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# Pharmacological inhibitors of cyclin-dependent kinases

Marie Knockaert, Paul Greengard and Laurent Meijer

Cyclin-dependent kinases (CDKs) regulate the cell division cycle, apoptosis, transcription and differentiation in addition to functions in the nervous system. Deregulation of CDKs in various diseases has stimulated an intensive search for selective pharmacological inhibitors of these kinases. More than 50 inhibitors have been identified, among which >20 have been co-crystallized with CDK2. These inhibitors all target the ATP-binding pocket of the catalytic site of the kinase. The actual selectivity of most known CDK inhibitors, and thus the underlying mechanism of their cellular effects, is poorly known. Pharmacological inhibitors of CDKs are currently being evaluated for therapeutic use against cancer, alopecia, neurodegenerative disorders (e.g. Alzheimer’s disease, amyotrophic lateral sclerosis and stroke), cardiovascular disorders (e.g. atherosclerosis and restenosis), glomerulonephritis, viral infections (e.g. HCMV, HIV and HSV) and parasitic protozoa (*Plasmodium* sp. and *Leishmania* sp.).

The discovery of protein phosphorylation and protein kinases by E. Krebs and E. Fischer in the 1950s opened the way to an amazing flow of data on how protein kinases are regulated and control cell fate. It is now clear that phosphorylation of serine, threonine and tyrosine residues plays a fundamental role in essentially

all molecular aspects of cell life and that protein kinases constitute major pharmacological targets [1]. As the sequencing of entire genomes is reaching completion in several organisms, we realize that all species are equipped with a rich set of kinase genes (e.g. humans: >850 kinases among 30 000 genes; *Caenorhabditis elegans*: 493 kinases among 19 099 genes; *Drosophila melanogaster*: 251 kinases among 13 601 genes; and *Saccharomyces cerevisiae*: 124 kinases among 6000 genes). Among Ser/Thr kinases, cyclin-dependent kinases (CDKs) have been characterized extensively during the past two decades. The pioneering work of L. Hartwell, P. Nurse and T. Hunt on cyclins and CDKs has recently received prominent recognition. Cdc2 (CDK1), the first known member of this family, was initially discovered for its cell cycle functions in yeast. Homologues were soon identified in species ranging from plants to mammals. Independently, the discovery of cyclins in sea urchin embryos was also followed by the unravelling of the extensive conservation of cyclins

## Box 1. Human CDKs and cyclins, and their functions

Cyclin-dependent kinases (CDKs) are Ser/Thr kinases, displaying the typical 11 subdomains of all kinases [a–c]. The strong sequence homology between CDKs suggests that their three-dimensional structures will be similar. CDK2, CDK2–cyclin A, CDK2–cyclin-A–p27<sup>kip1</sup>, CDK2–cyclin-A–peptides, CDK2–cyclin M, CDK2–KAP, CDK5–p25, CDK6–p19<sup>INK4D</sup> and CDK6–viral-cyclin have been crystallized. CDK1 and CDK4 have been modelled from the CDK2 structure. The overall shape of CDKs is shared by all kinases: a small N-terminal lobe consisting mostly of  $\beta$ -sheets and a large C-terminal lobe, formed essentially from  $\alpha$ -helices. The ATP-binding pocket is located between the two lobes. The binding of cyclin induces important changes in the CDK structure, forcing the kinase into an active conformation.

Among the 13 CDKs and 25 cyclin-box-containing proteins obtained from human genome sequencing, only those known to interact in a complex are presented in Fig. 1. CDKs are only active in association with a regulatory partner (i.e. cyclin or other protein). Other potential CDKs include the PCTAIRES, PFTAIRES, PITAIRES, KKIALRE, PISSLRE and NKIAMRE (named after the sequence of their PSTAIRES motif, a conserved motif located in the cyclin-binding domain). ‘Orphan’ cyclins include cyclins F, G, I, M, O and P.

### Functions of CDKs

CDKs play essential functions in cell cycle control (shown in red in Fig. 1), transcription (brown), neurones (cyan), differentiation (orange), cell death (black) or other events (purple).

### Cell division cycle

CDKs regulate progression through the G1, S, G2 and M phases of the cell division cycle. In early–mid G1, extracellular signals modulate the activation of CDK4 and CDK6 associated with D-type cyclins. These complexes phosphorylate and inactivate the retinoblastoma protein (pRb), resulting in the release of the E2F and DP1 transcription factors, which control the expression of genes required for G1/S transition and S phase progression. The CDK2–cyclin E complex is responsible for G1/S transition but also regulates centrosome duplication. During S phase, CDK2–cyclin A phosphorylates various substrates allowing DNA replication and the inactivation of G1 transcription factors. Around the S/G2 transition, CDK1 associates with cyclin A. Later, CDK1–cyclin B appears and triggers the G2/M transition by phosphorylating a large set of substrates. Phosphorylation of the ‘anaphase promoting complex’ by CDK1–cyclin B results in transition to anaphase and completion of mitosis. These successive waves of CDK–cyclin assembly, activation and inactivation are regulated tightly by post-translational modifications and intracellular translocations, and are coordinated and dependent on the completion of previous steps, through so-called ‘checkpoint’ controls.

