20 f,n,r	*n/t*	*n/t*	*n/t*	<20	*n/t*	*n/t*	*n/t*	*n/t*
	SRPK2	*n/t*	32 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
STE	MAP3K4,5	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	MAP3K8	*n/t*	42 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	GCK;ZC1,3	*n/t*	1-21 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	KHS1;ZC2	*n/t*	22 r (KHS1)	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	MST1,2	*n/t*	<9 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PAK1,5;SLK;LOK	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PAK3,4,6	*n/t*	<26 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	TAO1;YSK1	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MST3,4	*n/t*	11-13 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MAP2K1	*n/t*	<24 f,n,r	*n/t*	*n/t*	*n/t*	<20	*n/t*	*n/t*	*n/t*	>100 ai
	MAP2K2,6	*n/t*	<29 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
TK	ABL1	>100 e	6 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	ABL2	>1,000 c	13 r	>10	100 c,d	>10 b	*n/t*	>100 b,j	*n/t*	>10	*n/t*
	ALK	*n/t*	57 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	cMER;TYRO3	*n/t*	11-14 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CSK	*n/t*	10-<20 f,r	>10	*n/t*	*n/t*	***80***	*n/t*	*n/t*	>10	*n/t*
	CTK	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	EGFR	*n/t*	8 r	>10	440 d	*n/t*	*n/t*	*n/t*	25 o, 21 z	>10	*n/t*
	HER2,4	*n/t*	<8 r	>10 (HER2)	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10 (HER2)	*n/t*
	EphA1	*n/t*	12 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	EphA2,3,4,5,8	*n/t*	<19 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	EphA6,7	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	EphB1	*n/t*	15 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	2.2	*n/t*
	EphB2,3	*n/t*	6-7 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	EphB4	*n/t*	4 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	FAK	*n/t*	12 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	FER;FES	*n/t*	16-39 r	>10 (FER)	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10 (FER)	*n/t*
	FGFR1,2,3	*n/t*	6-13 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	FGFR4	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	IGF1R	*n/t*	13 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	INSR	*n/t*	17 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	IRR	70 c	16 r	*n/t*	400 c,d	5 b	*n/t*	2.2 b,j	*n/t*	*n/t*	11
	JAK1	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	JAK2	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	JAK3	*n/t*	13 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	cMET;RON	*n/t*	12-42 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MUSK	*n/t*	20 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PDGFRα;FMS	*n/t*	2-6 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PDGFRβ;FLT3;KIT	*n/t*	6-16 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	RET	*n/t*	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	ROS	*n/t*	9 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	BLK;SRM	*n/t*	<3 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	BRK	>100 e	4 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	FGR;LYN	*n/t*	7-11 r	>10	>1,000 d	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	FYN;HCK;YES;FR	*n/t*	3-10 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	LCK	*n/t*	10-30 f,n,r	>10	>2,000 d	*n/t*	78	*n/t*	*n/t*	>10	*n/t*
	SRC/c-src	250c	11-<20 n,r	*n/t*	>1,000 c,d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	SYK;ZAP70	*n/t*	12-21 r	>10 (SYK)	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10 (SYK)	*n/t*
	BTK;BMX	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	ITK	*n/t*	8 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	TIE2	*n/t*	9 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	TRKA,B,C	*n/t*	5-13 r	>10 (TRKA)	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10 (TRKA)	*n/t*
	VEGFR1	*n/t*	10 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*

Table 4 (continued)

		Oligo-specific							Pan-specific		
		Rosco			Olo	Purv A		Purv B	Flavo		Ind
Group	Kinase	IC_{50}, μM	Inhib. at 10 μM, %	K_d, μM t	IC_{50}, μM	IC_{50}, μM	Inhib. at 10 μM, %	IC_{50}, μM	IC_{50}, μM	K_d, μM t	IC_{50}, μM
	VEGFR2,3	*n/t*	9-14 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
TKL	IRAK4	*n/t*	27 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	LIMK1	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	MLK1	*n/t*	7 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	cRAF	*n/t*	<20-41 n,r	*n/t*	*n/t*	>1 b	*n/t*	>10 b,j	*n/t*	*n/t*	>100 ai
	BRAF	*n/t*	39 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	RIPK2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	>11
	ALK4	*n/t*	4 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
Other	AurA,C	600b, >100e	0 r (AurA)	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	AurB	>100e	0 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CaMKK1,2	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	0.019-0.32	*n/t*
	CDC7	>1,000 m	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	CK2	*n/t*	<20 f,n	*n/t*	*n/t*	>10 b	<20	>10 b,j	*n/t*	*n/t*	*n/t*
	CK2α1	>1,000 c	3 r	*n/t*	>2,000 c,d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	12 ai
	CK2α2;IKKβ;	*n/t*	<1 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	GAK;PLK4	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	1.1-3.1	*n/t*
	AAK1;MPSK1; BIKE;NEK6;9;TTK; ULK3	*n/t*	*n/t*	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	NEK2	*n/t*	0 r	>10	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	PKR	*n/t*	*n/t*	*n/t*	>500 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PLK1,2,3;NEK1	*n/t*	<9 r	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	dmFused	*n/t*	*n/t*	2.1	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	>10	*n/t*
	MYT1	*n/t*	47 a	>10	*n/t*	*n/t*	25 a	*n/t*	*n/t*	>10	*n/t*
	Wee1	*n/t*	0 a	*n/t*	*n/t*	*n/t*	9 a	*n/t*	*n/t*	*n/t*	*n/t*
Other kinases	NDPK	*n/t*	*n/t*	*n/t*	>1,000 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PI3K	>50 s	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	PDXK	binds v,x	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
Viral kinases	HSV-1 U,13	>26 aa	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	VZV ORF47	not inhibited ak	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
Phosphatases	CDC25A;Pyp3	*n/t*	*n/t*	*n/t*	>1,000 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
Other ATP-requiring enzymes	TOP1,2	*n/t*	*n/t*	*n/t*	>250 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	DNA polymerase α,δ	*n/t*	*n/t*	*n/t*	>500 d	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*
	MRP-1	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	activated ag	*n/t*	*n/t*
	GPa	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	2.5 ad	*n/t*	*n/t*
	GPb	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	***1 ad, 15.5***	*n/t*	*n/t*
	ALDH	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	binds but no inhibtion af	*n/t*	*n/t*
Other molecules	Duplex DNA	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	*n/t*	binds ah	*n/t*	*n/t*

For roscovitine (Rosco), light gray represents IC_{50}≥7 μM, % inhibition <80 and K_d >7 μM, dark gray shading represents 0.7 μM ≤IC_{50}<3.5 μM, % inhibition ≥93 and 0.7 μM ≤K_d<3.5 μM, and bold and italicized figures represent 3.5 μM ≤IC_{50}<7 μM, 80≤ % inhibition <93 and 3.5 μM ≤K_d<7 μM. For olomoucine (Olo), light grey represents IC_{50}>70 μM, dark grey shading represents 7 μM≤IC_{50}≤35 μM, and bold and italicized figures represent 35 μM<IC_{50}≤70 μM. For purvalanol A (Purv A) and purvalanol B (Purv B), light grey represents IC_{50}≥0.7 μM and % inhibition <80, dark grey represents 0.07 μM≤IC_{50}<0.35 μM and % inhibition ≥93, and bold and italicized figures represent 0.35 μM≤IC_{50}<0.7 μM and 80≤% inhibition<93. For flavopiridol (Flav), light gray represents IC_{50}>1 μM and K_d >1 μM, dark gray shading represents 0.1 μM ≤IC_{50}<0.5 μM and 0.1 μM ≤K_d<0.5 μM, and bold and italicized figures represent 0.5 μM ≤IC_{50}<1 μM and 0.5 μM ≤K_d<1 μM. For indirubin-3′-monoxime (Ind), light grey represents IC_{50}>4.5 μM, dark grey shading represents 0.45 μM≤IC_{50}<2.25 μM, and bold and italicized figures represent 2.25 μM≤IC_{50}<4.5 μM. The activity or binding of four oligo-specific pharmacological cyclin-dependent kinase inhibitors (PCIs; Rosco, Olo, Purv A and Purv B) and two pan-specific PCIs (Flav and Ind) against all human protein kinases, several non-human protein kinases, and other selected enzymes or biomolecules are presented. The activities against each protein are presented as 50% inhibition concentrations (IC_{50}, in μM), degree of inhibition at 10 μM of each drug (as percentage), or relative affinity (as K_d). n/t, not tested; a, 250 μM ATP (Kristjansdottir and Rudolph 2003); b, 15 μM ATP (Gray, *et al*., 1998); c, 15 μM ATP (Meijer *et al*., 1997); d, 15 μM ATP (Vesely *et al*., 1994); e, ProQuinase, as quoted in (Bach *et al*., 2005); f, 100 μM ATP (Bain *et al*., 2003); g, 15 μM ATP (Leclerc *et al*., 2001); h, (Knockaert, Gray *et al*., 2000); i, 15 μM ATP (Schang *et al*., 2002a); j, 15 μM ATP (Knockaert and Meijer 2002); k, 144 μM ATP (Wang *et al*., 2001); l, 100 μM ATP (McClue *et al*., 2002); m, 50 μM ATP (Caligiuri, Becker *et al*., 2005); n, 10 μM ATP; http://www.upstate.com/img/pdf/KinaseProfiler.pdf; o, 375 μM ATP (Losiewicz *et al*., 1994); p, 10 μM ATP (Pinhero *et al*., 2004); q, 400 μM ATP (CDK2), 20 μM ATP (CDK4; Carlson *et al*., 1996); r, http://www.invitrogen.com/downloads/SelectScreen_Data_193.pdf; s, (Whittaker, Walton *et al*., 2004); t, (Fabian *et al*., 2005); u, 15 μM ATP (De Azevedo *et al*., 1997); v, 15 μM ATP (Bach *et al*., 2005); w, 10 μM ATP (Chao *et al*., 2000); x, 15 μM ATP (Tang, Li *et al*., 2005); y, 50 μM ATP (Agbottah *et al*., 2005); z, (Senderowicz and Sausville 2000); aa, 10 μM ATP (Kawaguchi *et al*., 2003); ab, 144 μM ATP (Heredia *et al*., 2005); ac, 100 μM ATP (Zhou *et al*., 2004); ad, 1,000 μM ATP (Kaiser, Nishi *et al*., 2001); ae, (Oikonomakos, Schnier *et al*., 2000); af, (Schnier, Kaur *et al*., 1999); ag, (Hooijberg, Broxterman *et al*., 1999); ah, (Bible, Bible *et al*., 2000); ai, 15 μM ATP (Hoessel *et al*., 1999); aj, 50 μM ATP (Xie, Liu *et al*., 2004); ak, personal communication quoted in (Moffat, Taylor *et al*., 2003). A full version of Table 4 can be found online as an additional file (see additional files).

have proven to be the major challenge to the development of inhibitors that discriminate among them (Table 4). Although the ATP-binding pocket of CDK7 is more distantly related, most CDK1-, CDK2-, and CDK5-specific PCIs that have been evaluated in this respect also tend to inhibit CDK7.

