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Review Article

Cyclin-dependent kinase inhibitors: discovery, development and target rationale for different therapeutic applications

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Abstract

Members of the family of cyclin-dependent kinases (CDKs) fulfill important and partly overlapping roles in the regulation of the cell proliferation cycle and the RNA polymerase-II (RNAP-II) transcription cycle. Clinical development of CDK inhibitors as new anticancer agents has been under way for several years and the first signs of potential efficacy in certain indications have recently been reported. Furthermore, there has been a recent resurgence in the clinical entry of new CDK inhibitors for the treatment of cancer. However, there are numerous other therapeutic applications where inhibition of cell proliferation and transcription with pharmacological CDK inhibitors might be useful. Chief amongst these are proliferative disorders in the areas of nephrology, cardiovascular disease and neurodegeneration. Additionally, infectious diseases in which the invading microorganisms themselves possess CDK-like proteins or depend on the host cell proliferation and transcription machinery are attractive targets. The biomedical rationales for some of these applications of CDK inhibitors are discussed here, with emphasis on the CDK specificity of potential pharmacological agents. Furthermore, progress in the discovery and development of CDK-inhibitory agents is brought up to date.

Introduction

The discovery and development of cyclin-dependent kinase (CDK) inhibitors as a new therapeutic modality in oncology have been under way for some years and the first experimental drugs are now approaching the end of phase II clinical evaluation (1, 2). The original CDK target hypothesis was based on the idea that aberrant cell cycle regulation in cancer cells might be brought under control selectively with pharmacological inhibitors of the main mediators of the cell cycle checkpoints, *i.e.,* the CDKs (Table I).

The mitogenic signaling pathway, which recruits cells into the proliferative state –and which is constitutively activated in most transformed cells– converges on the complex of the retinoblastoma tumor susceptibility gene product pRb and the main cell cycle transcription factor E2F-1. It involves the D-type CDKs CDK4 and CDK6, which phosphorylate pRb and thereby initiate the processes leading to release of E2F-1 transcriptional activity, required for entry into the DNA synthesis (S) phase (3). Beyond the restriction point, where cells become insensitive to mitogenic signals and are committed to cytokinesis, cyclin E- and cyclin A-associated CDK2 activities are important for entry from the first gap phase (G1) into S phase, as well as for progression through and exit from S phase (4). At the transition between the second cell cycle gap phase (G2) and mitosis (M phase), cyclin B/CDK1 activity governs entry into M phase (5). Activation of the cyclin B/CDK1 complex requires another CDK, *i.e.,* the so-called CDK-activating kinase (CAK), a complex containing CDK7, cyclin H and MAT-1 (6).

Several of the cell cycle-related cyclins and CDKs previously thought to be unique sensors and facilitators of cell cycle entry and progression have recently been shown not to be crucial for cell proliferation (7-11). These findings, together with the demonstration that even certain cancer cells can proliferate in the absence of CDK2 activity (12), have led to the current debate regarding the validity of cell cycle CDKs as oncology drug targets (13-15).

CDKs also play important roles in the regulation of transcription, because certain CDKs, especially CDK7 (6)

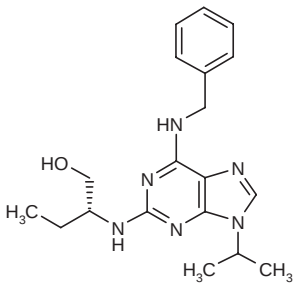
Table I: Main functions of human CDKs.

CDK	Main activator subunits	Functions	Main cellular phosphorylation targets	Main effects of inhibition
1	B-type cyclins	Cell cycle (G2/M)	Cytoskeleton proteins involved in mitosis, histones	Cell cycle arrest in G2/M
2	A- and E-type cyclins	Cell cycle (G1/S)	Pocket proteins, DNA replication proteins, E2F, histones	Cell cycle arrest in G1/S
3	C- and E-type cyclins	Cell cycle	Not known	Cell cycle arrest in G1/S
4	D-type cyclins	Cell cycle (G1)	Priming phosphorylation of pocket proteins	Cell cycle arrest in G1
5	p35 (p25) and p39 (p29)	Cell cycle	Neuroskeletal proteins	Neuronal cell functions
6	D-type cyclins	Cell cycle (G1)	Priming phosphorylation of pocket proteins	Cell cycle arrest in G1
7	Cyclin H, MAT1, TFIIE	Cell cycle and transcription	CAK; CTD of promoter-bound RNAP-II	Cell cycle arrest and inhibition of transcription
8	Cyclin C	Transcription	CTD of free RNAP-II	Activation of transcription (?)
9	K- and T-type cyclins	Transcription	CTD of stalled RNAP-II	Inhibition of transcription
10	Not known	Cell cycle	Not known	Cell cycle arrest in G2/M
11	Cyclin L	Cell cycle and transcription	RNAP-II	Inhibition of transcription (?)

Table II: Clinical and late preclinical small-molecule CDK inhibitors in oncology.

Compound	Structure (see Fig. 1)	Source	Comments (Ref.)	Phase	Administration route
Seliciclib (CYC-202, *R*-roscovitine)	**1**	Cyclacel	Selective CDK2 CDK7 CDK9 inhibitor (190)	Phase II	p.o.
Alvocidib (flavopiridol, HMR-1275)	**2**	Sanofi-Aventis/ NCI	Promiscuous kinase inhibitor with CDK-inhibitory activity (72)	Phase II	i.v.
UCN-01	**3**	Kyowa Hakko Kogyo/NCI	Promiscuous kinase inhibitor with CDK-inhibitory activity (191)	Phase II	i.v.
Indisulam (E-7070)	**4**	Eisai	G1/S cell cycle agent with indirect effects on CDK function (192)	Phase II	i.v.
SNS-032 (formerly BMS-387032)	**5**	Sunesis (Bristol-Myers Squibb)	CDK1/CDK2 inhibitor (193)	Phase I	i.v.
ON-01910.Na	**6**	Onconova	Dual-specificity non-ATP-competitive CDK1/polo-like kinase-1 inhibitor (194)	Phase I	Not known
AZD-5438	Not disclosed	AstraZeneca	(195)	Phase I	Not known
ZK-304709	Undisclosed pyrimidine	Schering AG	Pan-CDK and VEGF and PDGF RTK inhibitor (196)	Phase I	p.o.
PHA-690509	Not disclosed	Nerviano Medical Science	Not known	Phase I	Not known
PD-0332991	**7**	Pfizer	Highly CDK4-selective with G1/S activity (34)	Preclinical/phase I	p.o.
AG-024322	**8**	Pfizer	Pan-CDK inhibitor (197, 198)	Preclinical/phase I	Not known
RGB-286199	**9**	GPC Biotech	Pan-CDK inhibitor (199)	Preclinical/phase I	i.v.
JNJ-7706621	**10**	Johnson & Johnson	CDK inhibitor with G2/M activity (200)	Preclinical/phase I	Not known
Not known	Undisclosed 2,4-diamino-pyrimidine	Roche	Not known (201-203)	Preclinical/phase I	p.o.

and CDK9 (16) (Table I), control initiation and elongation of mRNA synthesis by phosphorylation of the *C*-terminal domain (CTD) of RNAP-II (DNA-directed RNA polymerase II) (17, 18). Initiation of transcription involves both specific and general transcription factors, many of which are linked directly to the cell cycle, *e.g.,* the E2F family of transcription factors that are required for the expression of genes essential for a cell to enter S phase and the later stages of the cell division cycle (19). A link also exists at the level of general transcription factors, where one of the