### Transcription

Four CDKs regulate transcription. CDK7–cyclin-H–MAT1 is a component of the transcription factor TFIIH. Both CDK7–cyclin H and CDK8–cyclin C phosphorylate the large subunit of RNA polymerase II, required for elongation. CDK9–cyclin T is a component of the transcription factor P-TEFb. It is required for the kinase-dependent HIV-1 Tat transactivation. PITLSRE kinases (CDK11) are also very important in regulating RNA processing and transcription, in association with the recently described cyclin L (Ania-6).

### Neuronal functions

CDK5, activated by p35 and p39, and their proteolytic cleavage products, p25 and p29, respectively, play numerous functions in the nervous system, including neurite outgrowth, neurone migration, and metabotropic glutamate receptor and dopamine signalling pathways. Ania-6 (cyclin L) appears to activate the PITLSRE (CDK11) kinases, in dopamine and glutamate signalling.

### Differentiation

Activation or inhibition of CDKs plays a central role in differentiation, although no general picture can be drawn at present. Examples include the positive effects of CDK5 and CDK9 and negative effects of CDK2 in the MyoD-mediated differentiation of muscle cells, the involvement of CDKs in the differentiation of lens cells, and the re-differentiation of leukaemic cells by selective inhibition of CDK2 and CDK6.

### Cell death

CDK1, CDK4 and CDK6 play essential functions in neuronal cell death, and inhibition of these kinases results in neuroprotection. By contrast, dividing cells undergo apoptosis when exposed to pharmacological inhibitors of CDKs. CDK5 and CDK11 also participate in apoptosis.

### Other functions

CDKs are also involved in Golgi membrane traffic, insulin exocytosis by pancreatic  $\beta$ -cells, and retinal phosphodiesterase regulation.

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across phyla. Thirteen CDKs and 25 proteins with homology in the cyclin box are encoded in the human genome (Box 1). Although CDKs were initially studied for their cell cycle functions, it gradually became clear that some members of the family played roles in totally different contexts (Box 1).

### Pharmacological inhibitors: diversity of structures, similarity of action

Frequent deregulations of CDKs in cancers [2] provided the main impetus to the active search for pharmacological inhibitors of these kinases.

The first reported pharmacological CDK inhibitors (6-dimethylaminopurine and isopentenyladenine) were neither particularly active nor selective. However, they provided the first grasp on inhibitory structures, and constituted the starting point for the search for more potent and selective inhibitors. More than 50 inhibitors have now been described, some being active at nanomolar concentrations. Their structures

were recently extensively reviewed [3]. In this article, only the compounds that have been co-crystallized with CDK2 (or modelled with a CDK) will be described (Table 1, Fig. 1).

Despite striking chemical diversity, all CDK inhibitors share some common properties: (1) they have low molecular weights (<600); (2) they are flat, hydrophobic heterocycles; (3) they act by competing with ATP for binding in the kinase ATP-binding site; (4) they bind mostly by hydrophobic interactions and hydrogen bonds with the kinase; and (5) the backbone carbonyl and amino side-chains of Leu83 act, respectively, as an H-bond acceptor and an H-bond donor to the inhibitors, whereas the backbone carbonyl of Glu81 often acts as an H-bond acceptor (Fig. 1). The atomic interaction between inhibitors and CDKs is extensively described in the articles listed in Table 1 [4]. Figure 1 also illustrates how the small ATP-binding site can accommodate a large diversity of structures. Interestingly, inhibitors of CDKs that act via

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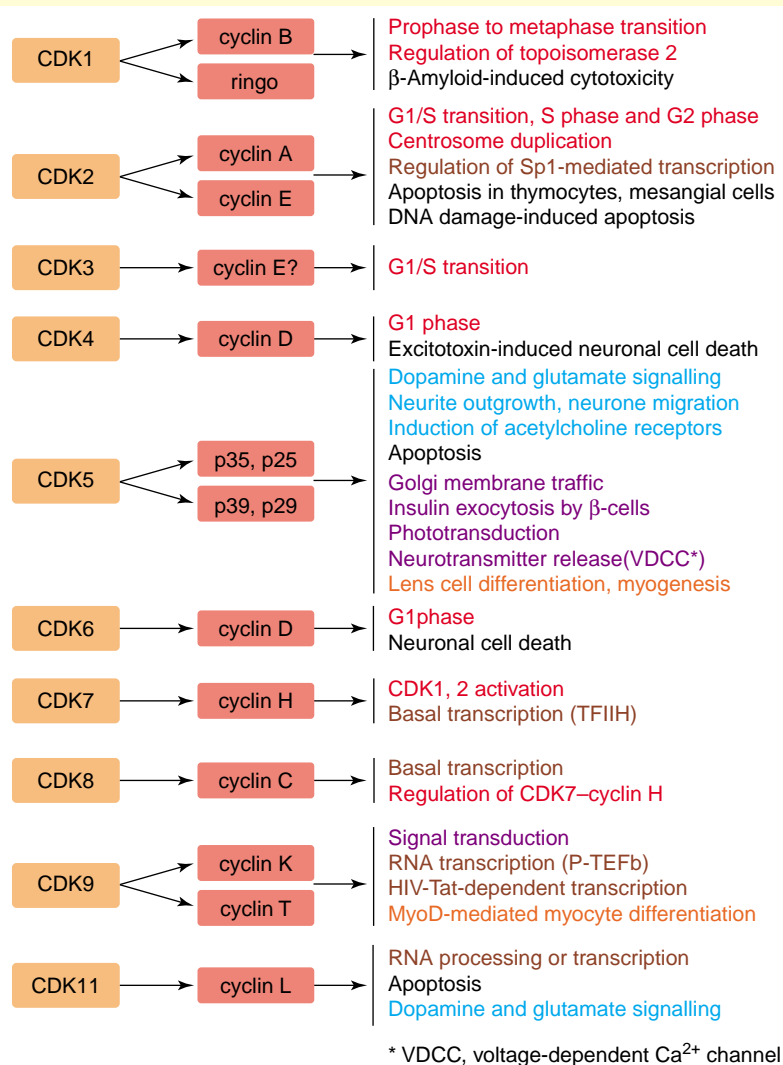


