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DESIGNING INHIBITORS OF CYCLIN-DEPENDENT KINASES

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■ **Abstract** Cyclin-dependent kinases (cdks) play a pivotal role in controlling progression through the cell cycle. The complex mechanisms that control cdks have been elucidated and, in the case of cdk2, explained with reference to X-ray crystal structures of the catalytically active and inactive kinase. Deregulation of the cell cycle is commonly observed in cancer, so cdks are potential targets for experimental therapeutic agents. A number of distinct structural classes of cdk inhibitors have been discovered. Good selectivity among these ATP competitive inhibitors for cdks over other kinases has been established, and selectivity between individual cdks is often observed. The crystal structures of a number of key inhibitors bound to cdk2 can be used to explain the observed structure-activity relationships within the compound series and to guide the design of more potent inhibitors.

INTRODUCTION

The protein kinase class of enzymes catalyse the transfer of the terminal phosphate group from an adenosine triphosphate (ATP) molecule to the hydroxyl group of a tyrosine, serine, or threonine residue on an acceptor protein. The phosphorylation of one or more residues of a protein can change its function within the cell. For example, enzymes may gain or lose catalytic activity on phosphorylation, or proteins may gain binding affinity for other proteins. Thus, phosphorylation is a biological switching mechanism. For this reason protein kinases regulate and coordinate many aspects of cell biology including metabolic processes, growth, movement, differentiation, and division.

Many distinct protein kinase domain sequences have been elucidated. Analysis of these sequences along with crystallographic studies has revealed that all such kinases have similar structures (1). Conserved features have been observed in 12 subdomain regions that are important structurally and functionally. This common

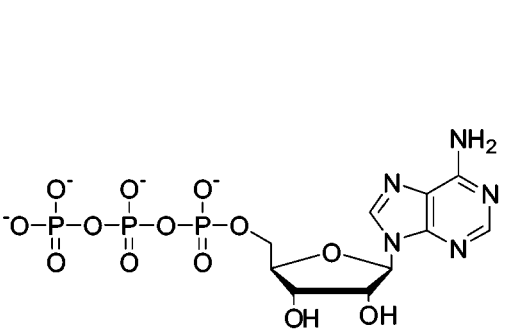
architecture is particularly apparent in the ATP binding domain, which contains three highly conserved residues in the catalytic region.

The cyclin-dependent kinase (cdk) family of enzymes is responsible for the orderly progression of cells through the various phases of the cell cycle. Unlike other protein kinases, cdks are regulated by binding to their cyclin protein partners, forming active heterodimeric complexes. Eight cdk family members (cdk1–cdk8) and nine cyclins (A–I) have been identified. The cdks have similar sizes (30–40 kDa) and share 40% homology, including the highly conserved catalytic core region of 300 residues. The cyclin subunits vary in size (30–80 kDa), but all contain a homologous 100–amino acid cyclin-box domain. The cdk subunits are not catalytically active unless bound to a cyclin partner, resulting in a complex with basal catalytic activity. Phosphorylation is required to yield the fully active complex. Regulation of cdk activity is essential for the stepwise execution of the many processes such as DNA replication and chromosone separation required for cell growth and division. Control of cdk activity throughout the cell cycle is achieved by a number of mechanisms. The levels of activating cyclin partners vary during the cycle, balanced by their synthesis and degradation. Likewise, levels of cdk inhibitor proteins vary as they are synthesized, bind to cdks, and target them for destruction. The phosphorylation required for activation of cdks occurs at a conserved threonine residue (Thr^{161} in cdk1) and is performed by a multisubunit enzyme called CAK. CAK itself consists of cdk7 and cyclin H. The action of two kinase enzymes, MYT1 and WEE1, deactivates the cdk-cyclin complex by phosphorylation of conserved threonine and tyrosine residues (Thr^{14} and Tyr^{15}, respectively, for cdk1). Dephosphorylation of these residues by CDC25 reactivates the complex. Deactivation of the cdk-cyclin complex is also regulated by the action of a diverse family of proteins called CKIs. Four major mammalian CKIs have been discovered. P21(CIP1/WAF1) and p27(KIP1) inactivate cdk2 and cdk4 cyclin complexes by binding to them. $P16^{INK4}$ and $p15^{INK4B}$ are specific for cdk4 and cdk6. They inhibit the formation of the active cyclin complexes by binding to the inactive cdk, and they can also bind to the active complex (2, 3).

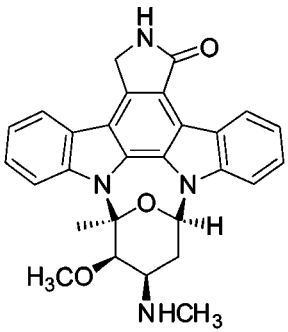
Cancer is often regarded as a disease of uncontrolled cell growth, so it is not surprising that many of the cell cycle regulatory mechanisms have been found to be altered in tumors. The $p16^{INK4a}$ inhibitor protein is a tumor suppressor and has been found to be mutated in approximately one third of human cancers. The action of $p21^{Cip1}$ is linked to that of p53. The *p53* gene is the most frequent site of mutation found in human cancers. Low levels of the related protein $p27^{Cip2}$ appear in many tumors and correlate with poor prognosis. Overexpression of cyclin D is commonly observed in breast cancer, and mutation of cdk4 has been observed in melanomas. In addition to this, the cell cycle machinery may be overdriven in response to many oncogenic signaling pathways.

The search for cdk inhibitors as potential cancer therapeutics is relatively new. It had been thought that the high degree of homology within the ATP binding domain of all protein kinases would make the design of selective ATP competitive agents impossible. This view was reinforced by the discovery from the screening of natural products of a number of ATP competitive protein kinase inhibitors, e.g.,

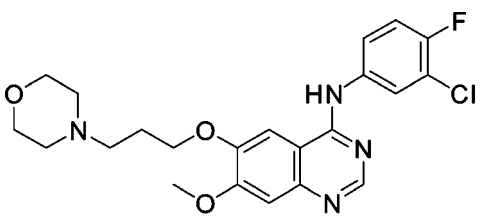
staurosporine [Structure *1*], which inhibited a wide range of kinases. However, the discovery of potent specific ATP-competitive inhibitors of epidermal growth factor tyrosine kinase, e.g., [Structure *2*] has encouraged the search for inhibitors against a wide range of protein kinase enzymes, including cdks. This review is concerned with the main classes of compounds that are selective for cdks. In particular, the structural basis for kinase inhibition with respect to the kinase ATP binding site is described.



ATP



Staurosporin (**1**)



Iressa (**2**)

STRUCTURAL STUDIES ON cdk2, ATP, AND CYCLINS

Extensive X-ray crystallographic studies have been carried out on cdk2 (4–7). The data have provided a detailed structural explanation for the activation of cdk2 by cyclins and CAKs. Cdk2 consists of the catalytic protein kinase subunit and little else. The tertiary structure of the protein is similar to other kinase enzymes, forming a fold between the ~80-residue N-terminal domain, a C-helix, and the ~120-residue α-helical C-terminal domain. The crucial ATP binding site is found in the cleft formed between the two domains. Importantly, the ATP binding site is present in the inactive monomeric cdk2. ATP binds to the protein, but the triphosphate moiety is aligned in a catalytically inactive conformation.

