MINIREVIEW

Cyclin-Dependent Kinase Inhibitors as Anticancer Therapeutics^S

Mary E. Law, Patrick E. Corsino, Satya Narayan, and Brian K. Law

Departments of Pharmacology and Therapeutics (M.E.L., P.E.C., B.K.L.), Anatomy and Cell Biology (S.N.), and the University of Florida Health Cancer Center (M.E.L., P.E.C., S.N., B.K.L.), University of Florida, Gainesville, Florida

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ABSTRACT

Cyclin-dependent kinases (CDKs) have been considered promising drug targets for a number of years, but most CDK inhibitors have failed rigorous clinical testing. Recent studies demonstrating clear anticancer efficacy and reduced toxicity of CDK4/6 inhibitors such as palbociclib and multi-CDK inhibitors such as dinaciclib have rejuvenated the field. Favorable results with palbociclib and its recent U.S. Food and Drug Administration approval demonstrate that CDK inhibitors with narrow selectivity profiles can have clinical utility for therapy based on individual tumor genetics. A brief overview of results obtained with ATP-competitive inhibitors such as palbociclib and dinaciclib is presented, followed by a compilation of new avenues that have

been pursued toward the development of novel, non-ATP-competitive CDK inhibitors. These creative ways to develop CDK inhibitors are presented along with crystal structures of these agents complexed with CDK2 to highlight differences in their binding sites and mechanisms of action. The recent successes of CDK inhibitors in the clinic, combined with the potential for structure-based routes to the development of non-ATP-competitive CDK inhibitors, and evidence that CDK inhibitors may have use in suppressing chromosomal instability and in synthetic lethal drug combinations inspire optimism that CDK inhibitors will become important weapons in the fight against cancer.

Introduction

There are 20 different cyclin-dependent kinase (CDK) family members in the human kinome (Manning et al., 2002). The CDKs control cell cycle transitions and other important cellular functions, including transcription. Cancer is a disease of uncontrolled proliferation, and since CDKs are a central component of the cell cycle engine, great effort has been expended in developing CDK inhibitors as anticancer agents. The purpose of this review is to provide a broad overview of the development of various classes of CDK inhibitors. A number of thorough and informative reviews on ATP-competitive CDK inhibitors exist (Wang and Ren, 2010; Jorda et al., 2012; Blachly and Byrd, 2013; Galons et al., 2013); therefore, this review will emphasize efforts that take new and varied approaches to the development of CDK inhibitors.

Pivotal Discoveries Leading to Our Current Understanding of the Cell Cycle

Early yeast genetics studies led to the discovery of the first CDK, then known as *cde2*, now referred to as CDK1 (Nurse and Thuriaux, 1980), as a protein involved in cell division control. Later, the prototype “pocket” protein, the retinoblastoma tumor suppressor (Rb), was found to be an important substrate for CDKs (Akiyama et al., 1992; Cobrinik et al., 1992) that in turn controls the activity of the E2F transcription factors in a phosphorylation-dependent manner (Chellappan et al., 1991; Nevins et al., 1991; Hiebert et al., 1992). E2F regulates genes important for transit through G1 into S-phase and beyond. Dysregulation of the cell cycle through a variety of mechanisms can lead to oncogenic transformation, including Rb mutation (Knudson, 1971), cyclin D1 (Matsushime et al., 1992; Sherr et al., 1992) or E (Keyomarsi and Pardee, 1993; Keyomarsi et al., 1995) overexpression, loss of expression or function of CDK inhibitory proteins (el-Deiry et al., 1994; Shiohara et al., 1994; Okuda et al., 1995; Spirin et al., 1996; Takeuchi et al., 1996), mutational deregulation of CDK4 (Soufir et al., 1998; Rane et al., 2002), or overexpression of E2Fs (Johnson et al., 1994). This has resulted in the conclusion that most, if not all, cancers exhibit one or more cell cycle defects (Sherr, 1996),

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ABBREVIATIONS: API-1, 4-amino-5,8-dihydro-5-oxo-8- β -D-ribofuranosyl-pyrido[2,3-*d*]pyrimidine-6-carboxamide; CDK, cyclin-dependent kinase; MK-2206, 8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2*H*-[1,2,4]triazolo[3,4-*f*][1,6]naphthyridin-3-one; Rb, retinoblastoma tumor suppressor; RNA Pol II, RNA polymerase II.

and that effective cancer therapy will require restoring normal cell cycle control.