Fig. 1

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mechanisms other than competing with ATP have not been described, despite intensive screening and despite the other obvious possibilities of kinase inhibition (e.g. competition with substrate, interference with cyclin binding, and simulation of the natural protein inhibitors).

#### The selectivity of inhibitors

Within the CDK group of kinases, CDK inhibitors fall into three classes: those that are not selective for any specific CDK [e.g. deschloroflavopiridol, flavopiridol, oxindole 16 (compound 3) and oxindole 91], those that inhibit CDK1,2,5 (and possibly CDK9) [e.g. olomoucine, (*R*)-roscovitine, purvalanol B, aminopurvalanol (NG97), hymenialdisine, indirubin-3'-monoxime, indirubin-5-sulfonate, SU9516 and alsterpaullone], and those that are selective for CDK4,6 (e.g. fascaplysin, Compound 66, PD0183812, Compound 26a, Compound 15b and CINK4). No inhibitor that is selective for a single CDK has been

discovered. This is probably due to the conservation of the amino acids lining the CDK ATP-binding pocket [5,6].

Although CDK inhibitors are primarily selected and optimized in CDK screens, what are their real intracellular targets? Most researchers address this question by running their compounds on panels of purified enzymes. This classical selectivity approach remains time-consuming and rather unsatisfying, when we think of the number of non-tested kinases (800+), and of other potential non-kinase binding proteins. It must be admitted that the more we investigate the selectivity of a compound, the less it appears to be selective! Nevertheless some compounds (e.g. flavopiridol and staurosporine) are definitely less selective than others (e.g. purvalanols and paullones).

As an alternative way to identify the targets of CDK inhibitors, we have developed an affinity chromatography method using immobilized inhibitors [7,8] (Fig. 2). Briefly, a linker chain is attached to a suitable place on the inhibitor (an inhibitor-CDK co-crystal structure is of great help in choosing the most appropriate attachment site). The linker is then covalently bound to an agarose matrix. Control, inactive analogues can also be bound to a resin. Extracts from various tissues or cells are loaded on the inhibitor matrix. After extensive washing the beads are analysed by SDS-PAGE for interacting proteins, which are identified by microsequencing. In addition to confirming the interaction with CDKs from cellular extracts, this method has led to the identification of additional, sometimes unexpected, targets of CDK inhibitors: extracellular signal-regulated kinase 1 (ERK1) or ERK2 (purvalanol) [7], mitochondrial malate dehydrogenase (paullones) [8], glycogen phosphorylase (flavopiridol) [9,10] and cytosolic aldehyde dehydrogenase (flavopiridol) [9].

When CDK inhibitors are used as pharmacological tools in cell biology to demonstrate the involvement of a particular CDK in a cellular process, selectivity is a key issue. By contrast, absolute selectivity might not be the best approach to cure complex disorders where multiple pathways are deregulated. Indeed, combinations of effects rather than a single effect might yield better therapeutic agents. Knowing their range of targets will help to develop agents with improved clinical efficiency by providing information both on the set of targets that need to be hit to obtain the desired pharmacological effect and on the enzymes the inhibition of which is undesirable. Identification of the 'good' and 'bad' targets might help to circumvent toxic side-effects.

#### Pharmacological inhibitors of CDKs: diversity of applications

Although the search for inhibitors of CDKs was initially directed towards applications against cancers, they are also being evaluated currently for other indications (Fig. 3).

