Bioorganic & Medicinal Chemistry Letters 24 (2014) 199–203

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Modulating the interaction between CDK2 and cyclin A with a quinoline-based inhibitor

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ARTICLE INFO

Article history:
Received 21 October 2013
Revised 13 November 2013
Accepted 15 November 2013
Available online 23 November 2013

Keywords:
Kinases
Tumor
CDK2
Modulator
High throughput screening

ABSTRACT

A new class of quinoline-based kinase inhibitors has been discovered that both disrupt cyclin dependent 2 (CDK2) interaction with its cyclin A subunit and act as ATP competitive inhibitors. The key strategy for discovering this class of protein–protein disrupter compounds was to screen the monomer CDK2 in an affinity-selection/mass spectrometry-based technique and to perform secondary assays that identified compounds that bound only to the inactive CDK2 monomer and not the active CDK2/cyclin A heterodimer. Through a series of chemical modifications the affinity (K_d) of the original hit improved from 1 to 0.005 μM.

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Multiple cellular signals can stimulate growth, differentiation, and apoptosis. A key mechanism for regulating these processes involves the cell cycle, which controls cell division by regulating passage through the G1, S, and G2/M phases of DNA synthesis and mitosis.[1,2] Progression through the eukaryotic cell cycle is controlled in part by cyclin dependent kinases CDK1 (cdc2), CDK2, CDK4 and CDK6.[3] These CDK proteins are serine/threonine kinases that regulate different phases of the cell cycle by binding to distinct regulatory subunits called cyclins and phosphorylation of a wide range of proteins. The temporal expression of cyclin A, cyclin B, cyclin D, and cyclin E, is tightly controlled to ensure successful progression through the cell cycle.[4] Loss of normal cell cycle regulation is thought to contribute to human proliferative disorders; therefore inhibition of unregulated CDK activity by small molecule inhibitors would be beneficial in the treatment of cancer. Although a large number of drug discovery programs have been directed toward developing CDK specific ATP-competitive inhibitors, only a few molecules have progressed into human clinical trials.[5–11] Therefore, there remains a need to develop additional CDK inhibitors for the treatment of human cancers.

CDK2 activation requires association with a cyclin (cyclin E at the G1–S phase transition and cyclin A during the S phase) as well as phosphorylation of Thr160 in the kinase activation segment. A structural comparison between the CDK2 monomer and the CDK2/cyclin A complex shows the activation loop moves ~25 Å following binding of the cyclin, stabilizing the complex and providing a structural realignment of the C-Helix to form the fully active conformation.[12] Thus far, most inhibitor screening research efforts have focused on screening the active (phosphorylated) form of CDK2 complexed with either cyclin A or cyclin E. The compounds developed using these strategies have led to ATP-competitive inhibitors. With the intent of finding new modes of inhibition we decided to screen unphosphorylated (inactive) CDK2 in the absence of a bound cyclin. It was hypothesized that the uncomplexed protein might be less rigid and able to undergo conformational

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http://dx.doi.org/10.1016/j.bmcl.2013.11.041

changes that would reveal new states capable of binding small molecules and that these inhibited states might have cellular function. Multiple inhibitory mechanisms were thereby enabled; including modulation of important protein–protein interactions (i.e., cyclin binding), decreasing phosphorylation (activation) of CDK2, or direct inhibition of kinase activity in ATP competitive, noncompetitive, and uncompetitive modalities.

To identify compounds with the above inhibition mechanisms an unbiased high-throughput screening campaign was performed using unphosphorylated CDK2 in the affinity-selection/mass spectrometry-based technique called Automated Ligand Identification System (ALIS) versus a library of 5 million compounds.[13] The majority of compounds that were identified from the ALIS screen were well known ATP competitive inhibitors (i.e., purine-based) or predicted to be ATP competitive. These compounds competed with an ATP-competitive fluorescent probe (B-Alexa-Fluor647) in a fluorescence polarization binding assay and also inhibited CDK2/cyclin A in the enzyme assay. One class of quinoline-based compounds had the desired binding/inhibition profile which demonstrated displacement of the fluorescent probe in the fluorescence polarization CDK2 assay but did not inhibit the CDK2/cyclin A enzyme assay and did not bind in the CDK2/cyclin A FP assay. Although these inhibitors did not inhibit the kinase activity of CDK2/cyclin A, it was anticipated that due to the temporal 'cyclic expression' of cyclin subunits in the cell that free CDK2 would exist as a monomer during the course of the cell cycle and thus monomer selective inhibitors would have the potential to be biologically active.

