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Inhibitors of Cyclin-Dependent Kinases as Cancer Therapeutics

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Abstract

Over the past two decades there has been a great deal of interest in the development of inhibitors of the Cyclin-dependent kinases (CDKs). This attention initially stemmed from observations that different CDK isoforms have key roles in cancer cell proliferation through loss of regulation of the cell cycle, a hallmark feature of cancer. CDKs have now been shown to regulate other processes, particularly various aspects of transcription. The early non-selective CDK inhibitors exhibited considerable toxicity and proved to be insufficiently active in most cancers. The lack of patient selection biomarkers and an absence of understanding of the inhibitory profile required for efficacy hampered the development of these inhibitors. However, the advent of potent isoform-selective inhibitors with accompanying biomarkers has re-ignited interest. Palbociclib, a selective CDK4/6 inhibitor, is now approved for the treatment of ER+/HER2- advanced breast cancer. Current developments in the field include the identification of potent and selective inhibitors of the transcriptional CDKs; these include tool compounds that have allowed exploration of individual CDKs as cancer targets and the determination of their potential therapeutic windows. Biomarkers that allow the selection of patients likely to respond are now being discovered. Drug resistance has emerged as a major hurdle in the clinic for most protein kinase inhibitors and resistance mechanism are beginning to be identified for CDK inhibitors in the clinic. This suggests that the selective inhibitors may be best used combined with standard of care or other molecularly targeted agents now in development rather than in isolation as monotherapies.

Keywords

Cyclin-dependent kinase; cell cycle; transcription; inhibitor

1 Introduction

The notion of the cell cycle and its regulatory restriction points was first proposed in the 1970s and early 1980s. The machinery components associated with this process were identified and characterized through many genetic and biochemical studies, mainly in yeast,

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Conflict of Interest

PAC, SRW, AM and PW are employees of The Institute of Cancer Research, which has a commercial interest in the development of CDK inhibitors.

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but also in sea urchin, xenopus, and eventually higher eukaryotic cells (Nurse, 2000). The core of this work resulted in the identification of the CDKs and their partner cyclins for which the Nobel Prize in Physiology and Medicine was awarded to Hartwell, Hunt and Nurse in 2001. The regulation of the growth and division of cells came to the attention of the biomedical research community when it became clear that unconstrained proliferation, in part due to a loss of cell cycle regulation, played a key role in the initiation and progression of cancer. More recently, sustained proliferation through the deregulation of cell cycle control has been recognized as one of the key hallmarks of cancer (Hanahan & Weinberg, 2011), and our understanding of how specific CDKs regulate transcription and maintain the oncogenic state has advanced considerably. This has led to considerable efforts to develop CDK inhibitors as cancer therapeutics, which is the subject of this review. Here we will review the role of CDKs in cancer and particularly those for which inhibitors have currently been identified. These inhibitors include the early non-selective inhibitors that suffered from toxicity and poor efficacy, but more importantly the more recent developments in selective CDK inhibitors that have led to the approval of palbociclib for the treatment of breast cancer.

The CDK family

The human genome encodes 26 serine/threonine protein kinases that form a CDK and CDK-like branch of the CMGC subfamily of the human kinome; of these, 21 are classified as CDKs (Malumbres, 2014; Malumbres, et al., 2009). The CDKs have specific or redundant roles in many aspects of cell growth, proliferation and transcriptional regulation in response to extracellular and intracellular signals. The evolutionary relationships between these CDK subfamilies have been identified (Figure 1) and indicate that the CDK subfamilies can be divided into subfamilies that directly or indirectly regulate the cell cycle (CDKs1-6, 11 and 14-18) or transcription (CDKs7-13, 19 and 20).

Similar to all protein kinases, the CDKs have a two-lobed structure comprising a beta sheet-rich amino terminus and an alpha helix-rich carboxy terminus, with the active site sandwiched between the two (Malumbres, 2014; Malumbres, et al., 2009). Members of the CDK family have a conserved catalytic core containing an ATP-binding pocket, a cyclin subunit - binding domain and an activating T-loop motif. Collectively these features participate in CDK activation. The CDKs are constitutively expressed but, as their name suggests, typically require association with a cyclin subunit in order to become active (Figure 1). Regulation of the CDKs predominantly occurs by means of the control of cyclin production and destruction, as cyclin binding displaces the T-loop, exposing the substrate binding site and realigning critical residues in the active site that primes the kinase for activity (Jeffrey, et al., 1995; Russo, Jeffrey, & Pavletich, 1996). In addition to the regulatory effects of cyclin-binding, phosphorylation also coordinates the activity of the CDKs in response to various stimuli (Mueller, Coleman, Kumagai, & Dunphy, 1995). Most CDKs have inhibitory phosphorylation sites in the P-loop of the active site which when phosphorylated interfere with ATP binding at the catalytic site (Mueller, et al., 1995). Some CDKs also have activating phosphorylation sites in their T-loops that are substrates of CDK-activating kinases that includes other CDKs. Phosphorylation of these T-loop sites enhances substrate binding and complex stability, promoting full CDK activation (Russo, et al., 1996).

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2 Cell cycle regulation by CDKs

The cell cycle has 5 distinct phases during which cells either have the capacity to grow (G1 and G2 phases), replicate their DNA (S phase), divide by mitosis (M phase) or can cease to proliferate and enter quiescence (G0 phase)(Figure 2). Cell cycle progression is governed by the activities of particular CDKs and interaction with their regulatory cyclin partners (Sherr, 1996; van den Heuvel & Harlow, 1993). The function and role of the key individual CDKs that regulate cell cycle progression are described in detail in the sections below.

2.1 CDK4/6

In normal cells the cell cycle is initiated when growth factor receptors are stimulated, propelling cells from a quiescent, non-cycling G0 state into an active cycling state (Figure 2). The mitogenic stimulation of RAS and RHO GTPase-dependent pathways, mTOR activation and steroid receptor-activation can all induce entry to the cell cycle (Aktas, Cai, & Cooper, 1997; Marshall, 1999; Rodgers, et al., 2014). Of these, the role of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) lipid kinase pathway has been the most extensively characterised. ERK1/2 activation promotes the transcription of the D-type cyclins, commonly through the activity of FOS- and JUN-related transcription factors (Balmanno & Cook, 1999; Lavoie, L'Allemain, Brunet, Muller, & Pouyssegur, 1996). The D-type cyclins associate with CDK4 and CDK6 to generate active kinase complexes that directly phosphorylate the retinoblastoma protein (RB) (Peeper, et al., 1997; Sherr, 1996). The predominant view is that this causes the dissociation of both HDAC1 and the E2F transcription factors from RB, allowing histone acetylation and activated transcription of many genes, including cyclin E (Blais & Dynlacht, 2007; H. S. Zhang & Dean, 2001) (Figure 2). Early studies showed that RB exists in a hypo-phosphorylated state in quiescent cells and that its phosphorylation in response to mitogens is associated with progression through the cell cycle (Classon & Harlow, 2002). While it was originally suggested that distinct CDK/cyclin complexes progressively target specific phosphorylation sites on RB, such as T821 by CDK2 and T826 by CDK4, this has not been comprehensively addressed (Mittnacht, Lees, Desai, Harlow, Morgan, & Weinberg, 1994; Zarkowska & Mittnacht, 1997). More recently, it has been proposed that RB may be mono-phosphorylated by CDK4/6 activity on any one of 14 sites (Narasimha, et al., 2014). Upon CDK2/cyclin E activation, the remaining sites are phosphorylated by CDK2 to give hyper-phosphorylated RB, to fully relieve suppression of E2Fs and permit cell cycle progression. Consequently, it may not be possible to use specific RB phosphorylation sites to monitor the activity of specific CDKs, rather the loss of mono-phosphorylated RB could be attributed to impaired CDK4/6 activity and loss of hyper-phosphorylated RB due to lack of CDK2 activity. In any case, RB remains phosphorylated throughout the S, G2 and M phases, and at the end of mitosis it is dephosphorylated by protein phosphatase 1 (Malumbres & Barbacid, 2009). Hence, there is very a strong link between the phosphorylation status of RB and cellular replication.

CDK4/6 also phosphorylate the transcription factor FOXM1, resulting in its activation and stabilization (Anders, et al., 2011). FOXM1 shares many of the functions of CDK4/6: it promotes the G1/S transition, suppresses senescence and is involved in tumorigenesis

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(Kalinichenko, et al., 2004; M. Liu, et al., 2006; I. C. Wang, et al., 2005; I. C. Wang, et al., 2008). Notably, FOXM1 is required for the expression of key genes that regulate G1/S phase progression, such as *CCNE1*, *CCNE2*, *E2F2*, *MCM2*, *MCM10* and *CDT1* (Anders, et al., 2011).

Genetic studies found most mouse cells proliferate in the absence of Cdk4 (Rane, et al., 1999; Tsutsui, et al., 1999), potentially because Cdk6 is able to compensate for the loss. Similarly, Cdk6 ablation is well tolerated: mouse embryos develop normally with modest impairment of haematopoiesis. In contrast, dual Cdk4/Cdk6 knockout embryos generally fail to survive to birth; those that are born die within a few hours, likely because of the limited proliferation of erythroid progenitors resulting in a lack of red blood cells (Malumbres, et al., 2004). However, cells from other tissues in these embryos proliferate normally, indicating that Cdk4/6 are primarily required for haematopoesis in early development. Simultaneous knockout of the D cyclins *Ccnd1*, *Ccnd2* and *Ccnd3* yielded comparable results to knockout of Cdk4/Cdk6 (Kozar, et al., 2004). Ablation of both Cdk4 and Cdk2 in adult mice is well tolerated and even highly proliferative tissues (oesophagus or intestine) are unaffected (Barriere, et al., 2007). The phenotype of conditional double knockout Cdk4/Cdk6 mice in adulthood and the effects on homeostasis have yet to be reported, but based on mouse embryonic fibroblasts derived from Cdk4/6 knockout mice, such events are anticipated to be tolerated in adult tissues (Malumbres, et al., 2004). Confidence in selective pharmacological CDK4/6 inhibition being well tolerated clinically has been boosted by such studies, and suggested the potential for a therapeutic window between tumor and normal tissue.

2.2 CDK2

The transcriptional program induced following the activation of E2F1 and FOXM1 by CDK4/6 includes increased expression of genes encoding cyclins E1 and E2. Further phosphorylation of RB (Figure 2) results from the newly synthesized cyclins E1 and E2 binding and activating, CDK2. E2F1 also stimulates the transcription of genes coding for proteins involved in DNA replication, including the expression of cyclin A, which accumulates during S phase and becomes the predominant cyclin bound to CDK2 (Harbour, Luo, Dei Santi, Postigo, & Dean, 1999; Helin, 1998). The activity of CDK4/6 and CDK2 coordinate progression into S phase, termed the ‘restriction point’, where the cell is no longer dependent on mitogens to complete the current cell cycle (Figure 2). CDK2 is capable of phosphorylating a number of additional substrates including NPAT, CDC6 and E2F1 (Asghar, Witkiewicz, Turner, & Knudsen, 2015). Specifically, while CDK2/cyclin E complexes promote entry into S phase through phosphorylation of RB and NPAT, CDK2/cyclin A complexes help to terminate S phase, by phosphorylating CDC6 and E2F1. The cyclin A protein remains present in the cell until mitosis when it is degraded in an APC-dependent manner prior to anaphase (Furuno, den Elzen, & Pines, 1999; Pagano, Pepperkok, Verde, Ansorge, & Draetta, 1992).

Cdk2 null mice are viable, suggesting that Cdk2 has little effect on the proliferation and survival of most cell lineages. In fact, the main phenotype displayed by Cdk2 null mice is defective gamete development attributed to impairment of the first meiotic division (Ortega,

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et al., 2003). These data are somewhat complemented by studies examining the effect of CDK2 inactivation in colon cancer cell lines, which have shown that inhibition of CDK2 through expression of $p27^{KIP1}$, DN-CDK2 or antisense mediated depletion does not inhibit cell proliferation (Tetsu & McCormick, 2003). However, recent data manipulating the gatekeeper residue in CDK2 to allow specific inhibition by adenine analogs resulted in reduced proliferation and indicates CDK2 may be required for cell proliferation in some circumstances (Merrick, et al., 2011). Consistent with this, loss of Cdk2 and/or cyclin A2 has been shown to inhibit the proliferation of mouse embryonic fibroblasts, promote premature senescence and delay tumorigenesis in a mouse liver cancer model (Gopinathan, et al., 2014). Notably, elevated CDK1 kinase activity may play a compensatory role following ablation of cyclin A2, suggesting that dual targeting of CDK1 and CDK2 may be a necessary strategy for cancer therapy. Currently our understanding of the precise contexts where CDK2 is required for cell proliferation is incomplete and further advances in this area would help direct the future development of this class of agents.

2.3 CDK1

Cyclin A also binds CDK1 during the late S/G2 phase. Activation of the transcription factors FOXM1 and FOXK2 by CDK1/cyclin A followed by CDK1/cyclin B promotes the expression of genes involved in mitotic progression (Laoukili, et al., 2008; Marais, et al., 2010; Sadasivam, Duan, & DeCaprio, 2012)(Figure 2). Cyclin B mRNA and protein increases during the G2 phase of the cell cycle, but is destroyed during mitosis at the metaphase-anaphase transition. Phosphorylation of the cyclin B cytoplasmic retention sequence, prior to mitosis, promotes the translocation of this protein to the nucleus and reduces its nuclear export. CDK1/cyclin B has a number of nuclear substrates: these complexes are known to phosphorylate lamin, leading to nuclear envelope breakdown (Peter, Nakagawa, Doree, Labbe, & Nigg, 1990a, 1990b), and nucleolin, regulating nuclear fragmentation and organization (Belenguer, Caizergues-Ferrer, Labbe, Doree, & Amalric, 1990; Peter, et al., 1990a). Thus, CDK1 activity is a major determinant of cell cycle progression, ensuring that critical events occur in the correct sequence so that cellular replication proceeds with high fidelity.

CDK1 has also been implicated in the DNA damage response. BRCA1 is a direct target of CDK1 kinase activity and mutation of the CDK1 phosphorylation sites on BRCA1, or a loss of CDK1 activity, reduces the number of BRCA1 foci. As a result, cells are sensitized to DNA damaging agents (Johnson, et al., 2009). This finding led to the hypothesis that CDK1 inhibition would induce a BRCA-deficient-like phenotype and that inhibition of PARP would present a novel therapeutic strategy for BRCA-wildtype cancers (Johnson, et al., 2011). Excitingly, this approach has shown selectivity for transformed cells and has been well tolerated in mouse models of $Kras^{G12D}$, $Trp53^{L/L}$ lung adenocarcinoma (Johnson, et al., 2011).

Genetic studies of Cdk1 ablation in mice have demonstrated that Cdk1 is required for cell cycle progression and that Cdk1 can functionally compensate for the loss of CDKs 2, 3, 4 and 6 by forming active complexes with cyclins D and E to drive the cell cycle. Knocking out Cdk1 yielded no viable homozygous mice or early stage embryos (E1.5-E2.5)

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(Santamaria, et al., 2007). Interestingly, an interaction between CDK1 and cyclins D and E is only observed in the absence of expression of the other CDKs, suggesting that phenotypic lethality may not occur if CDK1 were to be targeted by small-molecule inhibitors. However, given that CDK1 appears essential for cell proliferation, compounds that inhibit CDK1 directly, or indirectly via the depletion of cyclins A2 and B1, may display toxicity that limits their clinical utility (Brandeis, et al., 1998; Murphy, et al., 1997).

3 Transcriptional regulation by CDKs

The polymerase responsible for transcribing all protein-coding genes is RNA polymerase II. RNA polymerase II catalyzes the transcription of histone-associated genomic DNA, which is wrapped around nucleosomes and can be covalently modified to regulate the access of the transcriptional apparatus to the DNA (Li, Carey, & Workman, 2007). For gene-specific transcription to take place RNA polymerase II has to be recruited to, and then exit, the gene's promoter that is then and followed by productive transcription that elongates the mRNA. This is a complex process requiring chromatin modification, the recruitment of sequence-specific transcription factors and post-translation modification of the transcriptional machinery.

