



Published in final edited form as:

Expert Rev Anticancer Ther. 2019 July ; 19(7): 569–587. doi:10.1080/14737140.2019.1615889.

Recent advances with cyclin-dependent kinase inhibitors: therapeutic agents for breast cancer and their role in immunoncology

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Abstract

Introduction: Collaborative interactions between several diverse biological processes govern the onset and progression of breast cancer. These processes include alterations in cellular metabolism, anti-tumor immune responses, DNA damage repair, proliferation, anti-apoptotic signals, autophagy, epithelial-mesenchymal transition, components of the non-coding genome or onco-miRs, cancer stem cells and cellular invasiveness. The last two decades have revealed that each of these processes are also directly regulated by a component of the cell cycle apparatus, cyclin D1.

Area covered: The current review is provided to update recent developments in the clinical application of cyclin/CDK inhibitors to breast cancer with a focus on the anti-tumor immune response.

Expert opinion: The *cyclin D1* gene encodes the regulatory subunit of a proline-directed serine-threonine kinase that phosphorylates several substrates. CDKs possess phosphorylation site selectivity, with the phosphate-acceptor residue preceding a proline. Several important proteins are

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Supplemental data can be accessed [here](#).

Declaration of interest

R Pestell is the founder of LightSeed LLC, has issued patents in cancer treatments, and is both a shareholder and employee of CytoDyn Inc. M Cristofanilli has acted as a consultant for Novartis, Merus, and CytoDyn, and has received honoraria from Pfizer. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial or other relationships to disclose.

substrates including all three retinoblastoma proteins, NRF1, GCN5, and FOXM1. Over 280 cyclin D3/CDK6 substrates have been identified. Given the diversity of substrates for cyclin/CDKs, and the altered thresholds for substrate phosphorylation that occurs during the cell cycle, it is exciting that small molecular inhibitors targeting cyclin D/CDK activity have encouraging results in specific tumors.

Keywords

Breast cancer; CDK inhibitors; clinical trial; cyclin D1; chromosomal instability

1. Background

Breast cancer (BrCa) is the most common non-dermatological malignancy in women and represents about 30% of all malignancies diagnosed in women in the USA [1,2]. Breast cancers can be linked to gene mutations inherited from one close relative in more than 5–10% of cases. Historically, breast cancer was considered a disease of the developed World, but almost 50% of breast cancer cases and 58% of deaths occur in less developed Countries. Incidence rates vary greatly worldwide from 19.3 per 100,000 women in Eastern Africa to 89.7 per 100,000 women in Western Europe. The worldwide breast cancer incidence has increased by more than 20% and mortality has increased by 14% since 2008.

In an attempt to reduce mortality from breast cancer, precision medicine approaches have been deployed targeting specific abnormalities identified in the coding region of the genome. Using this approach genetic subtypes of breast cancer have been described in which specific patterns of expression were identified for the coding genome [3]. Growing evidence suggests both the coding and non-coding genome govern the onset and progression of tumorigenesis [4,5] and more recently subtypes of breast cancer have been also demonstrated using patterns of expression for the non-coding genome [6–8].

Using the coding genome, five distinct molecular subtypes were identified referred to as luminal A, luminal B, human epidermal growth factor receptor 2 (HER2)-enriched, basal-like, and claudin-low and normal-like [9]. The coding region classification identified potential genetic targets including the estrogen receptor (ER α) and/or progesterone receptor (PR) and Her2. Triple-negative breast cancer (TNBC), which lacks ER α , PR and Her2, is the most deadly form of breast cancer. TNBC is associated in 10- to 15% with mutations of DNA damage repair proteins (*BRCA1*, *BARD1*, *BRCA1*, *BRCA2*, *PALB2*, and *RAD51D*) [10], in 19% with PD-L1 expression [11] and in >95% with CCR5 overexpression [12]. CCR5 expression in TNBC endows the breast cancer cells with characteristics of cancer stem cells [13] and TNBC metastasis can be blocked with CCR5 inhibitors [12], the basis of a current phase 1b/II study ([Clinicaltrial.gov](https://clinicaltrials.gov), ID#:).

Breast cancer may be characterized based on either the coding [3] or non-coding (Gormley classification, G1-4) [6]. The *CCND1* gene, which encodes cyclin D1, has been identified as amplified in 29–58% of breast cancers (Cancer Genome Atlas Network, 2012). In addition, cyclin D1 protein overexpression, through gene amplification, transcriptional or post-transcriptional induction, is found in >50% of breast cancers. Cyclin D1 expression is believed to drive aberrant phosphorylation and inactivation of the retinoblastoma protein

(pRB), primarily in luminal A and luminal B where it is associated with increased chromosomal instability [14] (Figure 1).

