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Perspective

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Cyclin Dependent Kinase 9 Inhibitors for Cancer Therapy

Miniperspective

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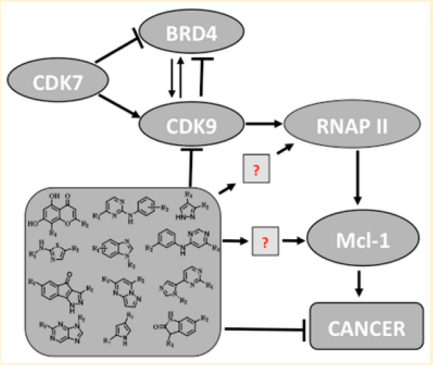
ABSTRACT: Cyclin dependent kinase (CDK) inhibitors have been the topic of intense research for nearly 2 decades due to their widely varied and critical functions within the cell. Recently CDK9 has emerged as a druggable target for the development of cancer therapeutics. CDK9 plays a crucial role in transcription regulation; specifically, CDK9 mediated transcriptional regulation of short-lived antiapoptotic proteins is critical for the survival of transformed cells. Focused chemical libraries based on a plethora of scaffolds have resulted in mixed success with regard to the development of selective CDK9 inhibitors. Here we review the regulation of CDK9, its cellular functions, and common core structures used to target CDK9, along with their selectivity profile and efficacy in vitro and in vivo.



CYCLIN DEPENDENT KINASE INHIBITORS

Since the approval of Gleevec, the U.S. Food and Drug Administration (FDA) has approved 28 small molecule kinase inhibitors.[1] Over the past 15 years, a number of cyclin dependent kinase (CDK) inhibitors (**2**−**13**)[2−11] have entered clinical trials for the treatment of cancer (Figure 1). In February 2015, FDA granted accelerated approval for the first ever CDK inhibitor palbociclib (PD-0332991, **1**)[12] for the treatment of metastatic breast cancer. **1** is selective for CDK 4/6 kinases over other CDKs by >2 orders of magnitude in vitro, and the selectivity is attributed to the aminopyridyl substituent at the C2 position.[13,14] This has energized the field and has given credence for the development of selective small molecule CDK inhibitors.

CDKs are members of the Ser/Thr kinase subfamily. Cyclins are regulatory subunits that bind to the CDK, resulting in the activation of the kinase. Most members of the CDK family form a CDK/cyclin complex and are involved in the regulation of either cell cycle or transcription. CDK5 is an exception, as it binds to a non-cyclin protein regulator and is not involved in the regulation of cell cycle or transcription/RNA processing. The human genome encodes 21 CDKs (1−11a, 11b−20) and over 15 cyclins (A−L, O, T, and Y). The major CDKs and cyclins involved in the cell cycle regulation are 1, 2, 3, 4, and 6 and A, B, D, and E, respectively. CDKs 2, 4, and 6 along with cyclins D and E are involved in the regulation of cell cycle through the G1 phase. CDKs 1, 2, 3 along with cyclins A, B, and E participate in the regulation of the S, G2, and M phases. CDKs 7, 8, 9, and 11 along with cyclins C, H, L, and T are key players in transcription regulation/RNA processing.[15,16] The high structural homology within the kinase domains particularly among the CDKs has posed problems for the discovery of CDK specific small molecule inhibitors. Despite this, >95% of all FDA approved kinase inhibitors target the ATP binding site within the kinase domain. Table 1 summarizes the in vitro selectivity profile of a panel of CDK inhibitors, which includes several that are/were in clinical trials.

CYCLIN DEPENDENT KINASE 9

CDK9 forms heterodimeric complexes with cyclins T1, T2a, T2b, and K. Cyclins T2a and T2b are splice variants with T2b having an additional 67 amino acids at its C-terminus.[17] CDK9 is expressed ubiquitously in all tissues, as are its activators cyclins T1, T2a, and T2b (www.proteinatlas.org).[18,19] Cyclin K is expressed predominantly in the testes, stomach, and bone marrow.[20] CDK9 is expressed in cells as two isoforms that are differentially localized: a lighter 42 kDa protein and a heavier 55 kDa protein.[21] The 55 kDa isoform has an additional 117 amino acid at the N-terminus of the 42 kDa isoform.[22] The association of a cyclin and the phosphorylation of the activation loop residue Thr^{186} are required for the activation of CDK9.[23]

Independent studies used orthogonal techniques to identify different kinases that phosphorylate the T-loop residue (Thr^{186}) on CDK9. The Rice lab used a RNAi screen in HeLa cells to identify calcium/calmodulin-dependent kinase 1D (CaMK1D) as a kinase that phosphorylates Thr^{186} on CDK9.[24] The Singer lab used in vitro kinase assays and siRNA knock-down studies in HeLa cells to show the atypical kinase

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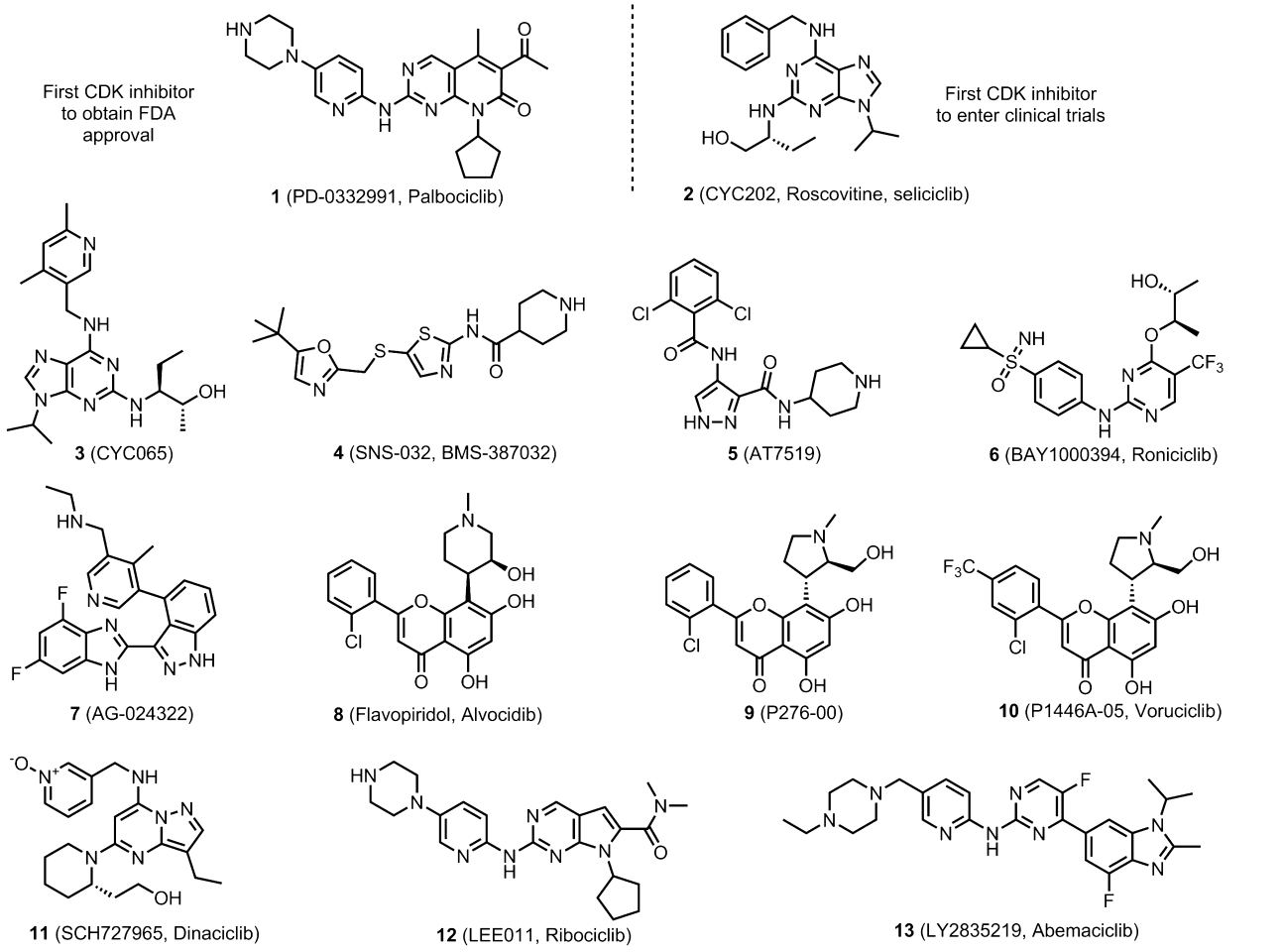

Figure 1. CDK inhibitors that are/were in clinical trials for the treatment of cancer.