Figure 1 shows the current model for how extracellular growth factors are thought to stimulate mammalian cells to initiate a round of replication. This model explains the molecular basis for the “restriction point” posited by Pardee (1974), whereby after a threshold duration of growth factor-induced mitogenic signaling has elapsed, cells are able to complete the remainder of a round of division in the absence of exogenous growth factor stimulation. The integrated feed-forward loops involving E2F-dependent cyclin E/A induction and cyclin E/CDK2-dependent degradation of the CDK inhibitory protein p27 (Sheaff et al., 1997) allow the antiproliferative actions of Rb family members and p27 to be overcome.

Because of the central role of CDK4/6 and CDK2 in overriding the built-in barriers to proliferation, their activities must be tightly regulated to prevent excessive proliferation that may result in cancer (Fig. 2). In general, CDK activation involves its binding to a cyclin and absence of a bound inhibitor. The INK4 family members p15, p16, p18, and p19 inhibit CDK4 and CDK6, whereas the Kip family of proteins p21, p27, and p57 exhibit broad CDK inhibitory activity (Canepa et al., 2007). CDK activity is increased by phosphorylation on the T-loop residue (Thr¹⁶⁰ in the case of CDK2) and suppressed by phosphorylation of residues within the GX₁GX₂X₃G motif involved in ATP binding, where the inhibitory phosphorylation sites Thr14 and Tyr15 are X₂ and X₃, respectively, in CDK2. These multiple requirements that must be met for full CDK activation ensure that these enzymes are tightly regulated.

Rationales for and against CDK Inhibitors as Anticancer Therapeutics

An early indicator that curing cancer may not be achieved by inhibiting CDKs was the observation that the proliferation of some cancer cell lines was not blocked by inactivating CDK2 function using a variety of methods (Tetsu and McCormick, 2003). Despite the dispensability of CDK2 for the mitotic cell cycle, CDK2 is essential for meiosis (Berthet et al., 2003;

Ortega et al., 2003; Viera et al., 2009), and both male and female *Cdk2*^{-/-} mice are sterile.

Other reports showed that mice develop normally in the absence of CDK2 (Ortega et al., 2003; Barriere et al., 2007) and CDK4 and 6 (Malumbres et al., 2004) expression, demonstrating a high degree of functional redundancy among the cell cycle CDKs. In fact, CDK1 is the only CDK essential for cell division (Berthet and Kaldis, 2006; Adhikari et al., 2012; Diril et al., 2012). Subsequent studies revealed the subtlety of cell cycle regulation by showing that select cyclins and CDKs are differentially required for transformation by specific oncogenes. For example, HER2-driven mammary tumorigenesis is suppressed by cyclin D1 or CDK4 deficiency (Reddy et al., 2005), leading to the conclusion that cyclin D1/CDK4 complexes mediate HER2-driven mammary tumorigenesis. However a more recent study demonstrated that CDK2 knockout also reduces tumor formation in mouse mammary tumor virus–HER2 transgenic mice (Ray et al., 2011). Interpretation of these results is complicated by the fact that cyclin D1 can bind and activate CDK2 under certain conditions (Jahn et al., 2013b), and CDK2 is a major binding protein for cyclin D1 in a number of tissues, including the mouse mammary gland and mouse mammary tumor virus–HER2 breast tumor tissues (Bienvenu et al., 2010). Interestingly, constitutively active forms of CDK2 (Corsino et al., 2007, 2008) or CDK4 (Sotillo et al., 2001) drive tumor formation in genetically modified mouse models. In sum, these observations suggest that, in certain situations, cancer cell proliferation, but not normal cell division, is suppressed by limiting CDK activity. This may indicate either that specific oncogenes drive proliferation through particular cyclin/CDK complexes, or alternatively, that a higher total threshold level of CDK activity is required to maintain aberrant proliferation than the normal cell replication required for development and maintenance of homeostasis.