2. The cell-cycle and human breast cancer

Abnormalities of the cell-cycle are a pervasive finding in human breast cancer and other malignancies. A number of recent studies have reviewed the application of the cyclin-dependent kinases (CDK) inhibitor therapy for breast cancer [8,15–17]. This review herein is intended to serve as an update of these studies with a focus on the non-canonical functions of cyclins, including the importance of the anti-tumor immune response, and thereby provide a rationale for additional approaches to further enhance the efficacy of current CDK targeting therapies.

Progression through the cell-cycle occurs in an orderly and precise manner, with descriptive terms related to changes in the cell which are visible by light microscopy, the Gap1 (G_1) phase, the S phase, during which time DNA synthesis occur, the G_2 phase [8] and cell division or mitosis (the M phase), resulting in the production of two identical daughter cells [18]. As noted above, two distinct processes contribute to cell-cycle progression, firstly changing substrate specificity of the cyclin/CDKs and secondly altered activity of the kinase during the cell cycle. In this regard, the substrates of mitotic kinases are phosphorylated in sequential, kinase-specific waves [19] and phosphorylation of different CDK substrates is temporally ordered in part by a wide dynamic range of differential substrate sensitivity to CDK activity [20].

The history of the current understanding on cyclins in cell cycle progression illustrates the importance of basic science contributions from a number of laboratories around the world [21]. Lee Hartwell's isolation of temperature-sensitive mutants of budding yeast that could not complete cell division, thus defining cell division cycle, or *cdc*, genes [22,23] and Mitchison's work on temperature sensitive budding yeast, in the early 1970s [24], led Paul Nurse, who studied fission yeast, Steve Reed, Kim Nasmyth, Ruderman and others to enter the cell-cycle field [21]. Importantly Nurse discovered *wee1*, which determined the length of G_2 , establishing a non- G_1 rate-limiting step for progression through the cell cycle [25]. *CDC28* one of the 'start genes' in budding yeast controlled G_1 phase [26] and both Reed and Nasmyth identified approaches to complement *CDC28* [27]. The key contributions of many scientists to the field in the 70s and 80s was recently well annotated [21]. The importance of studies on yeast *cdc13* [28–30], led ultimately to the complementation experiments and discovery of cyclin D1 by several independent laboratories. In 1989 Andrew Arnold identified a candidate oncogene (*PRAD1* or *D11S287E*) on chromosome 11q13 [31] and in April 1991 published that a human cDNA of this clone, now known as cyclin D1, conveyed the regulatory subunit enzyme activity to phosphorylate histone H1. Shortly thereafter David Beach's laboratory, who had previously shown cyclin and p34cdc2 are major components of the M-phase specific H1K in sea urchin [32] using a conditionally *CLN*-deficient yeast strain, identified a human cDNA that complemented the *CLN* genetic deficiency, designated cyclin D1 (*CCND1*) [33]. Furthermore, Beach showed that cyclin D1 associated with CDK4, CDK2, CDK5, PCNA and p21 [34]. Independent studies by Charles Sherr's laboratory showed that colony-stimulating factor-1 induced the abundance of a cyclin-like cDNA

(p36CYL) during G1 phase in murine macrophages and that p36CYL was phosphorylated and associated with a polypeptide antigenically related to p34cdc2 suggesting that CYL genes may function during S phase commitment [35].

CDKs are a family of conserved serine/threonine protein kinases with a phosphoacceptor site preceding a proline. The binding of CDKs to their cognate regulatory subunit results in the formation of a holoenzyme, which together with additional components, participates in phosphorylation of target substrates governing cell cycle progression [36–44] and other substrates including NRF1 [45], FOXM1 [46] and the basal transcription apparatus [47]. Thirteen CDKs (CDK1 - CDK13) exist in the human species [48,49]. Among the CDKs family, CDK4 and 6 (CDK4/6) together with their D type cyclins regulatory subunit, promote cell cycle progression [50].