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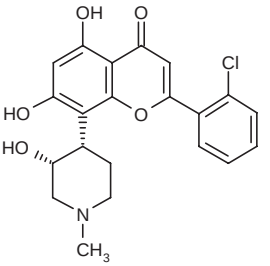

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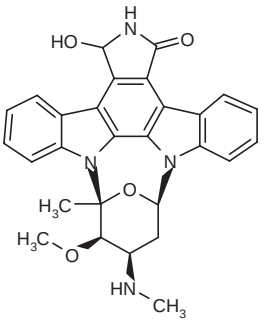

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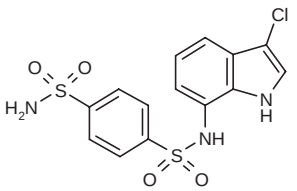

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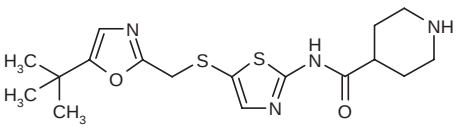

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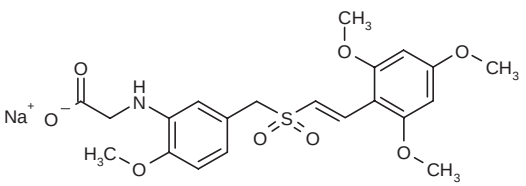

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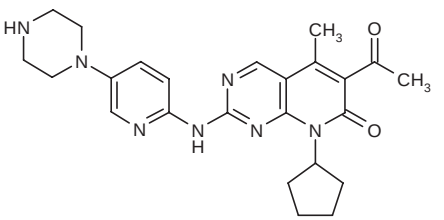

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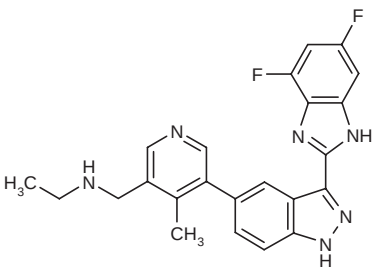

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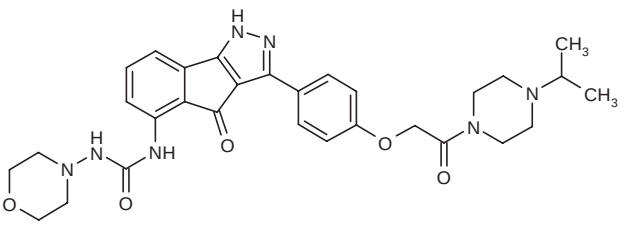

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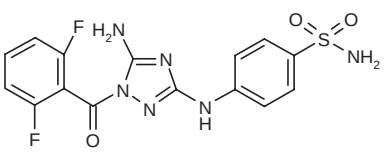

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Fig. 1. Structures of experimental CDK-inhibitory anticancer drugs (see Table II).

most important components for the initiation of transcription is TFIIH (transcription factor IIH), a complex that contains CDK7, which also has a role in the activation of other CDKs required for cell cycle progression (20).

Many genes are regulated specifically at the level of transcription elongation, and mRNA synthesis is under the control of the positive transcription elongation factor b (P-TEFb), which contains the CDK9/cyclin T complex (21). It is known that transformed cells require the continuous activity of RNAP-II to resist oncogene-induced apoptosis (22), and a new oncology CDK target rationale based on inhibition of transcription has been proposed (23). It turns out that inhibition of CDK activities in the RNAP-II transcription cycle is at least as important for the *in vivo* antiproliferative effects as for the cell cycle-related activities of the CDK inhibitor drug candidates seliciclib (**1**) and alvocidib (**2**) (Fig. 1, Table II), and probably for many other pharmacological CDK inhibitors as well (15, 24).

Thus, CDK inhibition may be a valid therapeutic strategy not only in cancer but also in other diseases where aberrant cell proliferation plays a role, although there are currently no CDK inhibitors in development outside oncology. Here I shall focus on some of these alternative CDK inhibitor concepts, as well as summarizing the latest development in the cancer field.

Strategies for pharmacological CDK inhibition

ATP antagonism

Of all the protein kinases that are currently being pursued as drug targets, CDKs have without doubt been the most fecund in terms of drug discovery: a bewildering array of pharmacophores, encompassing compounds with varying degrees of potency and specificity towards CDKs, are known. My intention here is not to enumerate these (for up-to-date reviews, the interested reader is referred to Refs. 1 and 25-28), but to highlight some of the outstanding issues, especially that of selectivity. Considering the fact that the human genome encodes more than 500 kinases, not to mention many other relevant mononucleotide-binding proteins, specificity is surely the major concern if we are to develop kinase inhibitor drugs that target the ATP-binding site and that will not exhibit promiscuous pharmacological effects (29). The fact that this can be achieved in practice has been demonstrated to a large extent with experimental late-stage drugs active against tyrosine kinases, and the currently approved small-molecule kinase inhibitor drugs imatinib (Gleevec/Glivec; Novartis), gefitinib (Iressa; AstraZeneca), and erlotinib (Tarceva; Genetech/OSI Pharmaceuticals), the main molecular targets of which are the Bcr-Abl (imatinib) and epidermal growth factor receptor (EGFR) kinases (gefitinib and erlotinib) (30-32).

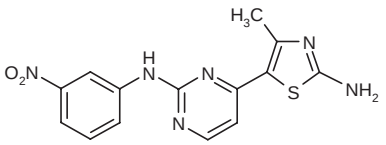
Practically all of the small-molecule CDK inhibitor leads and drug candidates reported to date are ATP antagonists (25). Monospecific CDK inhibitors, *i.e.,* molecules that preferentially inhibit one CDK at the exclusion of other CDKs and kinases in general, have not been reported so far. The fact that molecules with differing CDK specificity profiles are known, however, suggests that the discovery and design of such inhibitors may be possible. Schang (33) has proposed a useful nomenclature to classify currently known CDK inhibitors based on specificity: nonspecific molecules inhibit CDKs and a variety of other kinases; pan-specific molecules inhibit CDKs indiscriminately but (apparently) do not inhibit other kinases; and oligo-specific agents preferentially inhibit a subset of CDKs.

The most specific CDK inhibitor reported to date is probably the 8*H*-pyrido[2,3-*d*]pyrimidin-7-one **7** (Fig. 1, Table II), a low-nanomolar inhibitor of CDK4 and CDK6 that does not inhibit (at concentrations up to about 10 μM) CDK1, CDK2 and CDK5 or a host of non-CDK kinases (34). Although it is unclear if this compound inhibits the RNAP-II CDK7 and CDK9, the cellular mode of action presented strongly suggests that the compound does not interact with cellular antiproliferative targets other than CDK4 and CDK6.