Binding of cyclin A to cdk2 produces little structural change in the cyclin partner but induces significant structural modifications to the cdk that produce an optimized ATP binding site. The major conformational changes involve movement

of the C-helix toward the active site cleft and the reordering of the αL12 helix to form a short β-strand β9. These changes allow catalytically important residues into the correct position in the active site. These residues include Glu^{51} and Lys^{33} on the C-helix, which coordinate to the α-phosphate of ATP, and Asp^{145}, Phe^{146}, and Gly^{147}, which form the "DFG" motif of β9 responsible for inducing the correct conformation in the triphosphate moiety of ATP for catalytic transfer.

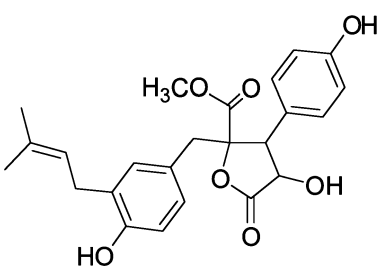
The cdk2/cyclin A complex requires phosphorylation at Thr^{190} to achieve full activity. Examination of the crystal structures shows that phosphorylation completes the structural changes in the active site by creating the peptide binding site. Phosphorylated Thr^{160} acts as an organizing center by making interactions with arginine residues to Arg^{50} in the C-helix, and to Arg^{126}, which is adjacent to the catalytic aspartate (Asp^{127}).

The crystal structure of monomeric cdk2 with ATP bound reveals important interactions within the active site cleft. ATP makes a number of hydrogen bonds to the backbone groups of the hinge region of the enzyme. Glu^{81} acts as an acceptor of a hydrogen bond from the N6 amino group of ATP, and N1 accepts a hydrogen bond from the backbone nitrogen of Leu^{83}. In addition to hydrogen bond interactions, favorable hydrophobic and Van der Waals contacts are formed between the enzyme and the adenine ring of ATP.

DISCOVERY AND DEVELOPMENT OF cdk INHIBITORS

Drug discovery programs in a number of centers have provided a variety of structural classes of compounds that are selective cdk inhibitors. The lead compounds have originated from a variety of sources including microbial and plant products, small-molecule screening collections, and combinatorial libraries.

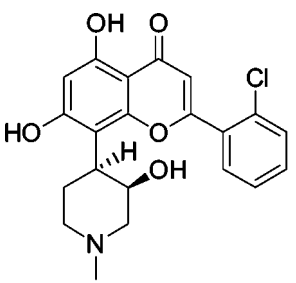
Butyrolactone I [Structure *3*] was identified from a culture of *Aspergillus* species, F-25799, as an inhibitor of cdk1 and cdk2 (IC_{50}'s of 2.6 and 0.82 μM, respectively) (8). Butyrolactone I [*3*] shows greater than 140-fold selectivity for cdk1/cyclin B ($IC_{50} = 0.68$ μM) over mitogen-activated protein kinase (MAPK) ($IC_{50} = 94$ μM) and other kinase enzymes. It is competitive with ATP, displaying a K_i of 0.36 μM against cdk1/cyclin B. Butyrolactone I [*3*] inhibits pRB phosphorylation in whole cell assays at high concentrations (IMR32 cells, 18h, $IC_{50} =$ 24 μM), possibly reflecting poor cellular uptake.



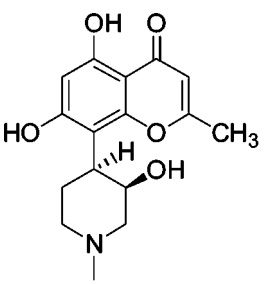
Butyrolactone I (**3**)

Flavopiridol [Structure *4*] is structurally related to a natural flavanoid alkaloid, rohitukine [Structure *5*], which was identified in a methanol extract of the stem-bark of the plant *Dysoxylum binectariferum* (9, 10). Flavopiridol [*4*] displays growth-inhibitory and cytotoxic activity in vitro, and it is growth inhibitory in human tumor xenograft models. Flavone [*4*] is active against a number of protein kinases including EGF-receptor tyrosine kinase, protein kinase A, and protein kinase C (IC_{50}'s of 21, 122, and 6 μM, respectively) but is most active against many cdk/cyclin complexes. Flavone [*4*] is active against cdk1/cyclin B ($IC_{50} = 0.3$ μM), cdk2/cyclin A ($IC_{50} = 0.1$ μM), cdk4/cyclin D ($IC_{50} = 0.4$ μM), and cdk7/cyclin H ($IC_{50} = 0.3$ μM) in an ATP competitive manner. Cells treated with [*4*] show a delay in S phase, consistent with inhibition of cdk2/cyclin B, followed by a block in the cell cycle at G_2, which may be caused by inhibition of cdk1/cyclin A and B and cdk7/cyclin H. Complete inhibition of cell cycle progression by [*4*] may be achieved at a concentration of 300 nM.

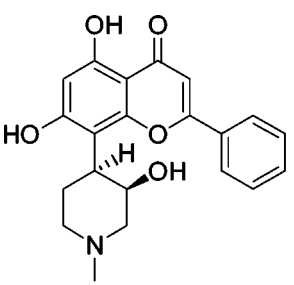
Comparison of the X-ray crystal structures of cdk2-ATP with cdk-L86 8276 [deschloroflavopiridol; Structure *6*] reveals the structural basis for inhibition. Compound [*6*] binds in the ATP binding pocket of cdk2, with the protein adopting a conformation almost identical to that observed with ATP bound. The benzopyran rings of [*6*] occupy the same space as the purine of ATP but with the benzopyran rotated 60° relative to the plane of the adenine. In this position the O5 hydroxyl group and the 4-carbonyl oxygen of [*6*] are close to the N6-amino and N1 in adenine. The binding of the heterocycle is predominantly hydrophobic and occupies similar space to adenine. The piperidinyl ring occupies part of the phosphate binding pocket. The phenyl ring, however, occupies space outside the ATP binding site and thus may provide a basis for understanding the observed selectivity. The main contacts in this region are with Leu^{83}, His^{84}, and Asp^{86}, and a repositioning of Lys^{89} is also observed. The chloro group of [*4*] is able to make additional contacts with Ile^{10}, Phe^{82}, and Leu^{93}, which could account for the observed 10-fold increase in potency for compound [*4*] compared with [*6*]. This area is highly conserved between cdk1 and cdk2, but in PKA there is no homology, which probably accounts for the 1000-fold difference in activity observed between cdk2 and PKA.