Pan-CDK Inhibitors

Several relatively nonspecific multi-CDK inhibitors such as flavopiridol and roscovitine have been reviewed elsewhere (Meijer and Raymond, 2003; Blagosklonny, 2004; Christian

Fig. 1. A brief overview of the G1 to S-phase cell cycle transition. Extracellular growth factors upregulate D-type cyclins through transcriptional, translational, and post-translational mechanisms, resulting in activation of CDK4 and 6 (*CDK activation). CDK4/6 initiates Rb phosphorylation, causing partial activation of E2F-dependent transcription, which leads to induction of cyclins E and A and the activation of other genes required for DNA synthesis. Cyclin E/CDK2 complexes phosphorylate p27, triggering its ubiquitination and proteasomal degradation. Cyclin/CDK2 complexes also phosphorylate the pocket proteins Rb, p107, and p130 on additional sites, further promoting E2F-dependent transcription. The two embedded positive feedback loops ensure that once cells have traversed the restriction point, they are committed to a round of replication.

Fig. 2. Mechanisms controlling endogenous CDK activity, using CDK2 as an example. CDK2 is only fully active if several conditions are met. These criteria include binding to a cyclin such as cyclin E; not having bound inhibitory proteins such as p21, p27, or p57; not being phosphorylated on the inhibitory sites within the N-terminal GX₁GX₂X₃G nucleotide binding motif (where X₂ and X₃ are the inhibitory phosphorylation sites Thr¹⁴ and Tyr¹⁵ for CDK2); and acquiring phosphorylation of the activating site, Thr¹⁶⁰.

et al., 2007; Wang and Ren, 2010; Jorda et al., 2012). These agents exhibited insufficient anticancer activity and significant toxicity. These limitations may have resulted from the facts that these compounds simultaneously block the activity of CDKs required for multiple processes such as transcription, translation, and cell proliferation, and that they may also have inhibitory actions against other classes of protein kinases. The variability in efficacy observed for some pan-CDK inhibitors may have resulted from a lack of knowledge of the relevant target(s) and therefore the absence of specific biomarkers that would allow rational patient selection for clinical trials. These difficulties have focused efforts toward the identification of CDK inhibitors with fewer off-target effects and the development of CDK inhibitors that selectively inhibit smaller subsets of CDKs. There are not currently enough CDK-selective agents available to comprehensively assess which of the many CDKs should be inhibited and in which combinations to block tumor growth. In this regard, the results of chemical-genetic screens (Bishop et al., 2000; Elphick et al., 2009; Enserink et al., 2009; Zimmermann et al., 2011; Horiuchi et al., 2012; Gravells et al., 2013) may be more informative than findings from knockout animals, since drug-inhibited CDKs may more closely resemble dominant-negative than null alleles because they likely still engage their cyclin partners and the rest of the CDK regulatory machinery. Additionally, future studies will be required to determine which combinations of subset-selective CDK inhibitors must be combined to overcome primary and acquired tumor resistance to these agents.

The advancement of the multi-CDK inhibitor dinaciclib into phase III clinical trials for the treatment of refractory chronic lymphocytic leukemia demonstrates the potential of multi-CDK inhibitors in cancer therapy, as dinaciclib inhibits the activities of CDK1, CDK2, CDK5, and CDK9. Dinaciclib is not truly a pan-CDK inhibitor, but rather a multi-CDK inhibitor because it does not inhibit CDK4, CDK6, or CDK7. It is currently unclear whether dinaciclib has been more successful than the earlier pan-CDK inhibitors, such as roscovitine or flavopiridol, because it inhibits a narrower spectrum of CDKs or has fewer non-CDK off-target effects. However, the observation

that dinaciclib also binds bromodomains (Martin et al., 2013) complicates the interpretation of the efficacy of dinaciclib.

CDK4/6-Selective Compounds

Much recent excitement has been generated by trials demonstrating the anticancer efficacy of CDK4/6-selective inhibitors in both preclinical studies and in a subset of patients in clinical trials (Michaud et al., 2010; Leonard et al., 2012; Dickson et al., 2013; DeMichele et al., 2015; Vora et al., 2014; Young et al., 2014). These agents appear to be particularly effective when combined with the aromatase inhibitor letrozole in patients with estrogen receptor-positive breast cancer (Finn et al., 2015). This led to Food and Drug Administration approval of the Pfizer CDK4/6 inhibitor Ibrance (palbociclib) in February 2015 for the treatment of estrogen receptor-positive, HER2-negative breast cancer. A number of clinical trials are currently underway to examine the utility of combining CDK4/6 inhibitors with other targeted agents or to test the efficacy of CDK4/6 inhibitors against other types of human cancer (Supplemental Table S1; Table 1).