Compound **1** (Fig. 1) had a K_i of 0.9 μM in the CDK2 fluorescence polarization (FP) binding assays[14] and an IC_{50} >10 μM in the CDK2/cyclin A enzyme assay. Two other binding assays were used to assess the binding affinity of ligands that bound preferentially to the inactive state of CDK2. The temperature-dependent circular dichroism assay (TdCD)[15] was used to determine the affinity of ligands bound to proteins by measuring the degree to which a bound ligand stabilized the thermal denaturation (change in secondary structure) of CDK2 as measured by following changes in the circular dichroism ellipticity at 220 nm. Isothermal titration calorimetry (ITC)[16] was also used for several compounds to obtain

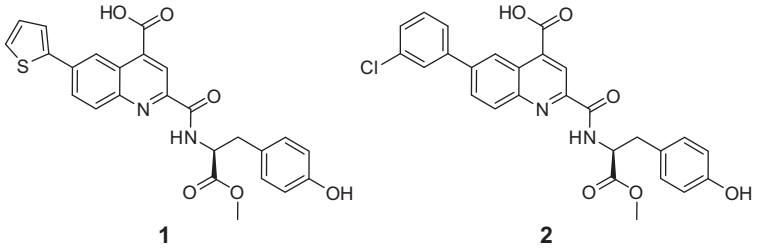
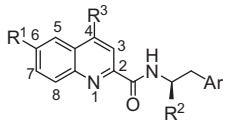


Figure 1. Quinoline-based compounds **1** and **2**.

Table 1
Structure–activity relationships of CDK2 inhibitors based on the quinoline scaffold[a,b]



Compd	R^1	R^2	R^3	Ar	FP K_i (μM)	TdCD K_d (μM)	ITC K_d (μM)
1	2-Thiophen	CO_2Me	COOH	4-OH-phenyl	0.90	1.1	1.00
2	3-Cl-phenyl	CO_2Me	COOH	4-OH-phenyl	0.14	0.30	0.30
7	2-Cl-phenyl	CO_2Me	COOH	4-OH-phenyl	0.7	0.8	
8	4-Cl-phenyl	CO_2Me	COOH	4-OH-phenyl	0.9	0.9	
9	3,5-di-Cl-phenyl	CO_2Me	COOH	4-OH-phenyl	0.2	0.32	
10	Phenyl	CO_2Me	COOH	4-OH-phenyl	10	12	
11	Morpholine	CO_2Me	COOH	4-OH-phenyl	2.8	2.4	
12	3-CF_3-phenyl	CO_2Me	COOH	4-OH-phenyl	<0.10	0.04	0.050
13	4-Cl-phenethyl	CO_2Me	COOH	4-OH-phenyl	<0.10	0.048	0.055
14	4-Cl-styryl	CO_2Me	COOH	4-OH-phenyl	<0.10	0.005	
15	*n*-Hexyl	CO_2Me	COOH	4-OH-phenyl	<0.10	0.076	
16	3-CF_3-phenyl	CO_2Me	CH_2OH	4-OH-phenyl	>40	>40	
17	2-Thiophen	CO_2Me	$CONH_2$	4-OH-phenyl	>40	>40	
18	2-Thiophen	CO_2Me	$CONHSO_2Me$	4-OH-phenyl	>40	>40	
19	3-CF_3-phenyl	CO_2Me	COOH	4-F-phenyl	2.5	4.0	
20	3-CF_3-phenyl	CO_2Me	COOH	4-OMe-phenyl	>40	>40	
21	3-CF_3-phenyl	CO_2Me	COOH	3-OH-phenyl	>40	>40	
22	2-Thiophen	H	COOH	4-OH-phenyl	>40	>40	
23	3-CF_3-phenyl	CO_2H	COOH	4-OH-phenyl	12	>10	
24	3-CF_3-phenyl	Me	COOH	4-OH-phenyl	2.5	4	
25	3-CF_3-phenyl	Propyl	COOH	4-OH-phenyl	0.3	0.4	
26	4-Cl-styryl	CONHMe	COOH	4-OH-phenyl	0.10	0.3	
27	4-Cl-styryl	$CON(Me)_2$	COOH	4-OH-phenyl	0.16	0.35	
28	3-CF_3-phenyl	5-Methyloxazole	COOH	4-OH-phenyl	0.20	0.15	
29	3-CF_3-phenyl	CH_2OMe	COOH	4-OH-phenyl	0.1	0.24	
30	4-Cl-styryl	CH_2OMe	COOH	4-OH-phenyl	<0.10	0.025	