Several potentially mono-specific PCIs have been reported in recent years. In most cases, however, the public information about the specificity of such compounds is limited to a relatively small number of CDKs. For example, the O^6-cyclohexylmethylguanine, NU6102, was tested in the reported experiments against CDK1, CDK2, and

CDK4 only. The results of these experiments demonstrated that this compound inhibited CDK1 with 50% inhibitory concentration (IC_{50}) only 1.5-fold greater than that against CDK2 (Davies *et al.*, 2002). CINK4 and fascaplycin are among the potentially mono-specific PCIs that were tested against the largest number of potential CDK targets. There is public information of these compounds having been tested against CDK1, CDK2, CDK4, CDK5 and CDK6 (but not against CDK3, CDK7, CDK8, or CDK9). CINK4 and fascaplycin both inhibited CDK6, with IC_{50} only 3.75-fold or 10-fold greater than those against CDK4, respectively (Soni *et al.*, 2000; Soni *et al.*, 2001).

Such difficulties in developing highly mono-specific PCIs could be considered as a major obstacle for their development as antivirals. However, recent knockout experiments strongly suggest that inhibition of a single CDK is unlikely to be sufficient to have major effects on cellular or viral functions. Contrary to previous expectations, deletion of CDK2, CDK4, CDK6, or their activating cyclins A1, E1, E2, D1, D2 or D3 did not result in cell-cycle arrest at the stages in which these CDKs were thought to be essential (recently reviewed in Santamaria & Ortega, 2006). These results have prompted a re-examination of the physiological roles of the different CDKs. It is now thought that most CDKs are highly redundant in their functions and that, consequently, inhibition of any single CDK is insufficient to inhibit most of their biological functions. This new understanding of the redundancy of CDKs casts further doubts on whether the potentially mono-specific PCIs that inhibit cell-cycle progression do so by inhibiting a single CDK.

The fine specificity of each purine-containing PCI differs to some extent, as expected, but as a group they preferentially inhibit CDK1, CDK2, CDK5 and CDK7, but not CDK4, CDK6 or CDK8 (Table 4) (Vesely *et al.*, 1994; Meijer *et al.*, 1997; Gray *et al.*, 1998; Davies *et al.*, 2002). At approximately 10-fold greater concentrations, many also inhibit CDK9, and at even higher concentrations (approximately 25–1,000-fold), ERK1, ERK2 and DYRK1a. In a recent kinomic assay, the purine-containing roscovitine bound to only 11 of 119 protein kinases tested (Fabian *et al.*, 2005). The 11 kinases included the two previously known targets in the assay, plus a CLK, a PCPK and CK1ε. All the kinases bound to roscovitine with low affinity, as expected for the CDKs because the inactive conformation of the kinase domain was used. Roscovitine binds with high affinity only to the active conformation of the target CDKs (De Azevedo *et al.*, 1997; Knockaert *et al.*, 2000).

Altogether, several purine-containing PCIs have been shown to inhibit or bind with high affinity only to CDK1, CDK2, CDK5, CDK7 and CDK9, and not to inhibit or bind with high affinity to a number of protein serine/threonine or tyrosine kinases, phosphatases, DNA polymerases or topoisomerases (Table 4) (Vesely *et al.*, 1994; Meijer *et al.*, 1997; Gray *et al.*, 1998; Leclerc *et al.*, 2000; Wang *et al.*, 2001; Schang *et al.*, 2002a; Bain *et al.*, 2003; Pinhero *et al.*, 2004; Bach *et al.*, 2005; Fabian *et al.*, 2005). Roscovitine, for example, has been tested against 246 proteins (mostly protein kinases), and found to inhibit with low IC_{50} only six CDK/cyclin combinations and a closely related protein kinase (CLK2). It also bound with relatively high affinity to six other protein kinases, four of which were not efficiently inhibited at 10 μM in the presence of low concentrations of ATP (Table 4).

It has been often stated that the specificity of any small molecule protein kinase inhibitor is inversely proportional to the time lapsed since its first description. Although this aphorism has been confirmed for many such inhibitors, roscovitine is still resisting the test of time. In fact, roscovitine and related PCIs have been repeatedly confirmed since 2001 to inhibit CDK activities (Table 4). Perhaps the most biologically relevant of these tests was an elegant CDK2 reporter *in vivo* luminescence assay, which was used to confirm that roscovitine inhibits CDK2 activity in undisturbed cells (Zhang *et al.*, 2004). As a result of their specificity, roscovitine and other purine-containing PCIs are more and more commonly used as probes to test the potential involvement of their target CDKs in cellular functions.

The potency of PCIs (and other protein kinase inhibitors) is customarily expressed as IC_{50}, which is a most sensitive parameter. PCIs compete with ATP, however, and kinase reactions are routinely performed at ATP concentrations approximately 100-fold to 1,000-fold below physiological levels. Moreover, the concentrations of any drug required to reduce enzymatic activity to background levels are typically 5-fold to 20-fold above the IC_{50}. It is thus not surprising that the concentrations of PCIs required to completely inhibit biological functions *in vivo* are significantly higher than their IC_{50} in kinase assays.

The purine-type PCIs that preferentially inhibit CDK1, CDK2, CDK5 and CDK7 are the PCIs for which the antiviral activities have been the most extensively characterized. Before 2001, the replication of only human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) had been shown to be inhibited by two such drugs, olomoucine and roscovitine (Bresnahan *et al.*, 1997; Schang *et al.*, 1998; Jordan *et al.*, 1999; Schang *et al.*, 1999, 2000). Considering the already known or expected roles of CDKs in the replication of several other viruses, and the similarities in the specificities of different purine-containing PCIs, it was nonetheless speculated in our original review that several such PCIs could also inhibit replication of several other viruses (Schang, 2001). Consistent with these speculations, several purine-containing PCIs have been demonstrated in the last 5 years to also inhibit replication of HSV-2 (Schang *et al.*, 2002a), varicella-zoster virus (VZV; Taylor *et al.*, 2004), Epstein–Barr virus (EBV; Kudoh *et al.*, 2004) and HIV

(Wang *et al*., 2001; Schang *et al*., 2002a; Agbottah *et al*., 2004; Heredia *et al*., 2005); purine-containing PCIs also inhibit specific viral functions of KSHV (Ghedin *et al*., 2004), human T-lymphotropic virus (HTLV; Wang *et al*., 2002), adenovirus (Fax *et al*., 2000) and several animal retroviruses (Bhattacharjee *et al*., 2001; Chao *et al*., 2003). A purine-containing PCI that was discussed in our original review, CVT-313, has also been shown to inhibit activation of a retroviral promoter (Bhattacharjee *et al*., 2001), but no further publications have since reported the use of this drug against viral functions.

Oligo-specific PCIs specific for transcription CDKs

Although this group includes the first identified PCIs, most of these early drugs, such as DRB, have since proven to have little specificity and will not be further discussed. A second group of drugs included in this category was discovered in 1997 (Mancebo *et al*., 1997; Flores *et al*., 1999), and discussed to great length in our previous review (Schang, 2001). Like the cell-cycle specific PCIs, this group includes chemically diverse molecules such as ribofuranosylbenzimidazoles, benzimidazoles, triazoles, isoquinoline sulphonamides, flavonoids and oxazoles (Mancebo *et al*., 1997; Flores *et al*., 1999). However, the specificity of these drugs has not been further characterized since the previous review was published and the last publication reporting the use of these compounds as potential antivirals dates to 2001 (Pisell *et al*., 2001). Consequently, these compounds will not be discussed any further either.

At the time of our previous review, roscovitine was classified as a 'strict' cell-cycle specific PCI (Schang, 2001; Schang, 2002). Since 2001, however, roscovitine has been shown to inhibit CDK7 and, at higher concentrations, CDK9 (Wang *et al*., 2001; Schang *et al*., 2002a; Pinhero *et al*., 2004). Roscovitine inhibited recombinant CDK7 (expressed in baculovirus) and native CDK7 highly purified from HeLa cells with the same IC_{50} as toward CDK1 and CDK2 (approximately 0.5 μM). It also inhibited immunoprecipitated CDK7 and CDK9 with an equivalent IC_{50}, although the purity of these kinases was not assessed. Whereas 0.5 μM roscovitine failed to inhibit to any significant extent baculovirus-expressed recombinant CDK9, it inhibited equally purified CDK7 by 50%. Roscovitine inhibited recombinant CDK9 only at 10-fold higher concentrations (5 μM). Roscovitine has a chiral carbon and thus exists as two stereoisomers (Figure 1). Using the *R*-isomer, Nelson and colleagues further reported an IC_{50} of 0.84 μM for CDK9, which was 10- and 1.5-fold higher than the IC_{50} reported in the same publication for CDK2 and CDK7, respectively (Gherardi *et al*., 2004). These IC_{50} are lower than those observed by other groups, which could be due to the use of the *R*-isomer, or to technical differences on the assessment of the IC_{50}.

In our own experiments, purvalanol (which is more potent and structurally related to roscovitine, but has lower specificity) failed to bind to CDK9 in extracts from cells in which it inhibited HSV-1 replication (Schang *et al*., 2002a). In the same cell extracts, purvalanol bound with high affinity to CDK2, CDK5, ERK1 and ERK2. As expected, it did not bind to CDK1 (Schang *et al*., 2002a) because the cells were infected under conditions in which no cyclin B was expressed. CDK1 was consequently in its inactive conformation, which does not bind to roscovitine with high affinity (Knockaert *et al*., 2000). These studies also provided a strong test for the specificity of purine-derived CDK inhibitors in cell extracts containing a large number of cellular (and viral) nucleotide-binding proteins, such as protein kinases, DNA and RNA polymerases, or G-proteins.

It is still unclear how significant the inhibition of CDK7 and CDK9 is for the ability of roscovitine to inhibit viral replication. CDK9 is required for early elongation of HIV transcription, which is inhibited by roscovitine (Wang *et al*., 2001). In contrast, roscovitine does not inhibit elongation of HSV-1 transcription (Diwan *et al*., 2004), which in any event is not known to require CDK9. It is of course possible that roscovitine inhibits transcription of different viruses through different mechanisms; however, it is also possible that it inhibits viral transcription as a result of its inhibition of CDK9 and other sensitive kinases. For example, CDK2 has been recently shown to be required, together with CDK7 and CDK9, for initiation and elongation of HIV transcription (Nekhai *et al*., 2002). Roscovitine was also shown to inhibit expression of HSV-1 genes in neurons that expressed CDK2, but not CDK7 (Schang *et al*., 2002a).