Table 1. Selectivity of pharmacological inhibitors of CDKs<sup>a,b</sup>

Inhibitor	Class	CDK1– cyclin B	CDK2–cyclin A, E <sup>c</sup>	CDK5– p25	CDK4– cyclin D <sup>c</sup>	ERK1,2 <sup>d</sup>	GSK-3	Refs
N <sup>6</sup> -Isopentenyladenine	Purine (adenine)	45	50 (A)	80	>100	90	?	[59]
Olomoucine	Purine (adenine)	7	7	3	>1000	30 (1), 50 (2)	100	[59]
(R)-Roscovitine	Purine (adenine)	0.45	0.7	0.16	>100	34 (1), 14 (2)	130	[60]
Purvalanol B	Purine (adenine)	0.006	0.006 (A), 0.009 (E)	0.006	>10	3.33 (1), ? (2)	>10	[61]
Aminopurvalanol (NG97)	Purine (adenine)	0.033	0.033 (A), 0.028 (E)	0.020	?	12 (1), 2.4 (2)	?	[62]
OL567	Purine (adenine)	0.23	?	?	?	?	?	[63]
H717	Purine (adenine)	0.23	0.050 (E)	?	2	?	?	[64]
NU2058	Purine (guanine)	5*	12*	?	?	?	?	[65]
NU6027	Pyrimidine	2.5*	1.3*	?	?	?	?	[65]
Hymenialdisine	Pyrrroloazepine	0.022	0.07 (A), 0.04 (E)	0.028	0.6	0.47 (1), 2 (2)	0.01	[66]
Deschloroflavopiridol	Flavone	?	?	?	?	?	0.45	[67]
Flavopiridol	Flavone	0.4	0.1 (A)	?	0.4	?	0.45	[67]
Staurosporine	Indololocarbazole	0.006	0.007	?	<10	0.020	?	[68]
Fascaplysin	–	>100	>50 (A), >50 (E)	20	0.35 (D1)	?	?	[69]
Compound 66	Pyridopyrimidine	0.675	0.129 (A), 0.410 (E)	?	0.032	?	?	[70]
PD0183812	Pyridopyrimidine	>40	0.21 (A), 0.17 (E)	?	0.008	?	?	[71]
Oxindole 16 (compound 3)	Oxindole (indolinone)	0.78	0.060	?	?	?	?	[72]
Oxindole 91	Oxindole (indolinone)	0.11	0.010 (A)	?	0.13	? (1), 21 (2)	0.056	[18]
Indolylmethylene-indolinone 8a	Indolinone	5.8	?	25	?	?	>100	[73]
Indolylmethylene-indolinone 8e	Indolinone	2.2	?	1.8	?	?	15	[73]
Indirubin-3'-monoxime	Oxindole (indolinone) (bis-indole)	0.180	0.44 (A), 0.25 (E)	0.10	3.33	>100	0.009	[74]
Indirubin-5-sulfonate	Oxindole (indolinone) (bis-indole)	0.055	0.04 (A), 0.15 (E)	0.065	0.3	38 (1), >100 (2)	0.28	[74]
SU9516	Oxindole (indolinone)	0.040	0.022 (A)	?	0.2	?	?	[75]
Compound 26a	Diarylurea	0.12	0.078	?	0.042	>1	?	[5,6]
Compound 15b	Diarylurea	1.80	0.44 (A)	?	0.0023	?	?	[5,6]
PKF049365	Arylpyrazole	1.3	1.6	?	?	?	?	[76]
PNU112455A	Aminopyrimidine	?	2* (E)	2*	?	? (1), >100 (2)	?	[77]
Alsterpaullone	Benzazepinone	0.035	0.015 (A) 0.2 (E)	0.040	>10	22 (1), 4.5 (2)	0.004	[78]
CINK4	Triaminopyrimidine	>100	>50 (A), >50 (E)	25	1.5 (D1)	?	?	[12]
Anilinoquinazoline 2	Quinazoline	1	?	?	?	?	?	[79]
Quinazoline 51	Quinazoline	?	0.65 (E)	?	>2.1	?	?	[80]

<sup>a</sup>Abbreviations: CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; GSK-3, glycogen synthase kinase 3.

<sup>b</sup>The IC<sub>50</sub> [or K<sub>i</sub> (indicated by an asterisk)] values of each compound tested on the indicated kinases are provided in micromolar concentrations. All compounds have been crystallized with CDK2, with the exception of SU9516 modelled with CDK2, alsterpaullone and indolylmethylene-indolinone 8a modelled with CDK1, and fascaplysin and compound 15b modelled with CDK4. Note that deschloroflavopiridol, not flavopiridol, was co-crystallized with CDK2.

<sup>c</sup>The specific cyclin is indicated in parentheses.

<sup>d</sup>The specific ERK is indicated in parentheses.

### Cancer

The effects of CDK inhibitors on the cell cycle and their potential value for the treatment of cancer have been extensively studied [2,11]. Three properties make CDK inhibitors attractive as potential anti-tumour agents. First, they are potent anti-proliferative agents, arresting cells in G1 [12] or G2/M [13]. Second, they trigger apoptosis, alone or in combination with other treatments [14]. Third, in some instances, inhibition of CDKs contributes to cell differentiation [15].