[a] The CDK2/cyclin A enzyme IC_{50} is greater than 10 μM for all of these compounds.
[b] Binding activity reported for the inactive CDK2 monomer.

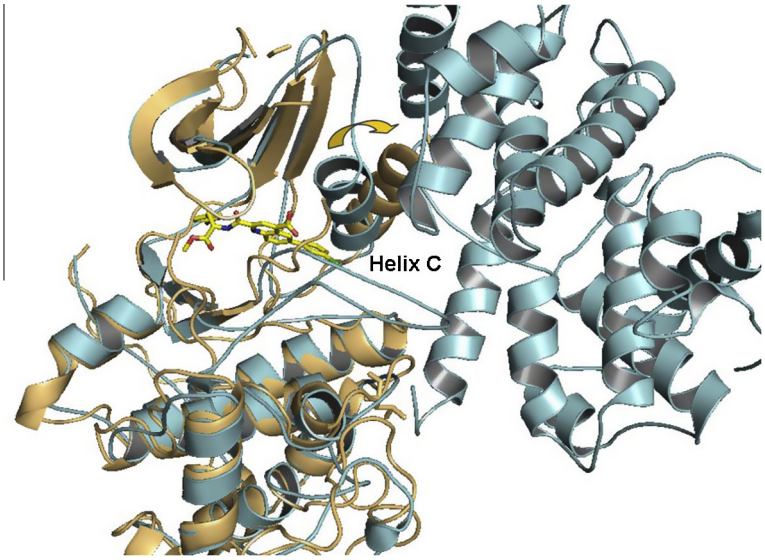


Figure 2. X-ray crystal structure of **2**/CDK2 complex superimposed on the CDK2/cyclin A complex (ATP removed). The **2**/CDK2 complex is shown with the kinase in brown and the compound in yellow. The CDK2/cyclin A complex is in blue. The yellow arrow identifies the shift in conformation for the C-Helix in the **2**/CDK2 complex versus the location of the C-Helix in the CDK2/cyclin A complex. Compound **2** binding to the kinase domain induces a conformational change that would cause steric interference in binding cyclin A. PDB code is 4NJ3.