Pan-specific PCIs

Although the pan-specific PCI flavopiridol was discovered before roscovitine, its abilities to inhibit CDK activities were discovered almost simultaneously to those of roscovitine. However, the inhibition of viral transcription by flavopiridol was discovered only in 2000 (Chao *et al*., 2000), 3 years after the same discovery was found in roscovitine (Bresnahan *et al*., 1997; Schang *et al*., 1998). Flavopiridol used to be thought specific for cell-cycle CDKs such as CDK1, CDK2, CDK4 and CDK6, with IC_{50} of approximately 0.04 μM. However, it was later found to be a much more potent inhibitor of CDK9 (IC_{50}=0.006 μM) (Chao *et al*., 2000). An early publication reported that flavopiridol most likely inhibited CDK7 and its activation *in vivo* (Sausville *et al*., 1999). More recently, the IC_{50} of flavopiridol toward CDK7 has been experimentally evaluated to be in the order of 0.2–0.3 μM (Zhou *et al*., 2004; Heredia *et al*., 2005), which is significantly higher than that toward CDK9 (evaluated in parallel in one of these reports to be around 0.001 μM; Zhou *et al*., 2004), or CDK1,

CDK2, CDK4 or CDK6 (approximately 0.04–0.1 μM; Losiewicz *et al*., 1994; Carlson *et al*., 1996).

Although flavopiridol competes with ATP for CDK1 (Losiewicz *et al*., 1994) and binds to the ATP-binding pocket of CDK2 (De Azevedo *et al*., 1996), it inhibits CDK9 in a non-competitive manner (Chao *et al*., 2000). The crystal structure of CDK9 has yet to be resolved and, consequently, there is no co-crystal structure of flavopiridol bound to CDK9. In the absence of such data, the binding of flavopiridol to CDK9 was modelled (de Azevedo *et al*., 2002). Surprisingly for a non-competitive inhibitor, the predicted binding site is in the ATP-binding pocket. It is possible that flavopiridol binds to this pocket with such high affinity that it cannot be outcompeted by relevant concentrations of ATP. However, the modelled binding does not provide any obvious energetic explanation for such tight binding (de Azevedo *et al*., 2002). Clearly, the mode of inhibition of CDK9 by flavopiridol needs to be further evaluated.

Flavopiridol has several biological effects in cultured cells at concentrations that are below its IC_{50} for most CDKs (for discussions, see Sausville *et al*., 1999; Schang, 2001; Schang, 2002; Schang, 2004). Flavopiridol may be concentrated inside cells by, for example, active transport mechanisms. Alternatively, its major intracellular target may be CDK9, which is inhibited at these low concentrations (Chao *et al*., 2000). It is also possible that the biological effects of flavopiridol result from inhibition of other of the cellular proteins that are targeted by this drug (Table 4). In support of this last hypothesis, Fabian and colleagues observed that flavopiridol bound with high affinity to 22 different protein kinases (Fabian *et al*., 2005). These kinases included the two CDKs (2 and 5) and three of the four CLKs included in the assays. However, in contrast to roscovitine, flavopiridol also bound with high affinity to several unrelated protein kinases, such as CamK2A (calcium and calmodulin-dependent protein kinase 2), or the receptor tyrosine kinases EphA5 or EphB1 (Fabian *et al*., 2005). Flavopiridol has also been shown to inhibit several other purified or recombinant protein kinases, and to bind or modulate the activity of other proteins, or even DNA (Table 4; discussed in Schang, 2001; Schang, 2002).

The indirubins are another group of pan-specific PCIs that had just been described when our first review was published; they were extensively discussed in it (Schang, 2001). They are components of a traditional Chinese medicine, Danggui Longhui Wan, used against leukaemia (for references on indirubins, see Hoessel *et al*., 1999; Schang, 2001; Meijer *et al*., 2006). The indirubins are specific inhibitors of CDK1, CDK2, CDK4, CDK5, and CDK9. Indirubin-3′-monoxime (3-[3-(hydroxyamino)-1H-indol-2-yl]indol-2-one) displays an IC_{50} of 0.18 μM for CDK1 and 0.1 μM for CDK5 (in 15 μM ATP; Hoessel *et al*., 1999). CDK2 was inhibited at approximately twofold higher concentrations and CDK4, at approximately 20-fold higher concentrations. Indirubin-3′-monoxime was recently shown to also inhibit CDK9 with an IC_{50} of approximately 0.05 μM (Heredia *et al*., 2005). In contrast, it did not inhibit CDK7 (IC_{50}>4 μM), and it is thus one of the few PCIs that discriminate between CDK1, CDK2 and CDK5 versus CDK7. Indirubin-3′-monoxime also inhibits protein kinase C β2 and cAMP-dependent protein kinase at concentrations 20- and 40-fold higher than those that inhibit CDK1, respectively. It does not significantly inhibit 15 other kinases (Table 4) (Hoessel *et al*., 1999). All indirubins inhibit GSK-3β, with an IC_{50} of 0.022 μM for indirubin-3′-monoxime (Leclerc *et al*., 2000). As with all other PCIs studied, indirubins bind to the ATP-binding pocket of the target kinases and the inhibition is competitive for the ATP co-substrate (Hoessel *et al*., 1999).

Based on their specificity, we had speculated in 2001 that the indirubins were likely to also have antiviral activities (Schang, 2001). Indeed, indirubins have recently been shown to inhibit HIV transcription and replication, centrosome abnormalities induced by oncogenic human papillomavirus (HPV), and induction of pro-inflammatory cytokines by influenza virus (Duensing *et al*., 2004; Mak *et al*., 2004; Heredia *et al*., 2005). In our own unpublished results, indirubin-3′-monoxime has also shown to be effective at inhibiting HSV-1 replication.

Antiviral effects of PCI in cultured cells

As discussed in the introduction, only a handful of PCIs had been shown to inhibit replication of HCMV, HSV-1 and HIV when the first review was published. In the following section, we will review the information about the antiviral activities of PCIs that has been published since then.

HSV-1 and HSV-2.

PCIs were already known in 2001 to inhibit the accumulation of several HSV-1 transcripts (Schang *et al*., 1998), even in the presence of the viral transactivators of gene expression (Jordan *et al*., 1999; Schang *et al*., 1999). However, it was not clear then whether these drugs inhibited initiation or elongation of viral transcription, or perhaps destabilized fully synthesized viral transcripts. To directly address these issues, we analysed the effects of roscovitine, flavopiridol and purvalanol on run on transcription in nuclei extracted from HSV-infected cells (Diwan *et al*., 2004; Lacasse *et al*., 2005). Surprisingly, roscovitine had no effect on ongoing viral transcription, or on activation or elongation of cellular transcription. It instead prevented the activation of viral transcription. Flavopiridol inhibited HSV-1 transcription elongation to some extent, as expected from inhibition of CDK9. But like roscovitine, it far more efficiently prevented activation of transcription (Lacasse *et al*., 2005). Thus, both these PCIs act at a level at which neither CDK7

nor CDK9 are known to play limiting roles. It is possible that these kinases also play other, yet unknown, roles in viral transcription. Alternatively, PCIs may inhibit protein kinases specifically required to activate transcription from extra-chromosomal viral genomes.

To test for the last possibility, a recombinant cell line was constructed in which the HSV-1 ICP0 promoter drives transcription of a reporter red-fluorescent protein gene (Diwan *et al*., 2004). When these cells were infected with HSV-1 in the presence of roscovitine, transcription driven by the ICP0 promoter in the viral genomes was inhibited, as expected. However, transcription driven by the ICP0 promoter in the cellular genome was surprisingly not inhibited (Diwan *et al*., 2004). Such specificity was dependent on the specificity of the PCI tested. The less specific purvalanol still preferentially inhibited transcription from the viral genome but, unlike roscovitine, it also inhibited transcription from the cellular genome (Diwan *et al*., 2004). The least specific drug tested, the pan-specific flavopiridol, inhibited to similar extent transcription from the cellular or viral genomes (Diwan *et al*., 2004).

Such specificity for viral genomes, not for specific promoter sequences, suggested that PCIs should be active against HSV-2, as well as against HSV-1 mutants that are resistant to conventional antiviral drugs. Indeed, roscovitine and purvalanol inhibited replication of HSV-2 or HSV-1 mutants resistant to acyclovir or phosphonoacetic acid as efficiently as they inhibited replication of wild-type virus (Schang *et al*., 2002a). Phosphonoacetic acid is structurally related to, and has similar antiviral mechanisms as, the clinically used foscarnet. Such specificity for viral genomes may also be a major cause of the difficulties encountered in selecting for resistance against PCIs (for examples, see Schang *et al*., 1998; Wang *et al*., 2001). If PCIs indeed prevent activation of transcription of extra-chromosomal genomes, then no combination of mutations in the extra-chromosomal viral genomes would lead to resistance. Although very attractive, this mechanism does not offer an obvious explanation for the inhibition of transcription of the integrated proviral genomes of retroviruses. It is perhaps possible that the effects on retroviruses are mediated exclusively by inhibition of CDK9. This is another area that still requires further study.

It is perhaps somewhat disappointing that the molecular targets of PCIs that are required for activation of HSV-1 transcription still remain incompletely defined. Although the strong circumstantial evidence still suggests a major role for CDK2 (for example, see Schang *et al*., 2002a; Schang *et al*., 2002b), knockout experiments have clearly indicated that CDKs are functionally redundant (Rane *et al*., 1999; Berthet *et al*., 2003; Ortega *et al*., 2003; Malumbres *et al*., 2004). Thus, deletion of a single CDK often results in only subtle phenotypes. It is therefore likely that several CDKs may also be able to provide the cellular functions required for activation of HSV-1 transcription. Under such scenario, inhibition of a single CDK may not suffice to inhibit viral replication. This redundancy may result in a change in the approaches followed to develop and optimize antiviral PCIs. Contrary to the standard approaches followed until recently, less specific PCIs (that is, oligo-specific) may actually be better suited as potential antivirals than the so-intensively searched highly mono-specific ones.

HCMV

Olomoucine and roscovitine had been demonstrated before 2001 to inhibit DNA replication of HCMV (Bresnahan *et al*., 1997). Since then, the effects of PCIs on HCMV functions have been characterized to a far larger extent. The effects of roscovitine on several HCMV functions are now known to present many similarities with those previously described on HSV-1 functions. The concentrations of roscovitine required to achieve full inhibition of HCMV replication are in the order of 25–50 μM, which were not obviously cytotoxic but did show cytostatic effects (Sanchez *et al*., 2004). Roscovitine inhibited HCMV replication even when added at late times after infection (up to 48 h for HCMV). Like for HSV-1, however, roscovitine also inhibited immediate-early and early functions, such as the expression of the viral transcriptional regulators and viral DNA synthesis (Sanchez *et al*., 2004). In addition, the effects of roscovitine on DNA synthesis, late viral functions and viral replication were similar whether the drug was added in the absence or in the presence of the viral regulators of transcription (Sanchez *et al*., 2004).

There were also some important differences in the effects of roscovitine on HSV or HCMV replication. For example, whereas roscovitine inhibited the activation of transcription of all tested (and likely all) HSV-1 genes (Diwan *et al*., 2004), it had differential effects on the expression of different HCMV genes (Sanchez *et al*., 2004). Roscovitine inhibited to great extent the expression of the so-called IE1-72 protein, while activating that of IE2-86. In this respect, there is also a major difference in the regulation of HSV-1 and HCMV gene expression. Whereas most HSV-1 proteins are expressed from non-spliced transcripts, the regulatory proteins of HCMV are expressed from alternatively spliced ones. IE1-72 and IE2-86, for example, are expressed from alternatively spliced transcripts from the same transcriptional unit. Roscovitine also had differential effects on the expression of two other alternatively spliced IE genes of HCMV, UL31x1 (which was inhibited) and UL37 (which was induced; Sanchez *et al*., 2004). As expected, these effects on the expression of IE proteins were not observed when roscovitine was added at later times. The pan-specific flavopiridol also inhibited the alternative splicing of IE1-72 and IE2-86, but its effects on viral replication were apparently not evaluated (Sanchez *et al*., 2004).