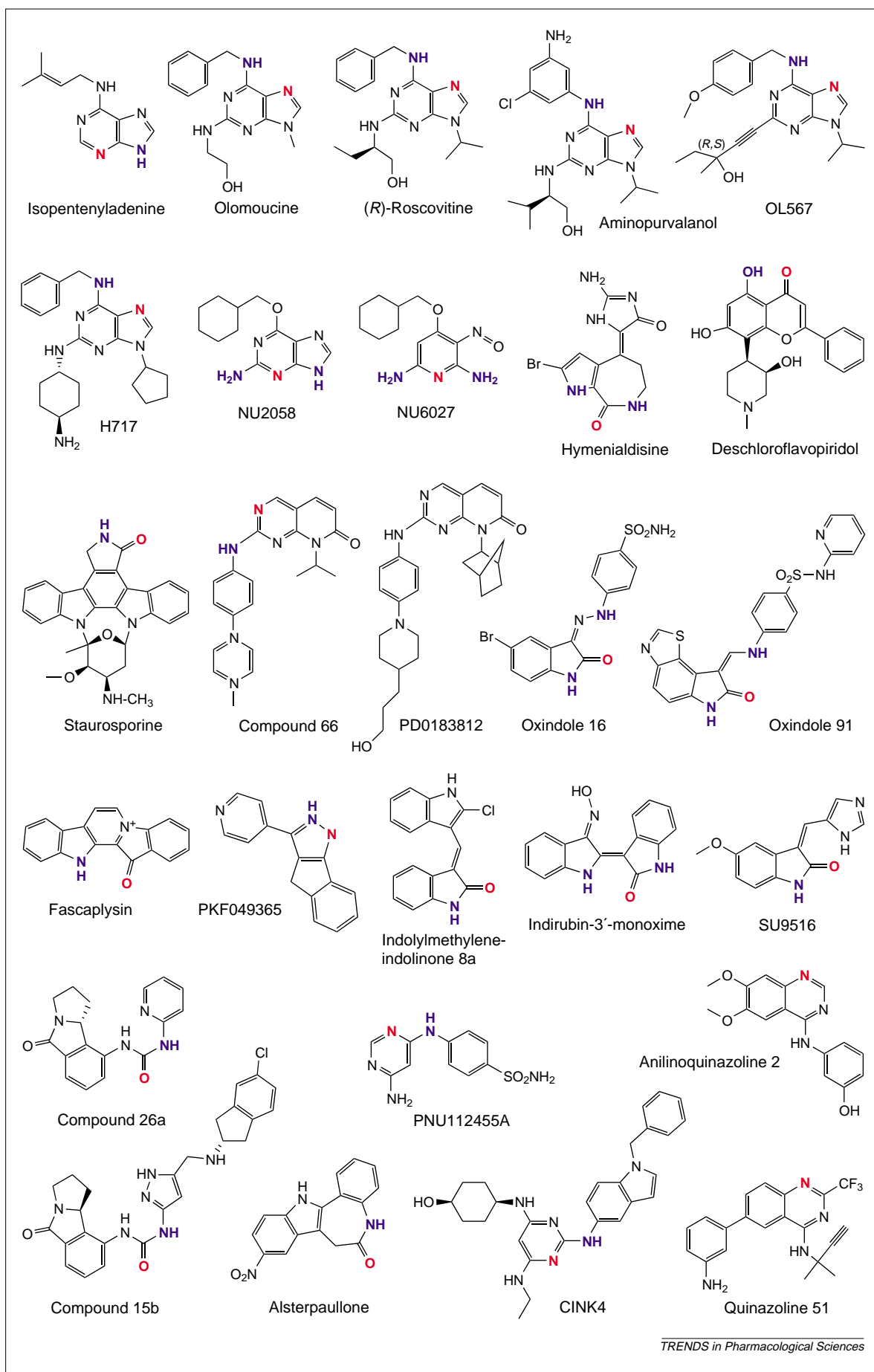
Only reports on the clinical trials of flavopiridol and UCN01 (7-hydroxystaurosporine) are available, but several other CDK inhibitors are currently making their way in the clinic. It seems likely that CDK inhibitors will be useful in the clinic in combination with compounds acting on other pathways.

To date CDK1,2 and CDK4,6 have been the most explored targets, but CDK7,8,9 clearly represent attractive targets. However, apparently selective compounds turn out to hit other targets as described above. Combined inhibition of targets can have either additive (inhibition of CDK1,2,7,9 and ERK1,2 by

roscovitine) [7] or opposing [inhibition of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and CDK1,2 by paullones] [16] actions on cell proliferation. At present no therapeutic agent used to treat cancer has been shown unequivocally to act through CDK inhibition. For example, flavopiridol, a definitive CDK inhibitor, interacts with many other targets and even acts as a global transcription inhibitor [17]. Surrogate markers clearly need to be developed for CDK inhibitors.

Effects on both cell cycle and apoptosis are necessary for effective cancer chemotherapy. In this respect the effects of CDK inhibitors are complex because they induce apoptosis in dividing cells but they protect normal cells from apoptosis induced by some but not all cytotoxic agents. This latter observation has led to the proposal to use CDK inhibitors to protect normal cells from chemotherapy-induced damage. For example, CDK inhibitors appear to prevent alopecia induced by current chemotherapeutic agents [18]. In another example the nonselective inhibitor, staurosporine, is used to arrest normal cells in G1, while the

Fig. 1. Structures of some of the most studied pharmacological inhibitors of cyclin-dependent kinases (CDKs). Only those that have been co-crystallized with CDK2 or modelled with CDK2 (CINK4), CDK1 (PNU112455A and alsterpallone) or CDK4 (anilinoquinazoline 2 and quinazoline 51) are shown (see Table 1 for references). The molecules are grouped by chemical families. The two or three atoms that interact with the backbone nitrogen and oxygen atoms of Leu83 and Glu81 (or Glu94 and Val96 in CDK4) are shown in red (H-bond acceptors) and blue (H-bond donors). Note that H-bond acceptor and donor atoms are not identical in all purines because isopentenyladenine, olomoucine, roscovitine, purvalanol (H717) and NU2058 are bound in totally different orientations within the CDK2 ATP-binding pocket. PD0183812, derived from Compound 66, has not been crystallized, but is the compound that has been used for cellular studies.