Significant progress has also been made in the design of oligo-specific CDK inhibitors that target the G1 CDKs (28, 35), whereas true separation of, for example, CDK1 *versus* CDK2 inhibition has not thus far been possible (25, 36). The role of CDKs in the regulation of transcription has only recently been appreciated, and for most compounds reported to date no information is available regarding inhibition of CDK7 and CDK9. In the author's laboratory, the experience has been that CDK2 inhibitors frequently also inhibit CDK9 with similar potency. For example, the thiazolopyrimidine compound **11** (Fig. 2) inhibits CDK2 and CDK9 at very low nanomolar concentrations and is > 10-fold selective with respect to CDK1, CDK4 and CDK7 (37). At present, there is no structural information available on CDK9, while the first X-ray crystal structure of CDK7 was just recently reported (Fig. 3) (38). The comparison of this structure with other CDKs should now facilitate the design of CDK7-specific inhibitors (Fig. 4).

Targeting CDK protein-protein interactions

The complex crystal structure between CDK2/cyclin A and the CDK-inhibitory protein $p27^{KIP1}$ illustrates at least two ways in which substrate phosphorylation by CDK2 can be inhibited (Fig. 5a) (39). Apart from blocking the ATP-binding pocket, $p27^{KIP1}$ also occupies a surface groove in cyclin A. This substrate recruitment site is critical for the recognition of the physiological CDK2/cyclin A substrates, such as pRb and E2F-1 (40), and a conserved recognition motif (ZRXLYY', where Z and X denote basic residues and Y and Y' refer to hydrophobic residues [41]) exists not only in such substrates, but also in many CDK2-inhibitory proteins (42). The key interactions between cyclin groove inhibitor peptides and the substrate recruitment site are well understood (43-45)



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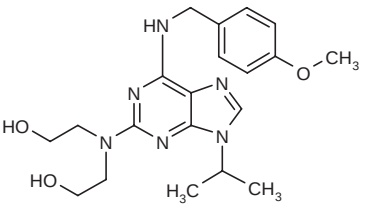

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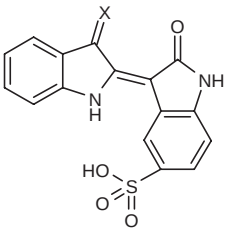
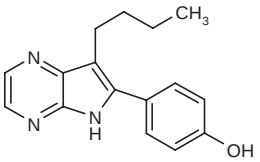


13a: X = O
13b: x = NOH



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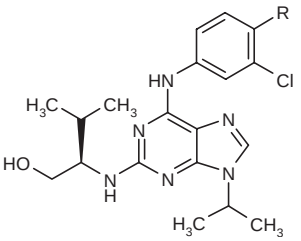
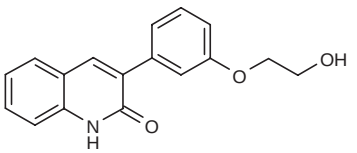


15a: R = H
15b: R = COOH



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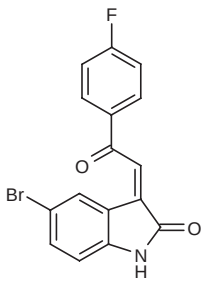

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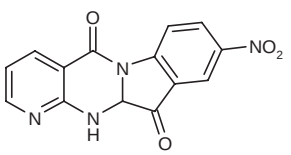

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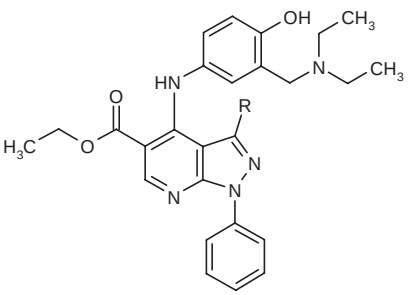

19a: R = Me
19b: R = Ph

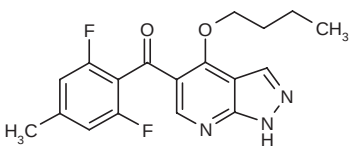

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Fig. 2. Preclinical human CDK inhibitors and inhibitors of parasite CRKs.

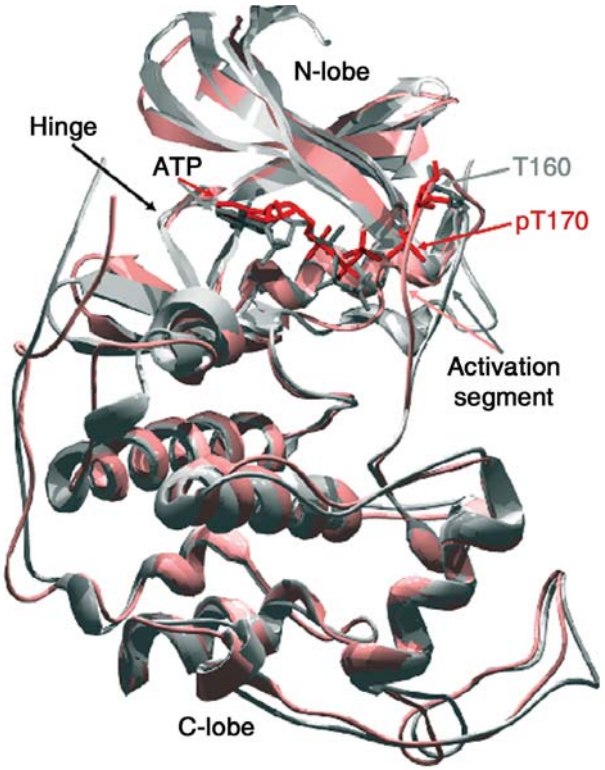


Fig. 3. Structural comparison of human CDK2 and CDK7. The recently determined X-ray crystal structure of CDK7 (pink ribbon; PDB # 1UA2 [38]) was aligned (overall 1.2 Å RMSD C^{α} fit) with the most similar structure of CDK2 (grey ribbon; PDB # 1B39 [204]), *i.e.*, an apoenzyme form lacking phosphorylation of the T160 residue in the activation segment, which differs significantly between the two structures. Structural features of the classical kinase fold are indicated.