RNA polymerase II is unique among the cellular polymerases as its largest subunit has a C-terminal domain (CTD) with an extended repeat comprised of a YSPTSPS heptapeptide that is present in a copy number ranging from 26 in budding yeast to 52 in humans (Corden, 2013; Eick & Geyer, 2013; Fisher, 2012; Jasnovidova & Stefl, 2013; Jeronimo, Bataille, & Robert, 2013; Jeronimo, Collin, & Robert, 2016). The CTD is not required for catalytic activity of the polymerase, but instead plays a key role in RNA processing and chromatin organization by acting as a landing pad for regulatory proteins, allowing the coordination of transcriptional and cotranscriptional events (Jeronimo, et al., 2013). The consensus heptapeptide can be phosphorylated at Tyr1, Ser2, Thr4, Ser5 and Ser7 and is a target of many kinases and phosphatases and post-translational modifying enzymes (Jeronimo, et al., 2016). With greater understanding of the role of the CTD it has become clear that specific and temporal post-translational modifications of the CTD regulate the activity of RNA polymerase II globally, and in a gene-specific manner, in response to environmental stimuli or the cellular state (Bataille, et al., 2012; Drogat & Hermand, 2012; Mayer, et al., 2010). Studies in yeast have determined a cycle of regulation (Figure 3). When RNA polymerase II is recruited to promoters it becomes phosphorylated on Ser5 and Ser7 before initiating transcription. Following the initiation of transcription Ser5 phosphorylation decreases while Ser2 and Tyr1 phosphorylation increases. When transcription terminates Tyr1 is the first residue to be dephosphorylated, closely followed by Ser5, Ser7 and Ser2. A similar pattern of CTD phosphorylation occurs in other higher eukaryotes (Figure 3), although possibly with the exception of the modification of Tyr1 (Corden, 2013; Eick & Geyer, 2013; Jasnovidova & Stefl, 2013; Jeronimo, et al., 2013; Jeronimo, et al., 2016). The multiple kinases that phosphorylate the CTD have been identified and, of relevance to this review, include CDKs (Jeronimo, et al., 2016).

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3.1 CDK7

The active CDK7 enzyme binds cyclin H and a ring finger protein MAT1 to form a subcomplex of the 10-subunit general transcription factor (TFIIH) complex. This complex has helicase, ATPase and protein kinase activity and is required for the earliest stage of transcription initiation (Figure 3)(Fisher, 2012). The first step in activating transcription is the formation of the pre-initiation complex that is required for promoter recognition and DNA unwinding. This requires RNA polymerase II to interact with multiple proteins, including the large multi-subunit Mediator complex and several general transcription factors, and may happen in a step-wise manner (Gupta, Sari-Ak, Haffke, Trowitzsch, & Berger, 2016). The binding of TFIID to the core promoter initiates the process and is followed by recruitment of additional general transcription factors and RNA polymerase II. This also involves structural rearrangements as the pre-initiation complex is initially in a ‘closed’ confirmation from which initiation cannot occur (Gupta, et al., 2016). The TFIIH complex is the last to be recruited and its helicase activity opens approximately one helical turn of DNA at the transcription start site. This leads to a conformational change that ‘opens’ the complex and positions the single strand DNA in the RNA polymerase II active site. The next step requires the kinase activity of the CDK7 subunit in the TFIIH complex to drive the escape of the polymerase from the promoter by breaking its interactions with other complex members. CDK7 primarily phosphorylates RNA polymerase II CTD Ser5, but can also phosphorylate Ser7 (Jeronimo, et al., 2016). Generally the RNA polymerase transcribes around 20-100 bases downstream of the promoter before pausing and in another regulatory process loses the remaining components of the initiation complex, yielding a fully functional elongation complex.

CDK7 may also influence cell cycle control by functioning as a CDK-activating kinase (CAK) and phosphorylating cell cycle CDKs, such as CDK1 and CDK2 (Fisher, 2012). *In vitro*, CDK7 can phosphorylate the T-loops of both cell cycle and transcriptional CDKs. Acute inhibition of CDK7 in human colorectal cancer cells prevented activation of CDK1 and 2, while genetic analyses in flies and worms implicated CDK7 in CDK1 activation. Cdk7 or Mat1 mouse knockouts are not viable as the loss of either gene is embryonically lethal (Ganuza, et al., 2012; Rossi, et al., 2001). Cells cultured from *Mat1*$^{-/-}$ mice were characterized by an inability to enter S phase and exhibited reduced phosphorylation of Ser5 and also Ser2 of the RNA polymerase II CTD. Despite this reduced phosphorylation knockout cells retained transcriptional activity, suggesting that residual Ser5 phosphorylation mediated by additional CTD kinases was sufficient to maintain transcription (Rossi, et al., 2001). Similar results were detected with Cdk7 deficient cells where Cdk7 was generally indispensible for proliferation, but not essential for global transcription. In adult Cdk7 conditional knockout models loss of Cdk7 had little effect in tissues with low levels of proliferation, in contrast, tissues with elevated cell turnover exhibited age-related loss of progenitor cells. At the molecular level loss of Cdk7 in mouse embryo fibroblasts resulted in reduced T-loop phosphorylation of CDKs suggesting that Cdk7 was acting as a CAK and expression of Cdk1^{T161E} and Cdk2^{T160E} T-loop phosphomimetic proteins partially restored proliferation.

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Over-expression of CDK7, and its cofactors cyclin H and MAT1, was recently reported in a cohort of >900 breast cancer samples. Expression of CDK7/cyclin H/MAT1 was greater in estrogen Receptor (ER) -positive breast cancer tumors versus ER-negative tumors. Loss of CDK7 activity resulted in decreased phosphorylation of ER^{SER118}, suggesting that CDK7 is responsible for ligand-dependent phosphorylation of this site and promotes ER activity. Hence, CDK7 inhibition is proposed as a potential treatment for ER-positive, high ER^{SER118} and CDK7-expressing breast cancers (Patel, et al., 2016). CRISPR/Cas9 mediated gene editing of CDK7 has also identified a dependency of triple-negative breast cancer (Y. Wang, et al., 2015). The cancer cells die by apoptosis as they are addicted to CDK7 and are dependent on an “achilles cluster” of survival genes regulated by super-enhancers that require CDK7. The CDK7 knockout studies suggest that CDK7 inhibitors would be tolerated for short periods, but may have effects on stem cell populations if given systemically resulting from cell cycle effects for an extended period of time (Ganuza, et al., 2012).

3.2 CDK9

Following transcription initiation, the pausing of the RNA polymerase II complex proximal to the promoter is an opportunity for mRNA processing and for the recruitment of further transcription factors (Figure 3). The transition to productive elongation requires the pTEFb elongation factor complex. This complex has a CDK9 subunit that phosphorylates the polymerase CTD at Ser2, and also the RNA polymerase II–associated negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) (Figure 3). Phosphorylation of the CTD at Ser5 by CDK7 is required for pTEFb recruitment and therefore inhibition of CDK7 can lead to reduced Ser2 phosphorylation by CDK9 (Larochelle, et al., 2012; Viladevall, et al., 2009).

CDK9 is ubiquitously expressed and forms heteromer complexes with cyclins T1, T2a, T2b and K (Figure 1). In addition to associating with a cyclin, CDK9 must be phosphorylated on activation-loop-residue Thr186 phosphorylated in order to function. Studies indicate that a number of different kinases can activate CDK9 and suggest that the cellular context of CDK9 critically influences the mode of its activation (Sonawane, et al., 2016). CDK7 may directly activate CDK9, as there is evidence that CDK7 can phosphorylate the T-loop of CDK9 (Larochelle, et al., 2012; Viladevall, et al., 2009). However, data from CDK7 knockout mice suggest CDK9 can also be activated by other protein kinases. RNAi screening identified CAMK1D as a kinase that phosphorylates CDK9 at Thr186, while the atypical kinase BRD4 can inhibit or activate CDK9 by phosphorylating it at Thr29 on the P-loop or Thr186 on the T-loop respectively (Devaiah & Singer, 2012; Ramakrishnan & Rice, 2012).

CDK9 has been proposed as a therapeutic target in cancer as it influences the expression of a number of genes encoding short-lived anti-apoptotic proteins associated with drug resistance (Bose, Simmons, & Grant, 2013; S. Wang & Fischer, 2008). A study using a phenotypic screen to identify the effects of inhibiting transcriptional CDKs found that transient inhibition of CTD phosphorylation induced caspase-dependent apoptosis, but only in transformed cells (S. Wang, et al., 2010). Silencing of CDK9 expression by shRNA or

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siRNA results in a range of phenotypes, with CDK9 knockout having contrasting effects on proliferation and cell death depending on use as a mono- or combined therapy (Garriga, Xie, Obradovic, & Grana, 2010; C. H. Huang, et al., 2014; F. Lam, et al., 2014; Storch & Cordes, 2016; Z. Q. Wang, et al., 2014). In immortalized normal fibroblasts, primary human astrocytes or glioblastoma cells CDK9 inhibition significantly influenced the gene expression pattern, but the number genes affected by dominant-negative *CDK9* expression or *CDK9* siRNA silencing was small and cell line dependent (Garriga, et al., 2010). These observations provide a degree of validation for CDK9 as a therapeutic target that may affect sub-set of tumor specific genes required for proliferation and survival. However, a recent study exploring components of the CDK9 interactome identified many proteins with roles in processes such as splicing and translation, downstream of the accepted locus of CDK9 function (J. Yang, et al., 2015). In addition, BRD4, that targets the P-TEFb complex to the promoters of many genes, mediates a compensatory mechanism that is activated upon CDK9 inhibition. Hence, the therapeutic potential of CDK9 inhibitors may only be revealed with concomitant BRD4 inhibition (Sonawane, et al., 2016).

3.3 CDK12/13

In higher eukaryotes, CDK9 is often referred to as the CTD Ser2 kinase, and the requirement for CDK9 for efficient transcriptional elongation has led to an assumption that Ser2 phosphorylation is critical for overcoming the early elongation block. Moreover, CDK9 is often presented as a kinase that essentially merges the activities of the yeast Bur1 and Ctk1 protein kinases (Bartkowiak & Greenleaf, 2011; Drogat & Hermand, 2012). However, several other Ser2 kinases have now been identified including CDK12 and its close homolog CDK13 (Bartkowiak, et al., 2010). Based on work carried out in Drosophila and yeast, it has been suggested that CDK9 phosphorylates Ser2 early on in transcription, before passing on this role to CDK12, which phosphorylates Ser2 for the majority of the elongation phase (Jeronimo, et al., 2016). However, knockout or chemical inhibition experiments have had, at best, modest effects on global CTD phosphorylation, which can be interpreted as CDK12 either having no importance in human CTD Ser2 phosphorylation or having functional redundancy with CDK13 or other Ser2 kinases (Bartkowiak, Yan, & Greenleaf, 2015; Jeronimo, et al., 2016). One explanation is that the role of CDK12 in CTD phosphorylation is gene-specific. Deletion of mouse Cdk12 is embryonically lethal as a result of pluripotent cells losing their capacity for self-renewal and dying by apoptosis (Juan, Lin, Chen, & Fann, 2016). At the molecular level this was due to reduced expression of genes associated with self-renewal of stem cells. CDK12 has also been associated with the regulation of a subset of genes required for the cellular response to DNA damage (Blazek, et al., 2011). This was also observed in the CDK12 knockout model that exhibited reduced expression of gene encoding DNA damage repair proteins (Juan, et al., 2016). This may impact on the maintenance of pluripotency as mouse embryonic cells frequently have elevated DNA breaks that occur during replication and their repair is critical for successful embryonic development. Importantly, mice deficient for Rad50, Rad51, Atr and Brca1 have similar phenotypes to the Cdk12 knockout mice (Juan, et al., 2016).

Proteomic and biochemical studies of CDK12 partners and substrates identified interactions between CDK12 and several RNA processing factors found in subnuclear domains enriched

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with splicing factors (Bartkowiak & Greenleaf, 2015; H. H. Chen, Wang, & Fann, 2006; Eifler, et al., 2015; Ko, Kelly, & Pines, 2001; Liang, et al., 2015). Loss of CDK12 and CDK13, or their associated cofactor cyclin K, not only impeded the progress of RNA polymerase II, but also RNA processing. CDK12 binds to exon-junction complexes containing arginine–serine rich splicing factors, and the loss of CDK12 leads to mRNA splicing defects (Bartkowiak & Greenleaf, 2015). Furthermore, the recruitment of factors involved in the cleavage and polyadenylation of the 3'-end of mRNA takes place at the same time as CTD Ser2 phosphorylation and is dependent on CDK12 function. Finally, CDK12 depletion leads to a loss of Ser2 phosphorylation, reduced recruitment of splicing factors, and 3'-end processing defects in the transcripts of genes such as *FOS* and *MYC* (L. Davidson, Muniz, & West, 2014; Eifler, et al., 2015).

Considering that CDK12 regulates the expression of several cancer-related genes, such as *FOS* and *MYC*, it is not surprising that the deregulation of CDK12 has been identified in cancerous tissues. For example, breast cancers have *CDK12* and *ERBB2* genes are amplified in around 20% of cases (Kauraniemi, Barlund, Monni, & Kallioniemi, 2001; Kauraniemi, Kuukasjarvi, Sauter, & Kallioniemi, 2003). In contrast, inactivating mutants of CDK12 have been found in ovarian cancer and cells expressing a defective CDK12 have functional defects in homologous repair, which makes them sensitive to PARP inhibition (P. M. Joshi, Sutor, Huntoon, & Karnitz, 2014). Genetic siRNA silencing screens have identified CDK12 as a modifier of breast cancer cell sensitivity to tamoxifen through MAPK activation and have also found CDK12 as a determinant of sensitivity to inhibitors of the poly (ADP-ribose) polymerases PARP1 and PARP2 (Bajrami, et al., 2014; Iorns, Martens-de Kemp, Lord, & Ashworth, 2009).

3.4 CDK8/19

As outlined in section 3.1, the unphosphorylated RNA polymerase II assembles at core promoters, where it interacts with the Mediator complex: a highly conserved transcriptional coactivator. The Mediator complex is a large, multisubunit protein complex central to the regulation of transcription in eukaryotes (Allen & Taatjes, 2015; Poss, Ebmeier, & Taatjes, 2013; Yin & Wang, 2014). Acting as a molecular bridge, the Mediator transfers signals from DNA-bound transcription factors to the RNA polymerase II pre-initiation complex. It has a role in recruiting proteins required for transcription elongation (including pTEFb) and transcriptional pausing, and influences chromatin structure, facilitating the formation of enhancer-promoter gene loops (Allen & Taatjes, 2015; Donner, Ebmeier, Taatjes, & Espinosa, 2010; Poss, et al., 2013; Takahashi, et al., 2011; Yin & Wang, 2014).

A four-subunit kinase module containing CDK8, cyclin C and the Mediator subunits MED12 and MED13 also transiently associates with the Mediator complex to regulate transcription (Y. Liu, Ranish, Aebersold, & Hahn, 2001; Taatjes, Naar, Andel, Nogales, & Tjian, 2002). Evidence indicates that the binding of Mediator to the kinase module and to RNA Pol II is mutually exclusive (Tsai, et al., 2013), suggesting that the interaction between Mediator and the kinase module may be a key checkpoint in the control of transcription. CDK8 knockdown studies have demonstrated reduced CTD phosphorylation at Ser2 and Ser5, impairment of transcriptional elongation and reduced recruitment of super elongation

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factors to immediate/early-response genes activated in response to serum stimulation (Donner, et al., 2010). Separate work has shown that the HIF1alpha transactivation of gene expression requires the CDK8 module for the recruitment of super elongation factors and their binding to RNA polymerase II, to stimulate the transition from a paused complex to transcriptional elongation (Galbraith, et al., 2013) (Figure 4)

As a kinase that reversibly associates with Mediator, CDK8 can regulate gene expression through the phosphorylation of transcription factors. Phosphorylation by CDK8 can directly alter transcription factor activity (Bancerek, et al., 2013; Morris, et al., 2008; J. Zhao, Ramos, & Demma, 2013) or mark factors for degradation (Alarcon, et al., 2009; Fryer, White, & Jones, 2004; X. Zhao, et al., 2012). The CDK8-Mediator complex has been shown to assemble on p53 target genes, including $p21^{CIP1}$, after the exposure of cancer cells to DNA-damaging agents such as radiation therapy, doxycycline and fluorouracil (Donner, et al., 2010). CDK8 phosphorylates STAT1 at Ser727, activating STAT1-regulated transcription in response to IFN-gamma stimulation (Bancerek, et al., 2013). This regulatory pathway is typically associated with antiviral defense and tumor-suppressor functions. However, other evidence has also implicated the STAT1 pathway in cellular resistance to DNA-damage and aggressive tumor growth (Duarte, et al., 2012; Khodarev, et al., 2004; Khodarev, Roizman, & Weichselbaum, 2012). The phosphorylation of STAT1 at Ser727 by CDK8 was found to suppress natural killer cells and the expression of a dominant-negative CDK8 was shown to be more cytotoxic to cancer cells than healthy cells (Putz, et al., 2013).