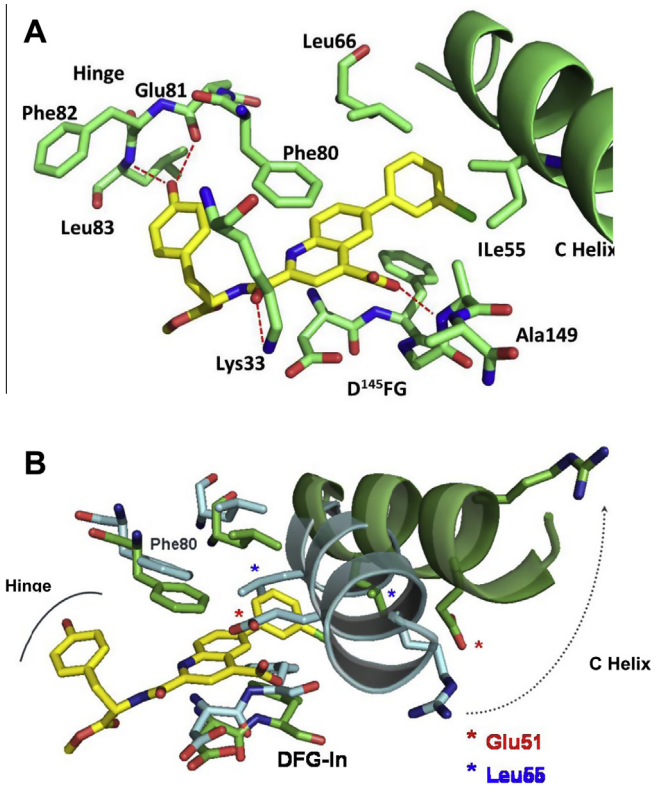


Figure 3. (A and B) Binding mode of **2** in CDK2. (3A) Compound **2** (yellow) is bound to the hinge region of CDK2 through the sharing of 2 hydrogen bonds with the carbonyl of Glu81 and the NH of Leu83. The DFG^{147} group is in the 'In' position and the quinoline-R^3 group extends over Phe146 forming an array of hydrophobic interactions toward the C-Helix. (3B) Overlay of the structure of **2** (yellow)/CDK2 (green) with the structure of CDK2/cyclin A (blue) (Ref. 15 pdb 1FIN). An 11.7 Å shift and rotation in Helix C is observed between the CDK2/cyclin A structure and **2**/CDK2 complex. This conformational change is required to bind the R^3-quinoline group otherwise there would be steric hindrance in binding **2** to the kinase domain.

a full thermodynamic characterization of the binding affinity to verify the affinity measured from FP and TdCD assays. The FP-K_i, TdCD-K_d and ITC-K_d values determined for **1** and the quinoline derived series of compounds are listed in Table 1. Interestingly, the corresponding R isomers of **1** and its analogs show no binding activities (data not shown).

A series of new synthesis led to **2** which contains a more electron-deficient 3-choloro phenyl appendage at R^1 and has a significant improvement in the FP-K_i to 0.14 μM, (consistent with lower K_d values in TdCD and ITC studies). The binding mode of **2** bound to the monomeric state of CDK2 determined by X-ray crystallography shows **2** binds not only in the ATP active site but also extends into a back pocket behind the gatekeeper and induces several conformational changes (Fig. 2). The Tyrosine phenol of **2** interacts with the hinge region (Fig. 3A) and shares a hydrogen bond with the carbonyl of Glu81 and the NH of Leu83 in the CDK2 hinge region, respectively. The carbonyl group of the amide bond at the 2-position of **2** forms a hydrogen bond with the conserved Lys33 residue. The carboxylic acid at the 4-position of the quinoline group forms a hydrogen bond with Ala149 adjacent to the DFG motif. The DFG motif is in the 'In' orientation with Phe146 buried in the core of the protein as found for active kinase conformations. The 3-chlorophenyl group at the 6-position of **2** binds deeply into a hydrophobic pocket formed by the side chains of Leu55, Leu58, Val123 and, Phe146. Comparison of the **2**/CDK2 structure with that of the active pThr160-CDK2/cyclin A/ATP complex (Figs. 2 and 3B, CDK2 green, CDK2/cyclin A blue) reveals several protein conformational changes between the two structures. First, the activation loop is displaced starting from the Gly147 in the DFG motif. More significantly the distal chlorophenyl pushes against the C-terminal cap of the C-Helix. The helix rotates such that the N-terminal end moves by 2.0 Å. The rotation occurs in a direction opposed to the C-Helix motion induced by cyclin binding such that the Glu51 Cα in the **2**/CDK2 complex is about 12 Å away from its position in the cyclin bound kinase structure (Fig. 3B). The C-Helix partly overlaps with cyclin in this new orientation (Fig 2). Hence the locking of the C-Helix in this orientation and steric hindrance of binding cyclin A provides a structural rationale as to why cyclin A cannot bind to the pre-formed **2**/CDK2 complex. At the beginning of the T-Loop, Phe146 (of the DFG motif) overlaps in the two structures and thus the **2**/CDK2 complex has a classical DFG-In conformation.