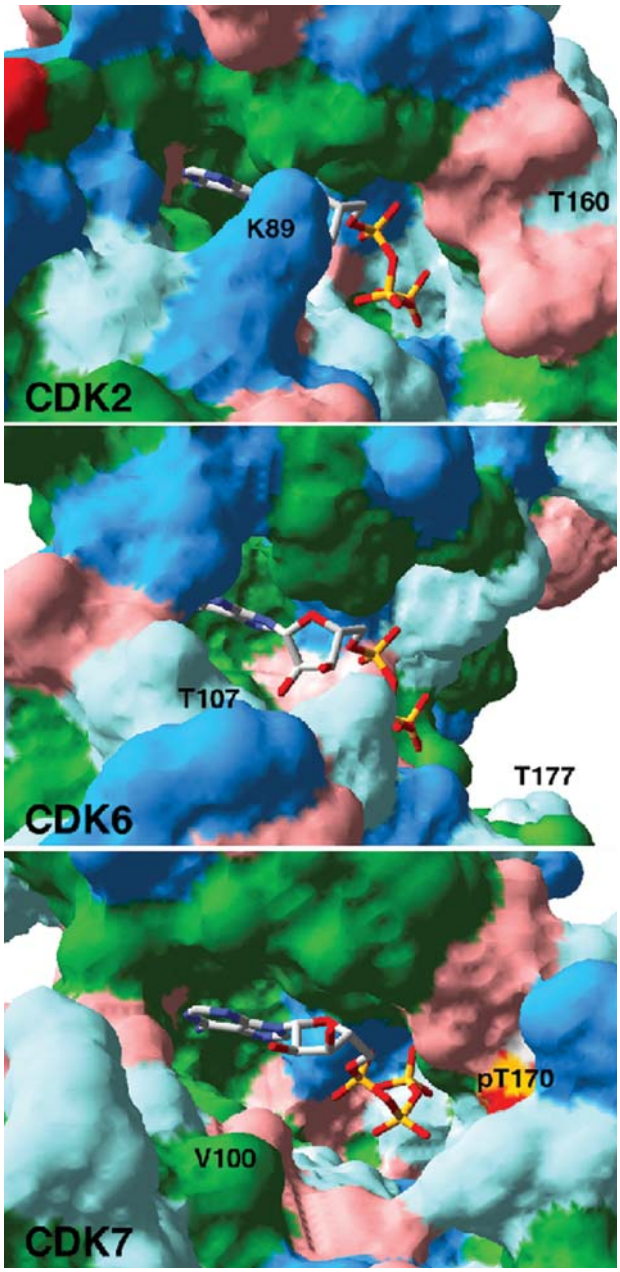


Fig. 4. Comparison of the ATP-binding sites in CDK2, CDK6 and CDK7. The structural coordinates of CDK2 (PDB # 1B39 [204]), CDK6 (PDB # 1JOW [205]) and CDK7 (PDB # 1UA2 [38]) were aligned. The ATP-binding sites are shown as molecular surfaces, with residues color-coded as follows: green = hydrophobic; light blue = polar; dark blue = basic; pink = acidic. ATP is depicted as CPK stick models. The corresponding residues at the opening of the binding cleft that differ significantly (K89, T107 and V100 in CDK2, CDK and CDK7, respectively) are labeled, as are the Thr residues in the activation segments that are phosphorylated.

and this protein-protein interaction thus offers a good starting point for peptidomimetic design of CDK2- and CDK4-selective drug leads (46, 47). This approach is currently being assessed and progress with peptide minimization and conformational constriction (Fig. 5b) suggests that drug-like peptidomimetics may be feasible (41, 43, 48).

A recent report suggests yet another way in which CDK activity may be inhibited. It is known that cyclin A binding induces a structural reorganization in CDK2 that is necessary for subsequent phosphorylation of Thr^{160} by CAK and full activation (49). The structural details of this process are best understood in the context of CDK2 and cyclin A, but similar processes probably apply in the activation of other CDK/cyclin pairs (50). The reorganization is believed to be a two-step process (51). First, the PSTAIRE helix of CDK2 is bound between the α3 and α5 helices of cyclin A, followed by additional contacts of cyclin A with the *C*-terminal lobe of CDK2 (Fig. 5b). Together these interactions lead to exposure of the activation segment T-loop and formation of a catalytically competent ATP-binding cleft and active site (52). The contact surface between cyclin A and CDK2 in the final activated complex is extensive and one would therefore expect that pharmacological interruption of this association might be difficult. Nevertheless, it has recently been

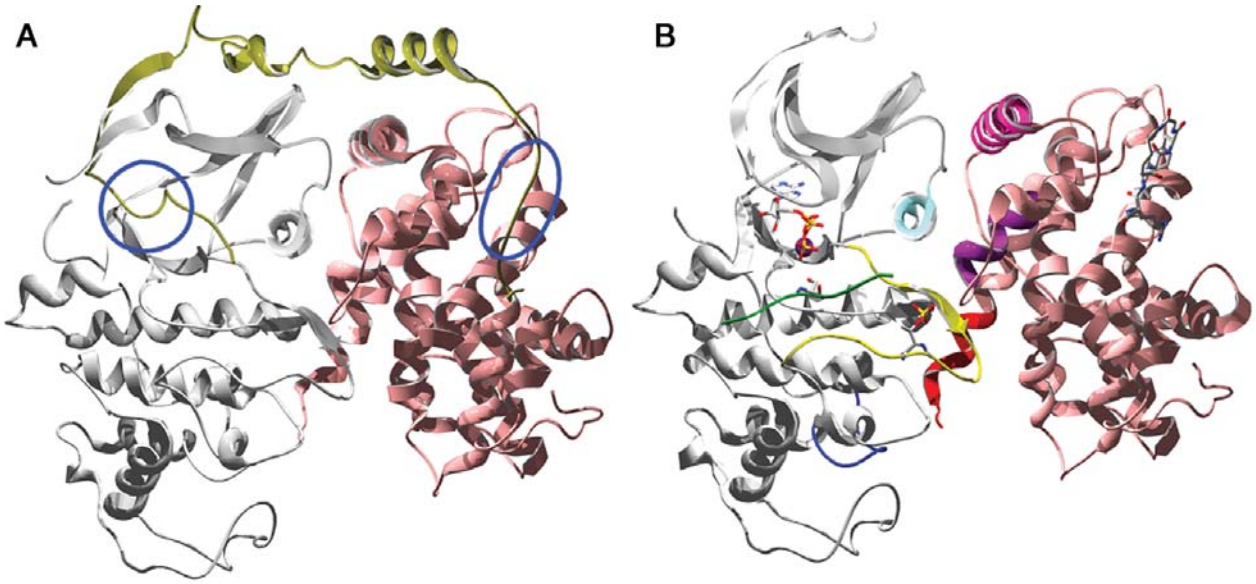


Fig. 5. Inhibition of CDK function through ATP antagonism or blockade of protein-protein interactions. A. The tumor suppressor protein $p27^{KIP1}$ (yellow) inhibits the complex between CDK2 (grey) and cyclin A (pink) by blocking the ATP-binding site in the kinase subunit, as well as the substrate recruitment site of cyclin A (PDB # 1JSU [39]). B. The PSTAIRE helix (light blue) of CDK2 is clamped between the α3 (magenta) and α5 (purple) helices of cyclin A. Rearrangement of the CDK2 structure following this interaction involves contacts with the activation segment (T-loop; yellow) and results in further interactions between the *N*-terminal helix of cyclin A (red) and parts of the *C*-terminal lobe of CDK2 (blue). In B, ligands for both the ATP-binding site and the substrate recruitment site are shown (CPK stick models), *i.e.*, Mg^{2+}/ATP and a cyclic inhibitor peptide derived from $p27^{KIP1}$. Constructed from PDB # 1QMZ [44,204] and 1URC (48).

reported that a peptide derived from residues 285-306 in the α5 helix of cyclin A specifically inhibits CDK2/cyclin A kinase function (53). Furthermore, when linked to a cell-penetrating vector, this peptide blocked tumor cell proliferation. Although the molecular mode of action remains unclear, it was found that the peptide does not compete with cyclin A for CDK2 binding, but in fact forms stable complexes with CDK2/cyclin A.

Applications in different therapeutic indications

Proliferative disorders

1. Oncology

The four most advanced clinical-stage CDK inhibitors are the 2,6,9-trisubstituted purine seliciclib, the flavonoid alvocidib, the 7-hydroxystaurosporine derivative UCN-01 (**3**) and the indolylbenzenedisulfonamide indisulam (**4**) (Fig. 1, Table II) (1, 54). Of these agents, only seliciclib is a selective inhibitor of CDKs (CDK2, CDK7 and CDK9) (55), although both seliciclib and alvocidib are thought to exert their antiproliferative and proapoptotic effects on tumor cells predominantly through inhibition of CDKs that act on the CTD of RNAP-II, especially CDK9 (15, 56-58).