Other transcriptional activator proteins phosphorylated by CDK8 include the SMAD proteins and NOTCH1. CDK8 phosphorylates an interdomain linker region within the SMAD proteins, which results in their transcriptional activation and primes them for ubiquitin-mediated degradation (Alarcon, et al., 2009). The NOTCH1 protein is a transmembrane receptor that undergoes cleavage when activated to release its intracellular domain, ICN1, which then translocates to the nucleus to activate gene-specific transcription. CDK8 regulates ICN1 activity by phosphorylation, resulting in increased ICN1 ubiquitination and proteasome-driven degradation (Fryer, et al., 2004). CDK8/cyclin C has also been shown to be a negative regulator of the lipogenic pathway, through its phosphorylation of nuclear SREBP-1c at a conserved threonine residue that enhances SREBP-1c ubiquitination and protein degradation (X. Zhao, et al., 2012). Although, much is known about the role of CDK8 in regulating transcription through the repression and activation of transcription factors, CDK8 may also regulate transcription at the chromatin level. CDK8 phosphorylates histone H3 at Ser10 (Knuesel, Meyer, Donner, Espinosa, & Taatjes, 2009), a mark associated with the transcriptional activation of immediate/early-response genes (Strelkov & Davie, 2002). CDK8 also interacts with GCN5L to generate a dual Ser10/Lys14A mark on histone H3 (Meyer, et al., 2008).

Our understanding of the role of CDK8, and the Mediator kinase module, in the control of transcription is complicated by the fact that vertebrates express paralogues of CDK8, MED12 and MED13 (CDK19, MED12L and MED13L respectively). Despite a high degree of sequence similarity, CDK8 and CDK19 can interact with different partners *in vitro* (Tsutsui, et al., 2008) and seem to perform some distinct roles *in vivo* (Galbraith, et al., 2013), which may explain why Cdk19 fails to compensate for the embryonic lethality of the

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Cdk8 knockout in mice (Westerling, Kuuluvainen, & Makela, 2007). In contrast, conditional Cdk8 knockout in adult mice has no major effects on the homeostasis of normal tissue (McCleland, et al., 2015).

The biological function of CDK8 varies by cell type and in response to different stimuli (Allen & Taatjes, 2015). This is particularly true in the development of cancer, where evidence suggests CDK8 can function as both an oncogene and tumor-suppressor depending on the context. Consistent with a role as a tumor-suppressor, *CDK8* expression is reduced in a subset of bladder cancers (Mitra, et al., 2006) and deletion of the *CDK8* gene is frequently observed in oesophageal squamous cell carcinomas (Chattopadhyay, et al., 2010). In endometrial cells the ectopic expression of *CDK8* blocked xenograft tumor growth and inhibited cell proliferation, migration and invasion; whereas CDK8 knockdown had the opposite effect (Gu, et al., 2013). Similarly, deletion of *Cdk8* in an Apc^{Min} murine tumor model led to an increase in tumor growth rate and size (McCleland, et al., 2015).

In contrast, it has been reported that *CDK8* may function as an oncogene, particularly in the development of colorectal cancer. *CDK8* is frequently amplified in colorectal cancer with copy number gains in ~60% of tumors (Firestein, et al., 2010; Seo, Han, & Lim, 2010), while CDK8 knockdown reportedly reduces the growth of human colorectal cancer tumor xenografts harboring *CDK8* gene amplification (Adler, et al., 2012; Firestein, et al., 2008). Furthermore, studies have indicated that *CDK8* expression is required to maintain colorectal cancer xenografts and embryonic stem cells in an undifferentiated state (Adler, et al., 2012). Importantly, NIH3T3 cells overexpressing wild-type *CDK8* have a malignant phenotype, but those overexpressing a kinase-dead *CDK8* mutant do not (Firestein, et al., 2008). More recently, both CDK8 and CDK19 have been identified as potential therapeutic targets in advanced prostate cancer, where siRNA knockdown or small molecule inhibition decreased invasion and migration (Bragelmann, et al., 2016).

One way in which CDK8 and the Mediator kinase module may promote oncogenesis is through activation of the canonical WNT signaling pathway. The WNT signaling pathway is critical to metazoan development and misregulation of this pathway has been implicated in a variety of cancers. *CDK8* expression correlates with the activation of β-catenin, a core transcriptional regulator of canonical WNT signaling, in gastric and colon cancers (Firestein, et al., 2010; M. Y. Kim, Han, & Lim, 2011) and shRNA screens have identified a requirement for CDK8 for the activation of WNT-signaling in colorectal cancer cell lines (Firestein, et al., 2008). While MED12 and MED13 stimulate WNT-signaling via a direct interaction between β-catenin and MED12 (Carrera, Janody, Leeds, Duveau, & Treisman, 2008; S. Kim, Xu, Hecht, & Boyer, 2006; Rocha, Scholze, Bleiss, & Schrewe, 2010), CDK8 acts by phosphorylating E2F1, preventing it from promoting the degradation of β-catenin (Morris, et al., 2008; J. Zhao, et al., 2013). Therefore the Mediator kinase module has the ability to drive cancer cell progression by both facilitating the transcription of *β*-catenin target genes and repressing an opposing degradation pathway.

As outlined in this section, the Mediator-associated kinases CDK8 and CDK19 have been proposed as context-dependent drivers or suppressors of tumorigenesis. Given the role of these proteins in regulation of signal-dependent gene expression and, in particular, their

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effect on the super-enhancers that regulate gene expression controlling cell identity and disease, inhibiting CDK8/19 would be predicted to have pleiotropic lineage-dependent effects. However, the impact of this on the clinical utility of CDK8/19 inhibitors is unclear, particularly in regard to the size of the therapeutic window between cancerous and healthy tissue.

4 Additional CDKs with a role in cancer

The main focus of this review is the CDKs for which inhibitors have been reported. However, other members of the CDK family may have a role in cancer and in some cases may represent future targets for therapeutic intervention.

4.1 CDK10

CDK10 binds the N-terminal domain of the ETS2 transcription factor and suppresses the ETS2 transactivation domain (Bagella, Giacinti, Simone, & Giordano, 2006; Kasten & Giordano, 2001), consistent with CDK10 functioning as a tumor suppressor, inhibiting the oncogenic potential of MAPK signaling. In a screen designed to identify modifiers of tamoxifen sensitivity in breast cancer, siRNA knockdown of *CDK10* mRNA relieved ETS2 repression, resulting in ETS2-mediated expression of *c-RAF* and increased MAPK-signaling (Iorns, et al., 2008). The result of this MAPK-activation was circumvention of ERα signaling in the tumor cells and continued proliferation in the presence of the anti-estrogen tamoxifen. Correspondingly, the same study provided evidence that breast cancer patients with ERα-positive tumors expressing *CDK10* at low levels are more likely to relapse. Recent studies have identified cyclin L2 as the regulatory cyclin for CDK10, and silencing of *CCNL2* phenocopies *CDK10* silencing, leading to an increase in the level of c-RAF and conferring tamoxifen resistance. CDK10/cyclin L2 was found to phosphorylate ETS2 and induce its degradation by the proteasome (Guen, et al., 2013).

4.2 CDK11

Unlike the other CDK family members, human CDK11 is encoded by highly homologous genes, *CDK11A* and *CDK11B*, unlike mice, which has a single gene (Zhou, Shen, Hornicek, Kan, & Duan, 2016). CDK11 has conserved cyclin-binding domains, a C-terminal catalytic domain and three regulatory phosphorylation sites. In addition, there is an N-terminal regulatory region containing multiple nuclear localization signals, a 14-3-3 consensus binding site, an arginine/glutamic acid domain thought to associate with RNA processing factors, and a poly-glutamic acid domain, which is a potential cytoskeleton-interacting domain. CDK11 binds L-type cyclins and has multiple roles in coordinating transcription and splicing, developmental signaling, cell cycle regulation, neuronal function, autophagy and apoptosis (Loyer, et al., 2008; Zhou, et al., 2016). Multiple approaches have been taken to identify CDK11-interacting proteins. Aside from the L cyclins, these studies have identified splicing factors, and multiple transcriptional initiation and elongation factors (Hu, Mayeda, Trembley, Lahti, & Kidd, 2003; Loyer, et al., 2008; Loyer, Trembley, Lahti, & Kidd, 1998; Trembley, et al., 2002).

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Different isoforms and splice variants of CDK11 have been identified, namely CDK11p46, CDK11p58 and CDK11p110. The larger CDK11p110 kinase is expressed throughout the cell cycle and is a nuclear protein that mainly associates with the transcriptional and splicing apparatus (Hu, et al., 2003; Trembley, et al., 2002; Trembley, et al., 2004). CDK11p58 is produced during M phase and in HeLa cells inhibition of *CDK11* expression using siRNA leads to abnormal spindle assembly, mitotic arrest and cell death (Franck, et al., 2011; Petretti, et al., 2006; Rakkaa, Escude, Giet, Magnaghi-Jaulin, & Jaulin, 2014; Yokoyama, et al., 2008). The CDK11p46 isoform is associated with apoptosis and is generated in response to apoptotic signaling by caspase 1 or 3 activity (Beyaert, et al., 1997). In contrast to the other CDK11 isoforms, CDK11p46 localizes predominantly to the cytoplasm when ectopically expressed. Several non-cyclin partners have been proposed to interact with CDK11p46, including the eukaryotic initiation factor EIF3E and the Ran-binding protein RanBP9 (Mikolajczyk, Shi, Vaillancourt, Sachs, & Nelson, 2003; Shi, et al., 2003).

CDK11 is highly expressed in triple negative breast cancers and is associated with both an advanced stage of disease and a poor clinical prognosis (Zhou, et al., 2015). Silencing of *CDK11* expression significantly inhibits migration, inhibits proliferation and induces apoptosis in breast cancer cell lines (Kren, et al., 2015). Similar results have been observed in multiple myeloma cells where two screens have independently identified *CDK11*A/*B* as crucial survival genes and their proteins as potential targets for therapeutics (Tiedemann, et al., 2012; Tiedemann, et al., 2010). Furthermore *CDK11* expression has been shown to be increased in primary multiple myeloma samples compared to normal primary tissues. Elevated *CDK11* expression, associated with a poor clinical outcome is observed in osteosarcoma cells and patient biopsies (Zhou, et al., 2016). Moreover, as described for breast cancer and multiple myeloma cells, shRNA and CRISPR/Cas9 genetic knockouts of *CDK11* in osteosarcoma cancer cells significantly reduced cell viability, proliferation, migration, and invasion, and induced cell death (Duan, et al., 2012; Feng, et al., 2015). *CDK11* is also highly expressed in liposarcoma tissues compared with expression in benign lipoma tissues - another type of malignancy that originates in mesenchymal tissue. SiRNA or shRNA inhibition of *CDK11* expression has been found to reduce cell proliferation and induce apoptosis in liposarcoma cells, in addition to enhancing the cytotoxic effect of doxorubicin (Jia, et al., 2014). Overall, *CDK11* is highly expressed in several types of human malignancies and is associated with a poor outcome. Consequently, CDK11 has potential as target for cancer therapy.

4.3 The cyclin Y binding CDKS

CDK14-18 bind cyclin Y, a membrane-associated cyclin whose cellular role remains elusive (Malumbres, 2014)(Figure 1). CDK14 participates in the regulation of WNT signaling. The WNT ligands are a family of secreted or cell surface glycoproteins that regulate cell proliferation, survival, migration, polarity, cell fate specification, and stem cell renewal (Clevers & Nusse, 2012). There are multiple seven-pass transmembrane proteins and two single-pass transmembrane low-density receptor-related lipoproteins (LRP5 and LRP6) capable of binding WNT in a ternary complex with Frizzled. LRP6 activation is a key regulatory node for WNT signaling and recent studies have identified multiple signals that can influence LRP6 activation, including inputs from the cell cycle (G. Davidson, et al.,

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2009). The cell cycle-dependent activation of LRP6 is mediated by the CDK14/cyclin Y complex, which phosphorylates LRP6 at the plasma membrane during G2/M. Phosphorylation increases the receptiveness of cells to incoming WNT signals and the peak of LRFP6 phosphorylation at G2/M appears to provide a clear mechanistic explanation for the increased activity of WNT signaling reported at G2/M. Cell cycle activation of LRP6 could be a regulatory mechanism to enhance signaling in particular proliferative regions, for example during development or in stem cells.

WNT signaling is closely linked to cancer progression: non-canonical WNT signaling is frequently increased in human hepatocellular carcinoma and CDK14 expression confers increased motility and metastatic potential in this setting (J. Huang, et al., 2012; Leung, et al., 2011; Sun, Co, & Wong, 2014). Recent studies show that CDK14 is highly expressed in several malignant tumors such as hepatocellular carcinoma, esophageal cancer, breast cancer, and gastric cancer, with roles in the regulation of the cell cycle, tumor proliferation, migration, and invasion (L. Yang, et al., 2015). Although there are many questions still to be answered regarding the role of CDK14 in WNT signaling, these links suggest that targeting CDK14 has therapeutic potential in the treatment of cancer.

Of the remaining cyclin Y binding CDKs, CDK15 has been found in a hepatitis B virus-gene fusion in hepatocellular carcinomas and may participate in hepatitis B virus driven transformation (Shiraishi, et al., 2014). CDK16 acts via mechanisms unknown to regulate p27Kip1 stability, mitosis, apoptosis, and growth in multiple cancer cell lines (prostate, breast, cervical cancers, and melanomas). Hence the inhibition of CDK16 may provide a strategy for the treatment of some human cancers with pathological elevations in the activity of this kinase (Yanagi & Matsuzawa, 2015).

4.4 CDKs with poorly defined functions

There remain classes of CDKs for which the underlying functions are unclear. CDK3 was found to be intrinsically important for cell cycle control based on cell-based experiments that used a dominant-negative version of CDK3 (van den Heuvel & Harlow, 1993). However, there are mouse strains that harbour an inactive CDK3 suggesting that its role in the cell cycle can be readily compensated for (Ye, Zhu, & Harper, 2001). CDK5 was largely viewed as a neuronal kinase; however, recent work suggests that it has functions similar to CDK4 and CDK2 in driving progression from G1–S and in RB phosphorylation in medullary thyroid cancer models and as such may be a therapeutic target at least in this cancer type (Pozo, et al., 2013). Finally, CDK20 interacts with cyclin H and was thought to phosphorylate and activate CDK2, suggesting a close relationship with CDK7; however, this suggestion remains controversial. Recent data suggest that CDK20 activates ICK or β-catenin-TCF signaling to stimulate cell-cycle progression (Malumbres, 2014).

5 Small molecule CDK inhibitors

The majority of protein kinase inhibitors developed to date are type I inhibitors: they bind at the ATP–binding site, are ATP-competitive and target the kinase in its active state, with the activation loop DFG motif in the ‘in’ position. In contrast, type II inhibitors can bind kinases that are in an inactive conformation, with the DFG motif flipped ‘out’. In addition to binding

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the ATP binding site, Type II inhibitors can also occupy a hydrophobic site, made accessible by the flipped "out" conformation of the DFG motif. Type II inhibitors are believed to be more selective than Type I inhibitors, however for both type of compounds there are examples of both highly selective and non-selective inhibitors (Treiber & Shah, 2013). The development of selective CDK/cyclin inhibitors was initially thought to be challenging since it was commonly believed that cyclin binding prevented the conformational change required at the ATP-binding site to generate a type II inhibitor-binding pocket, thus restricting the inhibitors to type I binding modes. In addition, the high degree of similarity between the ATP-binding sites of the CDKs was also predicted to be a challenge to generating isoform-selective inhibitors. Consistent with this the early type I inhibitors were generally found to be promiscuous across multiple CDKs. However, as described in this review it is now clear that it is possible to identify and develop potent CDK-selective type I inhibitors and also inhibitors that bind with a type II mode.

The first CDK inhibitors were developed predominantly against CDK2 and were relatively unselective, but acted as early pathfinder agents. These inhibitors encompassed heteroaromatic scaffolds including flavonoid, purine, indenopyrazole, aminopyrimidine, aminothiazole, indirubin, hymenialdisine, and paullone derivatives (Asghar, et al., 2015; Sanchez-Martinez, Gelbert, Lallena, & de Dios, 2015). Below we describe the early pan-CDK inhibitors and the improvements that have led to the current clinical studies with multi-target CDK inhibitors. We also discuss recent exciting advances in the development of isoform-selective CDK inhibitors.