Table 1. Selectivity Profile of Selected CDK Inhibitors That Are/Were in Clinical Trials

	selectivity (nM)							
inhibitor	CDK1/B	CDK2/A (CDK2/E)	CDK4/E (CDK4/D1)	CDK5/p25 (CDK5/p35)	CDK6/D	CDK7/H	CDK9/T	ref
1[a]	>10000	>10000	(11)	>10000	15			13
2[a]	2100	100	13500	160	23500	540	950	2
3[a]	578	5	232	21		193	26	3
4[a]	480	38 (48)	(925)	(340)	>1000	62	4	4
5[b]	190	44 (510)	(67)	(18)	660	2800	<100	4
6[a]	7	(9)	(11)	(<10)		25	5	5
7[a]	1−3	1−3	1−3					10
8[a]	27	405 (282)	(132)		395	514	11	6
9[a]	79	224 (2540)	(63)		396	2870	20	7
10[a]	25		(90)				22	8
11[a]	3	1		1			4	9
13[c]	1627	(504)	(2)		10	3910	57	11

[a]In vitro IC_{50}. [b]K_i. [c]K_i ATP cellular profiling.

bromodomain-containing protein 4 (BRD4) associates with CDK9. BRD4 acts on two different residues to either inhibit (Thr^{29}) or activate (Thr^{186}) CDK9. In the absence of BRD4 association, activation of CDK9 by autophosphorylation is miminal.[25] BRD4 kinase activity is inhibited by CDK7 mediated phosphorylation of BRD4.[25] Interestingly, the Fischer lab used chemical genetic studies and demonstrated CDK7 as another CDK9 activating kinase in vitro and on transcribed chromatin.[26] These studies indicate that CDK9 is activated by multiple kinases and suggest that the context of CDK9 is critical for the mode of activation. The existence of a CDK7-BRD4-CDK9 loop is also indicated by recent studies from the Zhou lab.[27] A BRD4 mediated compensatory mechanism is activated upon CDK9 inhibition and argues the need for simultaneous blockade of CDK9 and BRD4 as a therapeutic strategy. Similarly, the inactivation of CDK9 kinase activity is regulated through multiple mechanisms. Phosphorylation of Thr^{29} in the glycine rich loop of CDK9 by BRD4 inhibits its

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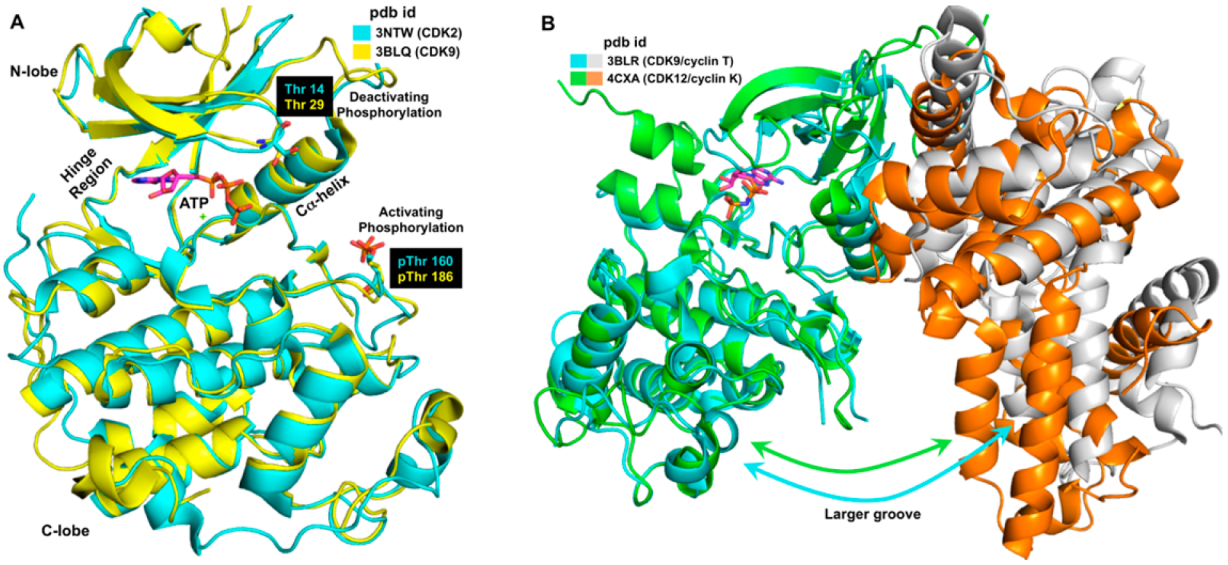

Figure 2. (A) Overlay of the kinase domains of CDK2 and CDK9 to show key residues that regulate the kinase activity through phosphorylation−dephosphorylation. (B) Overlay of CDK9 and CDK12 to show the larger groove created by the cyclin T complex when compared to the cyclin K complex.

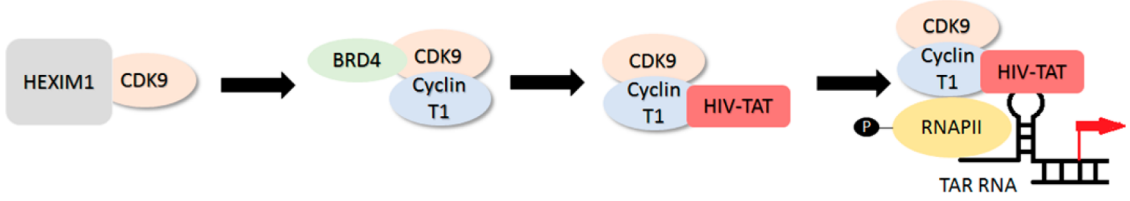

Figure 3. Flow diagram that describes the role of CDK9 in HIV transcription. The CDK9/cyclin T1 complex is sequestered by the HEXIM1 protein complex and is inactive in this state. It is pulled from the complex by BRD4 and becomes active. The HIV-Tat protein binds to the cyclin T1 subunit and recruits the complex to the TAR RNA, where it phosphorylates RNA polymerase II and moves it past elongation pausing.

kinase activity which is similar to that observed with the phosphorylation of Thr^{14} on CDK2 (Figure 2A).[25,28] An alternative mode for the inactivation of CDK9 kinase activity is through dephosphorylation of Thr^{186} by Mg^{2+}/Mn^{2+} dependent phosphatases 1A (PPM1A).[29] Mutation of conserved residues Tyr^{271} or Phe^{208} also adversely affects the kinase activity of CDK9.[23]

In HeLa extracts, CDK9 exists in a complex with cyclins T1, T2a, and T2b in an 8:1:1 ratio.[30] Following synthesis, the handoff of CDK9 through the chaperones Hsp70/Hsp90/Cdc37 leads to the proper folding and association with a cyclin to form the complex.[31] CDK9 binding to cyclin dramatically increases the stability of CDK9. In the absence of cyclin binding, CDK9 has a half-life of ∼6 h. Complexation with cyclin T increased the half-life of CDK9 by ∼6-fold. The CDK9/cyclin complexes are involved in a number of cellular functions. CDK9/cyclin K complex binds RNA and single stranded (ss) DNA, while CDK9/cyclin T complexes can bind double stranded (ds) DNA in addition to ssDNA/RNA. Figure 2B is an overlay of CDKs with cyclins K and T, and it illustrates that CDK/cyclin T complex has a larger groove when compared to CDK/cyclin K complex.[32,33] Structurally, this suggests that the difference in binding to DNA/RNA observed with the CDK9/cyclin K and CDK9/cyclin T can be attributed to the deeper groove observed in the CDK9/cyclin T complex when compared to CDK9/cyclin K complex.[32,34]

CDK9/cyclin heterodimers are a component of a larger protein complex called positive transcription elongation factor b (P-TEFb). CDK9 in the P-TEFb complexes phosphorylates the C-terminal domain of RNA pol II, a key regulatory mechanism during elongation.[17] In vitro, all four CDK9/cyclin T1, CDK9/cyclin T2a, CDK9/cyclin T2b, and CDK9/cyclin K complexes possess transcription elongation activity.[30] The CDK9/cyclin T2 complex phosphorylates retinoblastoma protein to regulate muscle differentiation.[35] The 55 kDa CDK9 isoform in complex with Ku70 and the 42 kDa CDK9 in complex with cyclin K plays a role in maintaining genome integrity.[36] Specifically, in response to hydroxyurea induced replication stress, CDK9/cyclin K complex is recruited to the chromatin which stalls the replication fork.[37]

■ ROLE OF CDK9 IN HIV

CDK9 was initially explored as a druggable target for human immunodeficiency virus (HIV) therapy. Approximately half of all cellular CDK9 is sequestered by binding to a ribonucleoprotein (snRNP) in the P-TEFb complex and regulates its activity through hexamethylene bisacetamide-inducible protein 1 (HEXIM1). HEXIM1 contains a peptide sequence that binds to the kinase to occlude the active site, thereby preventing CDK9 from binding to its substrates (Figure 3).[38] Protein phosphatase 2A dephosphorylates the C-terminal residues, which prevents CDK9 binding to the HIV TAR (transactivation response element) RNA and blocks transcription. Proteins involved in the phosphorylation−dephosphorylation mediated regulation of CDK9 are potential candidates to develop HIV therapies.[39]