The mechanisms by which the mitotic kinases govern progression through the cell cycle was thought to involve phosphorylation of the pRB and related proteins [42]. An increasing array of substrates has added greater complexity to this model [51,52]. Phosphorylation of the substrate pRB, by cyclin D1/CDK4 [53] and other kinases [54] results in the release of E2F/DP proteins (E2F-1 - 5). Thus, in mid/late G₁, D-type cyclins and cyclin E, together with cyclin-dependent kinases, pRB, and the related p107 and p130, which then releases E2Fs, which in turn govern the expression of genes promoting cell cycle progression [55]. Such a model suggests that E2F proteins would promote proliferation, however, genetic deletion analysis of E2F function in mice revealed hyper-proliferative phenotypes (reviewed in [56]) suggesting further complexities [8,57].

Despite having high homology with other CDKs, CDK5 is not activated upon binding with a cyclin, however, CDK5 has subsequently been implicated in activating pRb phosphorylation [49,58]. CDK5 has critical functions in both terminally differentiated and proliferating cells and has recently been implicated in diseases, including the development and progression of cancer and neurodegenerative diseases [59]. CDK5 also leads to phosphorylation of the retinoblastoma protein [49,58,60]. CDK5 inhibition is an additional therapeutic target agent to treat cancer [49]. pRB was discovered as a downstream target of CDK5. Expression of CDK5 leads to the phosphorylation of pRB.

CDK5, which has high amino acid sequence homology with other CDKs. Structural studies have shown the similarity of CDK5 (1H4L) with its closest structural homologs (CDK1) (5HQ0) and CDK2 (3QHR) (Figure 2(a)). CDK5 is upregulated in cancer and tamoxifen reduces both tumor growth and CDK5 activity. Cyclin D1 can attenuate CDK5 kinase activity by competing with p35 for binding with CDK5, thereby forming an inactive complex of cyclin D1 and CDK5 [61–64] (Figure 2(b)). In targeted drug design focus has been on targeting the ATP binding pocket. In Figure 2(b) the structural overlay of CDK5 is shown with the inhibitors R-roscovitine (1UNL) and the ATP analogue, [4-Amino-2-[(4-chlorophenyl)amino]-1,3-thiazol-5-yl](3-nitrophenyl)methanone (30OG), with a higher magnification view of the CDK5 active site in which R-roscovitine and the ATP analogue bind (Figure 2(c)).

3. Cyclin D1 is sufficient for the hallmarks and enabling characteristics of tumorigenesis

The cell cycle is critical in regulating the balance between the formation of new cells and programmed cell death. The induction and maintenance of tumorigenesis have been proposed to involve a variety of descriptive characteristics or ‘hallmarks’ of cancer (‘proliferative signaling’, ‘resistance to cell death’, ‘replicative immortality’, ‘induction of angiogenesis’, ‘evading growth suppressors’, ‘activating invasion and metastasis’) [65] and ‘enabling capabilities’ (‘avoiding immune destruction’, ‘tumor promoting inflammation’, ‘deregulated cellular energetics, genomic instability, and mutation’) [66] (Figure 1). It has also been proposed that a dynamic balance between oncogenes and tumor suppressors restrains tumorigenesis [8,48,67–70].

Although cyclin D1 was originally categorized within the presumptive ‘proliferative signaling’ category, the expression of cyclin D1 has been shown to be sufficient for the majority of these ‘hallmarks’ and ‘enabling characteristics’. In this regard cyclin D1 has been shown to induce proliferative signaling [33,71–74], resistance to cell death [71,75], replicative immortality [76], induction of angiogenesis [77], evading growth suppressors, activating invasion and metastasis [61,65] and enabling capabilities (‘avoiding immune destruction’ [63,64]), ‘tumor promoting inflammation’ [78], ‘deregulated cellular energetics’ [45,47,79,80], ‘genomic instability and mutation’ [51,52] (Figure 1). Although the above models of ‘cancer hallmarks’ and ‘enabling characteristics’ do not directly embrace the field of cancer stem cells, several studies have shown the importance of cancer stem cells in the onset and progression of cancer [81–83] and the importance of cyclin D1 in expanding the pool of stem [84,85] and/or cancer stem cells [86]. Osteopontin (OPN) was induced by cyclin D1 in fibroblasts, breast epithelial cells and in the murine transgenic mammary gland [78] and OPN was sufficient to induce stem cell expansion [78]. Thus, although cyclin D1 has historically been categorized as a gene product participating in proliferative signaling, given the diverse functions for cyclin D1 in tumorigenesis, it has been reassuring to find that drugs targeting the activity of cyclin D1 have shown promise in patients.