5.1 Early pan-CDK inhibitors

Among the first CDK inhibitors to advance to clinical trial were alvocidib (flavopiridol; **1**) and seliciclib (roscovitine/CYC202; **2**) (Figure 5). These are pan-CDK inhibitors: alvocidib inhibits CDKs 1, 2, 4, 6, 7 and 9 and seliciclib inhibits CDKs 1, 2, 5, 7 and 9. These agents produce G1 and G2 phase cell cycle arrest and apoptosis, an effect initially attributed to their inhibition of the cell cycle CDKs (Carlson, Dubay, Sausville, Brizuela, & Worland, 1996; Meijer, et al., 1997). However, later work indicated that many of the cellular activities of these inhibitors were probably the result of CDK7 or CDK9 inhibition including the transcriptional inhibition of cell cycle and apoptosis-related genes (L. T. Lam, et al., 2001; MacCallum, et al., 2005; Whittaker, et al., 2007; Whittaker, Walton, Garrett, & Workman, 2004). Alvocidib has demonstrated some clinical efficacy in hematological malignancies such as chronic lymphocytic leukemia (CLL), but responses were limited by toxicity (Aklilu, Kindler, Donehower, Mani, & Vokes, 2003; Burdette-Radoux, et al., 2004; Byrd, et al., 2007; Byrd, et al., 2005; G. Liu, et al., 2004; Schwartz, et al., 2001). Seliciclib was examined in two Phase I studies (Benson, et al., 2007; Le Tourneau, et al., 2010). The peak plasma levels of Seliciclib were not sufficiently sustained for antiproliferative effects, as modeled *in vitro* (Raynaud, et al., 2005). Consistent with this, attempts to measure RB phosphorylation and cyclin D1 expression biomarkers did not reliably show modulation on treatment (Benson, et al., 2007). While no objective responses were observed, disease stabilization was observed, with one ovarian cancer patient remaining on therapy for 18 weeks.

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The side-effects associated with these early pan-CDK inhibitors include nausea, vomiting, fatigue and hepatic dysfunction, with alvocidib also causing myelosuppression. Alternative dosing schedules were identified that permitted slightly higher, but intermittent, dosing frequencies (Le Tourneau, et al., 2010), and seliciclib has now been evaluated in 16 clinical trials, including combination studies. Ongoing studies are investigating activity in *BRCA* mutant tumors in combination with sapacitabine, a nucleoside analogue (Tolaney, et al., 2016; W. Yeo, 2009). Notably, this combination resulted in a disease control rate of 35.6%; mainly stable disease, in heavily pretreated patients with tumors of breast, ovarian and pancreatic origin. Mechanistically, suppression of BRCA protein expression and CDK2 and CDK9 activities by seliciclib is implicated in the potentiation of DNA damage induced by sapacitabine.

While data from mouse models has led some to question the requirement for specific CDKs to mediate cell cycle progression (see section 2), evidence suggests that CDK1 may be an essential cell cycle CDK (Santamaria, et al., 2007). However, because the biological consequences of knocking out a gene target and inhibiting protein activity with a drug can be phenotypically different, the development of more selective CDK inhibitors may help to delineate the mechanisms of therapeutic response and the cause of pan-CDK inhibitor toxicity.

5.2 Multitarget CDK inhibitors

Dinaciclib (**3**; Figure 5) is a near-equipotent inhibitor of CDK1, CDK2, CDK5 and CDK9, which blocks DNA replication in ovarian carcinoma cells with low single digit nM IC_{50}s. The compound displays good selectivity for CDKs over other kinases and is active in a broad range of cancer cell lines originating from multiple tumor types. Importantly, measurement of inhibition of RB phosphorylation in response to dinaciclib application has confirmed target engagement in cells, with apoptosis induced at between 12 and 500 nM. Interestingly, only 2 h transient exposures to dinaciclib are required to bring about these molecular changes, which are sustained for 6 h in the absence of the drug. Dinaciclib has shown good efficacy *in vivo* and is well tolerated, with daily intraperitoneal dosing inhibiting ovarian xenograft growth over 10 days. In terms of haematological effects, dinaciclib treatment has been shown to reversibly depress neutrophil and reticulocyte counts (Parry, et al., 2010). Dinaciclib has shown some activity in *MYCN*-driven neuroblastoma, attributed to inhibition of CDK2 and CDK9 (Z. Chen, et al., 2016). The overexpression of CDK2 in neuroblastoma tissue is associated with poor overall survival, suggesting a potential strategy for patient selection during clinical development of this drug (Z. Chen, et al., 2016).

Phase I testing of dinaciclib was conducted in 48 patients with advanced solid tumors receiving a 2 h infusion once a week for 3 weeks. Dose-limiting toxicities (DLTs) reported included orthostatic hypotension and elevated uric acid; however, overall, the drug was well tolerated, with stable disease observed in 10 patients (Nemunaitis, et al., 2013). Dinaciclib progressed into Phase II trials in patients with breast cancer and non-small cell lung cancer; however, in both studies time to disease progression was shorter with dinaciclib than the standard-of-care, and the trials were terminated early (Mita, et al., 2014). Given the

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promising results observed with the first generation CDK inhibitor alvocidib in relapsed and refractory CLL (Byrd, et al., 2007), the clinical activity of dinaciclib was also studied in this population (Flynn, et al., 2015). Strikingly, 54% responded to treatment with dinaciclib, with cytopenia and tumor lysis syndrome reported as associated adverse events. A followup Phase III trial of dinaciclib versus ofatumumab (an antibody targeting the CD20 antigen) in refractory CLL was terminated early, but did demonstrate activity (Ghia, et al., 2015). Additional studies of the activity of dinaciclib against other hematological cancers, such as multiple myeloma, have shown a response rate of 11% (Kumar, et al., 2015). Ongoing studies are looking at the efficacy of dinaciclib treatment in combination with immunotherapy (pembrolizumab) or PARP inhibition (veliparib) (Hossain, et al., 2016).

AT7519 (**4**; Figure 5) was discovered through a structure-guided, fragment-based, screen (Wyatt, et al., 2008). The compound inhibits CDK1, 2, 4, 5, 6 and 9. Cellular activity across a broad panel of human cancer cell lines has been observed, with GI_{50} values ranging from 40 to 940 nM. Evidence that inhibition of CDK4/6 were dispensable for activity came from observations that cell lines lacking functional RB retain sensitivity to AT7519 and the modulation of the CDK4/6 phosphorylation site Ser780 on RB was not affected by treatment (Squires, et al., 2009). Inhibition of NPM1 and other RB phosphorylation sites as early as 1 h post-treatment is consistent with the AT7519 having a direct effect on CDK2 activity; while the inhibition of PP1A phosphorylation was only reported at 24 h, suggesting that the effect of AT7519 on CDK1 activity in cells may be modest. Finally, treatment with AT7519 inhibits the phosphorylation of RNA polymerase II CTD Ser2 and blocks global transcription with an IC_{50} of 56 nM, reflective of an inhibition of CDK9 activity (Squires, et al., 2009).

AT7519 treatment causes cell cycle arrest at G0/G1 and G2/M. The compound has excellent antitumor efficacy in human colorectal cancer xenograft models, with extensive tumor regression, the modulation of pharmacodynamic biomarkers and elevated PARP cleavage indicating apoptotic cell death (Squires, et al., 2009). A Phase I trial of AT7519 in refractory solid tumors showed evidence of activity with 4 out of 28 patients experiencing stable disease and one a prolonged partial response, but had to be discontinued because of DLT, particularly an increase in cardiac QTc (Mahadevan, et al., 2011). A second Phase I study in advanced refractory solid tumors and non-Hodgkin's lymphoma did not report significant cardiac QTc prolongation and 10 of 19 patients had stable disease (E. X. Chen, et al., 2014).

Hematological cancers appear particularly susceptible to AT7519 treatment. These cancers are known to depend upon transcripts with short half-lives, such as *MCL1*, *BCL2* and *XIAP*, and AT7519 has been shown to inhibit *MCL1* expression in an HL60 xenograft model in which it induced tumor regression. Patient-derived CLL cells have also been shown to respond to a brief exposure to AT7519, undergoing apoptosis at concentrations of drug that can be safely achieved in patients for >12 h (Squires, et al., 2010). Further clinical investigation of AT7519 is underway in multiple myeloma, mantle cell lymphoma and chronic lymphocytic leukemia.

Recently, a role for AT7519 has also been described in the treatment of neuroblastoma. *MYCN*-amplified neuroblastoma cell lines were shown to be more prone to AT7519-induced

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apoptosis than non-amplified lines, despite similar levels of RB phosphorylation (Dolman, et al., 2015). Furthermore, AT7519 has shown activity in a *MYCN*-amplified AMC711T neuroblastoma xenograft model with 3 weeks treatment giving near complete inhibition of tumor growth and suppression of NPM and RB phosphorylation. AT7519 also suppressed the growth of tumors in Th-*MYCN* transgenic mice; the treated mice showed partial responses and survived significantly longer than the controls (Dolman, et al., 2015). Notably, this study did not resolve the contribution of specific CDKs to the activity observed in neuroblastoma, showing CDK2 substrates were clearly inhibited, but not assessing any CDK9-dependent effects.

CYC065 is a second generation CDK inhibitor optimised from seliciclib and CCT068127 (Wilson, et al., 2011). The compound shows greatest potency against CDKs 2, 5 and 9 (Frame, Saladino, Davis, Blake, & Zheleva, 2014). CYC065 has been tested in preclinical models of acute myeloid leukemia (AML) and acute lymphoblastic leukemia and is thought to restrict the proliferation and survival of leukemia cell lines by inhibiting CDK9, which reduces RNA polymerase II-mediated transcription and *MCL1* expression, inducing apoptosis. Furthermore, CYC065 can suppress *MEIS1* expression, a transcriptional cofactor, which is required for the induction and maintenance of mixed-lineage leukemic stem cells via promotion of cell cycle progression and inhibition of differentiation (Wong, Iwasaki, Somervaille, So, & Cleary, 2007). Oral dosing of mice with CYC065 led to a >90% reduction in the growth of EOL-1 and HL60 AML xenografts (Frame, et al., 2014). CYC065 and the related compound CCT068127 both inhibit *MCL1* expression, it has been suggested that co-treatment with BCL2 family inhibitors may be synergistic and drive cells into apoptosis (Frame, et al., 2014)(Whittaker *et al*, submitted). Consistent with this hypothesis, the effect of ABT-263 and CYC065 or CCT068127 on AML, acute lymphoblastic leukemia and colorectal cancer cell lines suggests that these inhibitors have synergistic activity (Frame, et al., 2014) (Whittaker *et al*, submitted).

CYC065-mediated CDK2 inhibition may have a role in solid tumors, for example in trastuzumab-refractory, HER2+ breast cancer, as treatment with CYC065 has been shown to induce apoptosis and suppress tumor growth in these xenograft models perhaps through a dependence on CCNE1 (Scaltriti, et al., 2011). *CCNE1* amplification is also a feature of uterine serous carcinoma and confers sensitivity to CYC065 in cell line models. *PIK3CA* mutation and/or amplification is also a common event in uterine serous carcinomas and treatment with CYC065 and GDC-0032, a selective class I PI3K inhibitor, has shown synergy *in vitro* and greater efficacy than monotherapy *in vivo* (Cocco, et al., 2016). While the activity of CYC065 was attributed to its inhibition of CDK2 in these latter two examples, the potential for CYC065 to inhibit CDK9, and hence decrease the transcription of genes involved in cell proliferation and survival, was not assessed. Transcriptional inhibition by CYC065 may be effective in neuroblastoma, as in leukemia, and warrant clinical investigation. Depletion of MYCN, a key driver of neuroblastoma, has been observed in human cancer cell lines, human tumor xenograft and mouse models treated with CYC065; similarly, CYC065 inhibited neuroblastoma cell proliferation and induced apoptosis, prolonging the survival of treated mice (Poon, et al., 2016). CYC065 has now entered a Phase I dose-escalation clinical trial in patients with advanced cancers.

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Several other multitarget CDK inhibitors have failed or not progressed beyond early clinical studies for example RGB-286638, ZK-304709, and P1446A-05 (**5-7**; Figure 5) were discontinued during phase I or phase II trials (Asghar, et al., 2015; Sanchez-Martinez, et al., 2015). P276-00 (**8**; Figure 5) ceased clinical studies following a commercial decision by the sponsor company (K. S. Joshi, et al., 2007). R547 (**9**; Figure 5) is an inhibitor of CDKs 1, 2 and 4, with reduced potency for CDK7, GSK3a and GSK3b, and was tested in a Phase I trial in 2007, but has not been progressed further despite reports of manageable toxicity (Diab, et al., 2007). SNS-032/BMS-387032 (**10**; Figure 5) was originally thought to be selective for CDK2 over CDKs 1 and 4, but is now known to also target CDK7 and CDK9. This compound was tested in two Phase I clinical studies, but these have not been followed up (Heath, Bible, Martell, Adelman, & Lorusso, 2008; Tong, et al., 2010). AZD5438 (**11**; Figure 5) is an inhibitor with selectivity for CDKs 1, 2 and 9 over CDKs 5 and 6, and was not tolerated in Phase I trials, halting the clinical development of this compound (Boss, et al., 2010; Byth, et al., 2009). Finally, AG-024322 (**12**; Figure 5), an inhibitor of CDKs 1, 2 and 4 failed in its initial Phase I study (Asghar, et al., 2015).

There are a number of other agents that display multi-targeted activity, including the inhibition of CDKs that are undergoing clinical investigation. PHA-848125 (milciclib, **13**; Figure 4) is a dual tropomyosin receptor kinase A and CDK2 inhibitor, with 4 to 10-fold selectivity versus other CDKs and receptor tyrosine kinases (Albanese, et al., 2010; Brasca, et al., 2009). Given the multitargeted nature of the compound, broad-spectrum antitumor activity was observed across a ~200 cell line panel. The compound was assessed in a Phase I trial with 2 of 37 patients experiencing a partial responses, whom both had thymic carcinomas, a disease associated with loss of CIP/KIP family proteins and possibly TRK activation (Weiss, et al., 2012) and a Phase II investigation in thymic carcinoma patients is ongoing. BAY-1000394 (roniciclib, **14**; Figure 4) is a low nM inhibitor of CDKs 1-5, 7, and 9 and shows sub-100 nM activity against 16 other kinases (Siemeister, et al., 2012). Broad activity in human cancer cell line panels was observed, with no cell line exhibiting a GI_{50} of more than 100nM. (Siemeister, et al., 2012). Clinical investigation in solid tumor populations showed signs of activity (10 of 25 patients had stable disease) with acceptable tolerability (Bahleda, et al., 2012). TG02 (**15**; Figure 4) is the most advanced compound to emerge from optimization of a macrocyclic structure (William, et al., 2012), and is a low nM inhibitor of CDK1, 2, 3, 5, 7 and 9 that inhibits a broad range of other kinases, including FLT3 and JAK2 and members of the SRC family (Goh, et al., 2012). As observed above, such multitargeted behavior also translates to broad antiproliferative activity in cell line panels and this is also true for TG02. Results of the Phase I trials of this compound are yet to be reported.

Overall, the clearest indication of clinical activity of CDK1/2/9 inhibitors has been in hematological malignancies. Preclinical data also supports the case for further clinical investigation into the effectiveness of CDK1/2/9 inhibitors in *MYCN*-driven or *CDK2* over-expressing neuroblastoma. Rational patient selection strategies should help to elucidate the critical targets of these multitarget inhibitors and inform attempts to define how best to further deploy these agents in the clinic.

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5.3 Selective CDK inhibitors

5.3.1 CDK4/6—Palbociclib (PD0332991) (**16**; Figure 6) was reported in 2004 as a specific, low nM inhibitor of CDKs 4 and 6 and no appreciable activity against 36 additional kinases. It was shown to potently inhibit RB phosphorylation at Ser780, with an IC_{50} value of 63 nM in MDA-MB-435 cells (Rae, Creighton, Meck, Haddad, & Johnson, 2007) and inhibit the proliferation of human breast, colon, lung and leukemia cell lines, with GI_{50} values ranging from 40 to 170 nM. Consistent with its kinase selectivity profile, palbociclib shows no antiproliferative activity in *RB*-deleted cell lines where the requirement for CDK4/6 is bypassed. As expected for a compound that inhibits CDK4/6 activity, palbociclib treatment led to a profound G1 arrest and cytostasis, which raised concerns about the ability of this approach to successfully induce tumor regression. However, notably in mice bearing human breast and colorectal xenografts oral treatment with palbociclib induced tumor regressions, with confirmed inhibition of RB phosphorylation in tumor lysates (Fry, et al., 2004). Subsequent studies of palbociclib focused on hematological cancers such as multiple myeloma and mantle cell lymphoma, in which cyclin D-dependent CDKs are deregulated (Baughn, et al., 2006; Marzec, et al., 2006). The most important advance came in 2009, where palbociclib was shown to display activity in luminal ER+ and *HER2* amplified breast cancer cell lines, including those with high levels of cyclin D1 and RB, and low levels of the CDK-regulator CDKN2A (R. S. Finn, et al., 2009). Importantly, this work established that the level of estrogen-induced cyclin D expression could be used to identify patients that may benefit from CDK4/6 inhibition. Correspondingly, the combined inhibition of estrogen-dependent signalling and CDK4/6 activity was shown to be particularly effective, with palbociclib and the antiestrogen tamoxifen showing synergy in breast cancer cell lines and palbociclib treatment able to overcome acquired resistance to tamoxifen (Finn, et al., 2009).