More recently (Tamrakar *et al.*, 2005), HCMV infection was shown to induce expression of CDK7, CDK9 and their activating cyclins, as well as hyperphosphorylation of the carboxy-terminal domain of RNA polymerase II in serines 2 and 5 (which are phosphorylated by CDK7 and CDK9). These hyperphosphorylations were inhibited to some extent by roscovitine. It was hypothesized that the enhanced hyperphosphorylation of RNA polymerase II provides docking sites for processing factors that then regulate the alternative splicing of the primary HCMV transcripts (Tamrakar *et al.*, 2005). Such model is in agreement with current models proposing that mRNA processing occurs co-transcriptionally. Roscovitine would thus inhibit the recruitment of the processing factors, thereby deregulating alternative splicing. Surprisingly, however, CDK7, CDK9, and hyperphosphorylation of RNA polymerase II were most significantly induced at later times after infection. Likewise, roscovitine had the most significant effects on RNA polymerase II phosphorylation at similar late times after infection (Tamrakar *et al.*, 2005). In contrast, roscovitine only affected alternative splicing of the IE1-72 and IE2-86 when it was added during the first 4 h of infection (Tamrakar *et al.*, 2005).

Even more recently, roscovitine was further shown to inhibit late HCMV functions (Sanchez & Spector, 2006). Roscovitine added 24 h after infection (at 20 μM) still inhibited the release of infectious HCMV virions, likely by downregulating the levels of the structural proteins pp150 and gB, inhibiting the expression of IE2-86 and inducing the mislocalization of pp69 (Sanchez & Spector, 2006).

VZV

Although the phosphorylation of a structural VZV protein was known before 2001 to be inhibited by roscovitine (Ye *et al.*, 1999), the importance of this inhibition for viral replication remains untested. Since the original review was published, however, PCIs have been shown to inhibit other VZV functions, as well as VZV replication (Taylor *et al.*, 2004; Habran *et al.*, 2005). Roscovitine (25–50 μM) inhibited VZV replication on MeWo cells in one-step growth or plaquing assays. The primary effects appear to be inhibition of expression and subcellular localization of several VZV proteins, including immediate-early proteins such as IE62 (Taylor *et al.*, 2004; Habran *et al.*, 2005). Roscovitine also inhibited phosphorylation and activity of IE63 (Habran *et al.*, 2005), which, when active, represses transcription of specific VZV genes. Surprisingly, inhibition of IE63 phosphorylation also resulted in its over-accumulation in the nucleus, where it normally exerts its transcriptionally regulatory activities (Habran *et al.*, 2005). As IE63 is a substrate *in vitro* for CDK1 or CDK5, but not for CDK2, CDK7 or CDK9, it was concluded that the relevant phosphorylation is performed by CDK1 in non-neuronal cells or by CDK5 in neuronal ones (Habran *et al.*, 2005).

EBV

It used to be hypothesized that activation of CDKs directed EBV toward the latent cycle of infection. It was thus surprising that, contrary to such model, CDK2 activity was recently shown to actually be required for the lytic cycle of EBV (Kudoh *et al.*, 2004). Consequently, *in vitro* EBV replication and reactivation from latency was inhibited by two PCIs, roscovitine and purvalanol A (Kudoh *et al.*, 2004). There were many unexpected similarities between the effects of PCIs on EBV functions with those previously shown on HSV-1. For example, similar concentrations of roscovitine were required to inhibit replication of EBV or HSV-1 (approximately 50 μM), and these concentrations also inhibited cell-cycle progression. As for HSV-1, EBV replication was insensitive to a variety of other protein kinase or cell-cycle progression inhibitors, which do not inhibit CDKs, and the inhibition by PCIs was fully reversible upon removal of the drug (Kudoh *et al.*, 2004). Roscovitine and purvalanol A still inhibited EBV replication when added at late times after induction, as they did for HSV-1 (Kudoh *et al.*, 2004). As for HSV-1, some inhibition of EBV replication was still observed when the drugs were added 24 h after induction, albeit only to a far lower level than when the drugs were added immediately after induction. Also as for HSV-1, PCIs inhibited the accumulation of the so called immediate-early or early EBV proteins, even in the presence of the required viral transactivators of gene expression (Kudoh *et al.*, 2004). This is an antiviral mechanism not shared with any conventional antiviral drug. Also like for HSV-1, the concentrations of PCIs that inhibited accumulation of EBV transcripts did not inhibit accumulation of representative cellular transcripts, and PCIs inhibited expression driven by EBV promoters transiently transfected into susceptible cells (Kudoh *et al.*, 2004).

The similarities of the effects of PCIs on HSV-1, HCMV, VZV, and EBV are even more impressive when considering the technical differences on the experiments. Most studies on HSV-1 were performed by infecting immortalized cells with cell-free virus. In contrast, the studies with HCMV were performed mostly using primary cells, and those with EBV were performed using an inducible promoter to regulate reactivation of latent EBV. Even more differently, the studies with VZV were performed mostly by infecting cultures with previously infected cells.

HIV

When the first review was published, CDK9 was just starting to be considered as a potential target in HIV therapy. Two groups of drugs had been shown then to inhibit HIV transcription elongation, the 'CDK9-specific' drugs (Flores *et al.*, 1999; Mancebo *et al.*, 1997) and the pan-specific PCI, flavopiridol (Chao *et al.*, 2000). Both

types of drugs inhibited purified CDK9 at low concentrations and inhibited elongation of HIV transcription, strongly suggesting that they acted primarily on CDK9. However, these drugs are now known to inhibit transcription or replication of HIV mutants that are not responsive to Tat transactivation (Pisell *et al*., 2001; Wang *et al*., 2001), and which are thus not known to require CDK9. Furthermore, flavopiridol inhibits *in vitro* transcription elongation from cellular or viral transcripts at similar concentrations (Chao & Price, 2001), whereas it inhibits HIV transcription and replication *in vivo* at concentrations that have no major effects on cellular transcription (Chao *et al*., 2000). The 'CDK9-specific' PCIs described by Flores *et al*., inhibited HIV replication even at late times after infection, in contrast to dominant negative CDK9 mutants, which only did so at early times post infection (Flores *et al*., 1999).

Since 2001, HIV replication has been further proven to be inhibited by oligo-specific PCIs. Roscovitine, purvalanol, and olomoucine inhibited HIV replication in primary or immortalized human cells, and HIV reactivation from latency in cultured cells (Nelson *et al*., 2001; Wang *et al*., 2001; Schang *et al*., 2002a; Agbottah *et al*., 2004; Hesselgesser J, Gibbs C & Shibata R (2004). Selective killing of HIV-1 infected cells by small molecule cyclin-dependent kinase inhibitors. *11th Conference on Retroviruses and Opportunistic Infections.* San Francisco, CA, USA, 8–11 February 2004. Abstract 546). Surprisingly, these drugs inhibited HIV transcription as efficiently as the pan-specific flavopiridol; this observation led to the discovery that purine type PCIs also inhibit CDK9 (Wang *et al*., 2001; Pinhero *et al*., 2004; Gherardi *et al*., 2004). As for the pan-specific PCIs, however, the inhibition of HIV transcription and replication by purine-type PCIs does not appear to be fully mediated by inhibition of CDK9. For example, roscovitine inhibited transcription driven by the HIV LTR promoter in the presence or absence of Tat (Wang *et al*., 2001), which is required to recruit CDK9 to nascent HIV transcripts. It also inhibited transcription of HIV mutants that are not known to require CDK9 (Wang *et al*., 2001).

Purine type PCIs have been shown to inhibit the replication of laboratory adapted or primary isolates of M- or T-tropic strains of HIV in immortalized or primary human cells. In an interesting, albeit yet unpublished, presentation at the *11th Conference on Retroviruses and Opportunistic Infections,* Hesselgesser and colleagues (from Gilead Sciences) demonstrated that nine PCIs of different chemical structures all efficiently inhibited HIV replication in primary T-lymphocytes and macrophages (Hesselgesser J, Gibbs C & Shibata R (2004). Selective killing of HIV-1 infected cells by small molecule cyclin-dependent kinase inhibitors. *11th Conference on Retroviruses and Opportunistic Infections.* San Francisco, CA, USA, 8–11 February 2004. Abstract 546). They further showed that combinations of different PCIs resulted in significantly better selectivity for HIV-infected over uninfected cells. As an unexpected, but highly desirable, side effect, PCIs also selectively killed HIV infected cells, presumably by inducing apoptosis. *S*-Roscovitine, for example, had no effect on cell viability at concentrations of up to 100 μM (CEM cells), concentrations at which it killed approximately 100% of chronically infected cells (Hesselgesser J, Gibbs C & Shibata R (2004). Selective killing of HIV-1 infected cells by small molecule cyclin-dependent kinase inhibitors. *11th Conference on Retroviruses and Opportunistic Infections.* San Francisco, CA, USA, 8–11 February 2004. Abstract 546). Alsterpaullone had a similar effect, but at significantly lower concentrations (approximately 10 μM). In more detailed experiments, Wang *et al*., (2001) also observed preferential killing by olomoucine, roscovitine or purvalanol of chronically infected ACH_2 or monocytic U_1 cells over parental (non HIV-infected) CEM or U937 cells. They further characterized the preferential killing of HIV-infected cells as a result of differential induction of apoptosis (Wang *et al*., 2001), although the mechanisms for this induction remain yet to be characterized.

More recently, the effects of PCIs on the reactivation of HIV from chronically infected cells were evaluated in greater detail (Agbottah *et al*., 2004). *R*-roscovitine inhibited tumour necrosis factor (TNF)-α induced reactivation of HIV in chronically infected OM 10.1 or U_1 cells. Similar to previous experiments with HSV-1, in which the drugs were added after the viral inocula were removed (Schang *et al*., 1998), roscovitine was added only after TNF-α was removed (Agbottah *et al*., 2004). Thus, the observed effects were not a result of inhibition of expression of cellular genes induced by TNF-α. Also as it had originally been tested for olomoucine and HSV-1 (Schang *et al*., 1998), these experiments included a methylated derivative of roscovitine that is inactive against CDKs (Agbottah *et al*., 2004). This methylated version did not inhibit HIV reactivation, thereby demonstrating that the activities against HIV correlate with inhibition of CDKs rather than with the core purine structure.

Purine type PCIs inhibit the replication of multidrug-resistant HIV strains and wild-type HIV equally well. Roscovitine efficiently inhibited replication of HIV strains L10R/M46I/L63P/V82T/I84V and RTMDR/MT-2, which are resistant to multiple protease or reverse transcriptase inhibitors (including nucleoside reverse transcriptase inhibitors and non-nucleoside reverse transcriptase inhibitors), respectively (Schang *et al*., 2002a). These findings have been confirmed and extended for the *R*-isomer of roscovitine (cyc202 or seliciclib), which efficiently inhibited replication of HIV strains resistant to zidovidine, lamivudine, TIBO, or proteinase inhibitors (strain PR-V82F/ 184V; Agbottah *et al*., 2004).