Because tumor cells depend on antiapoptotic proteins, *e.g.,* certain members of the Bcl-2 family and inhibitor of apoptosis (IAP) proteins, in order to be able to proliferate despite a compromised genome, inhibition of mRNA transcription can give rise to selective antitumor effects (22). Recently, the induction of apoptosis observed in many tumor cells in response to UCN-01 treatment has also been linked to transcriptional suppression of the antiapoptotic protein Bcl-x_L (59). Although UCN-01 inhibits CDKs and also other kinases, including checkpoint kinase 1 (CHK1) (60) and 3-phosphoinositide-dependent kinase 1 (PDK1) (61), the observed downregulation of Bcl-x_L does not apparently result from effects on the PDK1/protein kinase B (PKB)/Akt survival pathway, but could be due to CDK inhibition.

Clinical results with the four most advanced experimental CDK inhibitor drugs mentioned above were recently summarized (1, 54, 62-64). Apart from early safety and dose-finding monotherapy studies, the majority of trials to date have examined combinations with various existing chemotherapies. Based on preclinical data suggesting drug synergy, both seliciclib and alvocidib are being studied in combination with gemcitabine (Gemzar®; Lilly) (64-66) and docetaxel (Taxotere®; Sanofi-Aventis) (1, 67-71), especially in non-small cell lung cancer (NSCLC). Although some clinical responses have been observed, it is too early to say if CDK inhibitors will be truly useful when added to existing chemotherapy regimens.

The most promising clinical results observed to date, however, concern a mature dose-escalating phase I study in which alvocidib was administered alone as an i.v.

bolus followed by a 4-h continuous infusion to refractory, relapsed and genetically high-risk patients suffering from chronic lymphocytic leukemia (CLL). To date, 23 patients have been treated and an overall response rate of 43% was reported, with dose-limiting toxicity of acute tumor lysis syndrome (72). The dramatic effects of alvocidib in this study are probably related to the transcriptional inhibition mode of action discussed above, as well as a more effective dosing regimen compared to earlier clinical studies. Hematological malignancies appear to depend heavily on antiapoptotic proteins, especially Mcl-1 (73), which is downregulated by alvocidib (74-77). Seliciclib has similar effects on B-cell hematological malignancies *in vitro* and is also currently undergoing clinical trials in such indications (58, 78, 79).

In addition to the above-mentioned CDK inhibitors which have been in clinical development for a number of years, no fewer than five new compounds are currently in phase I clinical trials and many more are at the late preclinical development stage (Table II). The majority of these appear to be pan- and oligo-specific CDK inhibitors, but some also inhibit other Ser/Thr and Tyr kinases.

2. Nephrology

The glomerulus is the functional unit of the kidney responsible for blood filtration. It contains three different cell types: mesangial cells, glomerular endothelial cells and podocytes. Aberrant proliferation of these cells as a result of cell cycle dysregulation is implicated in a number of kidney diseases (80), including collapsing glomerulopathy, often associated with HIV infection. In this disorder, podocytes that normally are fully differentiated and nonproliferating undergo proliferation, dedifferentiation and apoptosis. Since the cell cycle machinery controls these latter processes, CDK inhibition is thought to offer a new therapeutic strategy for renal diseases (81). Indeed, it has been shown that seliciclib inhibits podocyte proliferation in experimental glomerular diseases and that this effect is associated with improvement in renal function (82). Patients with this HIV-associated nephropathy present with nephrotic syndrome and suffer from rapid deterioration of renal function. Histologically, the kidney presents a picture of collapsing glomerulopathy. Amelioration of nephropathy in a well-characterized HIV-1 transgenic mouse model of collapsing glomerulopathy was demonstrated with alvocidib (83) and seliciclib (84). In the latter case, reversal of collapsing glomerulopathy was observed in 8 of 12 treated (75 mg/kg p.o. every day for 20 days) animals, compared with progression in 10 of 12 control animals, demonstrating a significant therapeutic benefit. This effect did not correlate with decreases in kidney HIV-1 transgene expression, suggesting that suppression of HIV-1 transcription was not a prerequisite for the antiproliferative activity. These results provide proof of concept that targeting aberrantly proliferating renal parenchyma may be an effective therapeutic strategy for collapsing glomerulopathy.

Glomerulonephritis constitutes another group of glomerular diseases where abnormal proliferation of glomerular cells, especially mesangial cells, plays an important role in the pathology of the disease (85). Using a rodent nephrotoxic nephritis model of crescentic glomerulonephritis (86), it was recently reported that seliciclib reduced glomerular cell proliferation (87). Furthermore, seliciclib was effective in reducing the severity of renal impairment, acute glomerular inflammation and crescent formation, even when treatment was started after onset of the disease.

Lupus nephritis is yet another form of autoimmune renal disease where immune cell dysfunction is prominent, leading to the formation of autoantibodies. Since the cellular cell cycle inhibitor $p21^{WAF1}$ regulates CDK/cyclin complexes, controls T-cell proliferation and lupus development (88), the CDK inhibitor seliciclib was investigated in an animal model of lupus nephritis (89). It was found that in both preventive (early phase of disease) and therapeutic (established disease) models, seliciclib markedly prolonged survival and delayed the onset of the proteinuria characteristic of this disease.

3. Cardiovascular disorders

CDKs also represent potential drug targets for such vascular occlusive diseases as atherosclerosis, restenosis after angioplasty, transplant vasculopathy and graft atherosclerosis after bypass (90). Here, physiological and pathological insults to differentiated vascular smooth muscle cells (VSMCs), which are normally quiescent, cause cell cycle reentry and hyperplastic neointimal lesion growth, which contributes to pathogenesis. VSMC proliferation is regulated by the CDK-inhibitory tumor suppressor proteins $p27^{KIP1}$ and $p21^{WAF1}$ (91), and several of the gene therapy strategies based on these proteins, originally developed for oncology therapy, are now being studied successfully in preclinical models of vascular occlusive diseases. Similarly, antisense oligonucleotide approaches targeting cell cycle CDKs and cyclins have been studied in such models (reviewed in 90). At least two small-molecule ATP-antagonist CDK inhibitors –alvocidib (92) and CVT-313 (**12**) (Fig. 2) (93)– have been evaluated successfully in balloon angioplasty models. There is also an interest in CDK inhibitors to treat human coronary in-stent stenosis (94) and the feasibility of drug-eluting stents has been demonstrated with alvocidib (95).

Neurodegenerative disorders

Just like cancers, neurodegenerative diseases can occur spontaneously as a result of successive multiple somatic genetic lesions, or they can be of familial origin when neurons with pre-existing germ-line mutations acquire further lesions. However, unlike dividing stem cells where neoplastic transformation leads to uncontrolled proliferation, the fate of compromised terminally

differentiated neurons which have exited the cell cycle irreversibly is cell death (96). Both cases involve forced activation of the cyclin D/CDK4/6/pRb/E2F pathway, but with very different outcomes (97). The likely explanation for this difference has to do with the dual pro- and antiproliferative role of E2F transcription factors (98).

Apoptosis is an integral part of the development of the central nervous system and about half of all neurons eventually die. Similarly, unscheduled apoptosis is involved in various neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease and neuronal trauma (97, 99). Cell cycle regulators have been implicated in mediating neuronal cell death as a result of such diverse stimuli as neurotrophic factor deprivation, DNA damage, exposure to β-amyloid and stroke. Of particular therapeutic interest is Alzheimer's disease, which represents the leading cause of senile dementia. This disorder is associated with the extracellular accumulation of β-amyloid plaques and intracellular neurofibrillary tangles (99). Aberrant expression of cell cycle proteins, including cyclin D and CDK4, has been closely correlated with the pathology of Alzheimer's disease (100).