Non-Cell Cycle CDKs as Drug Targets

Several non-cell cycle CDKs may have potential value as therapeutic targets in the treatment of cancer, including CDK5, 8, and 9. Although the expression of CDK5 was originally considered to be restricted to the nervous system, recent studies suggest that CDK5 may play an important role in tumor progression (Goodyear and Sharma, 2007; Feldmann et al., 2010; Liang et al., 2013; Pozo et al., 2013). CDK5 has been proposed to contribute to a variety of procancer functions, including cell migration, proliferation, and survival (Goodyear and Sharma, 2007; Demelash et al., 2012); maintenance of Ras-Ral signaling (Feldmann et al., 2010); and promotion of the TGFβ-induced epithelial to mesenchymal transition (Liang et al., 2013). Likewise, accumulating evidence indicates a role for CDK8 in some human cancers (Firestein et al., 2008; Gu et al., 2013; He et al., 2013; Li et al., 2014a,b,c; Xu et al., 2015).

TABLE 1

CDK inhibitor clinical trials on the ClinicalTrials.gov website

Agents with more than one established mechanism of action or undergoing testing for applications other than cancer therapy have been excluded. See Supplemental Table S1 for more detailed information on the individual clinical trials.

Drug	ClinicalTrials.gov Entries
Palbociclib (PD-0332991)	12
Dinaciclib	9
P276-00	4
Ronaciclib (BAY 1000394)	4
P1446A-05	2
AT7519M	2
SNS-032	2
SCH 727965	2
AG-024322	1
Sum	39

AG-024322, *N*-[5-[(3*E*)-3-(4,6-difluorobenzimidazol-2-ylidene)-1,2-dihydroindazol-5-yl]-4-methylpyridin-3-yl]methyl]ethanamine; AT7519M, 4-[[2,6-dichlorobenzoyl]aminol-*N*-piperidin-4-yl]-1*H*-pyrazole-5-carboxamide; methanesulfonic acid; P1446A-05, voruciclib, 2-(2-chloro-4-(trifluoromethyl)phenyl)-5,7-dihydroxy-8-[(2*R*,3*S*)-2-(hydroxymethyl)-1-methylpyrrolidin-3-yl]-4*H*-chromen-4-one; P276-00, 2-(2-chlorophenyl)-5,7-dihydroxy-8-[(2*R*,3*S*)-2-(hydroxymethyl)-1-methylpyrrolidin-3-yl]chromen-4-one; SCH 727965, dinaciclib, 2-[(2*S*)-1-[3-ethyl-7-[(1-oxidopyridin-1-ium-3-yl)methylamino]pyrazolo[1,5-*a*]pyrimidin-5-yl]piperidin-2-yl]ethanol; SNS-032, *N*-[5-[(5-tert-butyl-1,3-oxazol-2-yl)methylsulfanyl]-1,3-thiazol-2-yl]piperidine-4-carboxamide.

CDK8 can associate with the mediator complex that in turn regulates RNA polymerase II (RNA Pol II)–mediated gene transcription. The mechanisms by which CDK8 controls this complex is an area of active research (Allen and Taatjes, 2015). CDK9 is a component of the super elongation complex that phosphorylates the RNA Pol II carboxy-terminal domain to promote RNA Pol II release and transcript elongation. Thus, CDK8 and CDK9 control different steps in RNA Pol II–mediated transcription. CDK9 has also been suggested to be a useful therapeutic target, and CDK9 inhibitors may be selectively cytotoxic to cancer cells compared with normal cells (De Falco and Giordano, 2002; Nowicki and Walkinshaw, 2010; Polier et al., 2011, 2015; Liu et al., 2012; Wang et al., 2014).

CDK7 is an interesting outlier in the CDK family because it has dual functions as a subunit of the general transcription factor Transcription Factor II Human (TFIIH), and is a component of the cyclin-dependent kinase activating kinase that is responsible for phosphorylating other CDKs on their stimulatory, T-loop sites (see Fig. 2) (Fisher, 2005). Several reports suggest that, as with CDK8 and CDK9, inhibition of CDK7 may be useful in the treatment of certain cancers (Manzo et al., 2012; Cao and Shilatifard, 2014; Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014).