Flavopiridol (**4**)

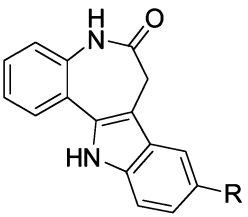


Rohitukine (**5**)



Deschloroflavopiridol (**6**)

The paullone class of compounds [Structure *7*] was discovered when flavopiridol [*4*] was used as the reference compound in a COMPARE analysis of the NCI compound collection in the Human Tumor Cell Line Anti-Cancer Drug Screen (11). The most potent compound identified was the 9-bromo substituted derivative kenpaullone [Structure *7a*], which has an IC_{50} of 0.4 μM against cdk1/cyclin B, and an apparent K_i of 2.5 μM, and is competitive against ATP. Compounds were also tested against a panel of 25 Ser/Thr and Tyr kinases, where [*7a*] displayed excellent selectivity for cdk1, cdk2, and cdk5 over other kinase enzymes. Unlike flavopiridol [*4*], [*7a*] displays no activity against cdk4/cyclin D1.

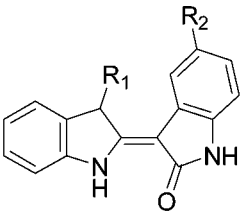


Paullones (**7**)
Kenpaullone R = Br (**7a**)

Indirubin [Structure *8a*] was identified as the active component of a traditional Chinese herbal remedy for chronic myelocytic leukaemia (12). Derivatives of indirubin, i.e., the 5-chloro derivative [*8b*], the 3′-monooxime [*8c*], and the 5-sulfonic acid [*8d*], are potent inhibitors of cdk1, cdk2, cdk4, and cdk5, but do not significantly inhibit other kinases (Table 1). Crystal structures of [*8c*] and [*8d*] bound to cdk2 have been determined. They show that both indirubin derivatives bind to cdk2 at the ATP binding site with a similar binding mode (Figure 1). The lactam amide nitrogen donates a hydrogen bond to the backbone carbonyl of Glu^{81}; the NH group of Leu^{83} donates a hydrogen bond to the lactam amide oxygen; and the indole NH acts as a hydrogen bond donor to the backbone oxygen of Leu^{83}. The latter hydrogen bond is not seen in the structure of ATP bound to cdk2. The indirubin derivatives occupy space similar to that of ATP in the binding site and make similar hydrophobic contacts. Both derivatives make an extra interaction with Phe^{80}, filling this area of the binding site more completely than ATP. The monooxime group of [*8c*] occupies the ATP-ribose binding pocket and does not make any direct interactions. In contrast, the sulfonic acid group of [*8d*] causes movement of the main chain at residues Asp^{145} and Phe^{146} to counter unfavorable interactions, which causes the enzyme to adopt a similar conformation to that seen when bound to cyclin A. Favorable ionic interactions between the sulfonic acid and Lys^{33} may account for the enhanced potency of this derivative.

A number of compounds of differing structures have been reported as cdk inhibitors following their discovery in a high throughput screen of small-molecule

TABLE 1 Inhibition of cyclin-dependent kinases by indirubin and analogues [Structure *8a–d*] [IC_{50} (μM)]



8a R_1 = O, R_2 = H; Indirubin
8b R_1 = NOH, R_2 = H; Indirubin-3′-monooxime
8c R_1 = O, R_2 = Cl; 5-Chloro-indirubin
8d R_1 = O, R_2 = SO_3H; Indirubin-5-sulfonic acid

Compound	cdk1/cyclin B	cdk2/cyclin A	cdk2/cyclin E	cdk4/cyclin D1	cdk5/p35
8a	10.0	2.2	7.5	12.0	5.5
8b	0.4	0.75	0.55	6.5	0.8
8c	0.18	0.44	0.25	3.33	0.1
8d	0.055	0.035	0.15	0.3	0.065

compound libraries [Structures *9–12*] for inhibitors of cdk4/cyclin D1 kinase activity (13). The active compounds were also assayed against cdk1/cyclin B and cdk2/cyclin E. The results are presented in Table 2.

Substituted pyrido[2,3-*d*]pyrimidin-7-ones have been reported as lead structures for cdk inhibitors (14). The initial 2-anilino compound [Structure *13a*] displayed modest potency against a range of cdk/cyclin complexes and some selectivity over other kinases, for example FGFr (see Table 3). Substitution at the

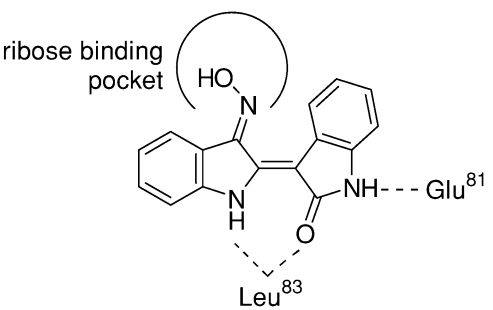
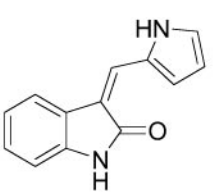


Figure 1 Binding mode of indirubin monoxime [*8b*] to cdk2.

TABLE 2 Inhibition of cyclin-dependent kinases by compounds [*9–12*] (IC_{50} (μM)



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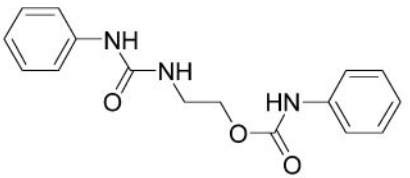

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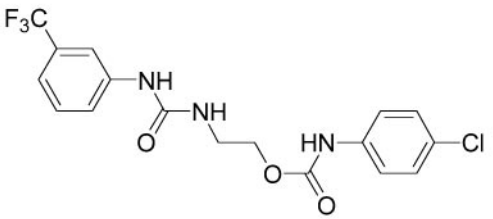

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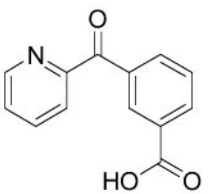


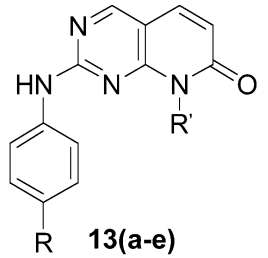
12

Compound	cdk4/cyclin D1	cdk1/cyclin B	cdk2/cyclin E
9	4.9	10.2	10.0
10	1.9	>30	>30
11	1.2	>30	>30
12	6.7	>30	>30

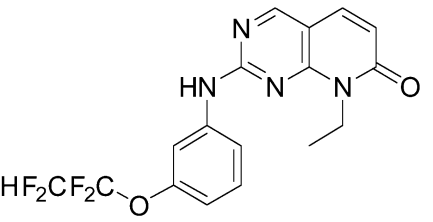
4-position of the anilino group with N-methylpiperazine [*13b*] resulted in an order of magnitude increase in potency against cdk4/cyclin D. In contrast, substitution at the 3-position with 1,1,2,2-tetrafluoroethoxy [*14*] reduced activity against cdk4/cyclin D. Optimization of the N8-position identified cyclohexyl [*13c*] and *exo*-1-bicyclo[2.2.1]hept-2-yl [*13d*] as the preferred substituents. Interestingly, substitution of the 2-anilino group with 4-(3-hydroxybutyl)piperidine and the N9-position with *exo*-1-bicyclo[2.2.1]hept-2-yl [*13e*] gave a compound with good selectivity for cdk4/cyclin D that was half as potent against cdk2/cyclin A and E and showed little activity against either cdk1/cyclin B or FGFr.