The concentrations of the purine type PCIs required to inhibit HIV replication, or induce preferential killing of HIV-

infected cells, have been generally reported to be in the 10–15 μM range, in close proximity to those required to inhibit cell-cycle progression (Wang *et al*., 2001; Schang *et al*., 2002a; Agbottah *et al*., 2004). Although HIV transcription and replication may actually be slightly more sensitive to roscovitine than cell-cycle progression by cultured cells (Wang *et al*., 2001; Agbottah *et al*., 2004), it is not clear yet whether these rather minor differences have major biological consequences.

A recent report has added the indirubins to the growing list of PCIs that inhibit HIV replication (Heredia *et al*., 2005). Indirubin-3′-monoxime displayed IC_{50} of 0.5 μM, >4 μM, and 0.05 μM against CDK2, CDK7 and CDK9 respectively, which were immunopurified from monocytic cells. Although the purity of the CDKs was apparently not assessed, the IC_{50} against CDK2 (Heredia *et al*., 2005) was in close proximity to that against the purified kinase (Hoessel *et al*., 1999). Indirubin-3′-monoxime inhibited basal CDK9 activity and CDK9 hyperactivated by purified HIV Tat. It also inhibited elongation of HIV transcription and HIV replication, although, as expected, at much higher concentrations. Thus, 8–16 μM were required to inhibit Tat-induced HIV replication in immortalized and chronically infected monocytes (Heredia *et al*., 2005). These concentrations had no effect on cell numbers after 48 h of treatment. Indirubin-3′-monoxime inhibited expression of full-length HIV transcripts, but not of the first 29 bases of the primary transcript, strongly suggesting that its effects against HIV are mediated by inhibition of CDK9 (Heredia *et al*., 2005). Similar concentrations of this PCI also inhibited replication of R5 or X4 HIV strains in permissive immortalized cells or primary PBMCs. No effect on cell proliferation or viability was observed on PBMCs incubated with 4 μM indirubin-3′-monoxime for 28 days, which is a concentration that significantly inhibited HIV replication (Heredia *et al*., 2005).

As it had been previously shown for other PCIs (Wang *et al*., 2001; Schang *et al*., 2002a; Agbottah *et al*., 2004), indirubin-3′-monoxime inhibited the replication of HIV strains resistant to conventional antiviral drugs as well as that of wild-type HIV. Indirubin-3′-monoxime efficiently inhibited replication of HIV strains RT MDR and CC101.19, which are resistant to multiple reverse transcriptase inhibitors and CCR5 antagonists, respectively (Heredia *et al*., 2005).

PCIs are thus known to inhibit HIV transcription in part as a result of inhibiting CDK9. As already discussed, however, PCIs have also shown to be capable of inhibiting HIV functions in the absence of the Tat-TAR interactions required to recruit CDK9 to nascent HIV transcripts. Furthermore, like HSV and HCMV, no HIV resistance to PCIs has been reported yet. PCIs may thus inhibit more than one HIV function. However, their effects on functions other than transcription elongation still remain to be thoroughly evaluated.

In summary, several PCIs have been demonstrated by different groups to inhibit replication of a variety of HIV strains in a variety of immortalized or primary cells. The effects against HIV were not affected by many different mutations that confer resistance to conventional antiviral drugs, including several mutations in the reverse transcriptase (which could have been considered a possible target for purine-containing PCIs; Wang *et al*., 2001; Schang *et al*., 2002a; Agbottah *et al*., 2004; Heredia *et al*., 2005).

Other oncogenic viruses.

The life cycles and pathogeneses of papillomavirus and KSHV depend on the activity of cell-cycle proteins such as CDKs (Ma *et al*., 1999; Van Dyk *et al*., 1999; van Dyk *et al*., 2000; Laman *et al*., 2001). The E7 protein of the oncogenic human papillomaviruses such as HPV16 activate CDK2 (He *et al*., 2003), for example, and induces DNA synthesis in the absence of growth factors. It also induces chromosome instability and centrosome duplication. All these activities may contribute to the oncogenic transformation during cervical cancer. All of them also require CDK2 activity and are consequently inhibited by roscovitine or indirubin-3′-monoxime (Duensing *et al*., 2000; Nguyen *et al*., 2002; Duensing *et al*., 2004). Indirubin-3′-monoxime inhibited centrosome multiplication and chromosome instability at 0.01–1.0 μM, which did not inhibit cellular DNA synthesis (in a short period) or physiological centrosome duplication during normal cell-cycle progression. The inhibition by indirubin-3′-monoxime was overcome by overexpression of cyclin A or E and CDK2, and it was mimicked by overexpression of small interfering RNA (siRNA) specific for CDK2 or by kinase inactive CDK2 mutants (so-called ‘dominant negative CDK2’; Duensing *et al*., 2004). A methylated derivative of indirubin-3′-monoxime, which does not inhibit CDKs, did not inhibit the abnormal centrosome multiplication either (Duensing *et al*., 2004).

A recent publication indicates that, like CDK2, CDK11 also plays a major role in centrosome duplication. CDK11 is expressed as two alternatively spliced forms (Petretti *et al*., 2006). One form is translated in to a protein that is expressed throughout the cell-cycle and is approximately 110 kDa in size, whereas the other is expressed exclusively at G2/M as a protein of approximately 58 kDa. This last isoform localizes to centrosomes (Petretti *et al*., 2006). Inhibiting the expression of the 58 kDa form by siRNA resulted in the activation of the mitotic checkpoint. This phenotype was rescued by overexpressing a GFP-CDK11 mutant resistant to the effects of the siRNA used (Petretti *et al*., 2006). As neither roscovitine nor indirubin-3′-monoxime activates the G2/M checkpoint, these results also strongly suggest that these PCIs do not inhibit CDK11 at the concentrations normally used in culture. However, the actual (lack of) effects of these drugs on CDK11 have yet to be experimentally tested.

KSHV encodes a viral cyclin that deregulates the activities of cellular CDKs, vCyc (Godden-Kent *et al*., 1997; Li *et al*., 1997; Swanton *et al*., 1997; Laman *et al*., 2001). Deletion of the *vCyc* gene in a murine herpesvirus related to KSHV inhibited reactivation from latency without affecting the lytic replication (Van Dyk *et al*., 1999; Van Dyk *et al*., 2000). KSHV vCyc preferentially activates CDK6, which is not inhibited by roscovitine (Godden-Kent *et al*., 1997; Li *et al*., 1997). However, vCyc activation of CDK6 most likely results in downstream activation of CDK2 (Duro *et al*., 1999; Mann *et al*., 1999), which would then mediate most of the downstream processes involved in cell transformation. There is circumstantial evidence to support such a model. Like HPV, KSHV transformation correlates with polyploidy and centrosome duplication and, as already discussed, HPV-induced centrosome duplication and polyploidy are mediated by CDK2. Although the actual requirement for CDK2 in these processes during KSHV-induced transformation has yet to be evaluated, the effects of CDK2-specific PCIs on KSHV replication have been evaluated. There are no efficient *in vitro* systems to evaluate KSHV replication, nor *in vivo* systems to evaluate its reactivation from latency. Thus, PCIs were used to inhibit CDKs during reactivation induced *in vitro* (Ghedin *et al*., 2004). Five micromolar *R*-roscovitine added together with the inducing phorbolesters inhibited the induction of expression of KSHV genes to the levels observed in non-activated cells (Ghedin *et al*., 2004). As a caveat of these experiments, however, it is not clear whether roscovitine inhibited the activation of viral gene expression or a phorbolester-induced pathway upstream of this activation.

Unfortunately, the inherent technical difficulties in studying the replication of HPV or KSHV in tissue culture have precluded a direct study of the effects of PCIs on their replication.

Other viruses

Having been demonstrated active against the viruses that are most likely to be sensitive to these drugs, PCIs are now starting to be tested against viruses that would not be so obviously expected to be sensitive. For example, 1–10 μM indirubin-3′-monoxime inhibited the overexpression of RANTES induced by influenza virus (Mak *et al*., 2004). Although the consequences of this effect in influenza virus replication or pathogenesis have yet to be evaluated, cytokine secretion is proposed to play a significant role in airway inflammation during influenza (Asai *et al*., 2001).

In contrast with the previously discussed viruses, PCIs failed to inhibit replication of vaccinia or lymphocytic choriomeningitis virus (Schang *et al*., 2002a), the replication of neither of which is thought to require CDKs. However, the pathogenesis of vaccinia virus does include unregulated cell proliferation and, therefore, may be inhibited by PCIs. Although this possibility remains untested, there is precedence for such an effect. Replication of vaccinia virus in cultured cells was not inhibited by gleevec, an inhibitor specific for cellular Abl and Kit protein tyrosine kinases. Gleevec did, however, inhibit the release of extracellular, enveloped virions (EEV), which led to a significant inhibition of viral pathogenesis in mice models (Reeves *et al*., 2005). In these interesting series of experiments, Reeves *et al*. (2005) first identified that Abl and Src localize to the actin tails, which mobilize vaccinia virions in the cytoplasm. Viral replication, cell-to-cell spread and release of EEV were subsequently evaluated in cells treated with 10 μM gleevec or PD-166326 (to inhibit Abl or Src kinases, respectively). Although these drugs did not inhibit vaccinia virus replication, they inhibited cell-to-cell spread (PD-166326) or release of EEV (gleevec; Reeves *et al*., 2005).

EEV are proposed to play important roles in the pathogenesis of vaccinia virus and gleevec is clinically used against chronic monocytic leukaemia. Therefore, the activity of gleevec against vaccinia pathogenesis was tested in a mouse model. Mice were implanted subcutaneously with an osmotic pump providing a continuous infusion of 100 mg/kg per day of gleevec, infected intraperitoneally with 2×10^4 PFU of vaccinia virus 24 h later and euthanized 4 days after infection. Gleevec inhibited the spread of the infection, resulting in a reduction of the number of vaccinia genomes in the ovaries from approximately 10^7 in the control animals to below 10^4 in all treated animals (and to below $10^{1.5}$ in half of them; Reeves *et al*., 2005). Mice equally treated with gleevec were also challenged intranasally with 10^4 PFU of vaccinia virus, strain IHD-J. Infected animals were euthanized when they lost 30% of their body weight. The control animals started to reach this endpoint on days 9–10 post infection; 50–75% of them had reached this endpoint by day 14. In contrast, none of the gleevec-treated mice had reached the endpoint when the experiment was terminated on day 14 (Reeves *et al*., 2005). A similar effect has also been observed for a small molecule inhibitor of the cellular Erb1 protein tyrosine kinase (Yang *et al*., 2005, further discussed below). Therefore, inhibition of protein kinases can inhibit viral pathogenesis even if the specific protein kinases play no direct role in viral replication.

