

Cyclin-Dependent Kinase Inhibitors as Anticancer Therapeutics

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Abbreviations:

Akt, Protein Kinase B (PKB); CAK, Cyclin-Dependent Kinase Activating Kinase; *Cdc2*, Cell division control-2; CDK, Cyclin-Dependent Kinase; CIN, Chromosomal Instability; CLL, Chronic Lymphocytic Leukemia; EMT, Epithelial to Mesenchymal Transition; FDA, U.S. Food and Drug Administration; HER2, Human Epidermal growth factor Receptor-2; INK4, Inhibitors of CDK4 and CDK6; MEK, Mitogen-activated protein kinase kinase; MMTV, Mouse Mammary Tumor Virus (promoter); Rb, Retinoblastoma Tumor Suppressor Protein

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 (Blachly and Byrd, 2013; Galons et al., 2013; Jorda et al., 2012; Wang and Ren, 2010), 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 Cdc2, 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; Hiebert et al., 1992; Nevins et al., 1991). 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 et al., 1995; Keyomarsi and Pardee, 1993) overexpression, loss of expression or function of CDK inhibitory proteins (el-Deiry et al., 1994; Okuda et al., 1995; Shiohara et al., 1994; Spirin et al., 1996; Takeuchi et al., 1996), mutational deregulation of CDK4 (Rane et al., 2002; Soufir et al., 1998), 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), 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 (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, while 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 (Barriere et al., 2007; Ortega et al., 2003) 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 (Adhikari et al., 2012; Berthet and Kaldis, 2006; 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 MMTV-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 MMTV-HER2 breast tumor tissues (Bienvenu et al., 2010). Interestingly, constitutively active forms of CDK2 (Corsino et al., 2007; Corsino et al., 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 (Blagosklonny, 2004; Christian et al., 2007; Jorda et al., 2012; Meijer and Raymond, 2003; Wang and Ren, 2010). 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 to 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; Gravelles et al., 2013; Horiuchi et al., 2012; Zimmermann et al., 2011) 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 (DeMichele et al., 2014; Dickson et al., 2013; Leonard et al., 2012; Michaud et al., 2010; 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 FDA approval of the Pfizer CDK4/6 inhibitor Ibrance (Palbociclib) in February 2015 for the treatment of ER-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 testing the efficacy of CDK4/6 inhibitors against other types of human cancer (Table I and Supplementary Table SI).

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 CDKs 5, 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 (Feldmann et al., 2010; Goodyear and Sharma, 2007; Liang et al., 2013; Pozo et al., 2013). CDK5 has been proposed to contribute to a variety of pro-cancer functions including cell migration (Demelash et al., 2012), proliferation and survival (Goodyear and Sharma, 2007), maintenance of Ras-Ral signaling (Feldmann et al., 2010), and promotion of the TGF β -induced Epithelial to Mesenchymal Transition (EMT) (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; Li et al., 2014b; Li et al., 2014c; Xu et al., 2015). 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; Liu et al., 2012; Nowicki and Walkinshaw, 2010; Polier et al., 2011; Polier et al., 2015; 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 TFIIH, and is a component of the Cyclin-dependent kinase Activating Kinase (CAK) 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 (Cao and Shilatifard, 2014; Chipumuro et al., 2014; Christensen et al., 2014; Kwiatkowski et al., 2014; Manzo et al., 2012).

Creative Approaches to CDK inhibition

While 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 employed. 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 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. 3E1, 2). A fourth novel strategy to inhibit CDK2 function involves

identifying molecules that occupy the substrate-binding groove of Cyclin A (Andrews et al., 2004) (PDB 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 in order to be phosphorylated by CDK2 (Fig. 3F). This general strategy has been extended using RELACE (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 causes 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 and API-1 are currently undergoing preclinical and clinical testing for anticancer efficacy (Hudis et al., 2013; Kim et al., 2010).

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 (Rong et al., 2010; Wirger et al., 2005). 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 (CLL) with flavopiridol or Dinaciclib (Flynn et al., 2015). Because of the issues raised above 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 (CIN) (Adon et al., 2010; Akli et al., 2004; Duensing et al., 2006; Hubalek et al., 2004; Jahn et al., 2013a; Kawamura et al., 2004). CIN may produce genetic diversity within cancers that favors either 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, comprised of p15, p16, p18, and p19, for selectively inactivating CDK4/6. Allosteric kinase inhibitors have gained traction for the inhibition of Akt, MEK, 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 co-inhibition of CDK2 and phosphatidylinositol 3'-kinase is also synthetically lethal (Cheng et al., 2012; Etemadmoghadam et al., 2013; Li et al., 2015; Molenaar et al., 2009).

In summary, CDK inhibitors finally appear to be poised to have 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: Law, M.E., Corsino, P.E., Narayan, S., and Law, B.K.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1- A brief overview of the G1 to S-phase cell cycle transition. Extracellular growth factors upregulate D-type cyclins through transcriptional, translational, and posttranslational mechanisms resulting in activation of CDK4 and 6 (CDK activation is denoted by "*"). CDK4/6 initiate 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.