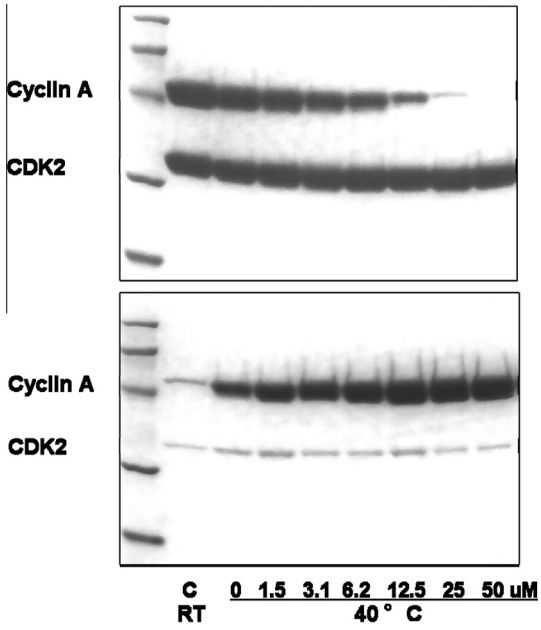


Figure 4. CDK2/cyclin A complex dissociation by **2** at 40 °C. CDK2/cyclin A at 1.5 μM was incubated with increasing concentrations of **2** at rt for 30 min and then heated at 40 °C for 30 min. The samples were centrifuged and the supernatant fractions run on an SDS–PAGE and stained with Coomassie stain (Fig. 4-top). The loss of cyclin A fractions at the higher concentrations of **2** is an indication of disruption of the CDK2/cyclin A complex. Figure 4-bottom is the centrifugation pellet fraction and demonstrates that cyclin A becomes dissociated in the presence of increasing **2** and is retained in the pellet fraction due to poor thermal stability at 40 °C. Sample C is a control sample with only 1% DMSO run at 40 °C under the same conditions as the compound treated samples. The final DMSO in all samples was 1%.

Compound **2** has been shown to bind to CDK2 by direct competition of the ATP-competitive fluorescently labeled compound (B-Alexa-Fluor647), binding in the TdF assay where bound **2** increases the thermal stability of CDK2, and binds in the isothermal titration calorimetry assay with a K_d = 0.3 μM. The unique binding mode of **2** with CDK2 suggests that **2** and its analogues may be selective against other kinases. Indeed counter-screening **2** against a panel of 310 kinases at 1 μM resulted in only one kinase with greater than 20% inhibition (PDK1, 30% inhibition) while control kinase inhibitors demonstrated the expected inhibition profile. No inhibition was observed for the active state of CDK1, CDK2, CDK5, CDK7, CDK8, and CDK9. We cannot rule out that compound **2** binds to the inactive (noncyclin bound) states of the CDK family. Binding studies would be needed to assess if compound **2** could bind to the inactive states of these CDKs. The bound conformation of **2** to CDK2 is similar to that observed for Lapatinib bound to EGFR (Type I½) where these compounds bind into the core of the protein toward the C-Helix but without inducing a DFG-out conformation.[17] It is interesting to note that Betzi et al.[18] have recently observed that two 8-anilino-1-naphthalene sulfonate (ANS) hydrophobic fluorescent probes (K_d was 37 μM) can also bind into the same region as the quinoline core and the R^3 group and induces a similar C-Helix conformational change. Selected compounds were also evaluated in a cell based proliferation assay but none showed cell proliferation inhibition possibly due to permeability issues due to the R^3 carboxylic acid group. Substitution of the ionic R^3 carboxylic acid group with bioisosteres that retain enzyme activity may improve permeability and cellular activity.