Animal and clinical studies on the toxicities of PCIs

Whether PCIs can be used as antivirals will eventually be determined by whether or not they inhibit viral replication or pathogenesis *in vivo* at doses that result in no major negative side effects. It is also clear that different degrees of tolerance of negative side effects are acceptable for different viruses. Risk-benefit analyses will thus be significantly different for drugs to be used against lethal and acute viruses, such as poxvirus, than for drugs to be used against mostly non-lethal and chronic viruses, such as herpes

simplex virus. Relatively high levels of negative side effects may be tolerated for the former, whereas only low levels may be tolerated for the latter.

Although it has been known since the very first experiments that PCIs inhibit viral replication *in vitro* at concentrations that are apparently well tolerated by animals, or even by human beings, these concentrations also suffice to induce cell-cycle arrest in cultured cells. Most cultured cells become irreversibly arrested, or even undergo apoptosis, when cell-cycle progression is inhibited for prolonged periods (Mgbonyebi *et al.*, 1998, 1999; Tsao *et al.*, 1999; Waheed *et al.*, 1999; Ishida *et al.*, 2000; Ramondetta *et al.*, 2000; Yang *et al.*, 2000). This represents a significant challenge in the development of PCIs as antivirals because their safety index (SI) cannot be assessed in culture. Paradoxically, however, several of the CDKs or cyclins that are apparently essential in cultured cells are not essential for mammals. Thus, single knockouts of CDK2, CDK4, CDK6, cyclins D1, D2, or D3 (which all activate CDK4 and 6), or E1, E2 or A1 (which all activate CDK2) are not lethal, but rather result in only limited phenotypes (recently reviewed in Santamaria & Ortega, 2006). Cells can therefore cycle (almost) normally *in vivo* in the absence of any of these cyclins or CDKs. This surprisingly non-essential nature of CDKs may explain in part the otherwise surprising limited toxicities displayed so far by PCIs in animals and humans, and further suggests that CDKs may be viable clinical targets. However, such non-essential nature in animals still does not provide an avenue to test the SI of PCIs in cultured cells. In most reported experiments, PCIs inhibited viral replication in cultured cells only at concentrations that also inhibit cell-cycle progression. As the most interesting exception, indirubin-3′-monoxime inhibited HIV replication at lower concentrations than those that inhibited cell-cycle progression (Heredia *et al.*, 2005). Even in this case, however, the apparent SI would only be approximately 4, which is far lower than what is normally considered promising for candidate antiviral drugs.

Like the effects of gleevec and CI-1033 on vaccinia virus pathogenesis, roscovitine and flavopiridol inhibited viral pathogenesis independently of their effects on viral replication (Nelson *et al.*, 2003; Gherardi *et al.*, 2004). Nelson and colleagues used the TG26 model of HIV-induced nephropathy (HIVAN) to test the potential therapeutic effects of PCIs in pre-clinical models. In this model, HIV Nef is expressed in TG26 transgenic mice from integrated and defective HIV provirus. These animals suffer from a progressive proliferative kidney disease similar to HIVAN.

Juvenile TG26 mice were treated for 20 days with either *R*-roscovitine or flavopiridol (Nelson *et al.*, 2003; Gherardi *et al.*, 2004). No negative side effects were observed – this is most remarkable considering that the animals were still growing, which is when CDKs would be expected to play their most critical roles. Both flavopiridol and roscovitine inhibited the clinical and histological kidney pathology of HIVAN. Both drugs also reversed the deregulation of gene expression observed in the kidneys, while having no effects on the expression of other cellular genes (Nelson *et al.*, 2003; Gherardi *et al.*, 2004). As a significant difference, however, flavopiridol inhibited expression of the integrated transgene, whereas roscovitine did not (Nelson *et al.*, 2003; Gherardi *et al.*, 2004). Such differential effects between the pan-specific flavopiridol and the oligo-specific roscovitine are remarkably consistent with their effects in cultured cells (Diwan *et al.*, 2004). In these cells, flavopiridol equally and efficiently inhibited gene expression driven by viral promoters in their natural location in the HSV-1 genome or when recombined into the cellular genome. In contrast, roscovitine inhibited expression driven by the promoters in the viral genome, but had only negligible effects on expression driven by the same viral promoters recombined in the cellular genome (Diwan *et al.*, 2004).

Despite the encouraging effects of PCIs on viral-induced pathology (Nelson *et al.*, 2003; Gherardi *et al.*, 2004), the TG26 model of HIVAN requires no viral replication. In fact, the defective integrated proviruses in the TG26 genome cannot replicate. Therefore, the actual antiviral effects of PCIs *in vivo* could not be tested in these experiments. Furthermore, these experiments only tested the apparent safety of PCIs in mice. Fortunately, the safety of PCIs in humans was already being tested during their development as potential anticancer agents. Although the risk-benefit analyses of drugs to be used against cancer or non-lethal viral infections are markedly different, the studies of PCIs against cancer still provide evidence of their apparent safety for humans.

Flavopiridol was the first PCI tested extensively in pre-clinical and clinical trials. When our previous review was prepared, it had completed Phase I trials and was apparently safe in limited Phase II clinical trials (Stadler *et al.*, 2000). However, more extensive Phase II clinical trials have since indicated an increased occurrence of blood clotting (Schwartz *et al.*, 2001; Shapiro *et al.*, 2001). It has been suggested that the excessive clotting could have resulted from the administration route. Due to the poor pharmacokinetics of the drug, flavopiridol was continuously administered intravenously for 72 h through a peristaltic pump (Schwartz *et al.*, 2001; Shapiro *et al.*, 2001). No such clotting problems were observed in a more recent Phase II clinical trial in which the drug was administered as bolus in intravenous injections (Kouroukis *et al.*, 2003). However, similar clotting problems have not been observed in clinical trials with other drugs administered through similar routes (extensively discussed in Schwartz *et al.*, 2001; Shapiro *et al.*, 2001). Although it is thus yet unclear whether flavopiridol itself leads to major negative side effects in humans, its poor pharmacokinetics, its association with increased clotting

events and its limited effects on cancer, have all subtracted from the previous enthusiasm about its potential as a clinical drug (Schwartz *et al*., 2001; Shapiro *et al*., 2001; Thomas *et al*., 2002; Kouroukis *et al*., 2003).

While the complications uncovered during the more advanced clinical trials with flavopiridol were curtailing the enthusiasm about this drug, the *R* isomer of roscovitine started to be tested in similar pre-clinical and clinical trials with promising successes (Buolamwini, 2000; Laurence *et al*., 2002b; Benson, C, White, J, Twelves, A, O'Donnell, A, Cruickshank, C, Tan, S, Gianella Borradori, A & Judson, I [2003]. A phase I trial of the oral cyclin-dependent kinase inhibitor CYC202 in patients with advanced malignancy. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA; Abstract 838; Pierga J-Y, Faivre S, Vera K, Laurence V, Delbaldo C, Bekradda M, Armand J-P, Gianella-Borradori A, Dieras V & Raymond E [2003] A phase I and pharmacokinetic trial of CYC202, a novel oral cyclin-dependent kinase inhibitor, in patients with advanced solid tumors. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA. Abstract 840; Fischer & Gianella-Borradori, 2003; for reviews, see Meijer & Raymond, 2003; Guzi, 2004; Fischer & Gianella-Borradori, 2005; Nutley *et al*., 2005; Raynaud *et al*., 2005). Roscovitine has shown several advantages over flavopiridol, perhaps the most important being its oral availability (Raynaud *et al*., 2005). The *R*-isomer of roscovitine (cyc202 or seliciclib) has undergone limited Phase I clinical trials indicating limited toxicities (Benson C, Raynaud D, O'Donnell A, Gianella–Borradori A, Westwood R, McClue SJ, Workman P & Judson I [2002]. Pharmacokinetics (PK) of the oral cyclin-dependent kinase inhibitor CYC202 (R roscovitine) in patients with cancer. *93rd Annual Meeting of the American Association for Cancer Research*, 6–10 April, San Francisco, CA, USA. Abstract 1354; Laurence *et al*., 2002a; Benson, C, White, J, Twelves, A, O'Donnell, A, Cruickshank, C, Tan, S, Gianella Borradori, A & Judson, I [2003]. A phase I trial of the oral cyclin-dependent kinase inhibitor CYC202 in patients with advanced malignancy. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA; Abstract 838; Pierga J-Y, Faivre S, Vera K, Laurence V, Delbaldo C, Bekradda M, Armand J-P, Gianella-Borradori A, Dieras V & Raymond E [2003] A phase I and pharmacokinetic trial of CYC202, a novel oral cyclin-dependent kinase inhibitor, in patients with advanced solid tumors. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA. Abstract 840; White JD, Cassidy J, Twelves C, Benson C, Pacey S, Judson I, McGrath H, Rose F & Frenz L [2004] A phase I trial of the oral cyclin-dependent kinase inhibitor CYC202 in patients with advanced malignancy. *40th Annual Meeting of the American Society of Clinical Oncology*, 5–8 June, New Orleans, LA, USA. Abstract 3042). It is currently undergoing several Phase II clinical trials in Europe (reviewed in Guzi, 2004) and more clinical trials in the Americas are scheduled (www.cyclacel.com). Although the results of these trials are promising, the real toxicities of roscovitine will be known only when these larger clinical trials are completed. It is even possible that such toxicities may be acceptable for anticancer drugs, but unacceptable for antiviral drugs.

The indirubins have been used in Chinese (although not Western) medicine for centuries, with no signs of major toxicities. Indirubin-3′-monoxime has recently been shown to be active against HIV in cultured cells (Heredia *et al*., 2005). A comprehensive book about indirubins, their history, potential clinical uses, experimental, pre-clinical, and clinical studies has just been published (Meijer *et al*., 2006); readers further interested in these compounds should refer to it.

Many novel CDK inhibitors are being developed, and several of them are entering human clinical trials, or scheduled to do so in the near future. Although the antiviral effects of these drugs are yet mostly unreported, any PCI that proves safe in clinical trials against any disease can promptly be evaluated for its antiviral activities.

Other cellular targets

The inhibitors of HIV entry, which target the interaction between viral and cellular proteins, were already being developed as novel anti-HIV drugs when our first review on PCIs, and cellular proteins as potential antiviral targets, was published (Schang, 2001). However, cellular proteins were then not commonly accepted as valid antiviral targets. They had been proposed on occasion as potential targets for antiviral drugs and even a few drugs that target cellular proteins had been tested as antivirals (perhaps the most prominent being dehydroxyurea, discussed in Schang, 2001; Provencher *et al*., 2004). Drs Albrecht, Jean, and Shugar, among many others, had explicitly discussed the potential of cellular proteins as antiviral targets before 2001 (Bresnahan *et al*., 1997; Shugar, 1999; Jean *et al*., 2000). Nonetheless, the consensus was still strongly influenced by the many antiviral drugs targeting viral proteins developed since the discovery of the first inhibitors of the HSV-1 DNA polymerase (Kaufman *et al*., 1962a; Kaufman *et al*., 1962b; de Rudder & Privat de Garilhe, 1965; Ch'ien *et al*., 1973; for a recent review, see Schang, 2006).