Since neuronal cell death can be suppressed upon inhibition of mRNA or protein synthesis, this process must require the activation of a specific genetic program. It has been demonstrated that elevation of cyclin D-associated CDK activity upon neurotrophic factor deprivation is instrumental in the induction of apoptosis in terminally differentiated neurons and that the G1 CDK-specific tumor suppressor protein $p16^{INK4}$ can protect neurons from apoptotic cell death (101). The phosphorylation by CDK4 and CDK6 of pocket proteins (such as pRb) leads to a reduction in transcriptional repression by complexes between E2F transcription factors and pocket proteins. The subset of genes derepressed in this manner includes not only cyclin E, the activation of which by CDK2 promotes S-phase entry, but also proapoptotic factors such as B- and C-*myb*, which are implicated in apoptotic neuronal cell death (102). Further phosphorylation of pocket proteins by CDK2/cyclin E permits E2F transactivational activity, which in turn induces many of the genes required for cell cycle progression (103). Because dominant-negative CDK4 and CDK6 protect neurons from apoptosis upon neurotrophic growth factor withdrawal, whereas dominant-negative CDK2 does not (104), derepression of proapoptotic genes as a result of cyclin D-associated kinase activity appears to underlie neuronal apoptosis. It follows from these arguments that a convincing rationale may exist for selective pharmacological CDK4/6 inhibition in neurodegenerative diseases.

Another CDK closely associated with the nervous system is CDK5 (Table I). Although expressed ubiquitously, it plays a central role in the development and functions of the CNS, as well as in neurodegeneration upon deregulation (105). The brain activators of CDK5 are cyclins known as p35 and p39, both of which are substrates for calpains, whose proteolytic activities give rise to the p35- and p39-derived *N*-terminally truncated fragments p25 and p29, respectively. Deregulated CDK5 activity on neuroskeletal proteins such as tau and on synaptic proteins as a result of neurotoxic insult is mediated by these fragments (106, 107).

The structural basis for hyperactivation of CDK5 by p25 has recently been elucidated and will hopefully lead to the structure-guided design of CDK5-specific inhibitors (108). CDK5 is closely related in structure to both CDK2 and glycogen synthase kinase-3 (GSK-3) and many pan-CDK inhibitors, including seliciclib and alvocidib, as well as indirubins (**13**) and aloisins (*e.g.,* aloisin A [**14**] [109]) (Fig. 2), also inhibit CDK5 (110, 111). However, there has been at least one report of a CDK5-selective inhibitor, *i.e.,* a compound with undisclosed structure referred to as indolinone D (112). Apparently this compound is > 15-fold selective for CDK5 with respect to CDK1, CDK2 and CDK4, and possesses neuroprotective properties due to a cytostatic effect resulting from crossinhibition of cell cycle CDKs. These results suggest that the development of truly CDK5-specific inhibitors for therapeutic application in neurodegenerative diseases may in fact be feasible. However, despite the exhaustive demonstration that ATP-antagonist CDK inhibitors may have neuroprotective properties in cell culture systems (113-118), it remains unclear if any of these compounds are actually capable of crossing the blood-brain barrier (119, 120), a property necessary for them to qualify as leads for CNS-related therapeutic application.

Heart failure

The latest potential therapeutic application for CDK concerns not abnormalities in the proliferative state of particular cells in diseased tissues, but rather is related to the involvement of CDK9 in hypertrophic growth and mitochondrial dysfunction of cardiomyocytes (121). Pathological hypertrophy is likely an initial adaptive compensatory response to offset wall stress and to maintain cardiac pump function, but ultimately it can lead to degeneration to decompensated dilated cardiomyopathy. Such heart failure is accompanied by increased myocyte apoptosis. It has been found that RNAP-II CTD phosphorylation by CDK9 is essential for cardiac growth and that CDK9 is genetically activated early in cardiac hypertrophy. Furthermore, CDK9 confers vulnerability to apoptotic cues and apoptosis induced by CDK9 has been attributed to a selective defect in the expression of genes for mitochondrial function due to suppression of a master gene for mitochondrial function, *i.e.,* peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) (122). As a consequence, partial CDK9 inhibition to restore baseline transcriptional activity has been proposed as a therapeutic strategy to combat cardiomyocyte hypertrophy (121).

Parasitic infection

Parasitic organisms are found in most eukaryotes and the parasites with the greatest impact on human health

belong to the protozoan phyla Apicomplexa and Kinetoplastida (123). Because of the evolutionary distance between the human host and these parasites, it has been suggested that conserved functional proteins, especially protein kinases, that have provided validated drug targets in humans, may also be amenable to the discovery and development of specific antiparasitic drugs (123, 124). There is an urgent need for new antimalarial drugs, as well as drugs for neglected tropical infectious diseases caused by trypanosomatids, such as sleeping sickness, Chagas' disease and cutaneous and visceral leishmaniasis (125, 126).

Protozoans contain CDK-related kinases (CRKs); in *Plasmodium falciparum* –the main plasmodial species causing malaria– the *P. falciparum* protein kinases 5 and 6 (PfPK5 and PfPK6), as well as MO15-related protein kinase from *P. falciparum* (Pfmrk), have been demonstrated to possess CDK activities (127-129). PfPK5 and 6 are most closely related to human CDK1 (60% identity in the case of PfPK5), whereas Pfmrk is most homologous with human CDK7 (40% identity) (130). Similarly, trypanosomatids such as *Trypanosoma brucei* and *Leishmania mexicana* also contain several CRKs (131-133).

To be regarded as a validated antiprotozoal drug target, the protein in question must be essential for parasite survival or development, and it must be divergent enough from related host proteins to permit specific interference (134). The latter condition may be met in a functional sense for CRKs, due to the major differences in the regulation of parasite *versus* human cell cycles. It remains to be seen, however, if there are sufficient structural differences between parasite CRKs and human CDKs to permit the design of truly selective drugs. In the case of trypanosomatids, gene disruption *via* homologous recombination showed that, for example, *CRK1* and *CRK3* of *L. mexicana* and *CRK2* of *T. brucei* are essential genes for parasite proliferation (135-137). Functional studies of essential genes in malaria parasites are more difficult (134), but plasmodial CRKs are nevertheless being pursued as novel antimalarial targets (138). Furthermore, target validation of *Plasmodium* kinases using the analogue-sensitive kinase allele (ASKA) reverse chemical genetics technique –for which proof of concept has already been established with human CDKs (139, 140)– has been initiated (123, 134).

One of the first studies aimed at the discovery of new parasite kinase inhibitors examined the effects of a range of purine derivatives –many of which were known as inhibitors of human CDKs– on the *in vitro* growth of *P. falciparum* (141). Several compounds were able to kill the parasite at low micromolar concentrations, including purvalanol B (**15b**) (Fig. 2), a highly potent inhibitor of human CDKs. Unlike purvalanol A (**15a**), this compound lacks antiproliferative activity against human cells, presumably because of its poor membrane permeability, ascribed to the presence of a carboxylic acid function in **15b** (123, 142).