Meijer and coworkers (15) identified N^6-Dimethylaminopurine [Structure *15*] and N^6-(Δ^2-Isopentenyl)adenine [Structure *16*] as lead compounds for cdk inhibition, with IC_{50} values of 120 and 55 μM against cdk1, respectively. Screening of a collection of 80 related substituted purines for activity against cdk1, cdk4, and cdk5 revealed that purines substituted at C2 with an amino alcohol, at N6 with

TABLE 3 Inhibition of cyclin-dependent kinases by compounds [*13*(*a–e*)] and [*14*] [IC_{50} (μM)]



13(a-e)



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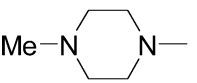
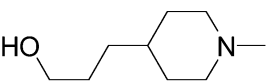
Ra = 

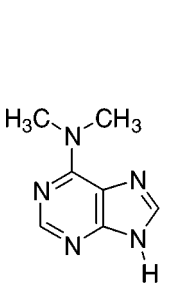
Rb = 

Rc = 

Compound	R	R′	cdk1/ cyclin A	cdk2/ cyclin A	cdk2/ cyclin E	cdk4/ cyclin D	FGFr
13a	H	Et	1.015	0.129	0.41	0.620	3.295
13b	Ra	Et	nr	nr	nr	0.085	nr
14	—	—	0.091	0.238	1.200	7.833	44.200
13c	H	cyclohexyl	nr	nr	nr	0.047	nr
13d	H	Rc	nr	nr	nr	0.038	nr
13e	Rb	Rc	>40	0.209	0.165	0.008	8.62

nr = not reported.

a benzyl or isopentenyl, and at N9 with a methyl or isopropyl group were inhibitors of cdk1 and cdk5 but not cdk4. Olomoucine [Structure *17*] inhibited cdk1 and cdk5 with IC_{50} values of 7 and 3 μM, respectively, but did not inhibit cdk4 and thus was selected for further study. Olomoucine [*17*] inhibits 1-methyladenine-induced G2/M transition in starfish oocytes in vivo and causes 50% growth inhibition in the NCI panel of 60 tumor cell lines at an average concentration of 60.3 μM, typically arresting cells at G1/S and the G2/M transitions. These cellular activities are consistent with inhibition of cdk1 and cdk2.



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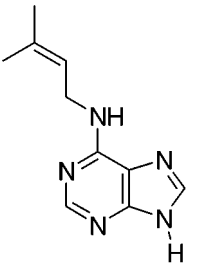

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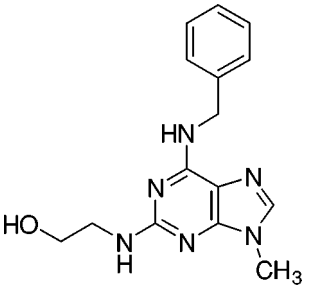
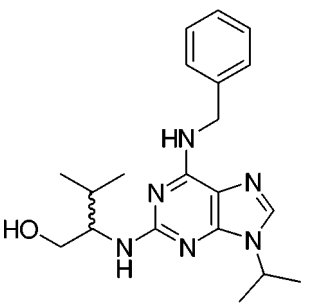


Olomoucine (**17**)

Other structure-activity studies have identified roscovitine [Structure *18*] as a more potent inhibitor of cdk1/cyclin B (16, 17). These studies defined the 2,6,9-substitution pattern around the purine heterocycle necessary for cdk1 inhibition. A polar side chain at the 2-position appears to be essential and results in a favorable increase in solubility. Interestingly, the roscovitine [*18*] enantiomer bearing the side chain with an (*R*) configuration is somewhat more active than the corresponding (*S*) isomer against cdk1/cyclin B (IC_{50} = 0.45 and 0.65 μM, respectively), but this difference was not seen for inhibition of cdk2/cyclin E. A benzyl group was the preferred substituent at N6 in this series. Roscovitine [*18*] displays similar selectivity to olomoucine [*17*] with IC_{50} values of 0.65 μM against cdk1/cyclin B, 0.7 μM against cdk2/cyclin A, 0.7 μM against cdk2/cyclin E, and 0.16 μM against cdk5/p35 but >100 μM against cdk4/cyclin D1 and cdk6/cyclin D2. The cellular effects of [*18*] have been investigated in a number of systems including the NCI 60 tumor cell line panel. All the tumor cell lines displayed similar sensitivity with an average IC_{50} of 16 μM (60.3 μM for olomoucine [*17*]). Analysis of the growth inhibitory activity of [*18*] in the NCI 60 tumor cell-line screen showed a pattern of activity that gave a strong correlation with data for flavopiridol [*4*] using the COMPARE analysis (17a).



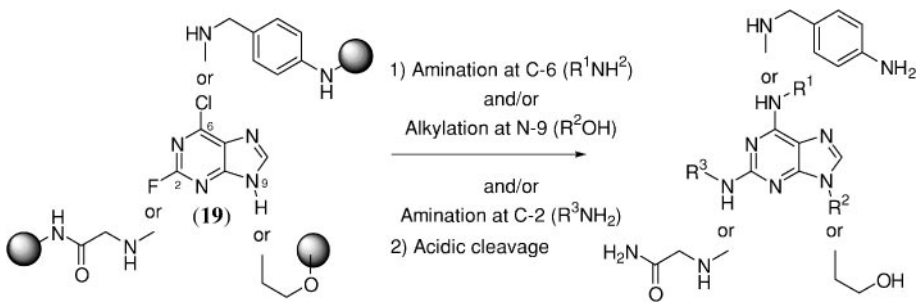
Roscovitine (**18**)

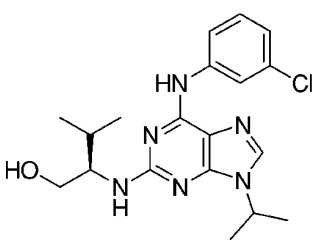
The X-ray crystal structures of olomoucine [*17*] and roscovitine [*18*], bound to cdk2, have been determined (18). The structures show the enzyme folded into its typical bilobal structure, and there are no significant differences between the

inhibitor-enzyme complexes and the ATP-enzyme complex. The inhibitors bind in the ATP binding site, in the cleft between the domains. The purine rings of the inhibitors occupy roughly the same plane as for ATP but in a completely different orientation, with N7 of [*18*] mapping close to N1 of ATP. There are two hydrogen bonds, between N6 and the carbonyl oxygen of Leu^{83} and between N7 and the backbone NH of Leu^{83}. This residue usually makes a hydrogen bond from the backbone nitrogen to N1 of ATP. In [*17*] and [*18*] the benzyl ring points away from the ATP binding site into unoccupied space in the ATP enzyme complex, and this makes Van der Waals contacts with Ile^{10}, Phe^{82}, and His^{84}. The polar side chains of [*17*] and [*18*] occupy the space filled by the ribose unit of ATP, although the orientation differs between the two. The N9 substituents occupy the region close to Lys^{33}, which is unoccupied in the ATP-enzyme complex.