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ROLE OF CDK9 IN CANCER

The carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAPII) contains heptad repeats with the following sequence: $YS^2PT^4S^5PS^7$.[40] Phosphorylation of serine and threonine residues in this sequence is tightly coordinated by kinases CDK7 and CDK9 to regulate transcription and mRNA splicing.[41−46] Phosphorylation of Ser^2 ($pSer^2$) on RNAPII by CDK9 is used as a marker in preclinical pharmacodynamics studies.[47] The case for CDK9 as a target in oncology is based on the observation that CDK9 regulates the RNA transcription of short-lived antiapoptotic proteins. Inhibition of CDK9 mediated phosphorylation of the CTD RNAPII results in reduced levels of antiapoptotic proteins such as Mcl-1 and XIAP, which reinstates the ability of cancer cells, particularly in lymphocytic leukemias, to undergo apoptosis.[48,49] Transformed cells are addicted to the sustained expression of antiapoptotic proteins. As a result, a hypothesis that transient inhibition of CDK9 blocks transcription of these antiapoptotic proteins was put forth. This was tested in an elegant cell based screening cascade designed to biochemically and phenotypically distinguish compounds that induce antiproliferative effects by inhibiting transcriptional CDKs from those that inhibit cell cycle CDKs. Protein levels of p53 and mitotic index were used as readouts for transcriptional CDK inhibition and cell cycle CDK inhibition, respectively. This study showed that transient inhibition of CTD RNAPII phosphorylation in both transformed and nontransformed cells to similar levels induced caspase-dependent apoptosis only in the transformed cells.[50]

Recent studies that used RNAi methods along with small molecule inhibitors to define the role of CDK9 in cancer are summarized here. Transient knock-down of CDK9 sensitized HeLa and A549 cells to the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) by down-regulating FLICE-like inhibitor protein (c-Flip) and Mcl-1. The combination treatment of a CDK inhibitor **4** (SNS-032)[51] and TRAIL showed synergistic antitumor effects in a lung cancer model. In hepatocellular carcinoma (HCC) lines, shRNA mediated knock-down of CDK9 showed a range of responses that were independent of the knock-down efficiency or proliferation rates. Moreover, in contrast to CDK9 inhibition by small molecules, CDK9 knock-down did not induce apoptosis but inhibited proliferation of HCC lines.[52] In head and neck squamous cell carcinoma cell lines, knock-down of CDK9 or inhibition by a small molecule inhibitor led to radiosensitization, while overexpression of CDK9 provided radioprotection.[53,54] In ovarian cancer cells (A2780), the shRNA knock-down of CDK9 or the treatment of cells with CDK inhibitors, flavopiridol (CDKI-73, **8**),[55] induced apoptosis. In a cell viability study, unlike wild-type A2780 cells, **8** had no effect in CDK9 knock-down cells at the 24 h time point, indicating selectivity for CDK9. However, CDK9 knock-down had no effect on **8** induced growth inhibition at the 72 h time point, likely due to off-target effects. These studies highlight that the anticancer effects associated with inhibition of CDK9 is dependent on how CDK9 function is inhibited, the cell type used in the study, and the kinetics associated with CDK9 inhibition.

The Graña lab conducted gene expression profiling in a glioblastoma cell line (T98G) to determine the differential effects of CDK9 inhibition through chemical (**8**) and genetic (dominant negative CDK9) methods. Expression of kinase dead CDK9 dominant negative (DN) mutant in cells displaced the wild-type CDK9 from the various cellular complexes to render it catalytically inactive. They observed significant differences on gene expression with inhibition of CDK9 by DN-CDK9 compared to **8** treatment. They concluded that in addition to CDK9, **8** targets other molecules to modulate gene expression, likely leading to the above observed 72 h growth inhibition in CDK9 knock-down cells.[56] In a follow-up study, they used immortalized normal fibroblasts and primary human astrocytes along with modulating CDK9 activity with siRNA to minimize inherent technical issues associated with the previous study. The results confirmed that the mode of CDK9 inhibition had a significant effect on the gene expression pattern. Although the number of common genes that were affected by **8** treatment and DN-CDK9 and CDK9 siRNA treatment was small, cluster analyses showed a higher correlation among the treatments when compared to the cell type used in the study.[57] These studies highlight the importance of multiple modes of perturbation of a target in multiple models during the validation of a potential target for therapeutic intervention.

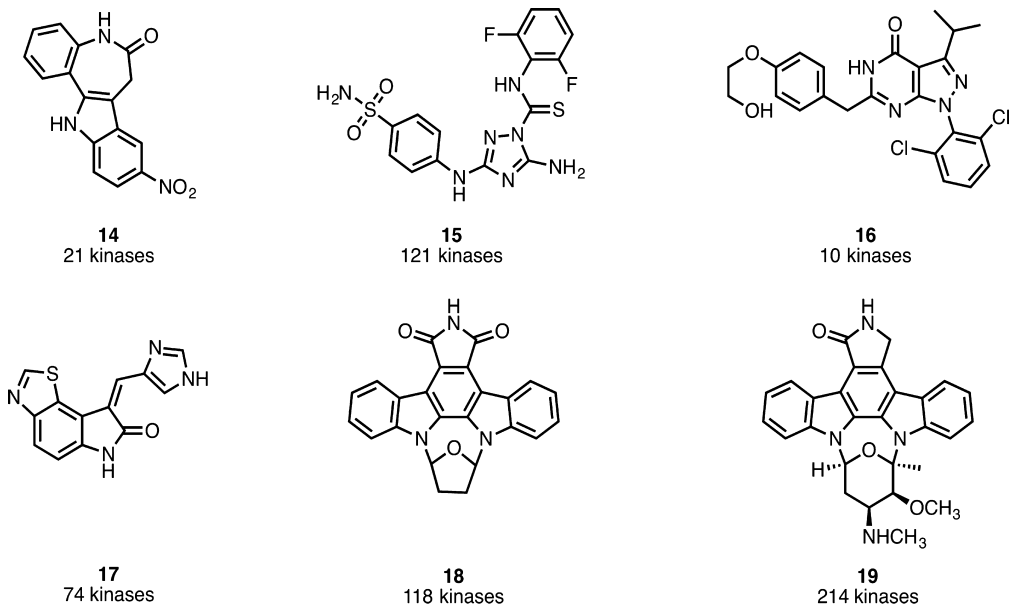

Figure 4. Compounds that inhibited the kinase activity of both CDK9/cyclin T1 and CDK9/cyclin K complexes. The numbers below the structure represent the number of kinases in the set of 300 that were inhibited >75%.

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The recent report from the Brasier lab adds another variable for the validation of CDK9 as a target. In resting cells, complexes with low and high CDK9 activity exists in a ∼1:1 ratio.[58] This distribution is altered by a number of stimuli including respiratory syncytial virus (RSV) infection, which triggers a switch in the composition of the complex to increase the population of the complexes with high CDK9 activity.[59] Differential quantitative proteomics in resting and poly(I:C) stimulated lung cancer (A549) cells were used to obtain a comprehensive profile of CDK9 interactome. 591 proteins in the CDK9 interactome were found, which includes ∼70% novel high-confidence proteins. This study expands the role of CDK9 to pre-mRNA splicing and to mRNA transport/translation.[60] These studies highlight the need for additional cellular readouts for the development and validation of CDK9 small molecule inhibitors. It is clear that CDK9 is involved in the regulation of a number of cellular functions, many of which are critical for cancer cell survival.

MINING LARGE-SCALE SCREENING SETS FOR KINASE INHIBITORS FOR CDK9 SELECTIVITY

Anastassiadis et al. reported a large-scale screen with 178 small molecule kinase inhibitors, which included FDA-approved drugs, small molecule inhibitors in preclinical development, and clinical candidates against a panel of 300 protein kinases representing all major kinase families.[61] The screen included the following cyclin dependent kinases and cyclins: CDKs 1, 2, 3, 4, 5, 6, 7, and 9; cyclins A, B, D1, D3, E, H, K, and T1 along with CDK5 activators p25 and p35. We analyzed these data and generated correlation coefficients among the CDKs (Table 2). A value of 1 would imply that inhibitors in the library could not distinguish between the corresponding CDK/cyclin complex. The highest r^2 value of 0.91 was observed for CDK2/cyclin A and CDK5/p35, and the lowest r^2 value of 0.12 was observed for CDK7/cyclin H and CDK9/cyclin T1.

In this set of 178 inhibitors, seven showed >75% inhibition for both CDK9/cyclin T1 and CDK9/cyclin K under the assay conditions. Six (**14**−**19**) of the seven inhibitors were common for both CDK9/cyclin T1 and CDK9/cyclin K (Figure 4). On the basis of the 75% inhibition criteria, the six compounds (**14**−**19**) identified as CDK9 inhibitors also inhibited 7%, 40.3%, 3.3%, 24.6%, 39.3%, and 71.3% of kinases, respectively. Among the six inhibitors, compound **16** is the most selective as it inhibits only 3.3% of kinases and compound **19** is the least selective as it inhibits 71.3% of the 300 kinases screened. Interestingly, of the 14 CDK/cyclin combinations in the 300 kinase set, 10 of them were inhibited (>75%) by compound **16**. This suggests that mining publically available large databases with in vitro screen data is a good source for small molecule inhibitors that can serve as hits that are suitable for optimization. Analysis of the data from this study also indicates that it is easier to develop CDK selective inhibitors but is more challenging to identify inhibitors that are selective among CDK/cyclin combinations.