4. Role of cyclin D1-CDK4/6 complexes in breast tumorigenesis

Cyclins are regulatory subunits that form holoenzyme complexes with the CDKs [37,41,42,55] in particular CDK4 [87] and CDK6 [88]. *Cyclin D1* encodes the regulatory subunit of the holoenzyme that is rate limiting in the proliferation of mammary epithelial cells [89] as well as fibroblasts [74,90] and neurites [91]. The inhibition of cyclin D1 expression leads to cell-cycle arrest, whereas cyclin D1 overexpression promotes G₁-S phase progression [74,92,93]. Cyclin D1 was also found to be a rate-limiting factor in the estrogen-induced proliferation of mammary epithelial cells [89]. Cyclin D1 overexpression is sufficient for the induction of mammary tumorigenesis [14,94] with high levels of chromosomal instability (CIN). Furthermore, the rate of mammary tumor onset, and the induction of CIN were identical with a kinase-dead mutant of cyclin D1 [51] suggesting kinase-independent functions may contribute to tumor onset *in vivo*. *In vivo* evidence for the requirement of cyclin D1 in mammary tumorigenesis was first identified using cyclin D1

anti-sense plasmids in ErbB2-induced mammary tumors [95]. In subsequent cyclin D1 gene knockout mouse experiments, cyclin D1 was shown to have a pivotal role in the induction of breast tumors by Ras or ErbB2 [96]. Furthermore, *cyclin D1* gene deletion evidenced a critical role in skin [97] and gastrointestinal tumorigenesis [98].

Cyclin D1 functions in a variety of cell types that participate in the onset and progression of tumorigenesis, in addition to the mammary epithelial cell, including adipocytes [99], lymphocytes [61], and stromal fibroblasts [78]. Cyclin D1 also affects the development of the mammary gland, in particular affecting the stem cells that may contribute to tumorigenesis [84]. Analysis of *cyclin D1* deletion in the mammary gland evidenced an important role for cyclin D1 in the expansion of progenitor cells that gave rise to tumors [84]. Therefore, mammary gland selective cyclin D1 inactivation was conducted in the mature fully developed mammary gland [79]. In contrast, with whole body *cyclin D1* gene deletion mice, mammary gland selective *cyclin D1* gene inactivation in the adult mammary gland, using inducible anti-sense, revealed a specific role in glucose metabolism. As an *in vivo* measurement of relative utilization of amino acids from the tricarboxylic acid cycle, the ratio of (glutamate + glutamine)/citrate was assessed using nuclear magnetic resonance. Mammary gland (glutamate + glutamine)/citrate was increased twofold by endogenous cyclin D1. These studies are consistent with a model in which cyclin D1 determines metabolic substrate prioritization toward amino acid synthesis from the tricarboxylic acid cycle, consistent with the known role for cyclin D1 in DNA synthesis.

5. Cyclin D1 and CDK activity in the anti-tumor immune response

Cyclin D1 has several significant effects on the immune system and the anti-tumor response. First, cyclin D1 is expressed in macrophages and is required for CSF-1 induced guided migration [61,62].

Secondly, cyclin D1 in stromal fibroblasts promotes the secretion of cytokines that govern the expansion of myeloid-derived stem cells (MDSC) [78]. Stromal cyclin D1 had a profound effect on the breast tumor microenvironment increasing the recruitment of F4/80⁺ and CD11b⁺ macrophages and increasing angiogenesis [78]. Myeloid-derived suppressor cells (MDSCs) constitute one population of inflammatory cells that suppress the anti-tumor immune response and thereby enhance tumor growth [100]. Cyclin D1 induced the expansion of the MDSC population [78]. Cyclin D1-conditioned medium induced expansion of CD34 positive hematopoietic stem cells (HSCs) and promoted differentiation of CD34 positive hematopoietic stem cells (HSCs) into MDSC [78]. Furthermore, stromal cyclin D1 induces secretion of factors that promotes the expansion of stem cells (breast stem-like cells, embryonic stem cells, and bone marrow-derived stem cells). Stromal cyclin D1 promotes secretion of pro-inflammatory cytokines (CCL2, CCL7, CCL11, CXCL1, CXCL5, CXCL9, CXCL12), CSF (CSF1, GM-CSF1) [78].