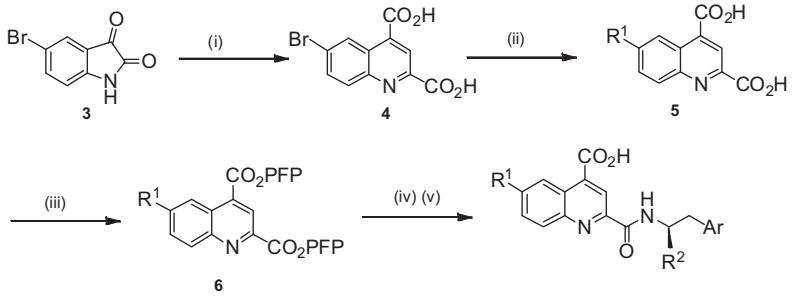
Evidence that **2**-induced conformational change prevents cyclin binding to CDK2 was obtained by incubating the compound with CDK2/cyclin A and measuring the amount of soluble cyclin A after incubating at 40 °C. Figure 4 is a Coomassie stained SDS gel that shows when CDK2/cyclin A is incubated at 40 °C without **2**, followed by centrifugation that both CDK2 and cyclin A remain in the supernatant fraction because the heterodimer complex is stable at 40 °C. However, a similar incubation with increasing concentrations of **2** results in the loss of cyclin A (which is insoluble as a monomer at 40 °C) from the supernatant fraction as evident by the decrease in the corresponding Coomassie band for the supernatant fractions and an increasing amount of cyclin A in the pellet fraction. Thus, CDK2-bound **2** inhibits cyclin A binding.

The synthesis of **2** and its derivatives with variations at the 2, 4 and 6-position is summarized in Scheme 1. 5-Bromoisatin **3**, was converted to the dicarboxylic acid **4** using pyruvic acid and potassium hydroxide.[19] The aryl group in **5** was introduced using standard coupling conditions with the corresponding boronic acid. The dicarboxylic acid **5** was then activated by converting to di-pentafluorophenol (PFP) ester **6** in the presence of DCC. It was found that the PFP ester at the 2-position of the quinoline was more reactive than at the 4-position and reacting di-PFP ester **6** with one equivalent of tyrosine methyl ester, followed by hydrolysis, generated **2** with ~60% yield. A similar approach yielded **7**–**30**.

The SAR for the analogs of **2** proved to be very consistent with these structural observations. Substitutions at the 6-position (R^1) of the quinoline showed that a meta substituent on the phenyl ring was preferred and that limited space around the *ortho* and *para*-positions was available due to steric hindrance from Phe146 and Leu 58 (**7**, **8**). The un-substituted phenyl group reduces the potency drastically (**10**). The R^1 group binds in a hydrophobic environment, and introduction of polar groups such as morpholine resulted in less potent compounds (**11**). Hydrophobic substitutions at R^1 (**12**, 3-CF_3-phenyl) or extended hydrophobic substitutions (**13** and **14**, 4-Cl-phenylethyl and 4-Cl-styryl, respectively) were favorable and resulted in single digit nM binding as determined by TdCD (K_d = 5 nM for **14**). These substitutions are keys for differentiating the binding of the quinoline compounds to CDK2 and not CDK2/cyclin A. In the CDK2/cyclin A binary complex, no binding pocket exists to accommodate the quinoline scaffold and substitutions at the 6-position (R^1). In the structure of CDK2 bound with **2** the C-Helix is pushed 11.7 Å outward which provides room for the compound binding but sterically inhibits the binding of cyclin A to CDK2.

The interaction of the quinoline 4-position carboxylic acid with the backbone of the protein proved to be very important. Reduction of the carboxylic acid to the hydroxymethyl group (**16**), conversion to an amide (**17**) or to an acylsulfonamide (**18**) completely abolished binding. In **2**, the hydroxyl group of the phenol (AR substitution) functions as both a hydrogen bond donor and an acceptor and shares two key hydrogen bond interactions with the hinge amide backbone (Glu81, Leu83). Replacement of the hydroxyl group with a fluorine group was 100 fold less potent (**19**). Methylation of the same hydroxyl group or relocating to a *meta*-position on the tyrosine ring resulted in complete loss of binding (**20** and **21**). The methyl ester at R^2 was found to be essential for binding since **22** (R^2 = H) and **23** (R^2 = COOH) bound poorly to CDK2. In anticipation of potential metabolic liabilities with the methyl ester (R^2), we decided to investigate its replacement with more stable functional groups. The crystal structure of **2** with CDK2 revealed a hydrophobic interaction between the methyl ester moiety and the side chain of Leu 134. This position tolerated a wide range of substitutions including alkyl (**25**), amide (**26** and **27**), heterocyclic (**28**) and methoxy–methyl groups (**29** and **30**).