SCAFFOLDS EXPLORED FOR THE DEVELOPMENT OF CDK9 INHIBITORS

The core structures explored thus far to develop CDK9 inhibitors include chromones, pyrimidines, pyrazoles, imidazoles, purines, and thiazoles. In addition, specific compounds that are in preclinical and clinical development will be discussed here.[62−77] Nearly all the core structures are anchored to the

Table 2. Correlation Coefficients of the Various CDK/Cyclin with a Diverse Library of Small Molecule Kinase Inhibitors

	CDK1/cy A	CDK1/cy B	CDK2/cy A	CDK2/cy E	CDK3/cy E	CDK4/cy D1	CDK4/cy D3	CDK5/p25	CDK5/p35	CDK6/cy D1	CDK6/cy D3	CDK7/cy H	CDK9/cy K	CDK9/cy T1
CDK1/cy A	1													
CDK1/cy B	0.73	1												
CDK2/cy A	0.70	0.79	1											
CDK2/cy E	0.67	0.68	0.88	1										
CDK3/cy E	0.73	0.61	0.73	0.76	1									
CDK4/cy D1	0.47	0.49	0 53	0.45	0.42	1								
CDK4/cy D3	0.63	0.62	0.59	0 53	0.52	0.66	1							
CDK5/p25	0.74	0 73	0.87	0.88	0.69	0 34	0 46	1						
CDK5/p35	0.67	0.76	0.91	0 89	0.75	0.47	0 52	0 86	1					
CDK6/cy D1	0.60	0.57	0 62	0.58	0 59	0.61	0 72	0 62	0.64	1				
CDK6/cy D3	0.49	0.50	0 51	0.47	0 50	0.53	0.58	0.52	0.56	0.81	1			
CDK7/cy H	0.50	0.43	0.41	0.45	0.50	0.16	0 32	0 46	0.48	0.38	0.34	1		
CDK9/cy K	0.49	0.52	0.60	0 61	0.54	0.45	0.48	0.56	0.60	0 66	0.49	0.30	1	
CDK9/cy T1	0.50	0 50	0 51	0.54	0.44	0.31	0 39	0.47	0.47	0 20	0.24	0.12	0 73	1

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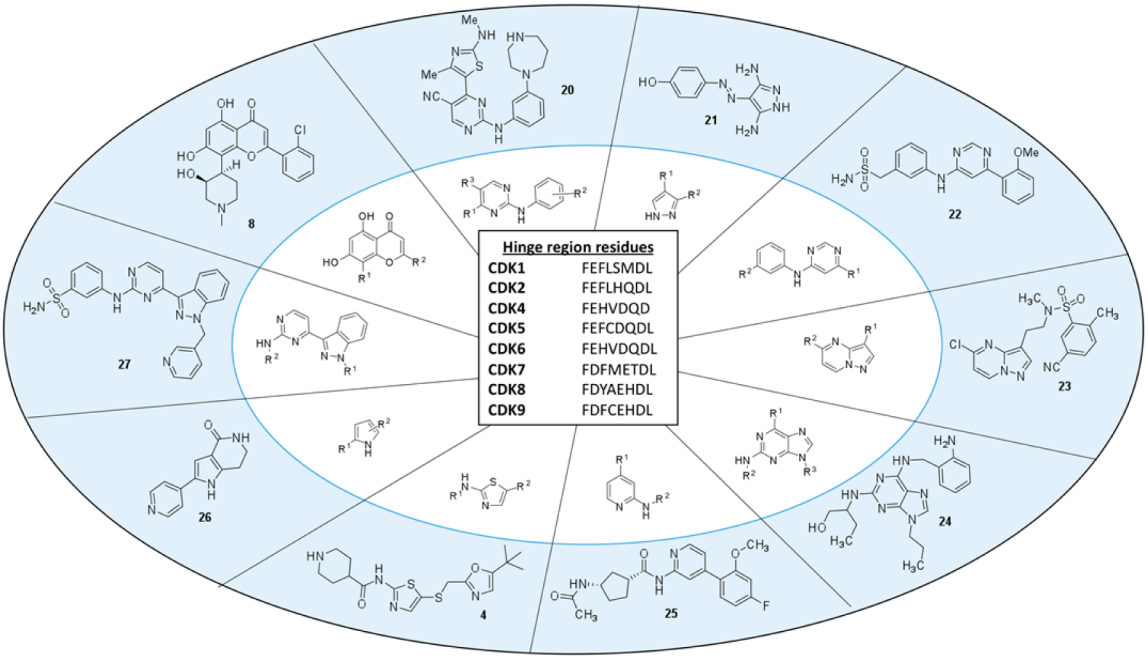

Figure 5. Structures in the inner and outer ring illustrate the core and specific CDK inhibitors. Shown are hinge region residues of CDKs (PDB codes 4YC6, 1VYZ, 3G33, 3O0G, 2EUF, 1UA2, 3RGF, and 3TN8).

CDKs through their interaction with the hinge region residues (Figure 5). The backbone oxygen atom on the carbonyl and hydrogen on the amide of the middle residues (a hydrophobic and an acidic/basic residue) are involved in anchoring small molecule inhibitors. Designing functional groups outside this region to target the clefts within the ATP binding site that are different among the kinases yields selective small molecule inhibitors. Nearly all the studies use in vitro cell free kinase assays to demonstrate selectivity. This is because the design principles use cocrystal structures to generate analogs with improved selectivity. Seldom do these studies show the selectivity in cell-based systems. This is important because most kinases are part of larger complexes and the conformations adopted by the kinase within these complexes may not be accurately captured by the in vitro kinase assays. Notwithstanding this caveat here, we will summarize structure−activity relationship (SAR) studies guided by in vitro assays.

Flavonoids. Compound **8** is an alkaloid natural product and was identified as a CDK inhibitor with growth inhibitory activity against a number of cancers.[78−81] Although preclinical studies with **8** were promising, results from the phase I/II clinical trials were not encouraging.[82,83] Acute myeloid leukemia (AML) is a hematologic malignancy characterized by an aberrant accumulation of immature myeloid precursor cells. Patients with AML respond poorly to the conventional chemotherapy agents. "7 + 3" [7 days of continuous infusion (CI) of cytarabine and 3 days of anthracycline] remains the standard induction therapy in the United States for newly diagnosed AML patients.[84,85] Recently, a phase II study demonstrated that induction therapy with FLAM [**8**, followed by cytarabine and mitoxantrone] showed improved remission rates with nearly 67−80% in newly diagnosed high-risk AML patients.[86−88] Compound **8** has been granted "orphan drug" status for treatment of AML.[89] Thus, far, ∼60 clinical trials were carried out with **8** against multiple myeloma, leukemia, lymphomas, sarcoma, and solid tumors as a single agent or in combination with other drugs.[83,90−93]

Cocrystal structure of **8** with CDK2 revealed it as an ATP-competitive inhibitor.[94] Subsequent studies showed that **8** inhibited transcription and studies from the Price lab suggested that **8** is a non-/un-ATP competitive inhibitor that binds tightly to CDK9.[47,95−97] Cocrystal structure of **8** with the CDK9/Cyclin T1 complex showed that **8** indeed binds to the ATP-binding site with only ∼8% of surface of **8** exposed (Figure 6A). The 2-chlorophenyl group in **8** made favorable electrostatic contacts with CDK9 over CDK2. In the CDK2−inhibitor **8** cocrystal structure, Lys^{89} (Figure 6C) residue was shifted to accommodate **8** and Gly^{112} made such a shift in CDK9 unnecessary. In addition, in CDK9, the glycine rich loop (residues 27−36, Figure 6B) folds over the active site and facilitated van der Waals contact between the hydrophobic (Ile^{25} and Val^{33}) residues with inhibitor **8** (Figure 6B). The Phe^{30} residue in the glycine rich loop moved to make additional hydrophobic contacts with the piperidinyl group of inhibitor **8**. Upon **8** binding, this major conformational change of the $\beta 3/\alpha c$ loop (residues 51−55, Figure 6B) locked the G-loop in a "closed" conformation, which explained the tight binding of CDK9.[32] This tight binding was reflected in a large change in melting temperature (ΔT_m = 5.03 ± 0.13 °C) measured by differential scanning fluorimetry (DSF).[98]

Compound **8** inhibited HIV-1 tat transactivation and viral replication by inhibiting CDK9 activity. However, in long-term replication assays, **8** showed a reduced antiviral efficacy and a greater cytotoxicity in physiologically relevant cell types, viz., peripheral blood lymphocytes (PBLs) and monocyte-derived macrophages.[99] To address this issue, a series of **8** analogs were synthesized and evaluated in cell-free and cell-based assays to improve the selectivity (Table 3).[100] Analogs of **8** inhibited CDK9 more potently than CDK2, suggesting that the size and the location of the substituent on the phenyl ring at the 2-position of the chromenone have an effect on in vitro activity and selectivity. Interestingly, decreasing the hydrophobicity at 2-position resulted in significant loss of activity in the cell-based cytotoxic assay. The lack of correlation between the cell-free