Thirdly, cyclin D1/CDK4 induces expression of the immune checkpoint regulatory target PDL-1 [63]. Targeting immune checkpoints such as the one mediated by programmed cell death protein 1 (PD-1) and its ligand PD-L1, have been approved for treating human cancers with durable clinical benefit [101,102]. PD-L1 protein abundance is regulated by cyclin D-

CDK4 and the cullin 3-SPOP E3 ligase via proteasome-mediated degradation. Inhibition of CDK4 and CDK6 *in vivo* increased PD-L1 protein levels [63]. Kaplan-Meier survival curves demonstrated the improved efficacy of combining PD-1 mAb with the CDK4/6 inhibitor, palbociclib in CT26 implanted tumor-bearing mice [63]. Transgenic mice (MMTV-rtTA/tetO-HER2 tumor-bearing mice) tumors treated with abemaciclib and anti-PDL1 combination therapy regressed to a greater degree compared with abemaciclib alone [64].

Fourthly, cyclin D1 triggers anti-tumor immunity [64] via activating endogenous retroviral element expression thereby stimulating type III interferon production and tumor antigen presentation, thereby suppressing regulatory T cell proliferation [64].

Like cyclin D/CDK4, CDK5 has also been implicated in regulating the immune checkpoint. CDK5 has been implicated in upregulation of IFN γ -induced programmed death ligand 1 (PD-L1), which allows certain cells to evade detection by the immune system. Decreased CDK5 expression led to increased expression of the PD-L1 transcriptional repressors IRF2 and IRF2BP and consequent decreased PD-L1 expression [103].

6. Cyclin D1 regulation of the non-coding genome

Micro-RNAs (miRNAs) are 21- to 22-nucleotide-long molecules that regulate a variety of cellular phenotypes by affecting the stability of targeted mRNAs. Yu *et al.* compared the miRNA expression in cyclin D1-induced mammary tumors and mammary tissues derived from cyclin D1 anti-sense or knockout mice. These studies identified the miR-17/20 cluster as an important regulator of mammary tumorigenesis. miR-17/20 repressed expression of cyclin D1 by targeting the cyclin D1 3' untranslated region [104]. Cyclin D1 associated in the context of chromatin with the miR-17/20 regulatory region. This was the first study to demonstrate cyclin-dependent regulation of a non-coding RNA [105]. miR-17/20 was shown to regulate the secretion of cytokines and plasminogen activator via the expression of α -enolase and cytokeratin 8. The inhibition of plasminogen activator by miR-17/20 required cyclin D1, thereby defining a mechanism by which cyclin D1 governs a non-coding genome-cytokine loop in breast cancer [105].

Cyclin D1 was subsequently shown to regulate miRNA biogenesis through the transcriptional induction of *Dicer* [106]. Cyclin D1 and *Dicer* both maintained heterochromatic histone modification (Tri-m-H3K9). Several functions of *Dicer* were cyclin D1-dependent. Cyclin D1-mediated cellular proliferation and migration were *Dicer*-dependent [106]. These findings suggest that cyclin D1 regulates both the expression of individual miRNAs (via binding to their regulatory regions) and can also modulate the processing of other miRNAs via the transcriptional induction of *Dicer* [106].

A cyclin D1-regulated miRNA signature, was identified in breast cancer cells that included several onco-mirs including miR-193b. When assessed in a superset of 459 breast cancer samples, the cyclin D1-regulated miRNA signature was associated with the Gormley 2 (G2) breast cancer miRNA subset, ER α^+ status and activation of the Wnt pathway [6]. Seed elements for cyclin D1-regulated miRNA were identified in 63 genes of the Wnt signaling pathway including DKK [6]. These findings were consistent with studies conducted

profiling a large number of different types of cancer cells that identified a distinct class of miRNAs (miR-193b, miR-193a-3p, miR-195-5p, miR-214-5p, miR-890) that target nearly all cyclins/CDKs. These miRNAs were very effective inhibitors of cancer cell proliferation [107].

7. Role of cyclin D1-CDK4/6 in transcription and DNA damage

A substantial body of evidence now supports the model in which cyclin D1 governs gene transcription in the context of chromatin (reviewed in Di Sante et al. [18]). These studies extended earlier findings in which cyclin D1 was shown to interact in a kinase-independent manner with transcriptional coactivators including p300 [108] and more the 30 distinct transcription factors including the ER α [109,110], then DMP1 [111], v-Myb [112] and others (reviewed in cyclins [36,113,114]. In chromatin cyclin, D1 was initially shown to alter recruitment of transcription factors in chromatin and affect local histone acetylation [98]. Subsequently, cyclin D1 was itself shown to be recruited into chromatin using distinct evolving technologies including by ChIP [99,115,116], then ChIP arrays [117] and ChIP Seq [14]. Recruitment into chromatin was independent of the cyclin D1 kinase function [51]. In subsequent studies, other D types cyclins [118,119] and CDK6 was shown to be recruited into chromatin [120,121]. Furthermore, the CDK inhibitors affected phosphorylation of the transcription factors to which CDK bound [122].