Studies of resistance to palbociclib have indicated that its efficacy can be limited by increased *CDK2* expression/activity and elevated *RBL1* expression (Dean, Thangavel, McClendon, Reed, & Knudsen, 2010). It has recently been disclosed that in response to palbociclib, CDK2 is able to bind cyclin D1 in ER+ breast cancer, mediate RB phosphorylation, promote S phase entry and enable adaptation to palbociclib. Furthermore, in a model of acquired resistance to palbociclib *CCNE1* amplification increased levels of CDK2/CCNE1, leading to sustained RB inactivation and resistance to CDK4/6 inhibition (Herrera-Abreu, et al., 2016). Screening of a drug library in palbociclib-resistant cells uncovered a role for PI3K inhibitors in preventing the emergence of palbociclib-resistant clones; blocking PI3K can suppress cyclin D1 expression, which in concert with CDK4/6 inhibition prevents resistance. Synergy between CDK4 and PI3K inhibitors has also been described in an independent study (Vora, et al., 2014). Finally, CDK4 inhibition has been shown to be a promising therapeutic strategy in preclinical models of RAS-driven melanoma, NSCLC and colorectal cancer (Kwong, et al., 2012; Lee, et al., 2016; Puyol, et al., 2010). These pharmacological studies show how the G1/S checkpoint can be compromised in oncogene-driven cancers to drive resistance, indicating new opportunities for therapeutic intervention.

As with the pre-clinical studies, the clinical development of palbociclib has been protracted. The first Phase I trial in RB+ tumors reported good tolerability with 1 testicular cancer

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patient achieving a partial response and 9 exhibiting stable disease (Schwartz, et al., 2011). Similar responses were observed in a second Phase I trial where, the only significant toxicity reported was neutropenia (Flaherty, et al., 2012). Pharmacokinetic/pharmacodynamic analysis of these trials assessed absolute neutrophil counts and platelet levels, which were shown to decrease with increasing exposure, consistent with the effects of CDK inhibition in rapidly proliferating cells. Direct target engagement biomarkers such as RB phosphorylation were not reported. Palbociclib was subsequently assessed in a Phase II trial for CDK4-amplified lip sarcoma and was deemed beneficial (Dickson, et al., 2013). Significantly, in a key Phase II trial of palbociclib (PALOMA-1) carried out in postmenopausal women with HER2-, ER+ breast cancer, the addition of palbociclib to treatment with the anti-estrogen drug letrozole nearly doubled progression free survival (PFS) (R. S. Finn, et al., 2015; Richard S. Finn, et al., 2014). Surprisingly, a second cohort of patients with *CCND1* amplification did not have a better response to the combined treatment than patients selected only on the basis of having ER+/HER2- tumors. This implies that ER status is a primary driver of the palbociclib response, and other lesions in the RB pathway (barring RB loss) do not enhance the therapeutic response and could potentially dampen it. A Phase III trial in patients with ER+/HER2- advanced breast cancer confirmed the Phase II data, with a median PFS of 24.8 months in the palbociclib plus letrozole cohort versus 14.5 months in the letrozole only cohort. The drug was generally well tolerated with grade 3 neutropenia the most common adverse event (R.S. Finn, et al., 2016). Palbociclib was also tested with an alternative hormonal therapy, fulvestrant, in ER+/HER- metastatic breast cancer that had progressed on prior endocrine therapy. Again palbocicilib improved outcome, with a median PFS of 9.5 months in the palbociclib plus fulvestrant group versus 4.6 months in the fulvestrant plus placebo group, with neutropenia again reported to be associated with palbociclib treatment (Cristofanilli, et al., 2016; Harbeck, et al., 2016; Turner, et al., 2015; Verma, et al., 2016). Intrinsic or acquired resistance to endocrine therapy is a near universal feature of ER+ breast cancer, including mutations in the *ESR1* gene (that encodes the ER). These mutations are enriched in patients following aromatase inhibitor therapy, hence it was important understand how this affects the response to palbociclib. Notably, the combination of palbociclib and fulvestrant demonstrated comparable activity in patients with either mutant or wildtype *ESR1* with median PFS of 9.4 versus 9.5 months respectively (Fribbens, et al., 2016). Palbociclib is now approved in combination with letrozole for the treatment of postmenopausal women with ER+/HER2- advanced breast cancer as an initial endocrine therapy and in combination with fulvestrant in ER+/HER2- patients showing disease progression following endocrine therapy.

Abemaciclib (LY2835219) (**17**; Figure 6) is a selective inhibitor of CDK4 and CDK6, with reduced potency against CDKs 1, 2, 7 and 9. Abemaciclib treatment inhibits RB phosphorylation, with IC_{50} values ranging from 60 to 120 nM, and induces a G1 arrest at similar concentrations. Notably, despite being relatively potent against CDK9 *in vitro*, abemaciclib was only able to inhibit the phosphorylation of RNA polymerase II at significantly higher concentrations of 3.5 μM, suggesting it may not be a relevant target in more complex systems. Treatment of mice bearing COLO205 colorectal xenografts with daily abemaciclib inhibited RB phosphorylation, gave maximal inhibition at 24 h post-dosing, and led to a decrease in the abundance of topoisomerase IIa and phospho-histone

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H3, markers of S and M phases of the cell cycle respectively. Treatment was well tolerated and tumor xenograft growth was significantly inhibited (Gelbert, et al., 2014).

Blocking CDK4/6 activity has been shown to overcome resistance to BRAF inhibition in melanoma, frequently through MAPK pathway reactivation and the expression of cyclin D1. BRAF inhibitor (vemurafenib)-resistant cells are highly sensitised to abemaciclib: treatment with abemaciclib results in a profound induction of apoptosis in vemurafenib-resistant cells, but only G1 arrest in parental cells. This is indicative of a greater dependency on cyclin D-associated CDK4/6 activity in vemurafenib-resistant cells (Yadav, et al., 2014).

Abemaciclib has been investigated in a Phase I dose escalation trial in patients with NSCLC, glioblastoma, breast cancer, melanoma or colorectal cancer. The DLTs were fatigue plus gastrointestinal, renal and hematological events, which occurred early and were reversible. Toxicity was milder than observed with palbociclib or ribociclib and in patients with stable disease or a measurable response; a significant biomarker response was detected for RB S780 phosphorylation inhibition and topoisomerase IIa abundance in skin biopsies (Patnaik, et al., 2016). The value of pharmacodynamics biomarkers was highlighted when serial monitoring of RB phosphorylation in keratinocytes following dosing enabled selection of a schedule that gave a more sustained pharmcodynamic inhibition between doses. Similar responses were reportedly observed in tumor biopsies. In particular, inhibition of RB phosphorylation by 60% or greater was able to predict for disease control (Patnaik, et al., 2016). The evaluation of abemaciclib in advanced ER+/HER2- breast cancer was performed in an expansion cohort of the Phase I trial, and in these heavily pretreated patients the disease control rate was 72%. Abemaciclib activity was also assessed in pretreated NSCLC patients, including patients bearing tumors with *KRAS* mutations. A disease control rate of 49% was achieved in these patients – 55% in the *KRAS*-mutant patients and 39% in the *KRAS* wildtype patients (Patnaik, et al., 2016). This is in agreement with data from preclinical studies that indicated a synthetic lethal interaction between *CDK4* loss and *KRAS*-mutation in a mouse model of NSCLC (Puyol, et al., 2010). Abemaciclib has shown clinical activity in other tumor-specific cohorts (ovarian cancer, glioblastoma, melanoma and colorectal cancer) and preliminary combination studies with hormonal therapy have reported activity in a small cohort of ER+ metastatic breast cancer patients treated with abemaciclib and fulvestrant, with no discontinuations due to toxicity (Amita Patnaik, 2014). Abemaciclib received breakthrough therapy designation from the FDA in 2015 and 28 clinical trials of this agents are currently underway (www.clinicaltrials.gov). These include combinations with tamoxifen or other targeted agents such as everolimus or trastuzumab, in metastatic breast cancer, *KRAS* mutant non-small cell lung cancer, various advanced cancers including melanoma and colorectal cancer and in dedifferentiated liposarcomas.

Ribociclib (LEE011) (**18**; Figure 6) is a selective inhibitor of CDK4/6 that lacks significant activity against CDK1 and CDK2, but, to our knowledge activity against other CDKs has not been reported. Initial work examined the activity of ribociclib in liposarcoma cells, because of the strong expression of *CDK4* and resulting inactivation of RB. Ribociclib treatment of liposarcoma cells inhibited proliferation, with IC_{50} values ranging from 130 to 240 nM, and produced a robust G0/G1 arrest within 24 h (Y. X. Zhang, et al., 2014). Loss of RB, via siRNA knockdown, reduced the sensitivity of cells to ribociclib. Three doses of ribociclib

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were sufficient to decrease RB phosphorylation in human liposarcoma, CDK4 amplified LP6 xenografts; while longer continuous treatment with ribociclib significantly reduced tumor growth (Y. X. Zhang, et al., 2014). Human primary liposarcoma xenografts displayed greater sensitivity to ribociclib than secondary tumors, showing near-complete inhibition of tumor growth and durable regressions. Interestingly, chronic exposure to ribociclib was associated with the restoration of RB phosphorylation, likely driven by re-expression of D-type cyclins, although cells retained sensitivity to ribociclib when rechallenged with the compound following a washout protocol (Y. X. Zhang, et al., 2014). Ribociclib has also shown good activity in cellular models of neuroblastoma. *CDK4*, *CDK6* and *CCND1* are all amplified or overexpressed in neuroblastoma cell lines, with significantly greater levels of RB phosphorylation observed in *MYCN* amplified cell lines. Knockdown of CDK4/6 by siRNA achieved greater antiproliferative effects in these *MYCN* amplified cell lines and was phenocopied by ribociclib treatment. The FOXM1 transcription factor has recently been described as a substrate of CDK4/6, where phosphorylation stabilises FOXM1 promoting expression of G1/S phase genes and limiting the accumulation of reactive oxygen species. Ribociclib-treated cells had elevated staining of the senescence marker SA-β-galactosidase, consistent with CDK4/6 inhibition causing a loss of FOXM1 activity. Xenograft models of human neuroblastoma were sensitive to ribociclib, commensurate with a loss of RB phosphorylation (Rader, et al., 2013). A large-scale screen of patient-derived xenografts has boosted optimism about more widespread clinical utility of CDK4/6 inhibition; ribociclib demonstrated synergy in combination with a number of agents (Gao, et al., 2015).

In a Phase I dose escalation trial of ribociclib in 132 patients with RB+ advanced solid tumors or lymphomas 3 patients had a partial response and 43 patients had stable disease (Infante, et al., 2016). DLTs were neutropenia and thrombocytopenia and common adverse events were neutropenia, leukopenia, fatigue and nausea and at higher doses cardiac QT prolongation was also observed. RB phosphorylation was reduced in skin biopsies but levels in tumor biopsies were inconsistent and not dose-dependent, potentially due to the labile nature of phospho-epitopes in tissue samples. Interestingly, *CCND1* amplification was associated with longer treatments, whereas *CDKN2A*/*2B* loss was observed in patients with shorter on-treatment times (Infante, et al., 2016). Ribociclib received FDA breakthrough therapy designation in 2016 and currently there are 42 clinical trials involving ribociclib underway (www.clinicaltrials.gov). Studies include a Phase II trial in patients with advanced liposarcoma, combination studies with letrozole in patients with HR+/HER2- advanced breast cancer and combination studies with BRAF, MEK or PI3K inhibitors.

Overall, the experience gained from the development of CDK4/6 inhibitors described has emphasised the importance of defining pharmacokinetic/pharmacodynamic relationships in early phase clinical trials for optimising dosing schedules and also patient selection biomarkers that can find potential for widening the clinical scope of these agents through combination, extending their use to other cancers. Clinic resistance to therapy is a common feature of all protein kinase inhibitors and strategies to prevent or overcome this will almost certainly be required for CDK4/6 inhibitors as resistant mechanisms are revealed. Early identification of resistance mechanisms or biomarkers is critical and *in vitro* and *in vivo* studies are beginning to elucidate the molecular mechanisms that may drive resistance to this class of inhibitor.

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5.3.2 CDK7—A number of compounds have shown promise as selective inhibitors of CDK7. These include LDC3140 (**19**) and LDC4297 (**20**; Figure 7), which when applied to tumor cells at low concentrations cause the rapid clearance of paused RNA polymerase II, alter gene expression and induce tumor cell death. At higher concentrations these inhibitors reduce phosphorylation of the RNA polymerase CTD at Ser5 and Ser7 (Kelso, et al., 2014). The recently developed CDK7 inhibitors, THZ1 and THZ2 (**21** and **22**; Figure 7), have also proved valuable tool compounds for exploring CDK7 function (Kwiatkowski, et al., 2014). The specificity of these inhibitors derives from their ability to interact with a conserved cysteine residue outside the catalytic domain of CDK7, which is absent in other CDKs. Treatment inhibits the phosphorylation of the RNA polymerase CTD at Ser2, Ser5 and Ser7 and has antitumor activity in multiple tumor types, including aggressive and heterogeneous cancers, such as neuroblastoma, small cell lung cancer and triple-negative breast cancer, with poorly defined oncogenic driver mutations (Chipumuro, et al., 2014; Christensen, et al., 2014; Kwiatkowski, et al., 2014; Y. Wang, et al., 2015). In general, these tumors had an amplification of one of the *MYC* family members, neuroendocrine lineage-specific factors and/or high levels of transcription of genes that promote an oncogenic phenotype. Interestingly, these regions of high transcription have clusters of enhancers, known as super-enhancers, suggesting that the responsiveness of cells to these is due to the sensitivity of super-enhancers to CDK7 inhibition.

THZ1 has also proved a valuable tool to explore the role and requirement for CDK7 during preinitiation. Treatment of nuclear extracts, and eventually cells, with THZ1 revealed the critical role of CDK7 in phosphorylating CTD Ser5 and Ser7 leading to an exchange of general transcription factors and recruitment of DSIF, NELF and the human capping enzyme (HCE) that generates the m^7G cap on the 5’ of mRNAs (Nilson, et al., 2015). The m^7G cap is subsequently required for efficient mRNA translation via cap-dependent translation. Inhibition of CDK7 with THZ1 resulted in defective CTD phosphorylation, co-transcriptional capping, promoter proximal pausing due to a block of DSF and NELF loading and finally the pTEFb-mediated transition into transcriptional elongation.

The findings in antitumor experiments described above were counter to the concern that basic processes such as transcription would make poor targets for therapeutics as they would not select for cancer cells and would have a poor therapeutic window. It is not clear why normal cells are insensitive, or at the very least less sensitive, to CDK7 inhibition. One explanation is that normal cells do not require the high levels of transcription driven by super-enhancers, or that in normal cells phosphorylation of the CTD at Ser5 can be independent of CDK7. In contrast, the tumor cells are dependent on or addicted to CDK7 for their survival. The identification of so-called ‘Achilles cluster’ of super-enhancer-regulated and CDK7-dependent genes, which are required for cancer cell survival, is consistent with this idea (Y. Wang et al., 2015). Although well tolerated in the mouse xenograft studies one concern is that long-term exposure to CDK7 will deplete pluripotent cells as observed in the CDK7 knockout mice (Ganuza, et al., 2012; Rossi, et al., 2001). This may not be due to the transcriptional effects following CDK7 inhibition, but instead as a result of the CAK activity of CDK7 required to maintain the activity of other CDKs including CDK1, 2 and 9 that may be required by pluripotent cells (Ganuza, et al., 2012; Rossi, et al., 2001). However, potential

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toxicological effects will only be revealed in dedicated tolerability studies carried out in higher animal species.

5.3.3 CDK9—Recently, CDK9 has emerged as a potential target for cancer therapeutics, as CDK9 regulates the transcription of genes encoding short-lived antiapoptotic proteins, such as MCL1 and XIAP, which are critical for the survival of transformed cells. Attempts to identify selective CDK9 inhibitors by screening focused chemical libraries containing diverse scaffold, have had mixed success (Sonawane, et al., 2016).

Wogonin (**23**; Figure 7), an active flavone from the herb *Scutellaria balcalensis*, induces apoptosis in a number of cell lines and has antitumor activity in xenograft models. Wogonin and structurally related flavones are similar in structure to flavopiridol, and block the CDK9-mediated phosphorylation of the RNA polymerase II CTD at Ser2, inducing apoptosis in leukemic T-cells (Polier, et al., 2011). This induction of apoptosis is associated with a decrease in *MCL1* expression, consistent with CDK9 inhibition. Pull-down and *in silico* docking studies have demonstrated that wogonin binds to CDK9, but does not appear to inhibit CDKs 2, 4 or 6 in cells, as assessed by measurement of RB phosphorylation, at concentrations that inhibit CDK9 activity.