Creative Approaches to CDK Inhibition

Although most efforts to develop antagonists of CDK function have focused on identifying and optimizing ATP-competitive CDK inhibitors, a number of studies have been

published in which new, creative strategies have been used. Most of these approaches focus on CDK2 inhibition. This is in part due to the fact that X-ray crystal structures of CDK2 and the cyclin A/CDK2 complex have been available longer than similar data for other CDK and cyclin/CDK complexes. A crystal structure of cyclin A/CDK2 in complex with ATP and a substrate peptide (Brown et al., 1999) (Fig. 3A) shows ATP bound in a cleft formed on one side by the GEGTYG nucleotide-binding motif (red- and blue-colored residues). The peptide substrate is bound in a cleft adjacent to the ATP binding site and in close apposition to ATP. Interestingly, binding of the endogenous CDK inhibitor p27 to cyclin A/CDK2 complex causes large-scale structural changes to the cyclin A/CDK2 complex (Fig. 3B) (Russo et al., 1996). p27 inserts itself into the ATP binding site and wraps around both the CDK2 and cyclin A subunits, occupying the cyclin A substrate binding groove that is thought to confer specificity of the complex to certain cell cycle substrates such as Rb. ATP-competitive compounds are the most heavily studied class of CDK inhibitors and are by far the most numerous. As shown in Fig. 3C, ATP competitors such as roscovitine partially or fully occupy the ATP binding pocket (De Azevedo et al., 1997). Allosteric inhibitors have been discovered that bind adjacent to the ATP binding pocket, but do not engage the GEGTYG motif (Martin et al., 2012) (Fig. 3D) and represent a second distinct class of CDK inhibitors. A third group of CDK2 inhibitors includes compounds that alter the folding of CDK2 such that it modulates cyclin binding (Deng et al., 2014) (Fig. 3E). A fourth novel strategy to inhibit CDK2 function involves identifying molecules that occupy the

Fig. 3. Innovative approaches to CDK inhibition. In the structures shown, CDK2 is in yellow, cyclin A is presented in magenta, and p27 is in green. The glycine residues of the GX₁GX₂X₃G motif are colored blue, and the X₁, X₂, and X₃ residues E, T, and Y, respectively, are shown in red to highlight the ATP binding pocket. (A) Structure of the cyclin A/CDK2 complex bound to two substrates (Subst.), the phosphate donor, ATP, and a phosphate acceptor peptide (PDB ID 1QMZ). (B) Crystal structure of the cyclin A/CDK2/p27 complex demonstrating the inhibitor p27 wrapping around the CDK2/cyclin A complex and disrupting the ATP binding pocket (PDB ID 1JSU). (C) Structure of CDK2 complexed with the ATP-competitive inhibitor roscovitine (cyan arrow) (PDB ID 2A4L). (D) Binding of an allosteric CDK2 inhibitor (cyan arrow) adjacent to the ATP binding pocket (PDB ID 4EZ3). (E) Structural perturbations induced by a compound that suppresses CDK2 association with cyclins (PDB ID 4NJ3). The CDK2 inhibitory compound (cyan arrow) resides in a cleft behind the GXGXG ATP binding motif. Two views, (E1) and (E2), are shown where the structures are rotated 90° with respect to each other. (F) Docking of a CDK2 inhibitor (cyan arrow) to the substrate recognition groove of cyclin A demonstrated by X-ray crystallography (PDB ID 1URC).

substrate-binding groove of cyclin A (Andrews et al., 2004) (PDB ID 1URC). An advantage of this approach is that it may allow CDK inhibition in a substrate-selective manner since not all proteins require binding to this cleft to be phosphorylated by CDK2 (Fig. 3F). This general strategy has been extended using REPLACE (REplacement with Partial Ligand Alternatives through Computational Enrichment; Andrews et al., 2006) to develop drug-like, peptidomimetic CDK2 inhibitors. A fifth approach to CDK2 inhibition includes efforts designed to mimic the conformational changes in CDK2 induced by p27 binding (Corsino et al., 2009). p27 association with CDK2 produces a pocket that is not present in its absence. Molecules predicted by molecular docking to bind to this pocket cause the selective aggregation and downregulation of CDK2 and CDK4, and evidence was presented that these compounds induce the degradation of CDKs via aggresomes (Corsino et al., 2009).