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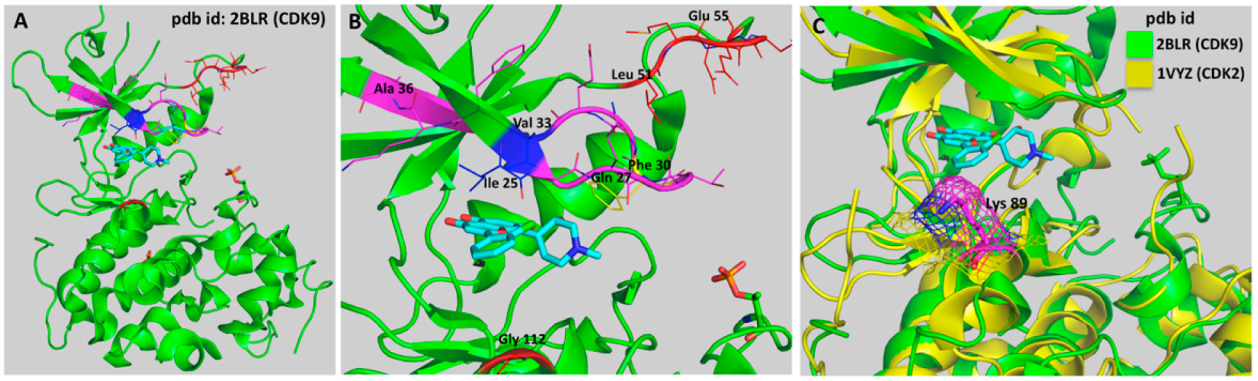
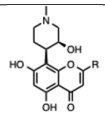


Figure 6. Binding mode for the inhibitor **8** in the ATP-binding sites of CDK9/cyclin T (green) and CDK2 (yellow): (A) cocrystal structure of CDK9 and inhibitor **8**; (B) close-up of binding interactions; (C) overlay of the CDK9 and CDK2 to show the effect of Lys^{89} in CDK2 and Gly^{112} in CDK9.

Table 3. Cell-Free and Cell-Based Assay with Analogs of Inhibitor 8

	 R =	IC_{50} (nM)		CDK9/CDK2	Cytotoxicity (μM)
		CDK9/cyT1	CDK2/cyA		
8	2-chlorophenyl	2.5	161.4	0.02 (1.0)	0.120
28	Phenyl	9.0	196.0	0.04 (3.0)	0.190
29	2-fluorophenyl	2.8	356.5	0.01 (0.7)	0.274
30	4-fluorophenyl	2.1	129.1	0.02 (1.1)	0.200
31	4-bromophenyl	5.5	223.5	0.02 (1.6)	0.280
32	4-vinylphenyl	9.5	206.2	0.05 (3.1)	0.219
33	5-methylisoxazole	20	238.3	0.08 (5.6)	0.645
34	4-hydroxyphenyl	9.6	196.0	0.05 (3.3)	20.92
35	4-pyridyl	6.5	208.2	0.03 (2.1)	3.350
36	4-trifluoromethylphenyl	19	301.9	0.06 (4.2)	1.200

target specific assay and the cell-based cytotoxic assay highlights the need for additionally assessing selectivity for kinase inhibition in cell-based systems.

Compound **9** (Figure 1, P276-00)[101] is a flavone based small molecule that selectively inhibits CDKs 1, 4, and 9 over the other CDKs. It inhibits cancer cell growth with nanomolar potencies ($IC_{50} \approx 300-800$ nM) and exhibits good selectivity over normal fibroblast cells.[102,103] Biochemical studies demonstrated that **9** inhibits RNA polymerase II phosphorylation and down-regulates Mcl-1.[104] Compound **9** has been subjected to 11 clinical trials either as a single agent or in combination with others to treat various cancers (www.cancer.gov).

Pyrimidines. Pyrimidine is a privileged core structure found in a number of kinase inhibitors.[105] AstraZeneca explored the aminopyrimidine core to develop CDK inhibitors. Virtual screening followed by structure-based design identified 4-(2,4-dimethylthiazol-5-yl)pyrimidin-2-amine (**37**)[106] as a CDK inhibitor. Using this as a starting point in a series of reports, the Fisher and Wang groups discussed their SAR results and CDK-small-molecule inhibitor cocrystal structures, which led to the discovery of a potent CDK2/CDK9 inhibitor **38**[67] with low-nanomolar (in vitro) K_i values and nanomolar (~300 nM) IC_{50} values in 3-day cell-based growth inhibitory assays in a panel of cancer cell lines (Figure 7). A follow-up study employed a cell-based phenotypic screening cascade to classify pyrimidine analogs into transcriptional, cell cycle, and mitotic inhibitor groups. SAR data support the hypothesis that inhibition of CDK9 results in reduced Ser^2 phosphorylation of RNAP-II, which is sufficient to inhibit transcription. Further optimization led to the identification of **39**[50] as a transcriptional inhibitor that is selective for CDK9 with antitumor activity in animal models (Figure 7). The trisubstituted pyrimidine core was subjected to additional SAR studies, which led to the identification of ~10- and ~20- fold selective CDK9 analog **40**[107,108] over CDK7 and CDK2, respectively (Figure 7). Further optimization yielded **20**[98] as a lead compound that is ~80-fold selective for CDK9 over CDK2.[76] Binding associated change in T_m of analog **20** ($\Delta T_m = 3.92 \pm 0.32$ °C) is comparable to flavopiridol. Importantly, the lead candidate **20** is ~31- and ~107-fold selective for primary chronic lymphocytic leukemia cells over normal B- and T-cells.[76]

The selectivity of **20** for CDK9 over CDK2 was explained through structural studies. For a large part, in the ATP binding

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J. Med. Chem. 2016, 59, 8667−8684

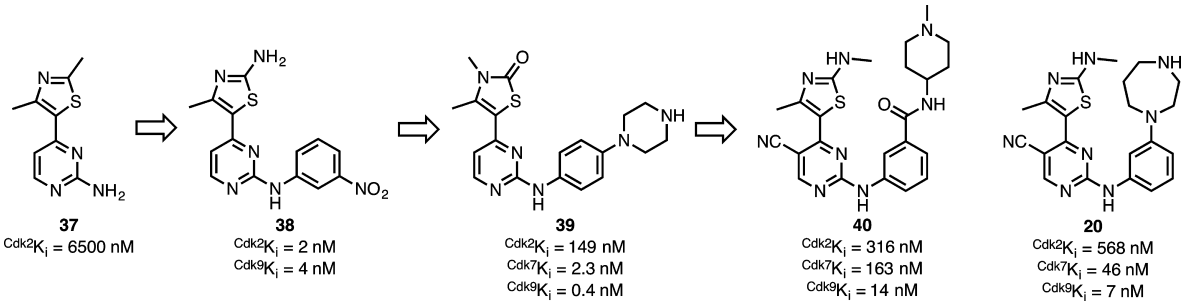

Figure 7. Aminopyrimidine analogs and their in vitro CDK inhibitory activities.

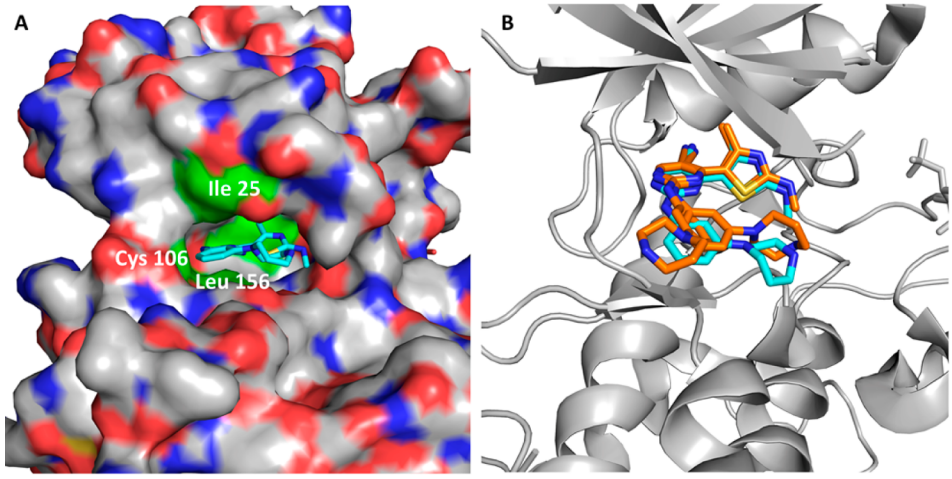

Figure 8. Binding mode for the inhibitor **20** in the ATP-binding sites of CDK9/cyclin T and CDK2/cyclin A: (A) cocrystal structure of CDK9 and inhibitor **20**; (B) cocrystal structure of CDK2 and inhibitor **20**. Data were extracted from PDB code 3BCP for CDK2 and from PDB code 4BCG for CDK9.