In separate work, a cellular screening strategy, using high content microscopy to determine effects on mitotic index and p53 induction, has been used to identify compounds that could inhibit transcription-regulating CDKs such as CDK7 and CDK9 (S. Wang, et al., 2010). This approach led to the identification of a range of inhibitors with different selectivity profiles. Subsequent optimization work resulted in the development of a CDK7/9 transcriptional inhibitor designated compound 14 in the publication (**24**; Figure 7). Treatment with his compound inhibited phosphorylation of the RNA polymerase II CTD at Ser2, repressed the expression of *MCL1*, induced apoptosis in several cancer cell lines and had anticancer activity in animal models. Subsequent work identified closely related compounds CDKI-71 and CDKI-73 (**25**; Figure 7) from a novel class of 5-substituted-4-(thiazol-5-yl)-2-(phenyl amino)pyrimidines (Shao, et al., 2013). Comparing the mechanism of action of CDKI-71 and alvodicib in human cancer cell lines, primary patient leukemia cells, B- & T-cells and embryonic lung fibroblasts, showed that both compounds were potently cytotoxic and induced caspase-dependent apoptosis (X. Liu, et al., 2012). Significantly, the CDK9-selective CDKI-71, but not the unselective alvocidib, preferentially affected cancer cells.

Chronic lymphocytic leukemia (CLL) is associated with the overexpression of genes encoding the BCL2 family of anti-apoptotic proteins. Inhibiting CDK9 expression is known to induce apoptosis in CLL cells and to increase sensitivity to fludarabine, a purine nucleoside analogue used as the standard of care for CLL (Walsby, et al., 2014). Treating primary human leukemia cells with CDKI-73 has been shown to lead to the dephosphorylation of CDK9, the dephosphorylation of the RNA polymerase II CTD at Ser2 and to induce caspase-dependent apoptosis. CDKI-73 was more potent than the pan-CDK inhibitor alvodicib, showing selectivity for primary leukemia cells over normal CD34+ cells and was synergistic with fludarabine (Walsby, et al., 2014).

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LDC000067 (**26**; Figure 7) is another phenylamino pyrimidine inhibitor selective for CDK9, with similar cellular activity to the derivative **25** described above (Albert, et al., 2014). Treatment with LDC000067 has been shown to increase the pausing of RNA polymerase II and to lead to a selective reduction in short-lived mRNAs, including those encoding regulators of proliferation and apoptosis. Phosphorous-containing analogues of the phenylamino pyrimidines with high selectivity for CDK9 have also been described, such as compound 93 (**27**; Figure 7) (Nemeth, et al., 2014). Overall, the data suggests there is scope to develop selective CDK9 inhibitors such as CDKI-73 or LCD000067 as anticancer therapeutics.

5.3.4 CDK8/19—The potential role of *CDK8* as an oncogene in colorectal cancer has raised interest in the development of CDK8 inhibitors (Rzymski, Mikula, Wiklik, & Brzozka, 2015). The CDK8 and CDK19 proteins have >90% similarity over the first 370 residues, which encompass the active site, but differ substantially toward the C-terminus in areas that may contribute to their non-redundant functions. This suggests that the active site of those two proteins is very similar and compound selectivity will be a challenge. Two studies have examined the ability of a range of compounds to bind a panel of human protein kinases and found a number of potential inhibitors of CDK8 that were selective over other CDKs (Davis, et al., 2011; Karaman, et al., 2008). These include BMS-387032/SNS-032, CP-724714, EXEL-2880/GSK-1363089, flavopiridol, PLX-4720, staurosporine and the type II inhibitors AST-487, BIRB-796, linifinib and sorafenib (**28**; Figure 8). Solving the crystal structure of CDK8/cyclin C subsequently revealed a unique helical recognition domain in cyclin c revealed a recognition helix, uniquely found in cyclin C, required for specific and tight binding between CDK8 and cyclin C. This work also revealed that sorafenib, a Type II inhibitor of CDK8, binds CDKs with the activation loop DF/MG-motif in an “out” conformation (Figure 9). This was the first example of a ligand binding to a CDK using this type of interaction (Schneider, et al., 2011).

Cortistatin A (**29**; Figure 8), a steroidal alkaloid isolated from the marine sponge *Corticium simplex*, has impressive antiproliferative activity in human umbilical vein endothelial cells compared to normal human dermal fibroblasts. This identified it as an attractive lead for drug discovery. Screening cortistatin A against a panel of 405 protein kinases identified a small number of kinases that it binds with high affinity, including ROCK (K_d = 220-250 nM, CDK8 (K_d = 17 nM) and CDK19 (K_d = 10 nM) (Cee, Chen, Lee, & Nicolaou, 2009). A recent follow-up study found that cortistatin A had anti-leukemic activity *in vitro* and *in vivo*, inhibited CDK8/19 activity and induced the expression of super-enhancer-associated genes in sensitive cell lines (Pelish, et al., 2015). Another screen, for inhibitors of p21-activated transcription, identified a group of compounds with a 4-aminoquinazoline scaffold as CDK8/19 inhibitors. Optimisation work yielded Senexin A (**30**; Figure 8), a CDK8 (K_d = 830 nM) and CDK19 (K_d = 310 nM) ligand that could inhibit beta-catenin dependent transcription, induce *EGR1* mRNA and increase the efficacy of chemotherapy against human lung carcinoma xenografts (Porter, et al., 2012). A patent (WO2013116786A1) has subsequently reported a second compound, Senexin B (**31**; Figure 8), with improved solubility and potency (CDK8 K_d = 140 nM, CDK19 K_d = 80 nM). This compound inhibits p21-activated transcription and oncogenic beta-catenin activity, as described for Senexin A

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(Rzymski, et al., 2015). Additional compound series have been identified and described in the patent literature by Selvita (WO2014072435), Roche (WO2014029726, WO2014090692, WO2014106606, WO2014154723, and WO2015049325), Nimbus (WO2014194201) and CNIO (WO2013001310).

We have previously reported the discovery and optimization of a singleton 3,4,5-trisubstituted pyridine inhibitor of WNT signaling using a high-throughput cell-based reporter assay (Mallinger, et al., 2015). This led to the identification of CCT251545 (**32**; Figure 8), a potent small-molecule inhibitor of WNT signaling with good oral pharmacokinetics. A chemoproteomic approach identified CDK8 (IC_{50} = 5 nM) and CDK19 (IC_{50} = 6 nM) as the targets of CCT251545 with >100-fold selectivity for CDK8/19 over 293 other kinases (including CDKs 1-3, 5-7 and 9) (Dale, et al., 2015). CCT251545 is a potent inhibitor of $STAT1^{SER727}$ phosphorylation, a robust biomarker of CDK8/19 inhibition, but does not inhibit $E2F1^{SER375}$ phosphorylation or RNA polymerase CTD phosphorylation. Microarray gene expression profiling has shown that, as expected, the expression of genes regulated by CDK8/19-dependent pathways is altered in cells treated with CCT251545.

The X-ray co-crystal structure of CDK8/cyclin C revealed CCT251545 to have a Type I binding mode, which translates into potent activity in a cell-based binding assay for CDK8 and 19, and a corresponding inhibition of CDK8/19 associated-biomarkers (Figure 9). In contrast, three Type II binders (linifinib, ponatinib and sorafinib), with potent activity against CDK8 in biochemical enzyme assays, do not have cellular CETSA-binding or biomarker activity against CDK8. This disconnect between the data obtained from enzymatic and cell-based assays has been corroborated in a second study that used sorafenib as a starting point to develop a series of Type II inhibitors of CDK8 (**33**; Figure 8) (Bergeron, et al., 2016). Overall the data suggests that the *in vitro* biochemical potency of type II inhibitors do not translate in cell-based activity, perhaps due to the association of CDK8 with MED12 and MED13. Additional CDK8/19 inhibitors have been reported in the literature, including a series of 6-aza-benzothiophene containing compounds that were developed into potent selective Type I inhibitors of CDK8 (**34**; Figure 8) (Koehler, et al., 2016; Rzymski, et al., 2015).

After the discovery of CCT251545 follow-up work yielded a 3,4,5-trisubstituted-2-aminopyridine series exemplified by CCT251921 (**35**; Figure 8). This compound is a potent selective and orally bioavailable inhibitor of CDK8, with equal affinity for CDK19 and optimal biochemical, pharmacokinetic, and physicochemical properties (Mallinger, et al., 2016). Further series of inhibitors were identified using scaffold-hop or high-throughput screening approaches, leading to the discovery of 2,8-disubstituted-1,6-naphthyridine-, 4,6-disubstituted-isoquinoline-, benzylindazole or imidazo-thiadiazole-based dual CDK8/19 ligands (**36**; Figure 8) (Czodrowski, et al., 2016; Mallinger, et al., 2016). Multiple cycles of structure-based design improved the microsomal stability, potency and kinase selectivity of an initial imidazo-thiadiazole scaffold, replacing it with a 3-methyl-1H-pyrazolo[3,4-b]-pyridine. This led to the identification of MSC2530818 (**36**; Figure 8), a compound with excellent kinase selectivity, biochemical and cellular potency, microsomal stability and oral bioavailability. MSC2530818 modulates $STAT1^{SER727}$ phosphorylation and inhibits tumor

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growth in an *APC* mutant SW620 human colorectal carcinoma xenograft model after oral administration. The identification of CCT251921 and MSC2530818 has provided two chemically-distinct compounds with suitable potency and selectivity for progress into preclinical efficacy and safety studies.

With potent and selective exemplar compounds from two structurally differentiated chemical series, CCT251921 and MSC2530818, and corresponding inactive control compounds, it was possible for us to investigate the therapeutic potential of dual CDK8/19 modulation. We found evidence of super-enhancer activation following CDK8/19 inhibition in xenograft models and also that systemic and xenograft AML tumor models were particularly sensitive to treatment with these compounds, as described by Pelish and colleagues (Clarke, et al., 2016; Pelish, et al., 2015). In an *in vivo* model of an oncogenically-activated stem cell compartment we found our CDK8/19 inhibitors altered the proportion of stem cells to proliferative TA cells that may in part be due to super-enhancer activation. We also detected inhibitory effects on bone progenitor stem cells and on immune and inflammatory models *in vitro* (Clarke, et al., 2016). Importantly, we found that these CDK8/19 inhibitors had a complex toxicological profile, making it impossible to identify a clear therapeutic window for biomarker inhibition and antitumor activity (Clarke, et al., 2016). Further clinical development of these compounds has been ruled out because of their major pleiotropic toxicity, suggesting others should be cautious when considering the clinical applicability of CDK8/19 inhibitors.

5.3.5 CDK12/13—CDK12 and 13 have a key role in the regulation of gene expression; however, in the absence of CDK12/13 tool compounds, obtaining data on the role of CDK12/13 in cancerous and normal cells has involved a genetic knockout approach. Recently, THZ531, a first-in-class selective inhibitor of CDK12/13 was described (T. Zhang, et al., 2016). This compound is a covalent inhibitor, which irreversibly binds to a cysteine located outside the kinase domain of CDK12. Treatment with THZ531 led to a broad loss of gene expression, associated with reduced transcriptional elongation and a loss of RNA polymerase II CTD phosphorylation. Genes encoding proteins involved in the DNA damage response and super-enhancer-associated genes were particularly affected. Importantly, treatment with THZ531 induced apoptosis in leukemic T cells, coincident with changes in gene expression. From a therapeutic standpoint, a compound such as THZ531 is an attractive prospect for clinical development, as targeting CDK12/13 may inhibit the aberrant transcription and genomic instability that are the hallmarks of cancer. As described earlier in this review, the synthetic lethal interactions identified for loss of CDK12 and sensitivity of human breast and ovarian cancers to tamoxifen or PARP inhibitors suggest potential stratified patient populations and combination regimes for CDK12/13 inhibitors (Bajrami, et al., 2014; Iorns, et al., 2008; P. M. Joshi, et al., 2014).

6 Conclusion and outlook

The CDKs are highly-validated targets for cancer therapeutics, because of their role in regulating critical cell cycle checkpoints and transcription. As kinases, CDKs are readily druggable and many CDK inhibitors have been described. The initially discovered ATP-competitive CDK inhibitors, such as alvocidib for example, have generally failed to progress

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beyond early clinical studies. Contributing factors were their poor CDK isoform selectivity, a lack of understanding of their precise mechanism of action and hence the absence of appropriate biomarkers. Particularly important was that this lack of knowledge led to a lack of patient selection biomarkers for use clinical studies, resulting in many trials being conducted in unstratified patient cohorts. Occasionally, activity in a particular tumor type has been observed in the clinic, but the underlying molecular mechanism, and hence the reason for the sensitivity of responding tumors, remains unknown. The lack of understanding of the mechanism of action of many early CDK inhibitors has also restricted their use in rational combinations with other targeted therapeutics. In addition, the inhibitors that target multiple CDKs frequently lack selectivity for cancer versus normal tissue and are not well tolerated at doses required for activity. Nevertheless, certain drugs with an appropriate mix of CDK selectivities – for example the orally-available, second generation inhibitor of cyclin dependent kinases (CDK) 2, 5 and 9 CYC065 derived from seleciclib – continue to be developed.

A real shift in attitude about the clinical use of CDK inhibitors has been the progression the CDK4/6-selective, ATP-competitive inhibitors palbociclib, abemaciclib and ribociclib – which are either approved or in advanced registration trials for several cancers. The discovery and clinical development of these drugs, and in particular, the FDA approval for palbociclib in ER+ breast cancer, has revived serious interest in inhibitors of the cell cycle CDKs. The identification and development of selective CDK4/6 inhibitors is a major breakthrough in the treatment of metastatic breast cancer and their activity in other cancer types will be defined by the outcomes of ongoing clinical trials. In addition, the identification of tolerated and active combination regimes, patient stratification biomarkers and resistance mechanisms will contribute to our greater understanding of their potential use. Furthermore, the utility of CDK4/6 inhibition in preventing the emergence of resistance to multiple targeted therapies across various cancer types is an area of intense clinical investigation, results of which are eagerly anticipated (www.clinicaltrials.gov) (Gao, et al., 2015). The selective CDK4/6 inhibitors are exemplars for inhibitors of other CDKs targets where the identification of highly selective compounds with pharmaceutical properties is critical. Strategies exploring alternative and non-competitive approaches to CDK inhibition – such as allosteric, covalent binding and peptidomimetic mechanisms – may uncover novel pharmacology and expand the therapeutic utility of these agents. For example, MMD37K (Sanchez-Martinez, et al., 2015), a peptidomimetic derived from p16, is the first alternative class of CDK4/6 inhibitor to enter clinical studies and the data generated will allow comparisons with existing ATP-competitive inhibitors.

Perhaps surprisingly, selective inhibitors transcriptional CDKs 7 and 9 have been found to exhibit exploitable therapeutic windows for tumors compared to normal tissue in preclinical mouse models. Covalently binding inhibitors of CDK7 have shown promise in driving increased selectivity and translation of these tool compounds into clinical candidates may be deeply instructive and provide further biological insight into the varied roles of CDKs. Highly potent CDK8/19 inhibitors have been discovered, but at least for two of the chemical series tested, a lack of tolerability and therapeutic window suggests great caution when considering their clinical development and also consideration of these as anti-targets to be avoided in other kinase inhibitors. Importantly, the availability of potent and selective tool

compounds for the transcriptional CDKs will allow further exploration of their function both in disease and normal tissue homeostasis.

Finally, the identification of predictive biomarkers of response will also allow the more recently discovered CDK inhibitors to be explored in particular genetically-defined contexts, for example by building on recent observations that *KRAS* mutant tumors are highly sensitive to CDK1 inhibition, and that CDK2 or CDK9 inhibition is synthetically lethal in MYC-addicted tumor cells (Costa-Cabral, et al., 2016; Poon, et al., 2016).