Many studies have found proteins with roles in both transcription and DNA repair (Figure 1), including Topoisomerase II α [123,124], PARP (Poly(ADP-ribose), TIP60, BRCA1, BRCA2, and the BRCA2 binding protein P/CAF. PARP-1, among other functions, promotes ER α activity [124]. Moreover, cyclin D1 has been shown to regulate the activity of each of these afore-mentioned dual-function proteins. We initially [36,125–129] and other groups subsequently [130], demonstrated that cyclin D1 regulates DNA damage repair (DDR) [129] and binds DDR proteins such as RAD51 [129] and BRCA1 [125]. In addition to binding Rad51, cyclin D1 binds other DNA damage repair proteins [130]. Estrogens can contribute to BrCa by inducing DNA damage [131]. Early BrCa lesions exhibit chromosomal instability with aneuploidy and estrogens lead to double-stranded DNA breaks and genomic instability [132]. Estrogens induce DNA damage via both the production of oxidative metabolites or other oxidative DNA damage [131]. Li *et al.* showed that cyclin D1 interacts with ER α and regulates a critical role in the E2-dependent DDR in human BrCa cells [128]. Cyclin D1 was recruited to γ H2AX foci by E2 and induced RAD51 expression [128] contributing to aberrant growth signaling [36]. The mechanisms governing recruitment of cyclin D1 into chromatin remains to be determined.

8. Stromal fibroblast cyclin D1 and its role in breast cancer

Although not formally included as either a cancer hallmark, nor an enabling characteristic of tumorigenesis [66], an overwhelming body of evidence has demonstrated the local microenvironment, including the tumor cancer-associated fibroblasts, contribute to the onset and progression of tumorigenesis [133–135]. Cyclin D1 is overexpressed, more than 30-fold in human breast cancer-associated fibroblasts, and reintroduction of cyclin D1 into fibroblasts augmented breast cancer tumor growth in mice [78].

Increased expression of cyclin D1 in the breast cancer stroma is also associated with poor prognosis [78]. When cyclin D1 was expressed in stromal fibroblasts breast epithelial cancer tumor growth was enhanced, apoptosis was restrained, and autophagy was increased [78]. These results demonstrate that stromal cyclin D1 drives tumor microenvironment heterocellular signaling, thereby promoting several key hallmarks of cancer [78].

9. Cyclin D1 regulation in other cancer types

In addition to gene amplification and transcriptional induction of cyclin D1 by Ras and other pathways [136–138], the abundance of cyclin D1 is regulated by posttranslational modification in a cell-type specific manner (reviewed in [139,140]). Posttranslational modification includes phosphorylation (T286, T288) and ubiquitylation. The ubiquitin hydrolase USP2 [141] and the deubiquitylase USP22, hydrolyzes ubiquitin at a set of specific lysine residues on cyclin D1 (K33, K46, 50, K112/114). Functionally, K33 ubiquitylation is implicated in nuclear localization of cyclin D1, and K112 and K114 ubiquitylation are linked to the interaction of cyclin D1 with partners, including CDK4/6 [142]. Mutation of all cyclin D1 lysines to arginine confers protection from proteasome-dependent degradation, while single mutations only offered a modest increase in the stability of cyclin D1 and the only mutation of K112 has been identified in human breast cancer. K112 affects interactions with a diverse array of proteins including Cdk4/6 and Rho proteins.