As the long journey to get CDK inhibitors into the clinic indicates, there are difficulties associated with the development of ATP-competitive inhibitors that inactivate CDKs, but not other kinases, or that selectively inhibit individual CDKs. Further investigation of alternative approaches to the development of CDK inhibitors such as those described here may produce new therapeutic agents. Consistent with this general notion, allosteric Akt inhibitors such as MK-2206 [8-[4-(1-aminocyclobutyl)phenyl]-9-phenyl-2H-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3-one] and API-1 [4-amino-5,8-dihydro-5-oxo-8- β -D-ribofuranosyl-pyrido[2,3-d]pyrimidine-6-carboxamide] are currently undergoing preclinical and clinical testing for anti-cancer efficacy (Kim et al., 2010; Hudis et al., 2013).

What is the Goal: Proliferative Arrest? Senescence? Cell Death? Suppression of Chromosomal Instability?

Inhibitors that target the cell cycle CDKs might be expected to exhibit the drawback that they arrest tumor cell proliferation in a reversible manner such that when they are not present, tumor growth resumes. However, depending on the individual cancer, various CDK inhibitors can induce cell cycle arrest or cell death (Wirger et al., 2005; Rong et al., 2010). In some settings, CDK inhibitor-mediated necrosis, termed tumor lysis syndrome, is a dose-limiting toxic effect, as has been observed in the treatment of patients with chronic lymphocytic leukemia with flavopiridol or dinaciclib (Flynn et al., 2015). Because of the aforementioned issues regarding the unclear kinase specificity and selectivity for individual CDKs, much work is needed to decipher the mechanisms by which inhibitors suppress tumor growth, and to identify which CDKs are most relevant in particular tumor types.

Further, it has been recognized that an important consequence of cell cycle deregulation is chromosomal instability (Akli et al., 2004; Hubalek et al., 2004; Kawamura et al., 2004; Duensing et al., 2006; Adon et al., 2010; Jahn et al., 2013a). Chromosomal instability may produce genetic diversity within cancers that either favors the pre-existence of drug-resistant clones or allows resistant strains of cancer cells to arise after treatment has been initiated. Therefore, it must be considered that CDK inhibitors may have use not only in suppressing tumor growth and inducing cancer cell death, but also in slowing tumor progression and the acquisition of drug resistance if chromosomal instability is halted.

Future Opportunities

Based on promising early results in the generation of novel classes of CDK2 inhibitors (Fig. 3), one could envision the design of small molecules that mimic the functions of the INK4 family of inhibitors, composed of p15, p16, p18, and p19, for selectively inactivating CDK4/6. Allosteric kinase inhibitors have gained traction for the inhibition of Akt, mitogen-activated protein kinase kinase, and other kinases, but have not been thoroughly investigated for the ablation of CDK activity. Further, chemical/genetic screens suggest that the concept of synthetic lethality can be applied to the use of CDK inhibitors against cancer. Specifically, inhibiting CDK2 in tumors that overexpress N-myc or c-Myc may induce synthetic lethality, and coinhibition of CDK2 and phosphatidylinositol 3'-kinase is also synthetically lethal (Molenaar et al., 2009; Cheng et al., 2012; Etemadmoghadam et al., 2013; Li et al., 2015).

In summary, CDK inhibitors finally appear to be poised to have a clinical impact, and this has been made possible through the development of more selective and potent ATP-competitive CDK inhibitors. This avenue will likely yield new and useful drugs for the treatment of cancer and other proliferative diseases. Additional CDK-selective agents may complement these ATP-competitive inhibitors based on their ability to disrupt substrate binding to cyclins, to block the binding of CDKs to their cyclin partners, or to abrogate ATP or protein substrate binding to the CDK subunit in an allosteric manner. These novel approaches for the identification of CDK inhibitors designed based on CDK2 structural information can potentially be implemented in the development of non-ATP-competitive agents targeting CDK4, CDK5, CDK6, CDK7, CDK8, CDK9, and other CDKs deemed important therapeutic targets in the treatment of cancer.

Authorship Contributions

Wrote or contributed to the writing of the manuscript: M. E. Law, Corsino, Narayan, B. K. Law.

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Address correspondence to: Dr. Brian Law, University of Florida, Department of Pharmacology and Therapeutics, 1200 Newell Drive, Academic Research Building, Room R5-210, Gainesville, FL 32610. E-mail: bklaw@ufl.edu