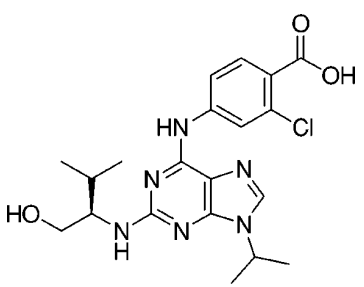
A combinatorial approach has been used to further optimize the 2,6,9-trisubstituted purines (19–21). Starting from 2-fluoro-6-chloropurine [Structure *19*], amine or anilino substituents were introduced sequentially, with one of the substituents attached to a solid support to facilitate rapid synthesis (Scheme 1). Compounds prepared this way were assayed against a variety of purified kinases in a high throughput manner. Two potent cdk inhibitors were identified, namely purvanalol A [Structure *20*] and B [Strucuture *21*]. The more potent of the two, [*21*], inhibits cdk2/cyclin A with an IC_{50} of 6 nM and thus is 1000-fold more potent than olomoucine. Both compounds show a high degree of selectivity for cdk1 and cdk2 (Table 4).

Scheme 1 Combinatorial synthesis of 2,6,9-trisubstituted purines.





Purvanalol A (**20**)



Purvanalol B (**21**)

TABLE 4 Inhibition of cyclin-dependent kinases by compounds [*20*] and [*21*] [IC_{50} (nM)]

Kinase	Purvanalol A [*20*]	Purvanalol B [*21*]
cdk1/cyclin B	4	6
cdk1/cyclin B (150 μM ATP)	40	50
cdk1/cyclin B (1.5 mM ATP)	500	250
cdk2/cyclin A	70	6
cdk2/cyclin E	35	9
cdk4/cyclin D1	850	>10,000
cdk5/p35	75	6
erk1	9,000	3,333

The increased potency of [*21*] over olomoucine [*17*] and roscovitine [Structure *18*] can be explained with reference to the crystal structure of the purvanalol B/cdk2 complex. The overall geometry of the purine ring for [*21*] is similar to that of [*17*] and [*18*], making hydrophobic and Van der Waals contacts with the enzyme. The purine ring also makes three hydrogen bond interactions with the enzyme; a pair of hydrogen bonds are seen for Leu^{83}, one between N7 and the backbone NH and another between the N6 amino group and the carbonyl oxygen (Figure 2). Additionally, a hydrogen bond is formed between the purine C8 hydrogen and the carbonyl group of Glu^{81}. The C2 side chain is bound in the ATP ribose binding pocket. The hydroxyl group makes a hydrogen bond with the backbone carbonyl of Gln^{131}, whereas the *R*-isopropyl packs closely to the backbone atoms of the glycine-rich loop.

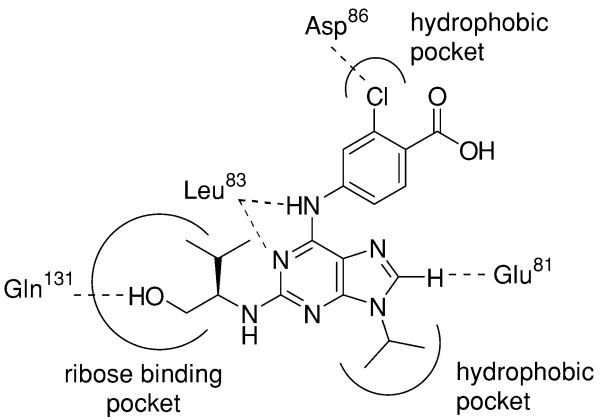
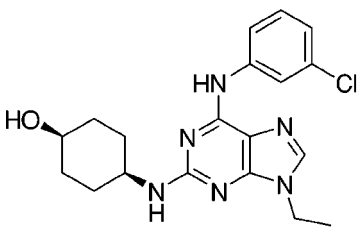


Figure 2 Binding mode of purvanalol B [*21*] to cdk2.

These interactions lead to the side chain adopting a different position compared with roscovitine [*18*]. The N6 anilino group points away from the ATP binding site into space not occupied in the ATP/cdk2 complex. The 3-chloroanilino group makes tight hydrophobic interactions with Ile^{10} and Phe^{82} side chains. Polar interactions occur between the chloro substituent and Asp^{86} in about two thirds of the molecules, whereas in the other conformation the ring is flipped by $\sim 160^{\circ}$. The N9 isopropyl group occupies a small hydrophobic pocket formed by the side chains of Val^{18}, Ala^{31}, Phe^{80}, Leu^{134}, and Ala^{144}. The improved potency of [*21*] may result from a combination of improved packing interactions, the steric constraints imposed by the 3-substituent, and the loss of conformational flexibility associated with the removal of the benzyl methylene group in [*17*] and [*18*].

A rational design approach based on modeling of the olomoucine-cdk2 crystal structure was employed by Imbach et al. (22). Optimization at N-9 showed ethyl and isopropyl substituents to be equipotent. Substitution at C6 with various anilines revealed that 3-chloro, 3-cyano, 3-fluoro, 4-fluoro, or 3-trifluoromethyl substituents gave the optimal activity. Substitution at C2 with either diamines or amino alcohols showed that optimal activity was achieved with conformationally restricted analogues, e.g., [*22*] and [*23*] (IC_{50} of 0.03 and 0.039 μM, respectively).



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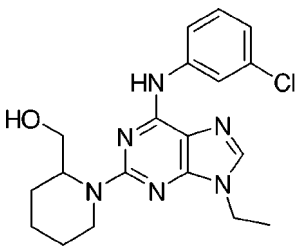
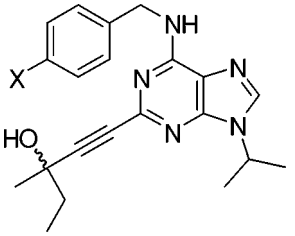
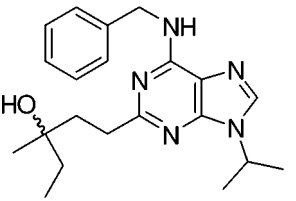


23

The observation from the olomoucine/cdk2 crystal structure that the NH at C-2 is not involved in binding interactions with the enzyme prompted the synthesis of a series of compounds bearing carbon substituents at C-2 (23, 24). Introduction of an alkynyl chain at C-2, e.g., [Structure *24*], gave compounds with increased potency over roscovitine [*18*] (IC_{50} against cdk1/cyclin B of 0.2 μM). Interestingly, reduction to the saturated alkane [Structure *25*] led to a significant loss of activity (IC_{50} against cdk1/cyclin B of 1.3 μM), suggesting that conformational flexibility in the ribose binding pocket is not favored. Substitution of the benzyl group with 4-OMe [Structure *26*] slightly reduced activity, but substitution with 4-Cl [Structure *27*] resulted in an increase in activity (IC_{50}s against cdk1/cyclin B of 0.23 and 0.06 μM). N-6 anilino compounds resembling purvanalol B [*21*] were also tested, the 3-chloro analog [Structure *28*] proving 10-fold less active than [*21*] (IC_{50} against cdk1/cyclin B of 0.06 μM).