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Abbreviations

AML	acute myeloid leukemia
CAK	CDK-activating kinase
CDK	cyclin-dependent kinases
CLL	chronic lymphocytic leukemia
CT	RNA polymerase II C-terminal domain
DLT	dose-limiting toxicities
DSIF	DRB-sensitivity inducing factor
ER	estrogen Receptor
LRP	low-density receptor-related lipoproteins
MAPK	mitogen-activated protein kinase
RB	retinoblastoma protein
NELF	RNA polymerase II–associated negative elongation factor
PFS	progression free survival
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase

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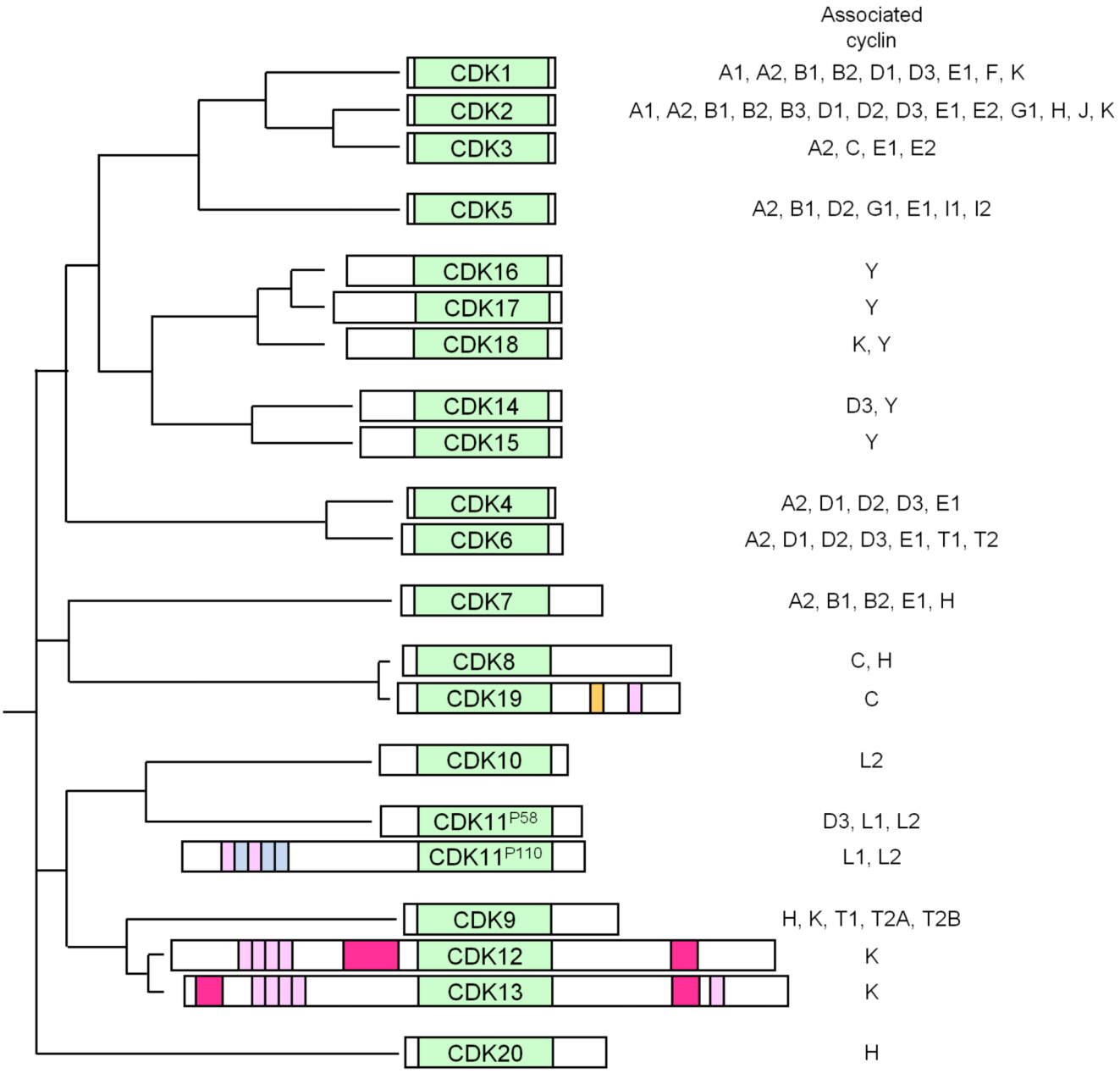

Figure 1. The evolutionary relationships between human CDK subfamilies determined by phylogenetic analysis based on gene sequence similarity.

Conserved domains are color-coded: green, kinase domain; pink, arginine/serine-rich domain; blue, glutamic acid-rich domain; yellow, glutamine-rich domain; red, proline-rich domain. CDK11 is encoded by two separate genes, *CDK11A* and *CDK11B*, which each encode two isoforms (adapted from (Malumbres, 2014). Cyclins required for CDK activation are also indicated.

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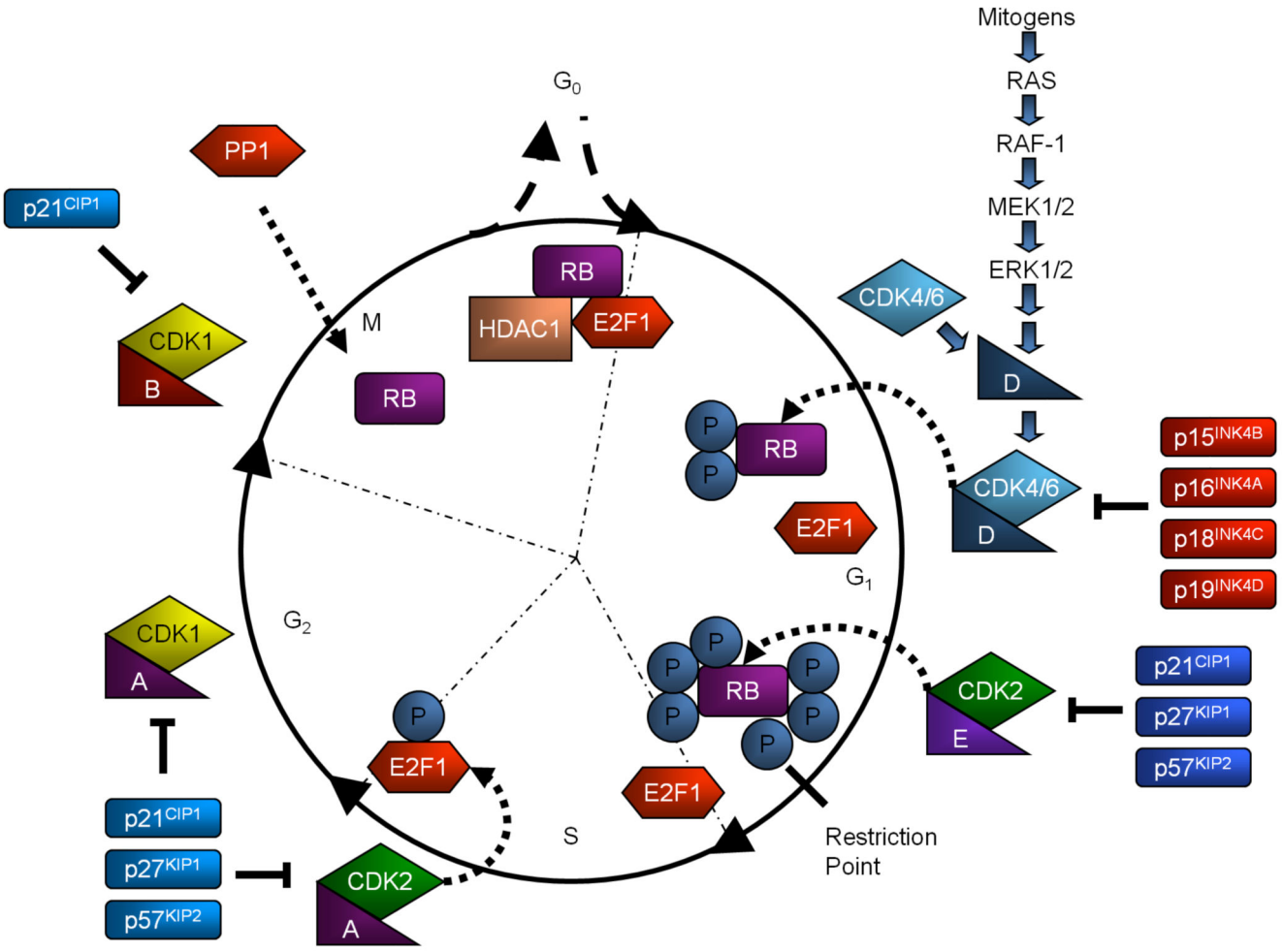

Figure 2. A simplified model of the mammalian cell cycle.

Mitogenic stimulation leads to the synthesis of D-type cyclins, activating CDK4/6 and ultimately CDK2. CDKs4/6 phosphorylate the RB protein (the dotted lines indicate phosphorylation or dephosphorylation), releasing histone deacetylase1 (HDAC), which relieves repression of the transcription factor E2F1. Cyclin E is transcribed, activating CDK2, enabling further phosphorylation of RB, allowing DNA synthesis to occur. S phase is terminated when CDK2/cyclin A phosphorylates E2F1, blocking its DNA-binding ability. CDK1/cyclin B activation triggers mitosis and RB is dephosphorylated by protein phosphatase 1 (PP1). The INK4 and CIP/KIP proteins that modulate CDK activity are also indicated. The CDKs are also regulated by two families of small inhibitory proteins, INK4 and CIP/KIP, which generally act by interfering with cyclin binding (Sherr & Roberts, 1999), for example, binding of $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$ and $p19^{INK4D}$ to CDK4 blocks the interaction with cyclin D.

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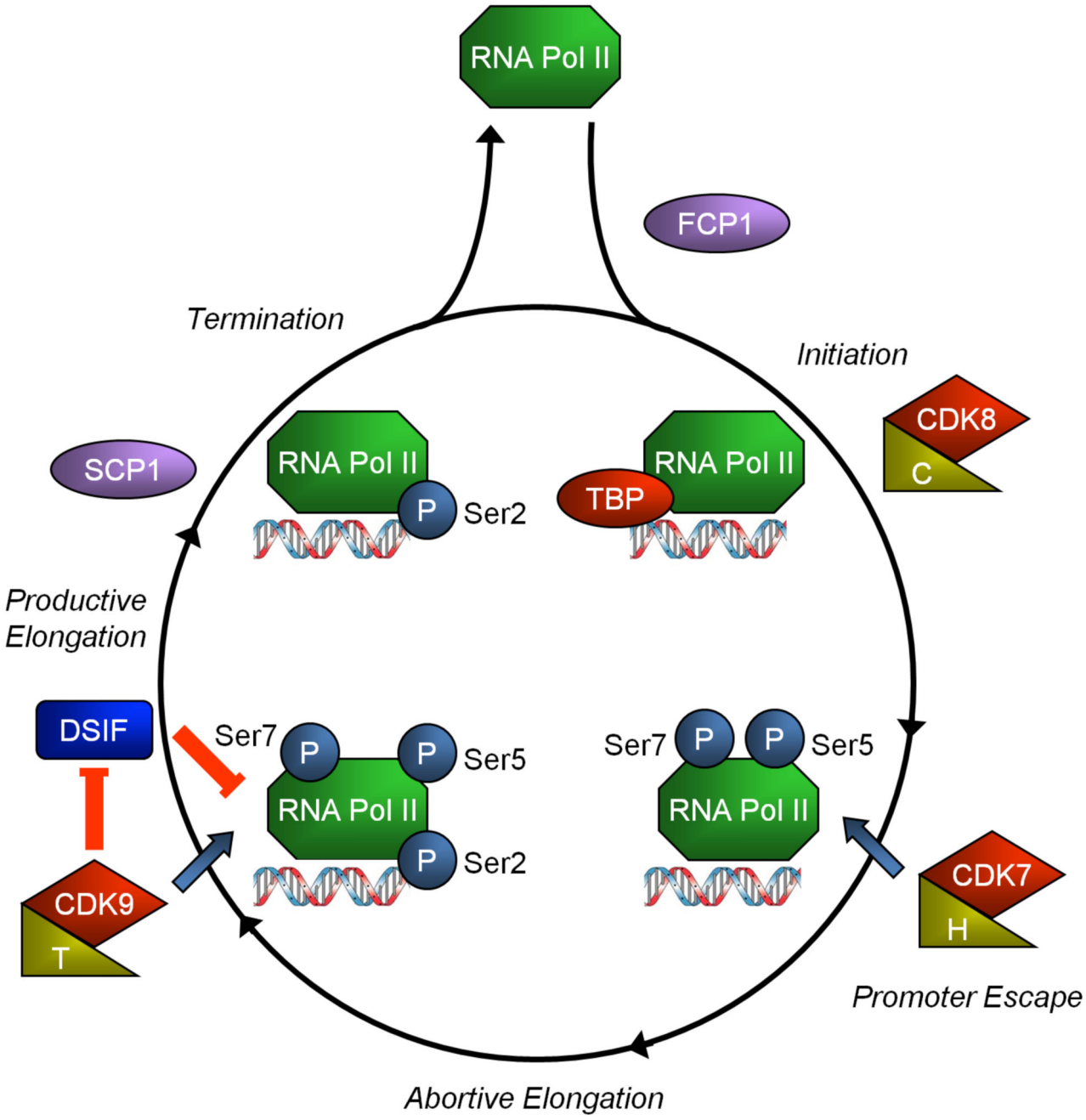

Figure 3. A simplified model of the transcriptional cycle of initiation, elongation and termination.

RNA polymerase II undergoes multiple rounds of phosphorylation and dephosphorylation in order to coordinate its activity and bring about the synthesis of mRNAs. CDK8 has positive and negative roles in regulating transcription through effects on specific transcription factors, super-enhancers and other transcriptional CDKs. CDK7 and CDK9 are involved in the elongation of mRNAs, while DSIF acts to block elongation and SCP1 promotes termination through dephosphorylation of Ser5 of RNA polymerase II.

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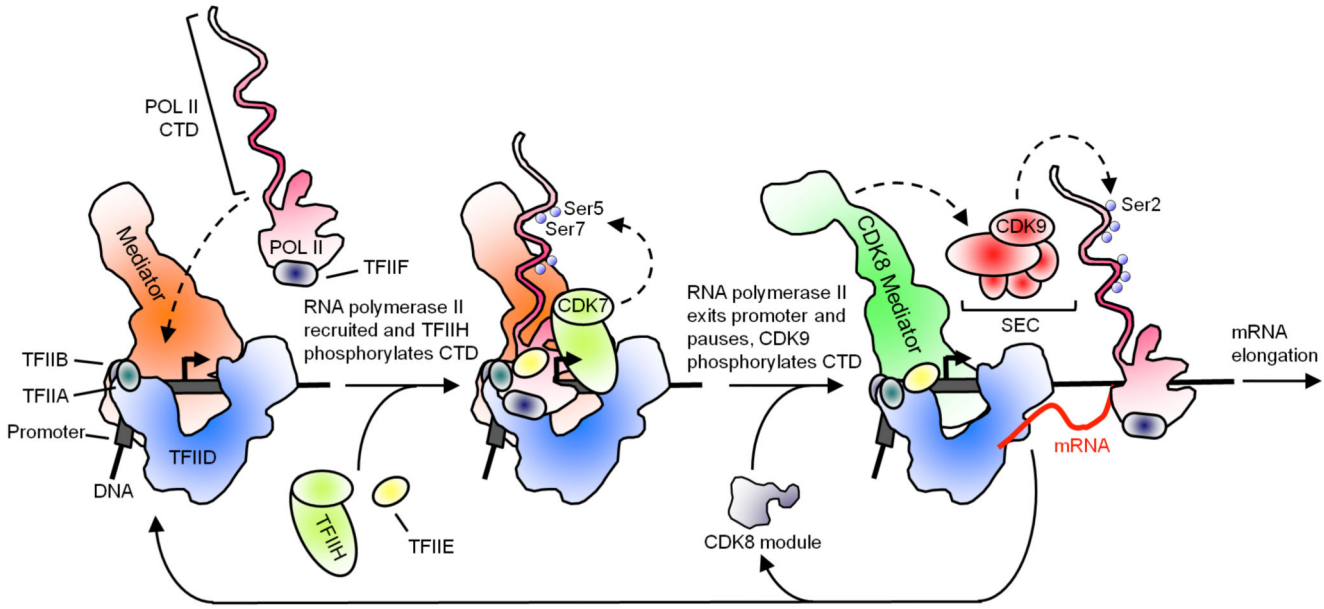
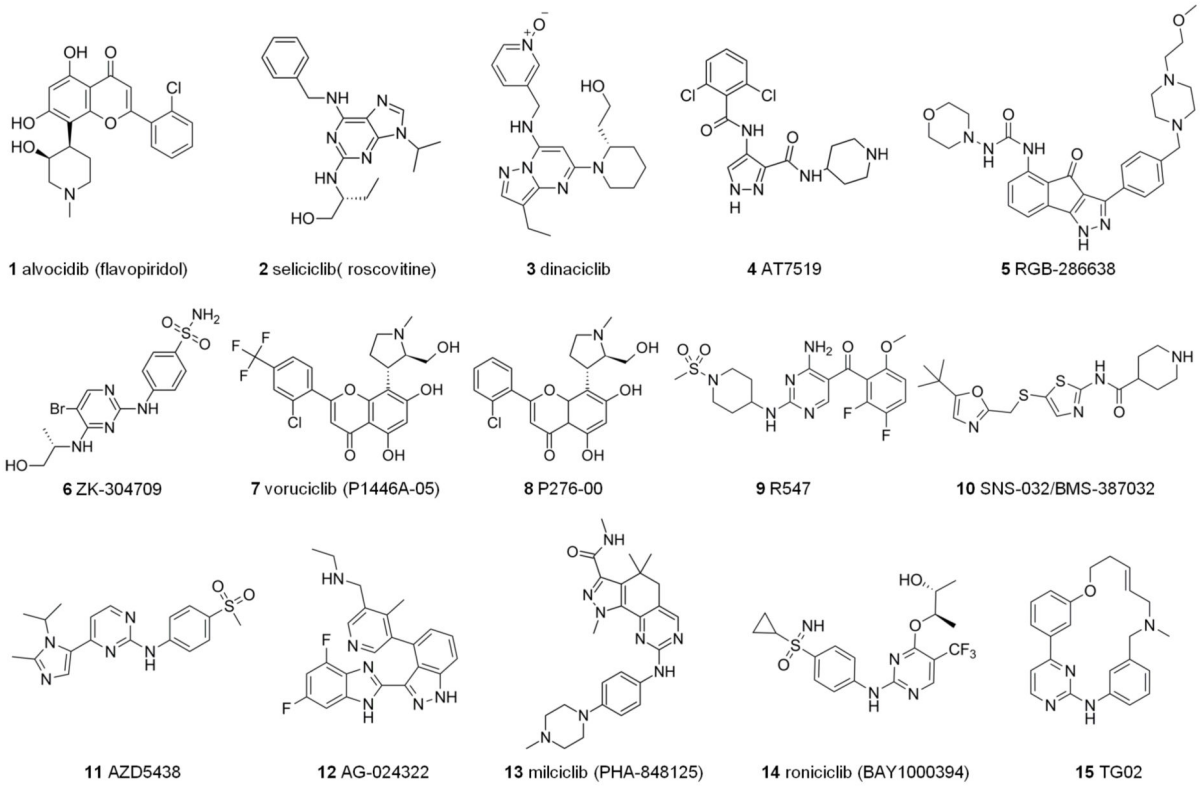


Figure 4. Simplified schematic of the role of the Mediator complex and CDK8 in the initiation of transcription.