It has been speculated (123) that **15b** may interfere with parasite growth due to the presence of efficient transporters (the parasite does not synthesize purines *de novo*) that may help to accumulate exogenous purines (143, 144), perhaps including **15b**. Alternatively, **15b** may target an essential parasite ectokinase located at the membrane of infected host cells. Affinity chromatography of *P. falciparum* extracts using immobilized **15b** in fact revealed not a CRK, but the parasite casein kinase-1 homologue PfCK1 as the major target (123, 145). The fact remains, however, that **15b** is a potent CRK inhibitor; it was reported to inhibit PfPK5 with an IC_{50} of 130 nM (corresponding human CDK2 IC_{50} = 6 nM) and to bind PfPK5 directly by co-crystallization (Fig. 6b) (146). The results from this study are particularly interesting because a crystal structure of the **15b**/human CDK2 complex is also available (Fig. 6a). Despite the evolutionary distance

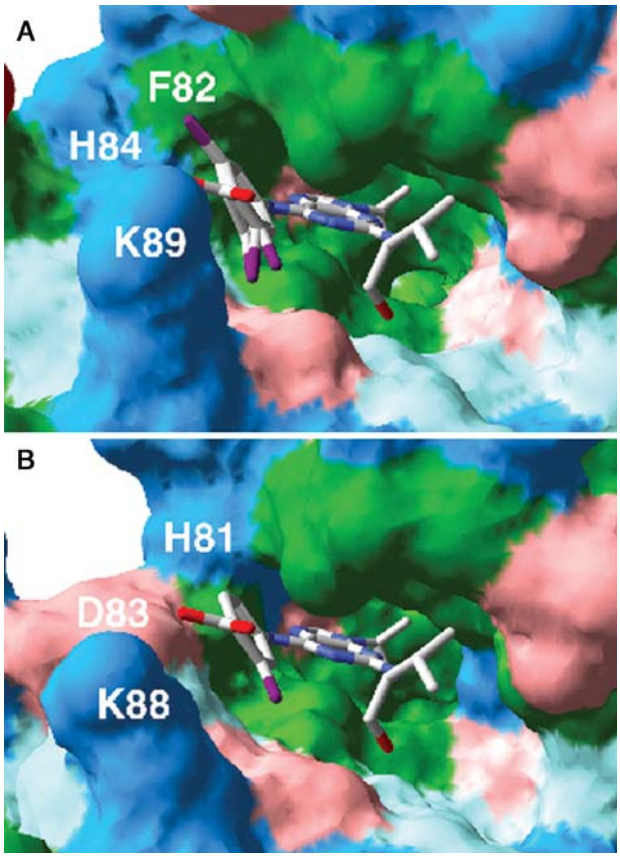


Fig. 6. Recognition of purvalanol B (15b) by CDK2 and PfPK5. The crystal structure coordinates of the complexes of 15b with CDK2 (A; PDB # 1CKP [142]) and PfPK5 (B; PDB # 1V0P [146]) were aligned (overall 0.93 Å RMSD C^{α} fit). The ATP-binding clefts are shown as molecular surfaces (residue coloring: green = hydrophobic; light blue = polar; dark blue = basic; pink = acidic). The most divergent residues lining the ATP-binding site are labeled. The ligand 15b is shown as CPK-colored stick models. Its chloroaniline ring adopts two alternative co-planar conformations when bound to CDK2 (both are shown and are superimposed with the PfPK5-bound conformation in A, whereas a single conformation is observed in PfPK5 (only PfPK5-bound pose shown in B).

between *P. falciparum* and humans, the conserved functional CDKs are closely related in structure; in fact the PfPK5 and CDK2 ATP-binding sites are very similar. Nevertheless, there are structural differences at the entrance to the ATP-binding pocket that may be exploited in structure-based drug design (Fig. 6) (147). Another well-known human CDK inhibitor, alvocidib, has also been shown to inhibit PfPK5 and the parasite *in vitro* (148).

On the other hand, the other plasmodial CRK, Pfmrk, is not inhibited by classical purine-based CDK inhibitors and a pharmacophore model has been presented to explain this result (127); unlike PfPK5 and human CDK1, CDK2 and CDK4, which contain a conserved Leu residue in the hydrophobic pocket responsible for inhibitor binding, Pfmrk has a bulkier Phe (F143) residue at the corresponding position (138).

A number of quinolinones have been reported as Pfmrk inhibitors; the most potent compound was the 3-phenylquinolinone **16** (Fig. 2), with an IC_{50} of 18 μM (149). Various oxindoles have been described as inhibitors of human CDKs (15, 150, 151) and this pharmacophore has also yielded comparatively selective Pfmrk inhibitors; the 3-alkylidene-1,3-dihydroindol-2-one **17** was reported to inhibit Pfmrk with an IC_{50} of 1.4 μM and to be > 100-fold and approximately 20-fold selective with respect to PfPK5 and human CDK1, respectively (152). The latest report on Pfmrk inhibitors discloses various active compounds from a pharmacophore model-driven screen of the 290,000-member Walter Reed Army Institute of Research compound collection (153), amongst them the 4-azaindolo[2,1-*b*]quinazoline-6,12-dione **18**, the most potent Pfmrk inhibitor reported to date (IC_{50} = 0.13 μM) (154).

The search for inhibitors of the trypanosomatid CRKs has also now been initiated. Based on the hypothesis that CRK3 is the most likely candidate for the functional homologue of human CDK1 in *L. mexicana* (132), and that it is inhibited by alvocidib (136), a microtiter plate-based assay was devised with this kinase and was used to screen a diverse collection of human CDK inhibitors (155). Numerous compounds were found to inhibit CRK3 and to possess antileishmanial activity in an *L. donovani*-infected mouse macrophage assay. Active compounds belonged to various well-known CDK-inhibitory compound classes, such as 2,6,9-trisubstituted purines, paullones and staurosporine derivatives. The most active compounds were the indirubins **13a** and **13b** (IC_{50} for CDRK3 and hCDK1 = 47 and 51 nM, and 16.5 and 5 nM, respectively). Indirubins have antitumor activity and have previously been shown to inhibit human CDKs, as well as the closely related GSK-3 (156 and references cited therein). Surprisingly, none of the active compounds exhibited specificity for CRK3; the indirubins were about equipotent against CRK3 and human CDK1.

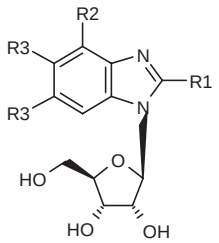
Very recently, 1*H*-pyrazolo[3,4-*b*]pyridines such as **19a** and **19b** (Fig. 2) were reported as potent antileishmanial compounds (*e.g.,* **19b** had an IC_{50} of 64 nM in an *L. amazonensis* promastigote assay) (157). Although the molecular target for these compounds in the parasite is not clear, it is likely to be a kinase, as pyrazolopyrimidines and pyrazolopyridines are frequently encountered ATP-competitive pharmacophores (158). The target may possibly be a CRK, since somewhat similar 1*H*-pyrazolo[3,4-*b*]pyridines, *e.g.,* **20** (Fig. 2), have been shown elsewhere to be potent inhibitors of human CDKs (159, 160).