X = H (**24**)
X = OMe; OL567 (**26**)
X = Cl; **27**



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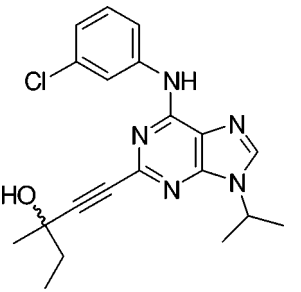
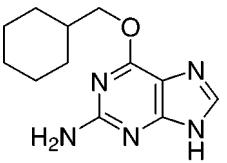


28

The crystal structure of compound [*26*] bound to cdk2 has been determined. The binding closely resembles that of olomoucine [*17*], roscovitine [*18*], and purvanalol [*21*], with similar hydrogen bonds being observed between N7 and Leu^{83} NH, and N6 and Glu^{81} backbone oxygen. The isopropyl group forms hydrophobic contacts with the side chain of Phe^{80}. The 3-methylpent-1-yn-3-ol side-chain group occupies the ATP ribose binding site.

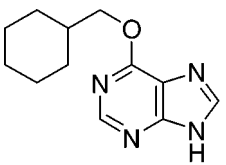
The data suggest that binding of the *S*-isomer is favored, with the methyl group occupying a small hydrophobic patch formed by Val^{18} and the backbone atoms of the glycine loop. The hydroxyl group of the *S*-isomer makes no direct hydrogen bonds but can bind to a water in the active site. The 4-methoxybenzylamino group on N6 binds outside the active site cleft, and the phenyl ring is surrounded by contacts from the side chains of Phe^{82}, Ile^{10}, Lys^{89}, and the cdk2 backbone at His^{84}. This region of the molecule shows the greatest differences between the enzyme-inhibitor structures. The greatest differences are observed in crystal structure [*26*], in which Lys^{89} adopts a different conformation to that seen in olomoucine [*17*] in order to avoid a steric clash with the OMe group.

Our group (25) has identified novel lead cdk inhibitors. The guanine derivative NU2058 [Structure *29*] is an ATP competitive inhibitor of both cdk1 and cdk2 with K_i values of 5 and 12 μM, respectively. The X-ray crystal structure of unphosphorylated cdk2 with [*29*] bound showed that the compound binds in the ATP binding pocket in a different orientation to other purine-based inhibitors, including olomoucine [*17*] and roscovitine [*18*]. The guanine ring forms three hydrogen bonds with the enzyme; N9 donates a hydrogen bond to the backbone carbonyl oxygen of Glu^{81}, N3 acts as a hydrogen bond acceptor from the NH group of Leu^{83}, and the 2-position NH_2 donates a hydrogen bond to the carbonyl group of Leu^{83}. Hydrogen bonding to Glu^{81} is also observed in the structures of cdk2 with both ATP and isopentenyl adenine bound, whereas the Leu^{83} residue is involved in hydrogen bonding to olomoucine [*17*], roscovitine [*18*], and purvanalol B [*21*]. The binding of the guanine ring of [*29*] is not coplanar with the adenine ring of ATP. The ATP ribose binding site is occupied by the cyclohexylmethyl group of NU2058 [*29*] despite lacking the ability to mimic the hydrogen bonding interactions of the ribose hydroxyls.

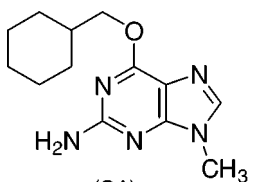


NU2058 (**29**)

Two compounds were designed to assess the relative importance of the hydrogen bonds at the 2-NH_2 and N9-positions. The O^6-cyclohexylmethylpurine [Structure *30*] and O^6-cyclohexylmethyl-N^9-methylpurine [Structure *31*], both lacking the ability to make one of the observed hydrogen bonds for [*29*], show reduced activity against cdk1 ($IC_{50} = 18$ and >100 μM, respectively) and cdk2 ($IC_{50} = 13$ and >100 μM, respectively). These results show that the hydrogen bond between the peptide oxygen of Glu^{81} and N9 is essential for activity, and the bonding between the 2-position NH_2 and Leu^{83} is not essential but is favorable.

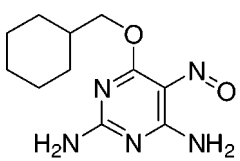


(**30**)



(**31**)

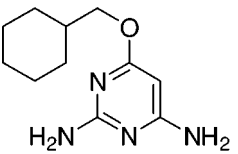
The pyrimidine NU6027 [Structure *32*] was designed as an alternative to the purine-based compounds. The capacity to form the three hydrogen bonds observed for [*29*] has been retained with the donor-acceptor-donor motif maintained. The 5-nitroso group was designed to provide an intramolecular hydrogen bond to the NH_2 group at the 6-position, thus ensuring that the remaining hydrogen on the nitrogen adopts the correct geometry for bonding to the peptide oxygen of Glu^{81}. NU6027 [*32*] is a potent ATP competitive inhibitor of both cdk1 and cdk2 with K_i values of 2.5 and 1.3 μM, respectively.



NU6027 (**32**)

The crystal structure for [*32*] bound to cdk2 has been determined. As predicted, the 6-NH_2 group donates a hydrogen bond to Glu^{81}, the NH group of Leu^{83} donates

a hydrogen bond to N1 and the C2 NH_2 group donates a hydrogen bond to the carbonyl group of Leu[83]. The interaction between the 5-nitroso and 6-NH_2 groups was confirmed to be an intramolecular hydrogen bond, and this was the sole interaction from the 5-nitroso group. The importance of this bonding in maintaining the 6-amino group in the correct orientation for binding to the enzyme was confirmed by the synthesis of [Structure *33*], which lacks the 5-nitroso group and did not display any inhibitory activity towards cdk1 or cdk2 at a concentration of 10 μM.