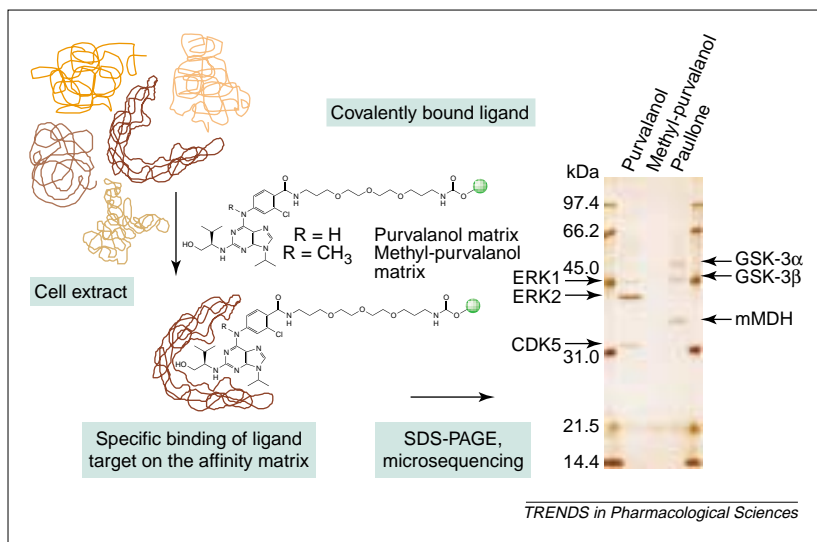


Fig. 2. Affinity chromatography purification of the targets of cyclin-dependent kinase (CDK) inhibitors. The inhibitors, purvalanol and paullone in this example, are immobilized to agarose beads through a polyethylene glycol linker. A control resin is also prepared using N<sup>6</sup>-methyl-purvalanol, a kinase-inactive compound. Cell or tissue extracts are then run on the matrices. After extensive washing, the affinity matrix-bound proteins are resolved by SDS-PAGE. The proteins are then excised from the gel and identified by microsequencing. In this example, using a brain extract, the targets of purvalanol were identified as extracellular signal-regulated kinase 1 (ERK1), ERK2 and CDK5. These kinases were absent from control beads. The targets of paullone were identified as glycogen synthase kinase 3 $\alpha$  (GSK-3 $\alpha$ ), GSK-3 $\beta$  and mitochondrial malate dehydrogenase (mMDH). Note a 21.5-kDa agarose-binding protein present in all lanes.

proliferation of tumour cells, lacking the G1 checkpoint controls, is unaffected [19]. Under these conditions, tumour cells are preferentially killed by doxorubicin or camptothecin. This idea of protecting normal cells has yet to be explored in the clinic.

#### Nervous system disorders

CDK5 is highly expressed in the nervous system where it phosphorylates a multitude of substrates [81]. Its multifunctional roles are well illustrated by the complex phenotypes of CDK5, p35 and p39 knockout mice [20]. CDK5 is normally activated by p35 or p39, and the basal activity of CDK5–p35 or CDK–p39 might be required for neuronal survival and development [21]. It has been reported that various neurotoxic insults trigger a Ca<sup>2+</sup>-dependent calpain-mediated conversion of p35 and p39 to p25 and p29, respectively, and that hyperactive CDK5–p25 or CDK5–p29 phosphorylates cytoskeletal proteins leading to neuronal cell death [22,23]. The reported deregulation of CDK5 observed in several neurodegenerative disorders suggests that CDK5 inhibitors might influence the outcome of some of these diseases.

One example of a disorder in which CDK5 might be deregulated is Alzheimer's disease (AD). A hallmark of AD is the intracellular aggregation of the microtubule-binding protein tau, following its abnormal phosphorylation (>20 sites) by several kinases, including CDK5 and GSK-3. Although the kinases that hyperphosphorylate tau constitute attractive targets for therapeutic intervention [24], the consequences of their selective inhibition for the development of AD remain elusive. Expression of cell cycle regulators (e.g. CDK1, cyclins B,D,E, CDC25, polo and CDK4) and mitosis-specific antigens (e.g. TG-3 and MPM-2) in post-mitotic neurones correlates with the onset of neurodegeneration in AD (references cited in [16,25]), suggesting that cell death is preceded by attempts of the injured cells to divide. Because many CDK inhibitors are active on GSK-3 [26] the combined inhibition of CDK1,2,5 and GSK-3

might constitute a pharmacological advantage. Another hallmark of AD is the extracellular accumulation of  $\beta$ -amyloid, initiated by presenilin- and BACE ( $\beta$ -site amyloid precursor protein cleaving enzyme)-dependent proteolytic cleavage of the  $\beta$ -amyloid precursor protein. Interestingly human CDK1 (and presumably CDK5) phosphorylates  $\beta$ -amyloid, and CDK inhibitors have been reported to prevent both  $\beta$ -amyloid phosphorylation [27] and  $\beta$ -amyloid-induced cytotoxicity [27,28].

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that leads to the loss of motor neurones, resulting in paralysis and death [29]. CDK5 has recently been implicated in ALS pathogenesis [30]. Twenty percent of the familial ALS cases are associated with missense mutations in Cu/Zn superoxide dismutase (SOD). Transgenic mice expressing a mutant SOD display an increase of the p25:p35 ratio in addition to abnormal localization and hyperactivation of CDK5. CDK5–p25 phosphorylates the neurofilament protein NF-H, leading to its accumulation, a characteristic ALS feature. Overexpression of NF-H in the mutant SOD mice leads to life span extension, suggesting that NF-H acted as a competitive substrate for CDK5, therefore making CDK5 inhibition an appealing approach to treat ALS.