Antiviral drugs targeting cellular proteins were thus mostly considered research tools or unexpected rarities. Such opinion has changed significantly since 2001, however, and cellular proteins are now often considered as valid alternative targets for antiviral drugs. For example, the basic science session of the *XV International AIDS Conference* (Bangkok, 11–16 July, 2004,) included a full day

Table 5. Selected potential antiviral drugs under development targeting cellular proteins (natural products and immunomodulatory agents omitted)

Compound	Target	Virus	Status	References
UK427,857	Cellular chemokine receptor*	HIV	Phase II/III	Door *et al*., 2003
Gleevec	Cellular Abl-family tyrosine kinases	KSHV (in AIDS patients)	Phase II	Reeves *et al*., 2005
CI-1033	Cellular Erb1 tyrosine kinases	VV	Experimental	Yang *et al*., 2005
RWJ67657	Cellular p38MAPK/JNK serine-threonine kinases	HIV	Experimental	Muthumani *et al*., 2004
PCIs	Cellular cyclin-dependent kinases	HIV, HSV	Experimental	†

*This compound targets the interaction between cellular CCR5 and viral gp120. †See text for details. HIV, human immunodeficiency virus; HSV, herpes simplex virus; KSHV, Kaposi's sarcoma herpesvirus; VV, vaccinia virus.

dedicated to cellular proteins as novel targets for antiretroviral therapy (for other few selected examples and discussions of cellular proteins as targets for antiviral drugs, see Schang, 2002; Richer *et al*., 2004; Sadaie *et al*., 2004; Liang *et al*., 2005; Schang, 2005a; Schang, 2005b; Yang *et al*., 2005; Baba, 2006; Schang, 2006). Several pharmaceutical companies and research groups are now exploring antiviral drugs that target cellular proteins (such as Cyclacel, Apath, Functional Genomic and the NCI, among many others), and a few drugs targeting cellular proteins are already being tested for their antiviral effects in humans (Table 5). Many of the concepts about the potential advantages of cellular proteins as targets for antiviral drugs discussed in 2001 are now commonly accepted. Drugs targeting cellular proteins are considered more likely to inhibit replication of several unrelated viruses, for example, or of strains that are resistant to conventional antiviral drugs. The number of potential cellular targets is commonly considered much larger than the number of viral ones. For the many virus-induced proliferative diseases, such as KSHV-induced Kaposi's sarcoma, HPV-induced cervical cancer or HIVAN, antiviral drugs targeting cellular proteins are now accepted to have the potential to simultaneously inhibit the replication of the etiological agent (that is, the virus), the expression of the transforming genes, and cell proliferation itself (Schang, 2002; Schang, 2004). Furthermore, antiviral drugs targeting cellular proteins are now commonly considered as potentially less likely to promptly select for resistance than drugs that target viral proteins. However, it is not sufficient that the target of a drug is a cellular protein to minimize the selection for resistance; the targeted cellular proteins must also be required for multiple viral functions. Viruses promptly evolve resistance to drugs targeting cellular proteins required for a single viral function, even if the cellular protein is essential for cell survival (for examples, see Murata *et al*., 2001; Rokyta *et al*., 2002; Crotty *et al*., 2004). In fact, resistance to inhibitors of HIV entry, or selection for pre-existing resistant quasispecies, has already been reported under experimental conditions both in culture and in patients (Trkola *et al*., 2002; Kitrinos *et al*., 2005; Kuhmann, 2005; for a recent review, see Briz *et al*., 2006).

The renewed interest in cellular proteins as targets for antivirals has prompted a number of studies aimed at identifying potential cellular targets. Due in part to the original studies on the antiviral activities of PCIs, the 'standard' approach is now to evaluate the potential antiviral activities of drugs that target cellular proteins already known to be involved in viral replication. Another approach, which has been named virogenomics, is to target cellular genes that are upregulated in infected cells (Früh *et al*., 2001; DeFilippis & Früh, 2006) and is being pursued for several viruses, including HIV (Krishnan & Zeichner, 2004). Yet another approach is to perform large-scale genomic screens using siRNA to identify potentially drugable targets (Liang *et al*., 2005). Each of these approaches has advantages and disadvantages. Testing known drugs that inhibit cellular functions already known to participate in viral replication has a significant advantage in that the pharmacological and toxicological analyses of the potential antiviral drug have often been already performed. Unfortunately this limits the analyses to only those cellular functions that are inhibited by available drugs. The advantage of virogenomics is that targeting cellular proteins upregulated specifically in infected cells may limit the potential toxicity of the drugs towards non-infected cells. However, many cellular proteins that play major roles in viral replication, such as CDK9, are not necessarily upregulated during infection. Furthermore, some of the upregulated proteins participate in antiviral responses, not in viral replication. Genome-wide screenings allow for the unbiased screenings for potential cellular targets, but are likely to identify many cellular proteins that are not easy pharmacological targets.

As an example of the first approach, the cellular receptor protein tyrosine kinases, Erbs, were identified as being involved in the pathogenesis of poxvirus infections using standard virological approaches (Buller *et al*., 1988; Opgenorth *et al*., 1993; Kim *et al*., 2004). Since Erbs are involved in human malignancies, many specific inhibitors of these protein kinases have been developed, and some are

being tested as potential anticancer agents (Baselga, 2002). Thus, the effects of such drugs on poxvirus replication and pathogenesis were tested (Yang *et al*., 2005). A specific inhibitor for Erb1, CI-1033, inhibited spread of vaccinia virus in cultured cells, without inhibiting viral replication in the primarily infected cells. Like the PCIs, the antiviral concentrations of CI-1033 in cultured cells were significantly higher than the IC_{50} toward the purified kinase (10 μM and 0.8 nM, respectively). Viral spread is essential for pathogenesis, and deletion of the vaccinia Erb1 ligand (VGF) results in significant attenuation (50% lethal dose is increased by approximately 2,000-fold). Therefore, the effects of CI-1033 were tested in a mouse model. Mice were infected intranasally with 10^4 PFU of vaccinia virus (WR strain) and treated intraperitonally with 1 mg/day of CI-1033, starting 6 h before infection. Treatment for 8 days resulted in 100% survival (in comparison to 100% lethality in untreated animals), whereas shorter treatments resulted in delayed (and reduced) lethality (Yang *et al*., 2005). These treatments did not decrease viral replication in the primary site (that is, the lungs), consistently with the effects observed in culture. Unfortunately, CI-1033 had no effect in lethality when the treatment was started 2 days after infection; effects at other starting times were not reported (Yang *et al*., 2005).

The mitogen-activated protein kinase pathway is thought to play major roles in proliferative or autoimmune diseases. Therefore, several small molecule inhibitors of this pathway have been developed. One such inhibitor, RWJ67657, has been tested for safety in humans and is currently undergoing further clinical trials against autoimmune disease (Bayes *et al*., 2003; Donnelly & Rogers, 2003; Parasrampuria *et al*., 2003). The mitogen-activated protein kinase pathway is also known to play major roles in HIV replication and reactivation from latency. Therefore, the activities of RWJ67657 on HIV replication were recently tested (Muthumani *et al*., 2004). Like the PCIs, RWJ67657 inhibited replication of laboratory-adapted and primary isolates of HIV in immortalized cells or primary human PBMC (Muthumani *et al*., 2004). Also like the PCIs, RWJ67657 inhibited the replication of HIV strains resistant to NRTI, NNRTI or PI (Muthumani *et al*., 2004). It is curious that both PCIs (Schang *et al*., 2002a; Agbottah *et al*., 2004) and RWJ67657 (Muthumani *et al*., 2004) appear to be the most potent against PI-resistant strains of HIV. Such a differential effect deserves to be further explored and, if confirmed, its mechanisms should be elucidated. Differing from the PCIs, however, RWJ67657 inhibited apoptosis of HIV-infected cells (Muthumani *et al*., 2004). The specific HIV functions inhibited by RWJ67657 remain yet to be analysed.

Protein kinases are also inhibited by molecules other than nucleosides. A group of prostaglandins, the cyclopentenone prostanoids (cyPGs), for example, bind to the activation loop of one of the subunits of the IKK kinase (Rossi *et al*., 2000), which results in the inhibition of IKK. Since IKK indirectly activates NFκB, cyPGs indirectly inhibit NFκB activation (Rossi *et al*., 2000). NFκB activity has long been known to be induced by, and required for replication of, several viruses, including HSV-1 and HIV (for examples, see Gimble *et al*., 1988; Patel *et al*., 1998a; Amici *et al*., 2001). The signalling pathway leading to NFκB activation in HSV-infected cells involves the IKK kinase (Amici *et al*., 2001; Gregory *et al*., 2004). Thus, Santoro and colleagues further evaluated the effects on HSV-1 replication of a natural cyPG, prostaglandin A_1 (PGA_1), (Amici *et al*., 2001). A single treatment with PGA_1 during the first 3 h of infection inhibited HSV replication for 72 h by 3 orders of magnitude (Amici *et al*., 2001). The dose-response and the kinetics of the inhibition of HSV-1 replication paralleled the effects on NFκB activation, suggesting that NFκB was the actual (although indirect) target of the drug (Amici *et al*., 2001). Based on these results, Fitzmaurice and colleagues further tested the potential antiviral activities of a synthetic cyPG analogue, CTC-8 (Fitzmaurice *et al*., 2003). Consistently with the effects of PGA_1, CTC-8 also inhibited HSV replication by ~2.5–3 orders of magnitude in a single-step growth assay (Fitzmaurice *et al*., 2003). The SI was estimated to be in the order of 10, but it was highly dependent on the specific conditions of the assay. A lower SI was observed in growing than in confluent cells, for example, presumably because the compound had cytostatic effects. The maximum anti-HSV effect was dependent on the multiplicity of infection, whereas the 90% effective dose was not (Fitzmaurice *et al*., 2003). The antiviral effects of CTC-8 were highly dependent on the time of treatment. Maximum inhibition was achieved when CTC-8 was added 6 h after infection and removed 6 h later, whereas surprisingly little inhibition was observed if CTC-8 was added before infection and maintained through the infection (Fitzmaurice *et al*., 2003). These results may reflect that the effects of certain cyPG such as PGA_1 on NFκB activation are only transient (Rossi *et al*., 2000). Alternatively, NFκB may be exquisitely required only at certain times after infection, while having antiviral activities at others. NFκB may also participate in an antiviral response, as well as in activation of viral gene expression and inhibition of premature apoptosis. Therefore, if NFκB were to be inhibited too early, it may actually prevent its antiviral activities, favouring viral replication. Regardless of the particulars of the mechanisms, however, such time-dependence results in CTC-8 being inactive in plaquing assays (Fitzmaurice *et al*., 2003). Supporting IKK as the most relevant target of the anti-HSV activity of cyPGs and their analogues, more recent experiments have shown that HSV replication is impaired in $IKK\beta^{-/-}$ or $NF\kappa B^{-/-}$ fibroblasts, or in cells overexpressing non-phosphorylable mutants of the direct target of IKK, IκB (Gregory *et al*., 2004; Taddeo *et al*., 2004).