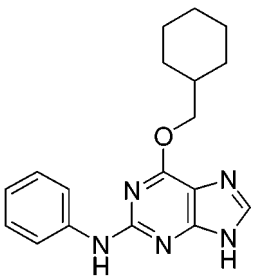


(**33**)

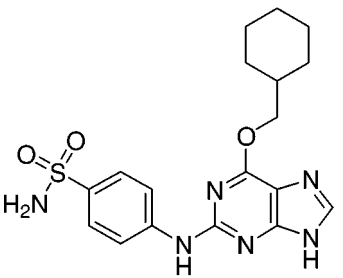
Compounds [*29–33*] were investigated in the NCI tumor cell line panel. Growth inhibitory data obtained for 57 of the cell lines showed a correlation between cdk inhibitory activity and cellular activity. The most potent compounds were [*29*] and NU6027 [*32*], which had mean GI_{50} values of 13 and 10 μM, respectively. COMPARE analysis for NU2058 [*29*] and NU6027 [*32*] did not reveal any strong correlation of activity with known antitumor agents. Of particular note was the lack of correlation with either of the known cdk inhibitors, flavopiridol [*4*] or olomoucine [*17*]. In contrast, COMPARE analysis showed strong correlations for both [*29*] with [*32*], and flavopiridol [*4*] with olomoucine [*17*]. The novel structural type and distinct binding mode of these inhibitors, together with the enzyme inhibition and cell growth inhibition data, indicate that these compounds are a novel structural class despite the apparent similarity to the other substituted purines.

Comparison of the binding mode of NU2058 [*29*] with that of olomoucine [*17*] indicated the possibility of introducing favorable substitutions to NU2058 [*29*] on the 2-amino group. Suitable aromatic substituents at the N2-position were expected to fill a similar area of the enzyme active site to that occupied by the N6-benzyl group of olomoucine [*17*]. The 2-anilino compound [Structure *34*] displayed increased activity against cdk1 and cdk2 (IC_{50}'s = 1.6 and 1.0 μM, respectively), as anticipated. A range of substituted 2-anilino compounds have been prepared; the most potent compound identified to date in this series is the 2-(4-sulfonamidoanilino) compound [NU6102; Structure *35*] with IC_{50}s of 9.4 nM and 6.9 nM against cdk1 and cdk2, respectively (26). The X-ray crystal structure of NU6102 [*35*] bound to cdk2 has been determined. As predicted, the purine ring makes the triplet of hydrogen bonds to Glu[81] and Leu[83] consistent with the structure of NU2058 [*29*] with cdk2 (Figure 3). Additional interactions occur between the phenyl ring and the region of hydrophobic residues, and two additional hydrogen bonds are formed. One of the sulfonamide oxygens accepts a hydrogen bond from the backbone NH of Asp[86], whereas the sulfonamide NH group donates a hydrogen

bond to the side-chain carbonyl of Asp[86]. It is likely that these interactions are responsible for the potency of NU6102 [*35*] compared with NU2058 [*29*].



(**34**)



(**35**)

The 3-(benzylidene)indolin-2-one [Structure *36*], which is an ATP competitive inhibitor of receptor tyrosine kinases, e.g., EGFR and Her-2 receptor kinases, provided the starting point for design of novel cdk inhibitors (27). The lead compound [Structure *37*], prepared as a homologue of [*36*], is a potent selective inhibitor of cdk2 ($IC_{50} = 60$ nM). Examination of the crystal structure of [*37*] bound to cdk2 provided information used to guide the design of analogues. The oxindole amide group forms two hydrogen bonds with the enzyme; the amide oxygen accepts a hydrogen bond from the backbone NH of Leu[83]; and the amide NH donates a hydrogen bond to the backbone carbonyl of Glu[81]. The sulfonamide group forms similar interactions to those of the sulfonamide [*35*], with the backbone NH of Asp[86] donating a hydrogen bond to one of the sulfonamide oxygens and the sulfonamide NH donating a hydrogen bond to the side-chain carbonyl. The 5-position is close to Lys[33], which indicated that hydrogen bond acceptors at this point could be favorable. Additionally, lipophilic substituents at the 4-position were expected

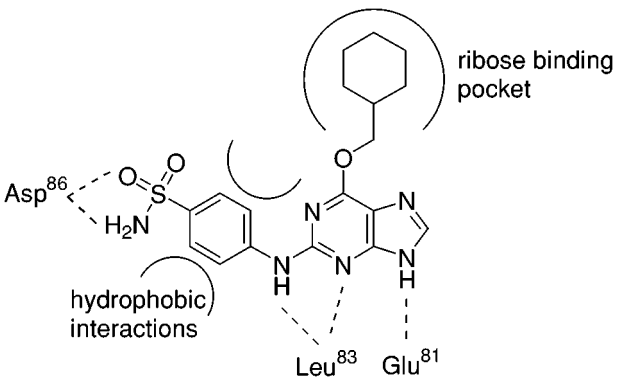


Figure 3 Binding mode of NU6102 [*35*] to cdk2.

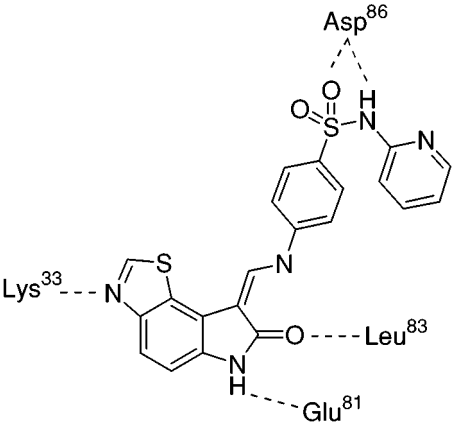
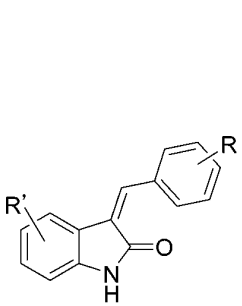


Figure 4 Binding mode of compound [*38*] to cdk2.

to provide favorable interactions with hydrophobic residues such as Val[18]. Substituents on the sulfonamide group were predicted to have minimal interactions with the enzyme and thus offered a position for the introduction of groups designed to optimize the pharmacological and physicochemical properties of the molecule. The analogue [Structure *38*] was designed according to these principles, and it is a potent and selective inhibitor of cdk2 ($IC_{50} = 10$ nM), a weaker inhibitor of cdk1 and cdk4 ($IC_{50} = 110$ nM and 130 nM, respectively), and a significantly weaker inhibitor of other protein kinases. The binding mode and predicted interactions were confirmed by the crystal structure of [*38*] bound to cdk2 (Figure 4). Compound [*38*] was found to block the progression through the cell cycle of treated cells ($IC_{50} = 2.5\ \mu$M), as measured by flow cytometry. The effect was attributed to a blockade of S-phase progression and was found to be reversible on compound withdrawal. The compound did not induce apoptosis.