*In vivo* excitotoxin-induced neuronal cell death leads to upregulation of CDK4 and cyclin D1 expression [31] and to conversion of p39 to p29 [32]. Global or focal cerebral ischaemia is accompanied *in vivo* by increased cyclin D1 and CDK4 expression [33–35] and decreased expression of the CDK inhibitor p16<sup>INK4A</sup> [36]. In cell cultures, oxygen–glucose deprivation is associated with an increase in CDK2 activity, and a decrease of p16<sup>INK4A</sup> and p27<sup>kip1</sup>. Pharmacological inhibitors of CDKs protect neurones from death *in vitro* but also *in vivo*, following focal cerebral ischaemia [34]. Infusion of CDK4 or cyclin D1 antisense oligonucleotides suppresses kainate-induced cell death [31]. These encouraging results are backed by numerous older studies that showed an overall association of CDK and GSK-3 activity with neuronal cell death and, conversely, the neuroprotective effects of pharmacological inhibitors of CDKs (and GSK-3) (references cited in [36]). Once again dual inhibition of CDKs and GSK-3 could be an advantage.

DARPP-32 (dopamine- and cAMP-regulated phosphoprotein 32 kDa) is a major neuronal intracellular protein that mediates the effects of many neurotransmitters such as dopamine. Among the kinases regulating its activity, CDK5 phosphorylates DARPP-32 on Thr75 and turns it into a cAMP-dependent kinase inhibitor [37]. Any CDK5 inhibitor will thus impinge on the neuronal events that are dependent on DARPP-32. Chronic cocaine administration to rats increases the level of the transcription factor  $\delta$ FosB, which triggers CDK5 overexpression. CDK5 thus appears to play a major role in the response of the brain to cocaine [38], and inhibitors of CDK5 might have clinical relevance.

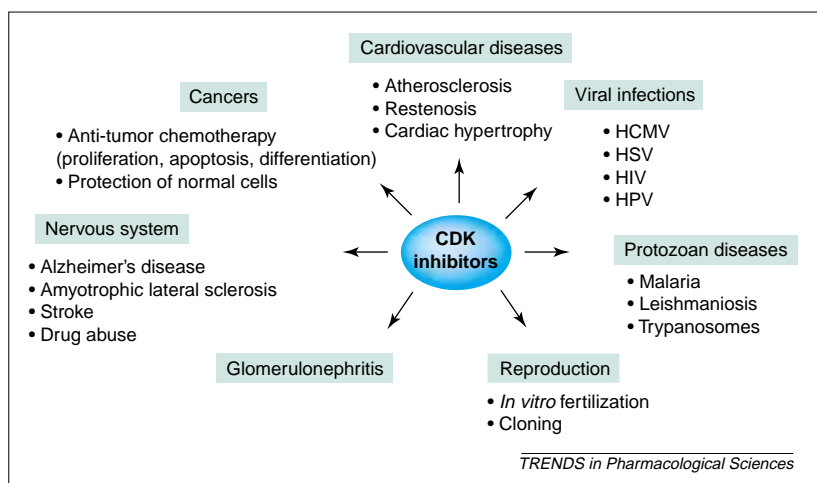


Fig. 3. The potential applications of cyclin-dependent kinase (CDK) inhibitors. Because CDKs are involved in cell proliferation, cell death, neuronal functions, transcription and other functions, pharmacological inhibitors of these kinases are evaluated in multiple therapeutic areas. Abbreviations: HCMV, human cytomegalovirus; HIV, human immunodeficiency virus; HPV, human papillomavirus; HSV, herpes simplex virus.

### Viral infections

The lack of potent antiviral drugs is aggravated by the resistance that rapidly develops with conventional drugs directed against viral proteins. Over the past few years, pharmacological inhibitors of CDKs have been reported to prevent viral replication *in vitro* [39]. The underlying mechanism of action, inhibition of cellular rather than viral targets, is unlikely to favour the appearance of resistant strains and could potentially be efficient against several unrelated viruses. Numerous viruses require active CDKs for their replication and some viruses actually encode their own cyclins, thereby regulating their host cell cycle.

Human cytomegalovirus (HCMV) activates CDK2 upon infection. Treatment with roscovitine or expression of a CDK2 dominant-negative mutant inhibits HCMV DNA replication, production of infectious progeny and late antigen production [40].

The best studied example is herpes simplex virus (HSV) [39,41,42]. Roscovitine inhibits transcription of HSV immediate-early, early and late genes and HSV DNA synthesis. Roscovitine interferes with the phosphorylation of the viral proteins ICP4 and ICP0, two global transcriptional activators of viral and cellular gene expression. As a consequence, the transactivating activity of ICP0 is reduced. The targets of roscovitine could be a combination of CDK1,2,7,9 and ERK1,2.