The chemical and structural SAR described above clearly defines the observed affinity SAR and why the quinoline series bind to the CDK2 monomer and not the CDK2/cyclin A heterodimer. Despite the improvement of binding affinity, none of the compounds described above show significant activity in a cell based proliferation assay or inhibition of pRb phosphorylation while CDK2 control compounds were active in these assays. Several reasons may exist



Scheme 1. Reagents and conditions : (i) Pyruvic acid, KOH, 40 °C, 12 h, 95%; (ii) 3-chlorophenyl boronic acid, $Pd(AcO)_2$, K_3PO_4, dioxane, water, 80 °C, 12 h, 85%; (iii) pentafluoro-phenol, DCC, rt, 4 h, 70%; (iv) tyrosine methyl ester, DIEA, 0 °C, 12 h; (v) water, DIEA, rt, 60%.

for the lack of cellular activity such as poor permeability resulting from the carboxylic acid functionality, difficulty of the current inhibitors to compete with cyclin binding to the monomeric CDK2 state (thus cyclin A would shift the equilibrium from free CDK2 to a CDK2/cyclin A complex incapable of binding compound **2**) or a combination of both. More experiments are needed to further understand how cyclin A regulates the activity of CDK2 and to improve the cell activity of this class of compounds.

In conclusion, we have identified and developed a series of compounds based on a quinoline scaffold which bind selectively to the CDK2 monomer and not the CDK2/cyclin A complex. The binding of the quinoline series has the potential functional consequence of not only inhibiting ATP binding to the active site but also the disruption of binding cyclins due to the conformational change in the critical C-Helix.

Acknowledgments

We would like to thank Drs. Huw Nash, and Hung Le for their helpful discussions.

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14. Zhang, R. M.; Mayhood, T.; Lipari, P.; Wang, Y. L.; Durkin, J.; Syto, R.; Gesell, J.; McNemar, C.; Windsor, W. *Anal. Biochem.* **2004**, *331*, 138. The reference compound BMS-250595 (BMS) is an ATP competitive compound that binds to CDK2 with a K_d = 9 nM. The deacetylated form of compound BMS was reacted with Alexa Fluor 647 to generate a compound (B-Alexa-Fluor647) that bound to both proteins and provided a fluorescence polarization signal that could be used to measure the affinity of compounds from the high throughput screening study. The binding assay was performed in 10 mM HEPES pH 7.5, 150 mM NaCl, 10% Glycerol, 1 mM DTT, 0.01% BOG with 200 nM CDK2 or 450 nM CDK2/cyclinA. The affinity of the fluorescence probe B-Alexa-Fluor647 was ~200 nM to both proteins. K_i values of test compounds were determined by adding increasing concentrations of unlabeled test compound to solutions containing 50 nM B-Alexa-Fluor647 and CDK2 or CDK2/cyclin A solutions. This displacement assay provided accurate affinities for inhibitors greater than ~100 nM. For compounds with a K_i of <100 nM other complimentary assays were utilized.
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16. ITC was used to obtain a full thermodynamic characterization of the binding affinity for several lead compounds. The procedure is similar to that reported by Mayhood and co-workers in Ref. 14. Briefly, human CDK2 was dialyzed in to 10 mM Hepes, 150 mM NaCl, 10% glycerol, 1 mM DTT, pH 7.5 overnight with 2 changes of buffer. Both protein and ligand samples were prepared in 2 ml volumes. To prepare the ligand solution 20 µl of a 10 mM compound stock (in DMSO) was added to 2 ml of buffer. The ITC runs were performed at 20 °C using approximately 10–15 µM CDK2 and 100–150 µM compound and mixed at a rate of 350 rpm. Injection volumes varied from 15 to 18 µl and 360 s of data was recorded for each titration. When a clear baseline was not observed a blank titration was performed to subtract it from the raw data. The MicroCal MCS ITC and included software were used for performing the titrations and analysis.
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