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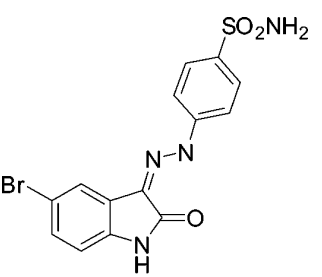

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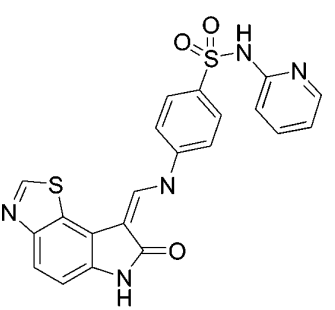
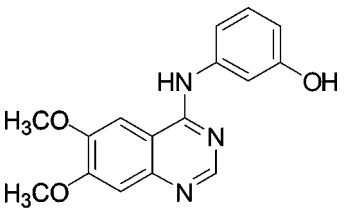


(**38**)

The quinazoline ring system has been explored as a template for cdk inhibitors. The 4-anilinoquinazoline [Structure *39*] is an inhibitor of cdk2 ($IC_{50} = 1$ μM) (28). A crystal structure of the 4-anilinoquinazoline [*39*] bound to cdk2 has been determined. The principle interactions observed are a hydrogen bond from the backbone NH of Leu^{83} to the quinazoline N1 and a pair of hydrogen bonds with the 3′-hydroxy group on the aniline, one to Asp^{145} and one from Lys^{33}. Additional interactions including aromatic CH to carbonyl oxygen interactions occur between C2 and C8 and the carbonyl groups of Glu^{81} and Leu^{83}, respectively.

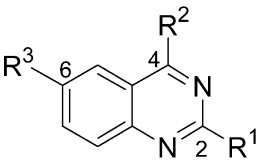


(**39**)

The substituted quinazoline [Structure *40a*] was discovered through screening as a lead inhibitor for cdks (29). Substituents at the 2-, 4-, and 6-positions were optimized successively. Substitution at the 2-position was found to favor small, electron-withdrawing, nonionizable groups such as trifluoromethyl [Structure *40b*] and trichloromethyl [*40c*]. Branched alkyl groups, e.g., *t*-butyl [*40c*] and *t*-amyl [*40d*] were found to be the optimal 4-substituents. Finally, the 6-position was explored; a number of differing groups were tolerated at this position including an amide [*40e*], trifluoromethyl [*40f*] and substituted aromatic [*40g* and *40h*] (see Table 5). A crystal structure of the most potent inhibitor [*40h*] bound to cdk2 has been determined. The inhibitor is bound to the ATP binding site of the enzyme, and significant movement of the side chains of Lys^{33} and Lys^{89} accommodate the inhibitor. Uniquely, inhibitor [*40h*] binds to the enzyme via a hydrogen bond between quinazoline N1 and a water bridging to Glu^{81} (Figure 5). Hydrogen bonding is also observed between the 6-anilino nitrogen and the side-chain oxygen of Asp^{86}. A stacking interaction is also observed between the 2-trifluoro group and Phe^{80}.

The natural product hymenialdisine [Structure *41*], isolated from a marine sponge, is a potent inhibitor of cdk1, cdk2, and cdk5 (Table 6) (30). In addition, it is a potent inhibitor of glycogen synthase kinase 3β (GSK3β) and casein kinase 1 (CK1). The crystal structure of [*41*] bound to cdk2 has been determined. The inhibitor binds in the ATP binding site and makes three hydrogen bonds with the enzyme; from the pyrrole, NH donates a hydrogen bond to the peptide oxygen of Leu^{83}; the azepine carbonyl oxygen O1 accepts a bond from the backbone amide

TABLE 5 Inhibition of cdks by quinazoline compounds [*40a–h*] [IC_{50} (μM)]



(**40**)

Compound	R1	R2	R3	cdk2/cyclin E	cdk4/cyclin D1
40a	CCl_3	$NHC(CH_3)_2CCH$	H	13.5	24.0
40b	CCl_3	NHtBu	H	6.1	11.9
40c	CF_3	NHtBu	H	1.5	14.3
40d	CF_3	$NHC(CH_3)_2CH_2CH_3$	H	1.4	16.8
40e	CF_3	NHtBu	NHCOH	0.925	14.0
40f	CF_3	NHtBu	CF_3	0.79	>37.0
40g	CF_3	NHtBu	Ph	1.8	7.7
40h	CF_3	NHtBu	3-NH_2Ph	0.6	>2.1

NH of Leu[83]; and the azepine amide NH donates a hydrogen bond to the carbonyl oxygen of Glu[81] (Figure 6). Additional hydrophobic interactions occur between the pyrroloazepine ring system and the side chains of residues Ile[10], Val[18], Ala[31], Val[64], Phe[80], and Leu[134] in the binding site. The guanidine ring makes a direct hydrogen bond from the 5-amino group to one of the side-chain oxygens of Asp[145]

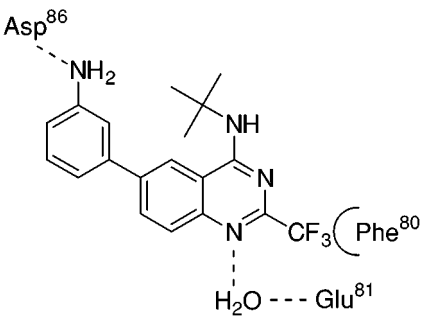
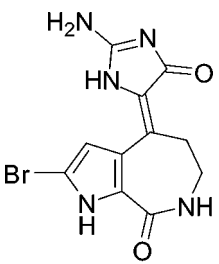


Figure 5 Binding mode of inhibitor [*40h*] to cdk2.

TABLE 6 Inhibition of kinases by hymenialdisine [*41*]

Enzyme	IC_{50} (nM)
cdk1/cyclin B	22
cdk2/cyclin A	70
cdk2/cyclin E	40
cdk3/cyclin E	100
cdk4/cyclin D1	600
cdk5/p25	28
cdk6/cyclin D2	700
GSK-3β	10
CK1	35

and two water-mediated hydrogen bonds, one from the peptide NH of Asp^{145} to the carbonyl O2, and another from the 5-amino group to the peptide carbonyl of Gln^{131}. Binding of the inhibitor to the kinase is accompanied by movement of Asp^{145}.



(**41**)

A recent review details other structural classes of cdk inhibitors for which detailed structure-activity or crystallographic data has not been published (31). Those compounds are not described in this review.

CONCLUSIONS

Compound screening and rational structure-based drug design have provided a range of structurally diverse cdk inhibitors. Many of the anticipated difficulties, such as potency and selectivity between the many similar kinases, have been overcome, and good selectivity has been achieved for cdks. Selectivity within the

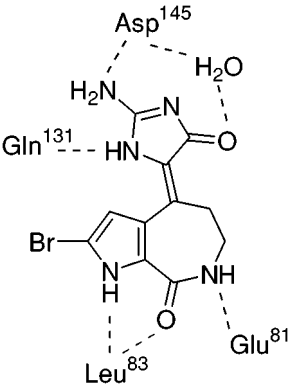


Figure 6 Binding mode of hymenialdesine [*41*] to cdk2.

cdk family has also been achieved, in particular for cdk4, cdk1 and 2. The design of compounds selective between cdk1 and cdk2 remains an attractive goal.

A number of compounds have progressed through preclinical evaluation and are in the early stages of clinical trials. Data from these trials will inform the design of compounds with optimized pharmacological and pharmacokinetic properties. These early trials will also generate proof of principle for the antitumor efficacy of cdk inhibitors.

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