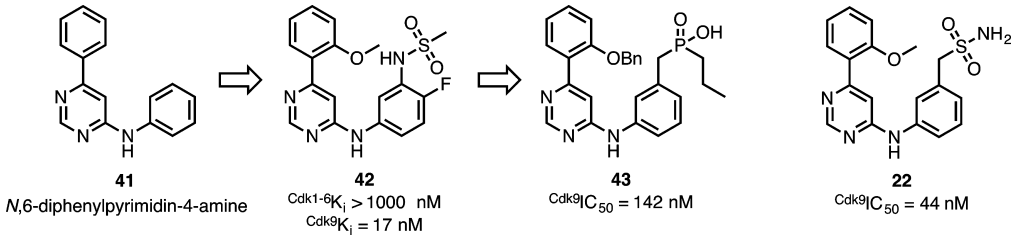

Figure 9. Phosphorus and sulfur containing *N*,6-diphenylpyrimidin-4-amine analogs as CDK9 inhibitors.

site, compound **20** has a similar binding mode for CDK9 (Figure 8A) and CDK2. The *N*1-pyrimidine and C2-NH pyrimidine interacts via hydrogen bonding with the backbone NH and CO groups of the hinge region residue Cys[106] in CDK9 (Leu[83] in CDK2). The C5-carbonitrile occupies the hydrophobic pocket near the gatekeeper residue (Phe[103] in CDK9 and Phe[80] in CDK2) and forms favorable lone pair−π interactions. The pyrimidine ring is sandwiched between the hydrophobic side chains of Ala[46] and Leu[156]. On the other hand, a different binding mode was observed for 1,4-diazepan-1-ylaniline moiety with CDK9 and CDK2 (Figure 8B). In the CDK9−inhibitor **20** complex, the 1,4-diazepane ring adopts an "inward" conformation oriented toward the thiazole ring, whereas in the CDK2-inhibitor **20** complex, the 1,4-diazepane ring adopts multiple conformations as inferred from the higher b-factors. Compared to CDK2, due to flexible backbone residues in the hinge region, **20** is accommodated better in CDK9 ATP binding site. Moreover, upon inhibitor binding, flexible G-loop in CDK9 induces a shift toward the ATP binding site, and such changes were not observed with CDK2, which indicate that the ATP binding site of CDK9 is larger and more flexible compared to CDK2.[98,109]

Screening a focused library of substituted *N*,6-diphenylpyrimidin-4-amine (**41**) analogs led to the identification of a remarkably selective CDK9 inhibitor **42**[110] with a low-nM CDK9 inhibitory activity and ∼3-fold less potency than **8**. Isosteric replacements of functional groups to improve ADMET properties are a common tactic used by medicinal chemists.[111] In a follow-up study, isosteric replacements of the sulfonamide group in **42** with phosphonamidates, phosphinates, and phosphonates were explored. SAR studies revealed phosphinic acid **43**[112] as a potent and selective ATP competitive CDK9 inhibitor.[113] Although a structurally similar analog with a sulfonamide **22** was previously reported as a potent CDK9 inhibitor, kinase profiling revealed **43** possessed superior selectivity (Figure 9).

Marine organisms constitute a promising and underexplored source of bioactive molecules. Indole alkaloids, a class of bioactive compounds (Table 4), are frequently found in marine invertebrates.[114] Meridianin is a family of 3-(2-aminopyrimidine)indoles

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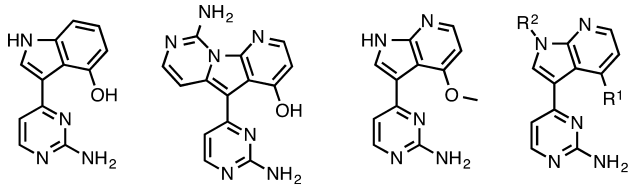
Table 4. Pyrimidine Containing Natural Products, Their Analogs, and the Kinase Inhibitory Activity

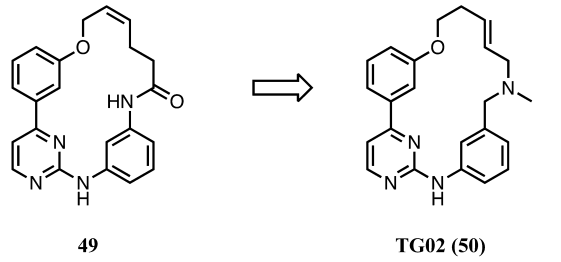


			IC_{50} (nM)			
	R^1	R^2	CDK1	CDK2	CDK5	CDK9
44	H	H	780	90	510	26
45	OH	H	57	18	50	18
46	OMe	H	170	11	170	6
47	OPr	H	7	3	3	6
48	OMe	Me	>10000	>10000	>10000	>10000

and was isolated from Ascidian *Aplidium meridianins*.[115] Subsequently, meridianin and its analogs were chemically synthesized and evaluated for kinase inhibitory properties.[116−119] Meridianins share structural homology with another natural product, variolins, extracted from the sponge *Kirkpatrickia variolosa*[120,121] and recently synthesized.[122−126] Echalier et al. synthesized and screened a library of 3-(pyrimidin-4-yl)-7-azaindoles (meriolins), a chemical hybrid of meridianins and variolins.[127] Meriolin binds to the hinge region of CDK2 via hydrogen bonding involving two nitrogen atoms within the pyrrolo[2,3-*b*]pyridine ring. Structural studies showed that both meridianins and variolin B occupy the kinase ATP binding site (CDK2 or CDK9). The inhibitory activity and selectivity for CDK9 among substituted meriolins are sensitive to substitutions at the R^1-position (**44**−**48**).[128] Interestingly indole NH (**46**) as opposed to the aminopyrimidine is involved in hydrogen bonding with the hinge region as evident by the loss of activity of *N*-methyl analog (**48**). Meriolin 3 (**46**) showed potent antitumor activity in animal models of Ewing sarcoma and colorectal cancer.

CDK9 MACROCYCLIC INHIBITORS

Macrocycles are an underexploited class of drug molecules that provide intrinsic structural preorganization and sufficient flexibility to mold to target sites for maximum binding interactions without a major entropic loss on binding.[129] Initial macrocyclic hit **49** was further optimized to yield SB1317 (TG02, **50**),[130] a pyrimidine-based multikinase inhibitor. Originally, **50** was reported as a CDK2, JAK2, and FLT3 inhibitor. It inhibits signaling pathways downstream of CDKs 1, 2, 5, 7, 9, JAK2, and FLT3 (Figure 10). Compound **50** showed antitumor activity via CDK9 inhibition, which led to the reduction of Mcl-1 levels and resulted in p53-independent apoptosis in a wide range of tumor cells. Compound **50** is currently evaluated in phase I clinical trial as a single agent in patients with chronic lymphocytic leukemia (CLL) and in combination with carfilzomib in patients with multiple myeloma (MM).[131] The cytotoxic mechanism of **50** and benzamide, 4-[4-[(4′-chloro[1,1′-biphenyl]-2-yl)methyl]-1-piperazinyl]-*N*-[[4-[[(1R)-3-(dimethylamino)-1-[(phenylthio)-methyl]propyl]amino]-3-nitrophenyl]sulfonyl]- (ABT-737) or benzamide, 4-[4-[[2-(4-chlorophenyl)-4,4-dimethyl-1-cyclohex-en-1-yl]methyl]-1-piperazinyl]-*N*-[[3-nitro-4-[[(tetrahydro-2*H*-pyran-4-yl)methyl]amino]phenyl]sulfonyl]-2-(1*H*-pyrrolo[2,3-*b*]-pyridin-5-yloxy)- (ABT-199 or venetoclax)[130] is complementary,



Kinase	IC_{50} (μM)	Kinase	IC_{50} (nM)
CDK2	2.6	CDK1, 2, 3, 5, 7, and 9	3-15
JAK2	1.7	JAK2	73
FLT3	1	$FLT3^{D835Y}$	21

Figure 10. Optimization of pyrimidine based macrocyclic pan CDK inhibitor.

therefore generating a robust synergistic activity in AML patient samples.[132]

Miscellaneous. Utilization of an in vivo screening approach with efficacy and tolerability as parameters resulted in identification of pyrazolo[1,5-*a*]pyrimidine based **11**[133] as a potent CDK inhibitor. Compound **11** (Figure 1) potently inhibits CDKs 1, 2, 5, and 9 activity in vitro with IC_{50} values of 3, 1, 1, 4 nmol/L, respectively.[9] Merck advanced **11** into phase III development for refractory chronic lymphocytic leukemia (CLL). **11** potently down-regulates the expression of Mcl-1 in CLL cells and antagonizes protection mediated by multiple soluble proteins important in the microenvironment of CLL.[134,135]

Recently, a novel pyrimidine based reversible ATP competitive inhibitor **51** (Figure 11, LY2857785)[136] was identified through

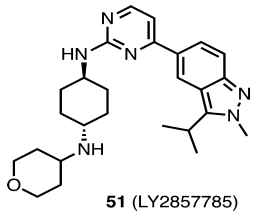


Figure 11. Pyrimidine based CDK9 inhibitor.

structure based design and SAR studies as a selective transcriptional CDK inhibitor (CDK9 IC_{50} = 11 nM, CDK8 IC_{50} = 16 nM, and CDK7 IC_{50} = 246 nM). Biochemical and cell cycle studies suggested transcriptional inhibition as its mode of action. Moreover, **51** possessed good overall selectivity against a panel of 115 kinases, high aqueous solubility, excellent stability in solution, and excellent physiochemical properties. Inhibitor **51** has comparable potency to **8** both in vitro and in vivo and demonstrated good inhibitory activity in a panel of tumor cell lines but had the highest activity in AML and other hematological cancer types (IC_{50} < 50 nM). **51** inhibited proliferation and induced apoptosis in a panel of cancer cell lines. Western blot analysis showed decreased levels of antiapoptotic proteins Mcl-1 and XIAP in hematologic cancer cell lines. However, in vitro human bone marrow colony formation assay and animal toxicity studies showed **51** inhibits proliferation of normal hematopoietic cells in dose-dependent and time-dependent manner.