The preinitiation complex forms following binding of the Mediator complex, TFIID and other general transcription factors in a step-wise manner that eventually recruits RNA polymerase II, and finally TFIIH, to the complex. The helicase activity of TFIIH opens the DNA to initiate transcription, and CDK7 activity contributes to promoter escape by breaking interactions with some factors through phosphorylation of RNA polymerase II CTD Ser5, and also Ser7. The RNA polymerase transcribes around 20-100 bases downstream of the promoter before pausing and in another regulatory process, following recruitment of the CDK8 kinase and CDK9 activation, phosphorylation of CTD Ser2 and other substrates, that loses the remaining components of the initiation complex, yielding a fully functional elongation complex (adapted from Allen & Taatjes, 2015).



CMPD	CDK1/CycB	CDK2/CycA (CDK2/CycE)	CDK4/CycD (CDK4/CycE)	CDK5/p25 (CDK5/p35)	CDK6/CycD	CDK7/CycH	CDK9/CycT
1	27	405 (282)	132		395	514	11
2	2100	100	(13500)	160	23500	540	950
3	3	1		1			4
4	190	44 (510)	67	(18)	660	2800	<100
5	480	38 (48)	925	(340)	>1000	62	4
6	2	(3)	4	(5)	55	44	1
7	50	(4)	61			85	5
8	79	224 (2543)	63		396	2870	20
9	2	(3)	1				
10	25		90				22
11	16	3 (10)	449	14	21	821	20
12	1-3	1-3	(1-3)				
13	398	45 (363)	160	(265)		150	
14	7	9	11	(<10)		25	5
15	9	5	>100	8	>100	3	37

Figure 5. Structure and activity of pan- or multitarget-CDK inhibitors.

Table indicates IC_{50} (nM) values for each compound, with the exception of compound 4 for which K_i (nM) values are given.

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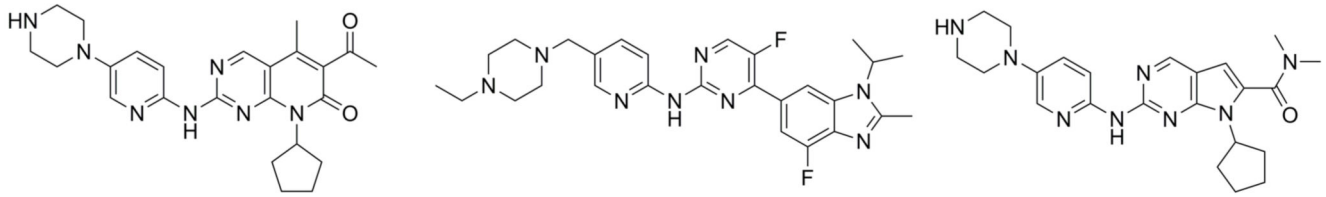

16 palbociclib **17** abemaciclib **18** ribociclib

CMPD	CDK1/CycB	CDK2/CycA (CDK2/CycE)	CDK4/CycD (CDK4/CycE)	CDK5/p25 (CDK5/p35)	CDK6/CycD	CDK7/CycH	CDK9/CycT
16	>10000	>10000	11	>10000	15		
17	1627	(504)	2		10	3910	57
18	>100000	>50000	10		39		

Figure 6. Structure and activity of selective CDK4/6 inhibitors.
Table indicates IC_{50} (nM) values.

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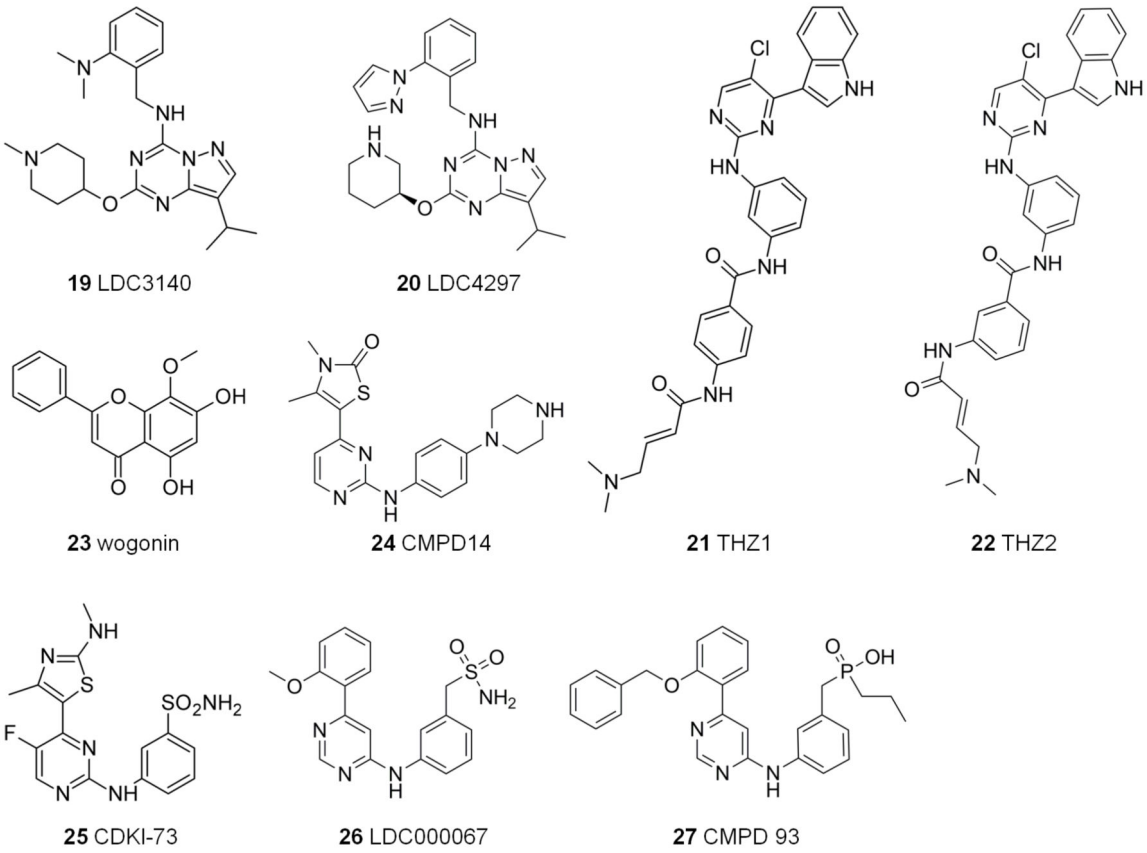

CMPD	CDK1/CycB	CDK2/CycA (CDK2/CycE)	CDK4/CycD (CDK4/CycE)	CDK5/p25 (CDK5/p35)	CDK6/CycD	CDK7/CycH	CDK9/CycT
19	>10000	3897	>10000		>10000	<5	7450
20	54	64	>10000		>10000	<5	1711
21						3.8	
22	97	222		134		14	194
23						190	12300
24	449	149	68			2	0.4
25	12	(4)			205	6	114
26	5513	2441	9242		>10000	>10000	44
27							142

Figure 7. Structure and activity of selective CDK7 and CDK9 inhibitors.
Table indicates IC_{50} (nM) values.

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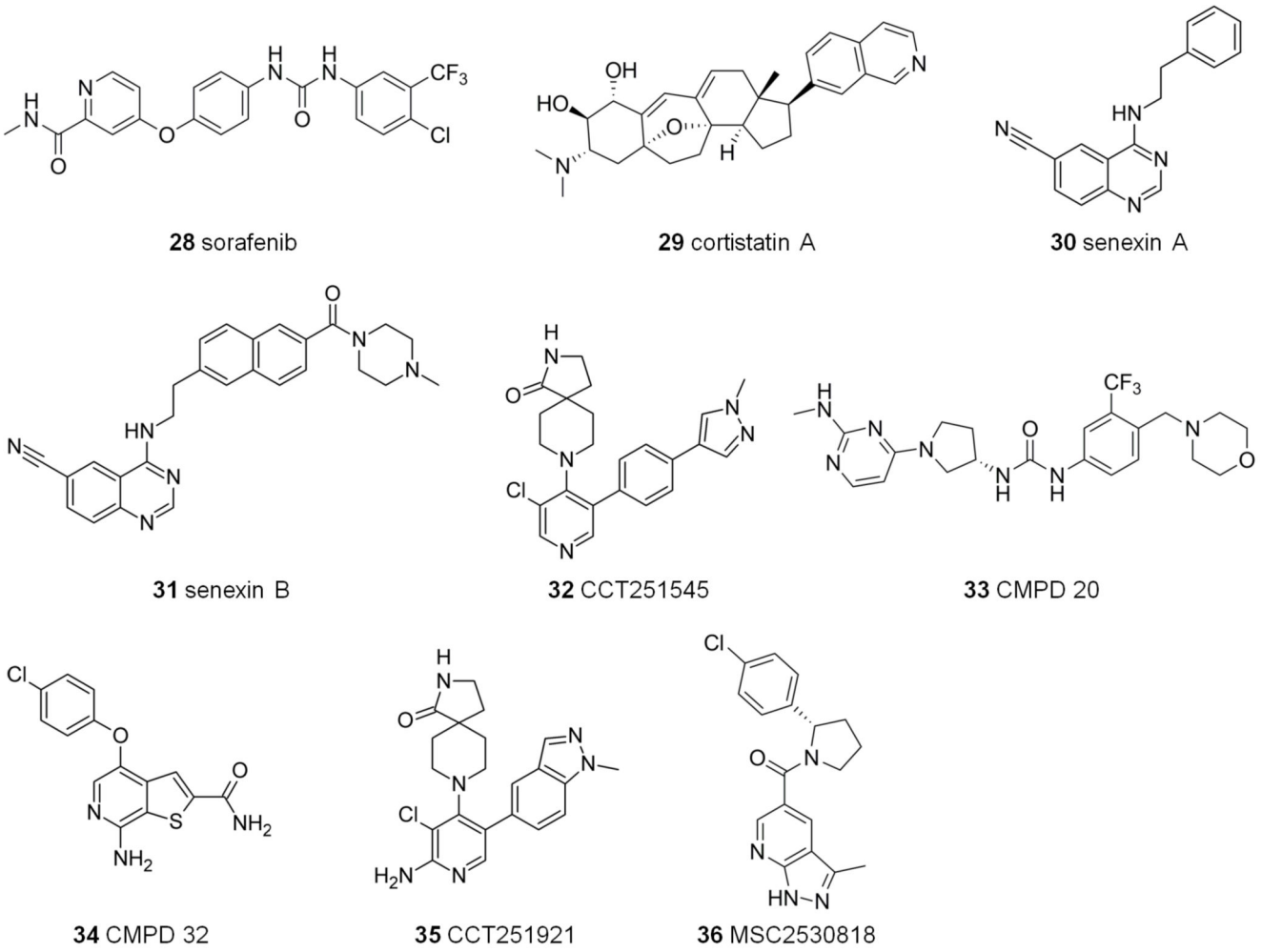

Figure 8. Structures of CDK8/19 inhibitors.

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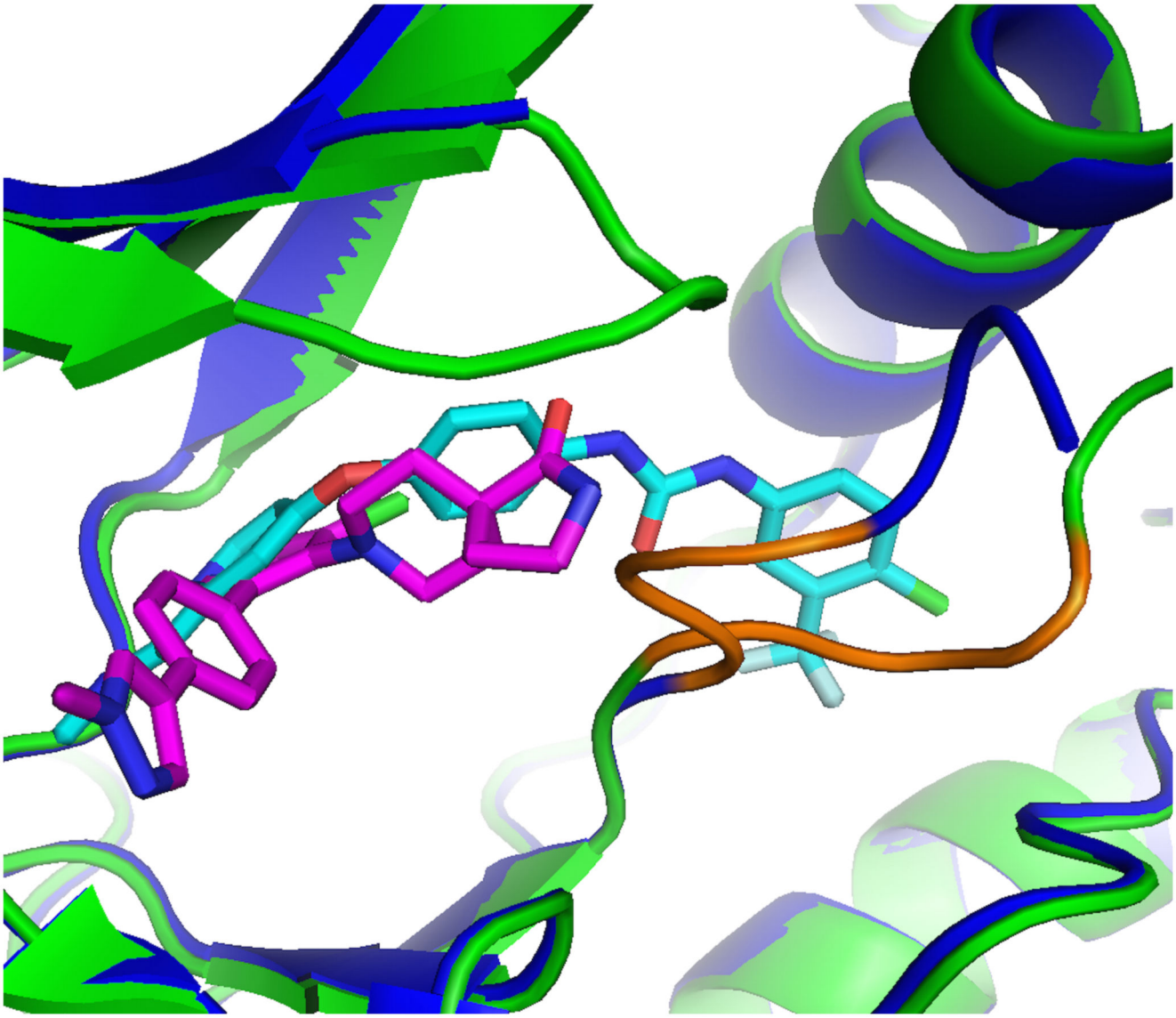

Figure 9. Type I and Type II inhibitor binding to CDK8/Cyclin C.

Diagram shows sorafenib (green structure, cyan compound) and CCT251545 (blue structure, magenta compound) bound to the CDK8/cyclin C complex. The DMG motif is shown in orange and is flipped “out” when bound to sorafenib and “in” when bound to CCT251545.