Cyclin D1 is polyubiquitinated and subsequently degraded through the 26S proteasome pathway. The formation of polyubiquitin-protein conjugates includes E3, a protein ligase, which attaches ubiquitin to a lysine residue on a target protein. Several distinct ubiquitin-protein E3 ligases have been identified for degrading cyclin D1 in distinct cell types [143]. The SCF E3 ubiquitin ligases FBXW8, (Fbw8/Cul7) [144], Fbx4 (scF^{Fbx4}/_{B-crystallin}) [145], β-Trcp [146], APC/C [147] and SCF^{Skp2} [143] have been shown to regulate cyclin D1 degradation. The F-box protein, SKP2, contributes to the SCF^{Skp2} complex, which has been shown to ubiquitinate cyclin D1 [143]. A splice variant of Skp2 is retained in the cytoplasm and fails to direct cyclin D1 ubiquitination in the uterine cancer cell line SK-UT [148]. For Fbx4 (SCF^{Fbx4}/_{B-crystallin}) of interest mutations in Fbx4 have been identified in esophageal squamous cell cancer, associated with increased abundance of cyclin D1 [145]. The kinases governing phosphorylation appear to be cell-type specific, with excellent studies showing the role of GSK3β [149], Ras/MAPK [144], p38SAPK2 [150], IKKα [146] and ATM/ATR [151]. In lymphoma, *cyclin D1* amplification is a frequent cause of overexpression, Eμ-cyclin D1 T-286, but not Eμ-cyclin D1 wt transgenic mice, develop lymphomas [152] and the cyclin D1 phosphorylation site mutant T-286 coprecipitates MEP50 and the methyltransferase PRMT5 [153], consistent with a role for Eμ-cyclin D1 T-286 [152] and PRMT5 in lymphomagenesis [154].

10. Environmental factors in human breast cancer

Molecular pathological epidemiology (MPE) is an integrative field that utilizes molecular pathology to incorporate interpersonal heterogeneity of a disease process into epidemiology [155]. The MPA approach defines interactive relationships between environmental exposure

and disease subtypes in determining disease incidence and mortality and can be used conceptually to integrate the impact of the microbiota as an ongoing exposure [156]. The microbiome influences the onset and progression of cancer [157]. The human gastrointestinal tract is colonized by a complex and diverse community of microorganisms including bacteria, viruses, archaea, and fungi [24]. Recent studies have suggested the microbiota of women with breast cancer differs from that of healthy women, and have proposed potential mechanisms including regulating estrogen-dependent mechanisms [158]. In this regard, alterations in the microbiota/estrobolome (the collection of the enteric bacterial genes whose products metabolize estrogen and its metabolites) can increase circulating estrogens and its metabolites, thereby increasing the risk of breast cancer.

Alternatively, the gut microbiome may affect breast cancer risk through estrogen-independent pathways, both locally and at a distance. In this regard, an enteromammary pathway has been described by which gut bacteria could reach the mammary gland. Furthermore, the gut microbiota differs in composition between healthy and breast cancer patients. The microbiome is present within the normal ductal lobular structure and some studies have identified different flora between individuals. Nipple aspirate fluid collected from breast cancer patients was also shown to have a distinct microbiota profile compared to healthy volunteers. Together these studies suggest an important interaction between the microbiome, food, and lifestyle to molecular pathologies and the influence of these factors on breast cancer and breast cancer therapies warrants consideration.

11. CDK inhibitors for breast cancer

Novel therapies targeting CDK activity have become increasingly precise in targeting cyclin D/CDK activity and have been recently reviewed [159–164]. The effectiveness of CDK inhibitors have been shown in human breast cancer, in which the regulatory subunit, cyclin D1 is overexpressed [15–17]. More than 10 CDK inhibitors have entered clinical trials since 2009, most of which target multiple kinases. Palbociclib (PD0332991), Ribociclib (LEE011), and Abemaciclib (LY2835219) are specific, orally administered, CDK inhibitors with a similar chemical structure (Figure 3) [165,166], that are FDA approved for metastatic breast cancer treatment. They selectively bind to the ATP-binding pocket of CDK4 and CDK6 [165,167–171]. Additional selective CDK inhibitors including G1T38, G1T28 and PF-06873600 are currently under investigation (Figures 3 and 4).

11.1 Pre-clinical studies

The anti-tumor effect of Palbociclib was investigated in neuroblastoma [172], non-small cell lung carcinoma [173] and breast cancer [174]. *In-vitro* studies demonstrated the effect of palbociclib reduced pRB phosphorylation, and G₁ phase cell cycle arrest. Mice carrying human colon cancer cells, treated with palbociclib, showed a reduction of pRB phosphorylation, downregulation of E2F-regulated genes and tumor regression [159,175]. Furthermore, palbociclib sensitized cancer cells to chemotherapy and ionizing radiation [176].