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Animal toxicology studies with **51** revealed dose dependent toxicity to key organs such as bone marrow and gastrointestinal tract, thereby limiting its clinical use.

Pyrazoles. Aminopyrazole is one of the many core structures that effectively mimic the adenine ring in ATP, and substituted aminopyrazoles have been explored as CDK inhibitors.[137,138] In house libraries screening identified an arylazo-substituted-1*H*-pyrazole-3,5-diamine as a CDK inhibitor.[69] A follow-up synthesis and evaluation of a focused arylazo-substituted-1*H*-pyrazole-3,5-diamine library identified **21**[69] as an ATP competitive CDK inhibitor that reduced phosphorylation of Rb and CTD RNAPII. The enhanced potency of **21** for CDK2 and CDK9 over the parent compound can be attributed to the hydrogen bond between the phenolic hydrogen and Glu[51] and Glu[66], respectively (Figure 12). The enhanced selectivity of

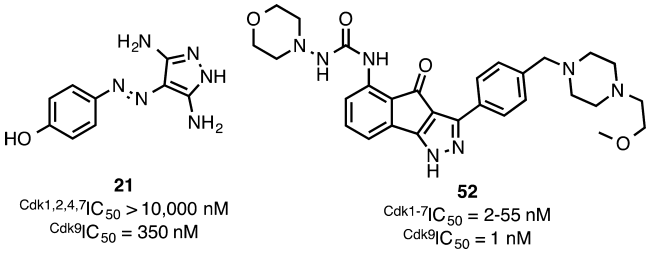

Figure 12. Pyrazole based CDK9 inhibitors.

21 for CDK9 is attributed to the apparent flexibility that enables better binding.[109]

Structure guided fragment-based studies led to the identification of an indazole core as an ATP competitive CDK inhibitor.[139] Truncation of indazole by removal of fused benzene ring led to the simplified pyrazole core with similar ligand efficiency (LE). Structure-guided optimization of the pyrazole core led to the discovery of a clinical candidate **5** (AT7519, Figure 1).[140] In multiple myeloma cells, **5** displayed potent cytotoxicity, induced apoptosis, and inhibited RNA polymerase II. In vitro kinase profiling showed that **5** is an ATP competitive inhibitor, and its cytotoxic effects were attributed to the inhibition of CDK9, CDK5, and GSK3b. The pyrazole-based CDK9 (IC_{50} < 10 nM) inhibitor **5** has been subjected to phase II clinical trials for the treatment of mantle cell lymphoma, chronic lymphocytic leukemia, and multiple myeloma.[140,141] Also, as a combination therapy with HSP90 inhibitor, compound **5** is in phase I clinical trials for the treatment of solid metastatic tumors that are not surgically resectable (clinicaltrials.gov).

Inhibitor **52** (RGB-286638)[142] is a pan-CDK inhibitor with the indenopyrazole core (Figure 12) that was evaluated in phase I clinical trials for the treatment of solid tumors. In vitro kinase profiling showed **52** as a potent CDK9 inhibitor.[143] Inhibitor **52** displayed caspase-dependent apoptosis, which correlated with down-regulation of RNA polymerase II and inhibition of transcription, suggesting CDK9 inhibition as the primary mode of action.

The Bcl-2 family member, Mcl-1, is a key driver of cell survival and apoptosis in diverse cancers. In AML patients, overexpression of Mcl-1 is linked to poor prognosis.[144] Moreover, Mcl-1 is critical for the initiation and sustained in vivo growth of mouse AMLs that harbor diverse genetic lesions.[145] These studies formed the basis for a screen to identify kinase inhibitors that down-regulated Mcl-1 levels. This study identified a PI3Kα inhibitor **53** (Figure 13),[146] which was previously known to inhibit CDKs, as an indirect Mcl-1 inhibitor. Biochemical studies showed that **53** is an ATP competitive inhibitor of CDK2 (K_d = 540 nM), CDK7 (K_d = 2.5 nM), and CDK9 (K_d = 4.1 nM). To improve the selectivity profile, SAR studies led to analogs **54** and **23**[147] with pyrazolopyrimidine core. Modeling studies indicated that pyrazolo[1,5-*a*]pyrimidines occupy the ATP binding site of CDK9 and 2,5-substituted aryl groups direct the orientation of the sulfonamide group. Key interactions include the halogen−π interaction with the Phe[103] residue. However, replacing the Br atom with the Cl atom resulted in significant reduction in the potency. This along with replacing the NO_2 group (**53**) with CN (**54**) flipped the selectivity. The metabolically labile hydrazone linker in **54**, when replaced with a stable aliphatic amino linker in **23**, reduced the potency while maintaining selectivity for CDK9.

Pyridines. In search of CDK inhibitors for cancer therapy, Novartis identified a pyrazinylpyridine class of compounds. A focused library of 278 compounds was synthesized and evaluated as a CDK9 inhibitor, which led to the identification of a low-nM inhibitor **55** (Figure 14).[148] A second iteration identified additional analogs (**56**, **57**, and **25**)[148−150] within this class as CDK9 inhibitors with improved selectivity (Figure 14). Novartis pharmaceuticals systematically optimized a bipyridine class (**58**−**61**) of compounds, which led to potent CDK9 inhibitors (Figure 14).[151]

Phenyltriazine. Phenyltriazines are a class of molecules containing diaryl linkage with a phenyl ring and a triazine ring. Substituted phenyltriazines have been explored as therapeutics for epilepsy and bipolar disorders.[152,153] Bayer research group identified phenyltriazine analogs as potent CDK9 inhibitors (Table 5). These inhibitors (**62**−**66**)[154,155] exhibited excellent cellular activities in a panel of cancer cell lines (HeLa, DU145, Caco-2, and B16F10).

Purines. The purine core has been extensively explored in the development of kinase inhibitors.[156] Trisubstituted purines were one of the first CDK inhibitors to be developed as cancer therapeutics.[157−159] **2** is a CDK inhibitor that targets CDKs 1, 2, 4, 5, and 9. **2** was one of the first CDK inhibitor

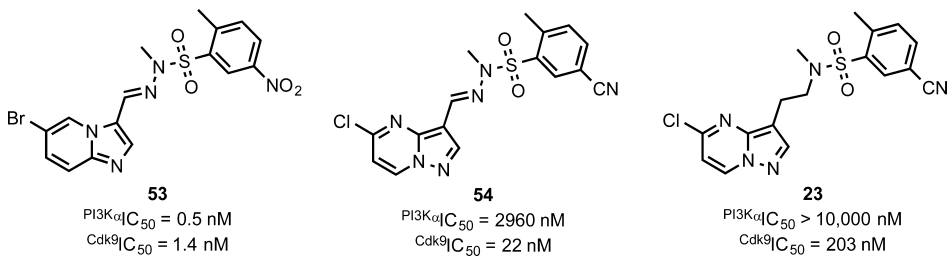

Figure 13. Fused pyrazolopyrimidine based CDK9 inhibitors.

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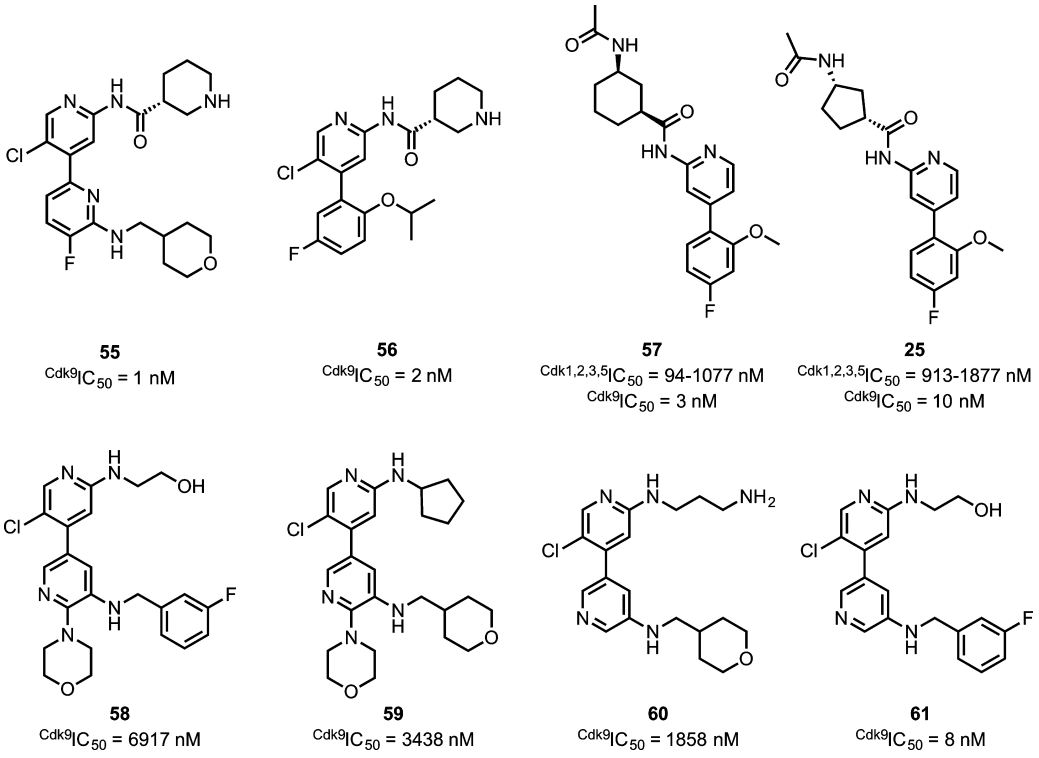
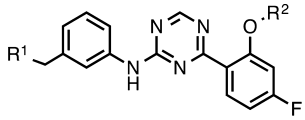
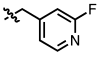
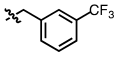
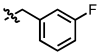


Figure 14. Pyridine based CDK9 inhibitors.