Transcription of the human immunodeficiency virus (HIV) is also inhibited by roscovitine [43] and flavopiridol [44]. This effect might be due to inhibition of CDK9–cyclin T1 [44,45], a cellular cofactor required for the HIV-1 transactivator Tat. As a consequence RNA polymerase II transcriptional activity and HIV-1 replication are potently inhibited. The use of CDK inhibitors for the treatment of glomerular lesions of HIV-associated nephropathy has been proposed recently [46]. Suppression of HIV-1 transcription by flavopiridol or roscovitine is accompanied by inhibition of podocyte proliferation and by re-expression of podocyte differentiation markers.

Human papillomavirus (HPV) is associated with 90% of cervical carcinomas. The HPV-16 E7

oncoprotein induces abnormal centrosome duplication, resulting in aberrant mitotic spindle poles and genomic instability, the probable cause of malignancy. This effect, potentiated by the E6 protein, is inhibited by expression of a dominant-negative CDK2 mutant or by roscovitine [47]. Interestingly, E7 stimulates the transcription of the CDK2 activating phosphatase CDC25A [48]. These early observations encourage the evaluation of CDK inhibitors for the control of HPV-induced tumours.

### Unicellular parasites

Unicellular parasites such as *Plasmodium falciparum* (malaria), *Leishmania major* (leishmaniasis), *Trypanosoma brucei* (sleeping sickness), *Trypanosoma cruzi* (Chagas disease) and *Toxoplasma gondii* (toxoplasmosis) are responsible for the world's most widespread diseases.

These microorganisms have genuine CDKs or CDK-related kinases. Despite 40–60% identity with human CDKs, their sequences diverge significantly from the closest mammalian homologues [49]. This opens a potential therapeutic window because structural differences between parasite and host CDKs might result in differential affinities for inhibitory molecules. The libraries of compounds synthesized to investigate the structure–activity relationship (SAR) of mammalian CDK inhibitors thus constitute an important resource to screen for protozoan CDK-selective inhibitors. This approach could be extended to other groups of parasites such as fungi or multicellular parasites (e.g. schistosomes, helminths and nematodes, among others).

Although mammalian CDK inhibitors have effects on protozoan CDKs and on the living parasites, their actual targets in the protozoan remain to be identified. For example, *PKCK1* (casein kinase 1) was recently identified as the major purvalanol target in *P. falciparum* [7].

### Glomerulonephritis

Certain forms of glomerular diseases, such as IgA nephropathy (the leading cause of glomerulonephritis worldwide) are characterized by mesangial cell proliferation and matrix production. Both are inhibited *in vivo* by roscovitine, resulting in improved renal function [50]. In human glomerular diseases associated with podocyte proliferation the expression of p27<sup>kip1</sup> and p57<sup>kip2</sup> increases. Moreover, glomerular disease in p21<sup>cip1</sup> and p27<sup>kip1</sup> null mice is associated with increased proliferation [51]. These diseases constitute potential targets for treatment with pharmacological inhibitors of CDKs, as illustrated in HIV-associated nephropathy [46].

### Cardiovascular diseases

Smooth muscle cell proliferation is common in atherosclerosis, the leading cause of coronary heart disease, and restenosis, a frequent consequence of balloon angioplasty, the main intervention for

symptomatic atherosclerotic lesions. These proliferative disorders constitute attractive indications for cell cycle inhibitors [52]. Flavopiridol [53] and an olomoucine derivative [54] inhibit vascular muscle cell proliferation *in vivo* following injury to the rat carotid artery. Cardiac hypertrophy is one of the complications that increases mortality as a result of cardiovascular diseases. The finding that overexpression of p16<sup>INK4A</sup> inhibits cardiac myocyte hypertrophy in culture but also *in vivo* [55] encourages the therapeutic evaluation of CDK inhibitors in this indication.

### Oocytes

The yield of *in vitro* production of domestic animal embryos is presently rather low because of the poor developmental capacity of *in vitro* matured oocytes. This is most probably linked to an incomplete terminal differentiation of the oocytes isolated from small ovarian follicles. Pharmacological inhibitors of CDKs are being evaluated as tools to prevent *in vitro* nuclear maturation (meiotic resumption) of mammalian oocytes, while allowing the cytoplasmic maturation (acquisition of fertilizability and subsequent developmental competence). Following reversible inhibition of nuclear maturation by CDK inhibitors and *in vitro* fertilization, the embryos can be cultivated with no loss of developmental

ability [56,57]. Work is in progress to define the 'premature' culture conditions that will allow inhibited oocytes to acquire maximal developmental competence after inhibitor removal.

Recently an improved survival rate of cloned calves was reported when the nuclei transferred to enucleated oocytes were obtained from roscovitine-synchronized adult somatic cells rather than from serum-deprivation synchronized cells [58].

### Concluding remarks

Substantial efforts from many research groups have led to the discovery, optimization and characterization of potent CDK inhibitors. Many have reached the nanomolar IC<sub>50</sub> level, and display an apparently good selectivity. However, their cellular targets remain to be identified. Most CDK inhibitors have anti-proliferative properties associated with apoptosis-inducing activity and display anti-tumoural activity. CDK inhibitors also prevent neuronal cell death associated with acute or chronic neurodegenerative disorders, and behave as neuroprotective agents *in vivo*. Chemical inhibitors of CDKs can also interfere with the replication of some viruses. Because CDKs are involved in multiple physiological pathways, it seems likely that numerous, currently unforeseen therapeutic indications will be discovered.

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