Figure 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 $GX_1GX_2X_3G$ nucleotide binding motif (where X_2 and X_3 are the inhibitory phosphorylation sites Thr¹⁴ and Tyr¹⁵ for CDK2), and acquiring phosphorylation of the activating site, Thr¹⁶⁰.

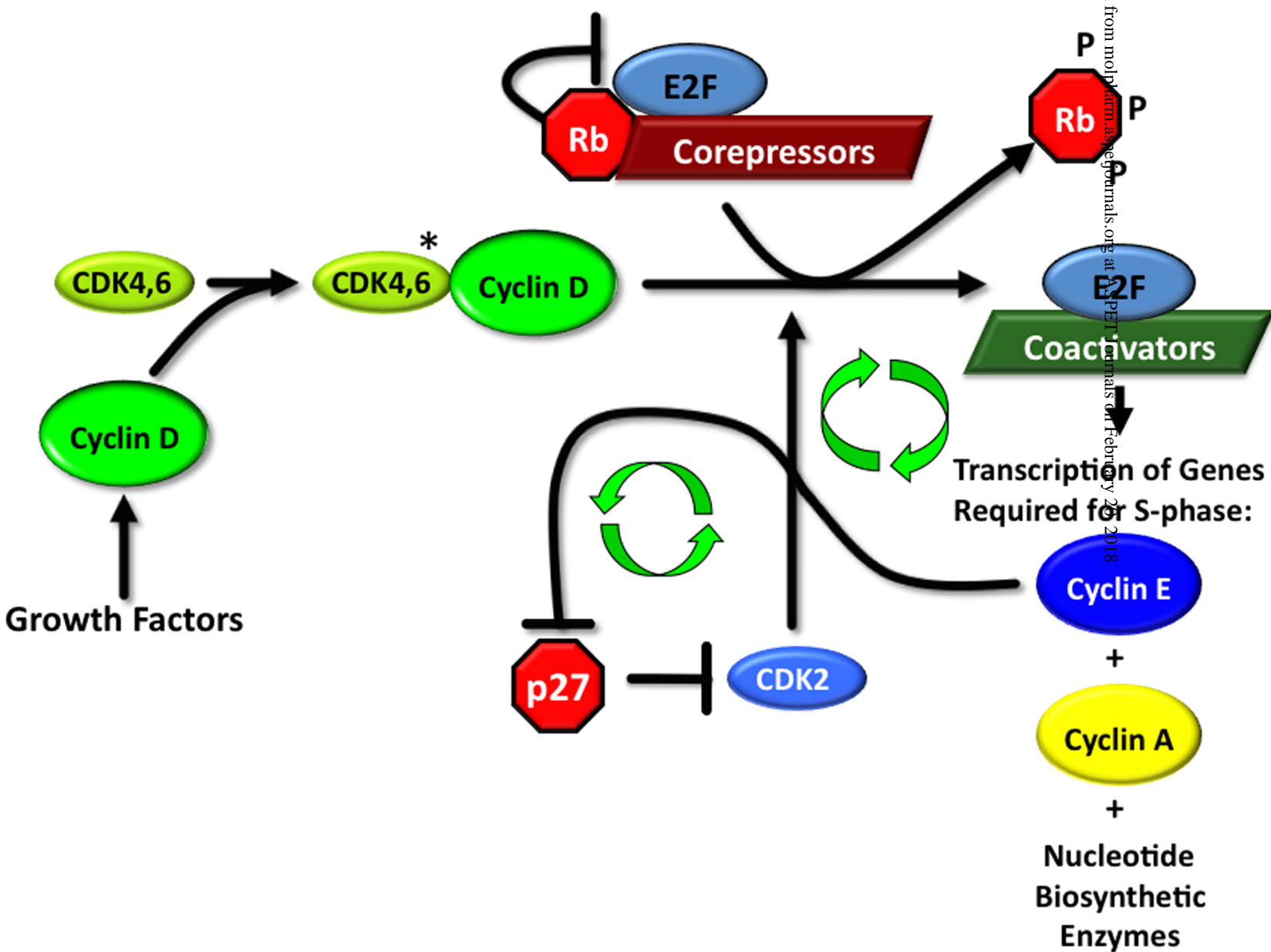
Figure 3- Innovative approaches to CDK inhibition. In the structures shown, CDK2 is in yellow, Cyclin A is presented in magenta, and p27 is in green. The glycine residues of the $GX_1GX_2X_3G$ motif are colored blue and the X_1 , X_2 , and X_3 residues E, T, and Y, respectively, are shown in red to highlight the ATP-binding pocket. A. Structure of the Cyclin A/CDK2 complex bound to two substrates, the phosphate donor, ATP, and a phosphate acceptor peptide (PDB 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 1JSU). C. Structure of CDK2 complexed with the ATP-competitive inhibitor Roscovitine (cyan arrow) (PDB 2A4L). D. Binding of an allosteric CDK2 inhibitor

(cyan arrow) adjacent to the ATP binding pocket (PDB 4EZ3). E. Structural perturbations induced by a compound that suppresses CDK2 association with Cyclins (PDB 4NJ3). The CDK2 inhibitory compound (cyan arrow) resides in a cleft behind the GXGXXG 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 1URC).