Table 5. Phenyltriazine Analogs and Their Inhibitory Activity



	R¹	R²	IC₅₀ (nM) (cell-free)		IC₅₀ (μM) (cell-based)				
			CDK9	CDK2	HeLa	HeLa/ MaTu/ ADR	H460	DU145	Caco-2	B16F10
62			3	89	0.1	0.1	0.15	0.14	0.14	0.13
63			1	75	-	-	-	-	-	-
64			4	1,300	1.3	-	-	-	-	-
65			2	260	0.17	0.10	0.11	0.11	0.08	0.10
66		-Me	2	300	-	-	-	-	-	-

to be subjected to multiple phase I–II clinical trials (clinicaltrials.gov). Short half-life and rapid metabolism were cited as reasons for the lack of success of **2** in the clinics. These issues were tackled through iterative SAR studies, which resulted in the development of multiple **2** analogs as clinical candidates.

Cocrystal structure of CDK9 with **67** (Figure 15)[158] shows that the N-7 and the amino group on C-6 of the purine ring interact via hydrogen bonding with the Cys[106] residue of the hinge region. The purine ring of **67** is sandwiched between hydrophobic residues Ile[25] and Leu[156], and the phenylpyridine is solvent exposed. Upon **67** binding, there is a downward

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J. Med. Chem. 2016, 59, 8667–8684

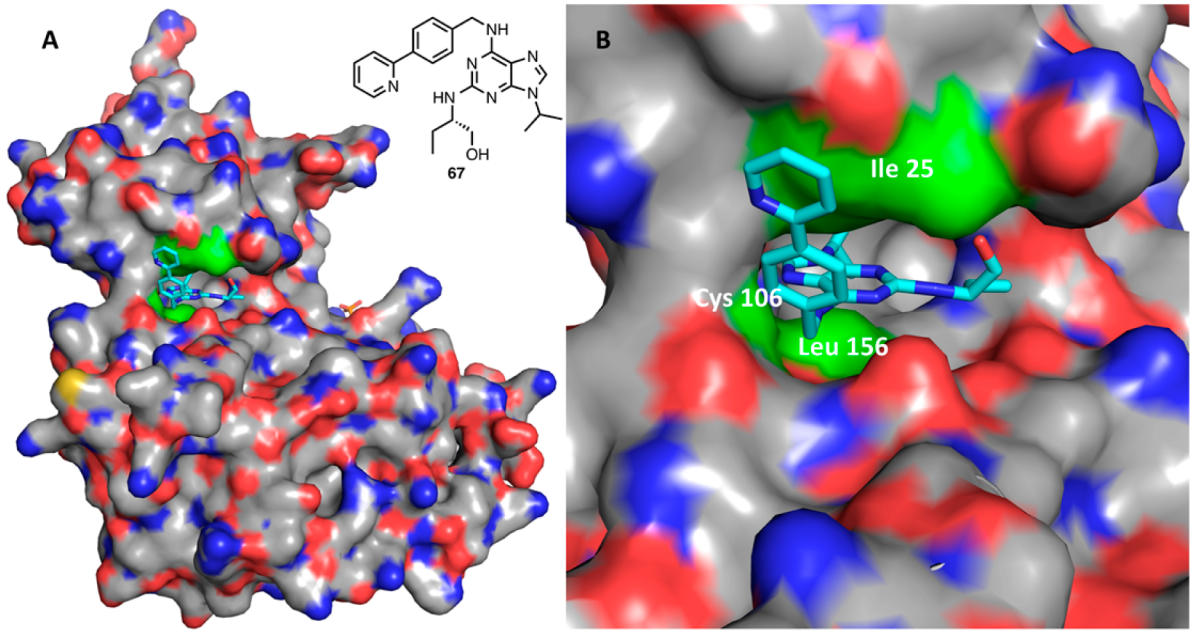

Figure 15. (A) Cocrystal structure of **67** and CDK9. (B) Binding interactions of **67** to Cys[106], Ile[25], and Leu[156] highlighted in green (PDB code for CDK9−**67** complex is 3LQ5).

movement of the glycine rich loop to occlude the inhibitor-binding site. Like other inhibitor−CDK9 structures, the conformational changes upon **67** binding partly explain the origin of selectivity for CDK9.

SUMMARY AND PERSPECTIVE

CDK9 is found in a myriad of cellular complexes that are important for the regulation of transcription. Several studies have described the role of CDK9 in different aspects of transcription. Given the central role played by CDK9 in transcription, it is likely that the different isoforms are localized to distinct complexes and possess cell type specific functions. At the present time, there is sufficient evidence to strongly support CDK9 as a cancer target. Structural studies that compare apo-CDK9 with inhibitor bound CDK9 indicate that several segments of the ATP binding site within CDK9 such as the hinge region, the G-loop, and the Cα-helix are more flexible than other CDKs, such as CDK2. This flexibility is probably critical for CDK9 to adapt to the various binding contexts found in the different complexes. Therefore, at any given time, small molecule CDK9 inhibitors might be targeting only a subset of complexes containing CDK9. This could be adapted into a screening system where potential therapeutics are tested against a panel of in vitro complexes to determine what effect it is likely to have on the cell.

The mechanism of anticancer effects coalesces around the idea that CDK9 is critical to maintain elevated levels of short-lived antiapoptotic proteins such as Mcl-1, and a subset of cancers require this for survival. Due to Mcl-1 dependence on CDK-9 function, it has been widely reported that synergism between CDK inhibitors and BH3 mimetic compounds exists, granting more value to targeted efforts toward CDK9.[160−163] We have previously shown that small molecules that indirectly modulate Mcl-1 sensitize cancers to direct inhibitors of Bcl-xL.[164] Chemical genetic screens to identify kinase inhibitors that induce apoptosis in either a Mcl-1 dependent manner or Bcl-xL dependent manner clustered CDK and PI3K inhibitors, respectively.[165] This is consistent with the recent report that demonstrated synergistic inhibition of pancreatic tumor growth and metastasis in patient-derived xenograft models by **11** (CDK inhibitor) and 8-(4-(1-aminocyclobutyl)phenyl)-9-phenyl[1,2,4]triazolo[3,4-*f*][1,6]-naphthyridin-3(2*H*)-one (MK-2206)[166] (Akt inhibitor).

The prevailing view within the community is that the improved selectivity for specific CDKs will lead to better cancer therapeutics.[109] Reviews have compared the clinical outcomes associated with **8** and **2**, which are pan-CDK inhibitors, with **1**, the FDA approved CDK4/6 inhibitor, to support the above statement.[16] However, a recent chemical proteomics study revealed that the recently approved CDK4/6 inhibitor, **1**, was a potent CDK9 inhibitor that also engaged lipid kinases, while a structurally related analog **12** did not.[167] These studies strongly argue against engineering out certain off target effects associated with CDK9 inhibitors, particularly those that will down-regulate the levels of other antiapoptotic proteins such as Bcl-xL. However, certain off target effects such as the inhibition of normal cellular growth would not be desirable and should be avoided. These observations along with poor correlation between cell-free and cell-based activities in SAR studies indicate the need for additional comprehensive preclinical/clinical studies to determine if improving the selectivity profile of a given CDK inhibitor will lead to an effective cancer therapeutic.

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■ ABBREVIATIONS USED

BRD4, bromodomain containing protein 4; CaMK1D, calcium/calmodulin-dependent protein kinase 1D; c-Flip, FLICE-like inhibitory protein; CTD, carboxy-terminal domain; cy, cyclin; DN, dominant negative; ds, double stranded; DSF, differential scanning fluorimetry; FLICE, FADD-like IL-1β-converting enzyme; HCC, hepatocellular carcinoma; HEXIM1, hexamethylene bisacetamide-inducible protein 1; LE, ligand efficiency; PBL, peripheral blood lymphocyte; PPM1A, protein phosphatase, $Mg^{2+/}Mn^{2+}$ dependent, 1A; P-TEFb, positive transcription elongation factor b; rb, retinoblastoma; RNAi, RNA interference; RNAPII, RNA polymerase II; RSV, respiratory syncytial virus; SAR, structure−activity relationship; shRNA, short hairpin RNA; siRNA, small interfering RNA; snRNP, small nuclear ribonucleoprotein; ss, single stranded; TAR, transactivation response element; TRAIL, tumor necrosis factor-related apoptosis inducing ligand

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