Many other cellular protein kinases are involved in viral replication and pathogenesis, and many specific protein kinase inhibitors have been developed. The protein kinases are thus one of the groups of cellular proteins that holds the most promise as potential targets for novel antiviral drugs (recently reviewed and discussed in Schang, 2004), and are indeed the first that have entered clinical trials (Koon *et al.*, 2005).

It is most likely, however, that the most important cellular targets for antiviral drugs will be eventually identified by a combination of all the approaches discussed above. The recent discovery of the activities of MGBG against HSV-1 provides a nice illustration of this possibility. Polyamines such as spermine have long been known to play major roles in the replication cycle of HSV-1 (Gibson & Roizman, 1971), and had even been shown to be required for efficient production of infectious HSV-1 virions (Pohjanpelto *et al.*, 1988). A proteomics study identified several proteins the expression of which was induced, or at least not inhibited, during HSV infection (Greco *et al.*, 2000). One of these proteins was later identified as ornithine decarboxylase, an enzyme in the metabolic pathway of polyamine synthesis (unpublished results quoted in Greco *et al.*, 2005). Small molecule inhibitors of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase were developed many years ago as potential anticancer agents, and have been tested on and off in clinical trials (recently reviewed in Basuroy & Gerner, 2006). On these bases, Greco and colleagues recently tested the antiherpesviral activities of two of these drugs, MGBG and DFMO. MGBG efficiently inhibited HSV-1 replication at 50–100 μM in a plaquing assay, but had no major effects on cell viability at concentrations <500 μM (Greco *et al.*, 2005). Like PCIs, the antiviral concentrations of MGBG were, however, cytostatic (Greco *et al.*, 2005). MGBG inhibited early and late HSV-1 gene expression and HSV DNA replication, but had no effect on the expression of the so-called immediate-early genes. Similar to the PCIs, MGBG was equally effective at inhibiting the replication of wild-type or drug-resistant HSV-1 strains (Greco *et al.*, 2005). As a caveat of these experiments, it should be noted that previous experiments had shown that depletion of polyamines resulted in either inhibition (Raina *et al.*, 1981; Pohjanpelto *et al.*, 1988) or no effect (McCormick & Newton, 1975; Tyms *et al.*, 1979) on HSV-1 replication.

A very large number of cellular proteins are involved in viral functions, and it is therefore impossible to properly discuss all potential cellular targets in one article. Therefore, we have focused this section only on very few selected examples of cellular proteins that are potential targets for antiviral drugs.

Future challenges

Much progress has been made in the 5 years since the first review on PCIs as potential antivirals was published (Schang, 2001). In 2001, the concept of developing antivirals that target cellular proteins was radical, so it is not surprising that much remains yet to be learnt before the true potential of PCIs as antivirals is known.

Four areas in need of development were discussed in the original review (Schang, 2001). The first was the identification of all the human pathogenic viruses that are sensitive to inhibition by PCIs. Major progress has been attained in this area. As expected, specific functions, replication and pathogenesis of many human viruses have now been shown to be inhibited by several PCIs (for examples, see Fax *et al.*, 2000; Wang *et al.*, 2001; Schang *et al.*, 2002a; Chao *et al.*, 2003; Agbottah *et al.*, 2004; Duensing *et al.*, 2004; Hesselgesser J, Gibbs C & Shibata R (2004). Selective killing of HIV-1 infected cells by small molecule cyclin-dependent kinase inhibitors. *11th Conference on Retroviruses and Opportunistic Infections*. San Francisco, CA, USA, 8–11 February 2004. Abstract 546; Kudoh *et al.*, 2004; Mak *et al.*, 2004; Taylor *et al.*, 2004; Heredia *et al.*, 2005). It has also been formally demonstrated that PCIs are effective against viral strains that are resistant to conventional antiviral drugs (Wang *et al.*, 2001; Schang *et al.*, 2002a; Agbottah *et al.*, 2004; Hesselgesser J, Gibbs C & Shibata R (2004). Selective killing of HIV-1 infected cells by small molecule cyclin-dependent kinase inhibitors. *11th Conference on Retroviruses and Opportunistic Infections*. San Francisco, CA, USA, 8–11 February 2004. Abstract 546; Heredia *et al.*, 2005), and that PCIs can inhibit viral pathogenesis even beyond their effects on viral replication (Nelson *et al.*, 2003; Gherardi *et al.*, 2004). We can expect that future studies may extend the analyses of the potential effects of PCIs to other viruses that, *a priori*, could be considered less likely to be inhibited by such drugs. For example, CDKs are already known to surprisingly play important roles in the pathogenesis or replication of several cytoplasmic RNA viruses (discussed in Schang, 2003). PCIs may thus have some activity against such viruses. Indeed, there is even some experimental, if preliminary, evidence that they may well do so (Mak *et al.*, 2004).

A second area identified as in need of further development was the identification of the major direct targets of PCIs in infected cells, and of the downstream effectors of these targets. Progress has been made in this area as well, although the full identification has yet to be completed. CDK9 has been confirmed as a major target for the anti-HIV effects of the pan-specific PCIs (Chao *et al.*, 2000; Chao & Price, 2001; Heredia *et al.*, 2005). It also likely plays a major role in the inhibition of HIV replication by oligo-specific PCIs, although it does not appear likely to be the only relevant target (Pisell *et al.*, 2001; Wang *et al.*, 2001; Schang *et al.*, 2002a). Downstream from their direct targets, PCIs have further been shown to prevent activation of transcription from extra-chromosomal viral genomes, independently of any given promoter-specific sequence (Diwan *et al.*, 2004). We can expect that future studies will complete the identification

and characterization of the cellular targets that mediate the antiviral activities of PCIs, and of the mechanisms of inhibition downstream from these primary targets.

A third area in need of development was the evaluation of the potential antiviral activities of non-toxic doses of PCIs on human beings. Two paths had been discussed in 2001 to achieve this goal (Schang, 2001), and a third path was proposed in 2002 and further discussed in 2004 (Schang, 2002; Schang, 2004). We proposed in 2001 that the safety of PCIs could be evaluated in more pre-clinical and clinical trials against proliferative diseases and on animal models of viral disease before their antiviral activities are tested in humans. As expected, significant progress has been made along this approach. PCIs (as well as gleevec and CI-1033) have been shown to inhibit viral induced pathogenesis (Nelson *et al*., 2003; Gherardi *et al*., 2004; Reeves *et al*., 2005), and PCIs have continued to show limited negative side effects in pre-clinical and clinical trials against cancer (Buolamwini, 2000; Laurence *et al*., 2002b; Benson, C, White, J, Twelves, A, O'Donnell, A, Cruickshank, C, Tan, S, Gianella Borradori, A & Judson, I [2003]. A phase I trial of the oral cyclin-dependent kinase inhibitor CYC202 in patients with advanced malignancy. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA; Abstract 838; Pierga J-Y, Faivre S, Vera K, Laurence V, Delbaldo C, Bekradda M, Armand J-P, Gianella-Borradori A, Dieras V & Raymond E [2003] A phase I and pharmacokinetic trial of CYC202, a novel oral cyclin-dependent kinase inhibitor, in patients with advanced solid tumors. *39th Annual Meeting of the American Society for Clinical Oncology*, 31 May–3 June, Chicago, IL, USA. Abstract 840; Fischer & Gianella-Borradori, 2003; for reviews, see Meijer & Raymond, 2003; Guzi, 2004; Fischer & Gianella-Borradori, 2005; Nutley *et al*., 2005; Raynaud *et al*., 2005). Consequently, the first clinical trials of PCIs as antivirals have now been tentatively scheduled for the near future (www.cyclacel.com).

The other avenue proposed in 2001, epidemiological studies of the frequency of viral disease in patients enrolled in clinical trials using PCIs against any disease (for a discussion, see Schang, 2001), has yet to be pursued. In contrast, the approach proposed in 2002, testing the antiviral activities of PCIs or similar drugs in patients suffering of virus-induced proliferative disease (Schang, 2002; Schang, 2004), has now been pursued to the beginning of Phase II clinical trials. This approach is based on the proposal that PCIs and related drugs would be expected to have a direct beneficial effect on the proliferative diseases themselves, thus allowing to evaluate their potential antiviral effects while the patients potentially benefit from their effects on pathogenesis. We had proposed as one example to test the antiviral activities of PCIs or related drugs against KSHV-induced Kaposi's sarcoma. Excitingly, gleevec (imatinib) was successful in a small pilot study against this disease in AIDS patients (Koon *et al*., 2005) and is currently undergoing larger Phase II clinical trials against this disease. We can expect that the antivirals effects of gleevec against KSHV will thus be known in the coming years. Protein kinases are involved in many different steps of viral replication and pathogenesis. The first clinical trials are for drugs that target protein kinases involved in pathogenesis, whereas other functions such as transcription may *a priori* appear to be less likely targets for virus-specific drugs. However, it is still too early to evaluate whether certain specific viral functions, such as entry or pathogenesis, will be more amenable to inhibition by drugs that target cellular proteins than others, such as transcription.

In sum, although PCIs or most other drugs that target cellular proteins have yet to show antiviral activities in human beings (the only exception being the HIV entry inhibitors that target cellular receptors or co-receptors), we can expect that the ongoing experiments and clinical trials will soon directly address this potential. Although it still remains to be shown that any drug targeting a cellular protein has antiviral activities in human beings at non-toxic concentrations, several groups are currently embarked on the analyses of such potential antivirals. We can thus expect to know the true potential of cellular targets for antivirals in the near future.

Conclusion

Although cellular proteins had been considered many times as potential targets for antiviral drugs, this possibility was not generally accepted before 2001. PCIs were in fact the first family of drugs tested as potential antiviral drugs because they target cellular proteins (required for viral replication). In great part due to the potential shown by PCIs, cellular proteins are now often considered as valid potential targets for antiviral drugs. Therefore, in this regard, PCIs have already had a significant impact on the development of novel antivirals.

Acknowledgements

LMS would like to thank Drs HEM Kaufman, Y-C Cheng and M Baba for their invitation in 2000 to present our results on basic virology to the antiviral community for the first time. Special thanks also go to Dr H Field for his invitation to submit the original review in 2001, his continuous support for that article and the invitation to submit this updated version. Many thanks also to all those colleagues who have so often shared exciting unpublished results.

LMS is a CIHR New Investigator, an AHFMR Scholar and a Burroughs-Wellcome Fund Investigator in the pathogenesis of Infectious Disease. JJL is supported by the AHFMR and the CIHR. MRSt.V is partially supported by NSERC. The studies in our lab are supported by CIHR operating budget grant MOP49551 and by the Burroughs-Wellcome Fund. Our laboratory was equipped with funds provided by the AHFMR and the Faculty of

Medicine and Dentistry of the University of Alberta, Edmonton, Alberta, Canada.

Additional files

The additional file 'Specificity profile of selected oligo- or pan-specific PCIs – full table', which is an expanded version of Table 4, can be accessed via the Volume 17 Issue 6 contents page of *Antiviral Chemistry and Chemotherapy*, which can be found at www.intmedpress.com (by clicking on 'Antiviral Chemistry and Chemotherapy' then Journal PDFs).

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Received 23 June 2006, accepted 22 October 2006