TABLES

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
P1446A-05	1
Sum:	39

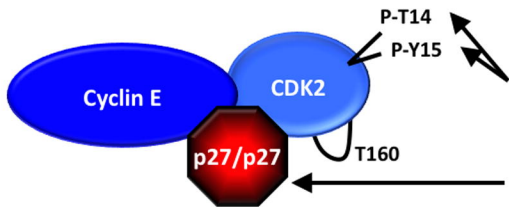
Table 1- CDK inhibitor clinical trials on the ClinicalTrials.gov website. Agents with more than one established mechanism of action or undergoing testing for applications other than cancer therapy have been excluded. See Supplemental Table SI for more detailed information on the individual clinical trials.



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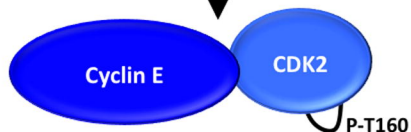
Law, et al., Figure 1

(Inactive Kinase)



1. Cyclin Binding
2. Inhibitory Phosphorylation:
Y15 and T14

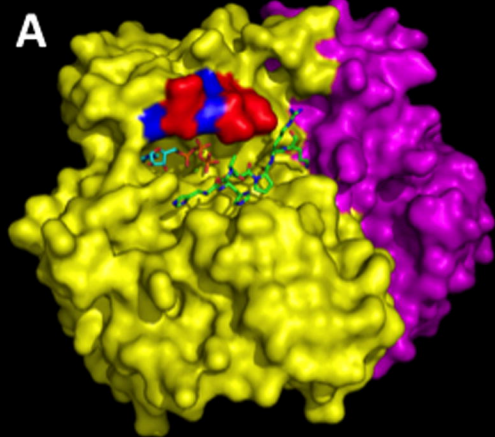
3. p21/p27/p57 Binding
(p15, p16, p18, and p19
binding to Cdk4 or Cdk6)



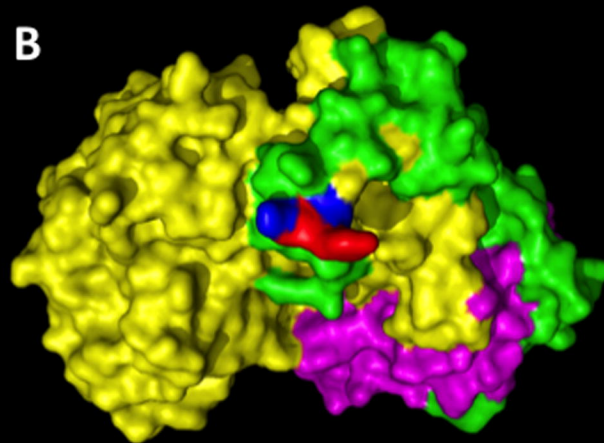
4. Activating Phosphorylation

(Active Kinase)

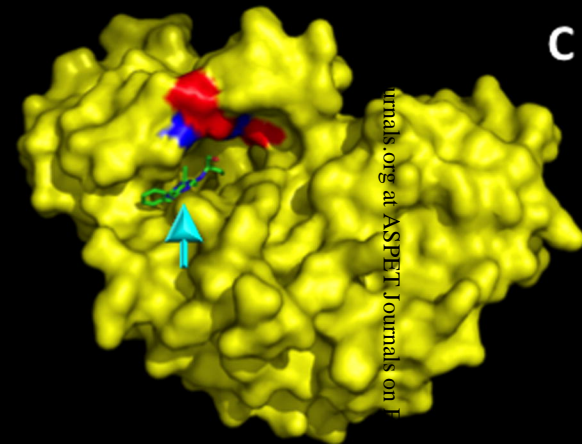
CDK2/Cyclin A/
ATP/Peptide Subst.



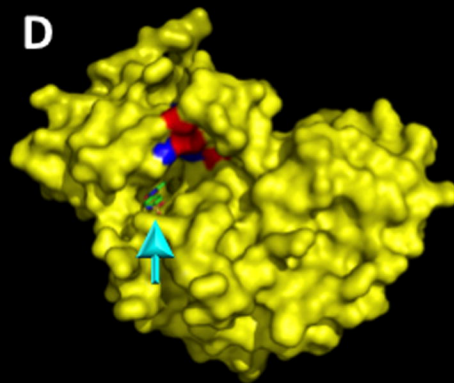
CDK2/Cyclin A/p27



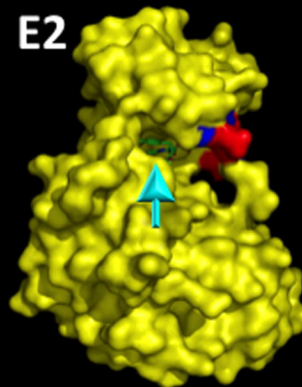
CDK2/Roscovitine
(ATP Competitive)



CDK2/
Allosteric inhibitor



Cyclin A/CDK2 Complex Disruptor



Cyclin Groove Inhibitor