Viral infection

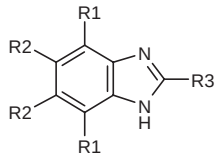
All human pathogenic viruses, regardless of whether or not they encode their own protein kinases, rely on host cell kinases to phosphorylate viral proteins and thus to regulate viral functions. CDKs are particularly prominent in this respect, since they are required for the nuclear replication of both DNA and RNA viruses. Some viruses require their host cells to cycle because they do not encode their own DNA polymerases, and cell cycle CDKs are thus important for their replication. However, several viruses that can replicate in both cycling and noncycling cells, such as herpes simplex viruses (HSV-1 and HSV-2), hepatitis B virus (HBV) and human immunodeficiency viruses (HIV-1 and HIV-2), also require CDK activities for viral gene expression. For these reasons, CDKs have been proposed as cellular targets for antiviral drugs in general (33, 161, 162). Indeed, seliciclib has been shown to inhibit the replication of a whole range of viruses, such as human cytomegalovirus (163), varicella-zoster virus (164), as well as HSV-1 and HIV-1 (165). Here, the discussion will be confined to the most promising viral application, *i.e.,* in HIV-1 infection (166).

The story of the potential application of CDK inhibitors in HIV therapy starts with and can be traced to the purine nucleoside analogue 5,6-dichloro-1-β-*D*-ribofuranosylbenzimidazole (**21a**; Fig. 7), a compound that has long been known as a specific inhibitor of mRNA transcription mediated by RNAP-II (167). Long before the discovery of the roles of CDKs in viral transcription, this compound was shown preferentially to inhibit the stimulation of transcription by the HIV-1 protein Tat (tansactivator of transcription) (168).

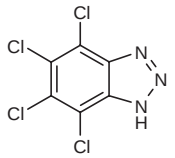
Tat enhances the processivity of RNAP-II elongation complexes, which initiate transcription in the HIV long terminal repeat (LTR) region. During elongation, Tat binds to the highly structured transactivation-responsive (TAR) RNA element present at the 5'-end of nascent viral transcripts (169). Independently, Tat was also found to bind P-TEFb (170), which contains a kinase activity that is responsible for the phosphorylation of the CTD of RNAP-II and is necessary for the promotion of transcription by P-TEFb (171). The picture that emerged from these and other findings shows that, before activation of infected cells, the HIV LTR produces mostly short transcripts because of low levels of P-TEFb. As cells are activated, cyclin T1 (one of the activating partners of CDK9) levels, and then Tat levels, rise. P-TEFb-Tat complexes form, associate with TAR, cause hyper-phosphorylation of the CTD and activate the LTR to produce full-length mRNAs (21). The recruitment of P-TEFb to TAR RNA is neces-



21a: $R^1 = R^2 = H$, $R^3 = Cl$
21b: $R^1 = I$, $R^2 = H$, $R^3 = Br$
21c: $R^1 = H$, $R^2 = R^3 = Cl$
21d: $R^1 = Me$, $R^2 = H$, $R^3 = Cl$



22a: $R^1 = H$, $R^2 = Br$, $R^3 = Cl$
22b: $R^1 = R^2 = Br$, $R^3 = I$



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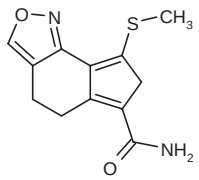

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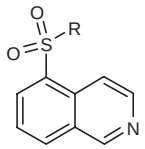

25a: R = 2-Me-1-piperazinyl
25b: R = 1-piperazinyl
25c: R = $NH(CH_2)_2NHC(=NH)NH_2$

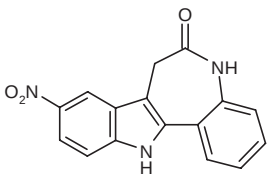

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Fig. 7. CDK inhibitors with antiviral activity.

sary and sufficient to activate transcription elongation from the HIV-1 LTR promoter, and recruitment of P-TEFb thus appears to be the sole function of the Tat/TAR axis in viral transcription (172). It turned out that the P-TEFb kinase activity is due to a cdc2-related kinase now known as CDK9 (173). Since both the function and kinase activity of P-TEFb are inhibited by **21a**, this compound can be regarded as the prototypical CDK9 inhibitor (171). In fact, **21a** was one of the hits in an early screen for new antiviral lead compounds using a Tat-dependent transcription assay, along with several other structurally related ribofuranosylbenzimidazoles (**21b**-**d**), benzimidazoles (**22**), a triazole (**23**), as well as the structurally comparatively unrelated isoxazole **24** and the isoquinoline sulfoanamides **25** (of which **25a**, known as H7, was a previously known cAMP-dependent kinase [PKA] inhibitor [174]), that were found to target the CDK9 kinase activity of P-TEFb (175).

Various cell-based studies using P-TEFb immunodepletion, P-TEFb knockdown through RNA interference (RNAi) and anti-cyclin T1 antibodies showed that host cell CDK9 activity is essential and limiting for HIV-1 replication (175-179). It follows that pharmacological targeting of this kinase may be an important new therapeutic antiviral strategy, especially since this approach might circumvent the problem of emerging resistance to therapies targeting genetically flexible viral components (165). The question remains, however, if the selective sensitivity of viral *versus* host cell transcription towards CDK9 inhibition –hinted at by cell-based studies with **21a**– will translate into a useful therapeutic safety margin. Findings with seliciclib and alvocidib, both of which are potent but comparatively unspecific CDK9 inhibitors, support this possibility. Both these experimental oncology drugs have been demonstrated to suppress HIV-1 replication in infected cells at concentrations that were not cytotoxic to uninfected cells (56, 180, 181). In the case of seliciclib, it was further suggested that this compound selectively killed HIV-1-infected cells without virion release (180). Alvocidib is the most potent CDK9 inhibitor reported to date, with a subnanomolar K_d value (56); a structural basis for the inhibition of this kinase has been proposed (182).

A recent study examining the effect of a number of different CDK inhibitors on uninfected or HIV-1-infected T-cell lines, as well as primary T-lymphocytes and macrophages, also showed selective killing of infected cells by some CDK-inhibitory compounds (183). The most potent compound was alsterpaullone (**26**) (Fig. 7), a CDK1/CDK2/CDK5 and GSK-3 inhibitor possibly with additional CDK9-inhibitory properties (184), which gave a CC_{50} (50% cytotoxic concentration) of 1.8 μM in chronically infected CEM (a human T-lymphocytic leukemia

cell line) cells and a selectivity of > 100 (CC_{50} infected/CC_{50} uninfected CEM cells). At present, it is not known if selective killing of HIV-1-infected cells can be achieved with CDK inhibitors *in vivo*, but this prospect is potentially very exciting, since it might enable viral load reduction without chronic drug administration. Furthermore, a number of CDK inhibitors have been shown to be effective against both wild-type and drug-resistant strains of HIV *in vitro*, apparently without selecting for drug resistance (185).

CDKs other than CDK9 have also been implicated in the regulation of Tat-dependent transcription. CDK2 has been suggested to form part of a transcription complex that is required for Tat-dependent transcription and CDK2/cyclin E is involved in phosphorylation of the RNAP-II CTD and Tat itself (186, 187). A very recent report showed that CDK2/cyclin E is loaded onto the HIV-1 genome *in vivo* and that seliciclib is able to inhibit this activity; it was argued that targeting CDK2, which has been shown to be dispensable for cell survival, is a compelling strategy to inhibit both wild-type and mutant HIV-1 strains (188). Both CDK2 and CDK7 fulfill important roles in host cell cycle control. It will therefore be important to determine what the optimal CDK inhibition profile is for achieving antiviral effects while sparing uninfected cycling cells (189).

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