The ribociclib anti-tumor effects have been studied in different types of cancer as well, including breast cancer. Studies on xenograft models determined the efficacy of Ribociclib

in many other types of Rb-positive cancers, such as ER α breast cancer [177] and neuroblastoma [172]. These studies showed that ribociclib induces pRB dephosphorylation that leads to G1 arrest in Rb-positive cells [172]. Ribociclib has been administered in combination with 3-phosphoinositide dependent protein kinase 1 inhibitor (GSK2334470). This study showed ribociclib suppressed proliferation and increase apoptosis in ER α -positive breast cancer cell lines [178].

The anti-tumor effect of abemaciclib has been assessed in several tumors, including breast cancer, and was shown to sensitize cancer cells over-expressing ATP Binding Cassette Subfamily B Member 1 (ABCB1) and ATP Binding Cassette Subfamily G Member 2 (ABCG2), which contribute to multidrug resistance in tumor chemotherapy [179].

11.2. Clinical pharmacology

The selective CDK4/6 inhibitors have been further tested in clinical trials. The updated list of completed and open clinical trials in breast cancer are shown in Supplemental Table 1 and Supplemental Table 2. The first phase I clinical study assessed the pharmacokinetics of palbociclib in patients with pRB-positive solid tumors [180]. To assess both dose-limiting toxicity (DLT) and maximum tolerated dose (MTD), palbociclib was administered once daily for 21 of 28 days. This phase I study () tested oral palbociclib in six dose-escalation cohorts. A daily dose of 125 mg over a period of 3 weeks, followed by 1 week off, was established to be the maximum tolerated dose (MTD) [180]. In the phase II clinical trial () of ribociclib, the drug was administered in an escalating dose from 50 mg to 120 mg daily. The MTD was 900 mg/day over a 3 weeks' period with 1 week off [181]. In the phase I trial () of abemaciclib the MTD resulted was 200 mg daily [182].

11.3. Toxicities

Each of the three CDK inhibitors induce dose-related neutropenia, with rare neutropenic fevers. Although abemaciclib is associated with less neutropenia, monitoring is required with the potential need to reduce the dose. Palbociclib and ribociclib are administered on a 3-week-on, 1-week-off schedule, a schedule necessitated by the greater neutropenia seen with these agents compared with abemaciclib. Ribociclib has been associated with QT prolongation, leading to a requirement for electrocardiogram monitoring. Gastrointestinal toxicity, in the form of diarrhea, is more common with abemaciclib. Diarrhea is dose-dependent and in the MONARCH 2 trial led to a decrease in the dose of abemaciclib from 200 mg to 150 mg. This diarrhea occurs early is readily manageable with conventional antidiarrheal agents, and in the MONARCH 2 trial and led to drug discontinuation in only 1.6% of patients.

Neutropenia was the primary adverse event that determined DLT for palbociclib. Other reported adverse events included fatigue, diarrhea, nausea, dyspnea, and arthralgia. During the Phase II and III trials a dose of 125 mg daily of palbociclib was well tolerated and the toxicity was consistent between the trials [180]. These adverse events caused an increased monitoring of the blood components during the treatment [181]. Abemaciclib showed a higher rate of diarrhea and fatigue as adverse events [182].

No biomarkers reliably define patients who will, or will not, benefit from the addition of a CDK4/6 inhibitor to their endocrine therapy. Adding CDK4/6 inhibitor to endocrine therapy prolongs PFS compared to endocrine therapy alone as first-line treatment in advanced breast cancer. The magnitude of PFS benefit is ethnicity-dependent but there were no interethnic differences in relative treatment-related toxicities [183]. Recently several biomarkers studies have been published with regards to the use of circulating tumor DNA (ctDNA) analysis performed either at baseline, shortly after treatment or at progression in the context of the PALOMA-3 study [184,185]. Longitudinal detection and persistence of *PI3KCA*, *ESR1*, and *Rb1* seems to predict resistance to the combination of palbociclib and fulvestrant in patients with endocrine-resistant disease [186].

11.4. CDK4/6 and CDK5 as therapeutic targets for breast cancer

Multiple studies have shown that the addition of CDK4/6 inhibitors confers prolonged progression-free survival (PFS) in the context of patients receiving endocrine therapy. In this regard, improvement has been observed in combination with aromatase inhibitors [169,171,187], and fulvestrant [167,188,189] for palbociclib, ribociclib, and abemaciclib therapy. In the Palbociclib: Ongoing Trials in the Management of Breast Cancer [PALOMA]-1), palbociclib resulted in a progression-free survival benefit in patients with previously untreated, ER α ⁺, HER2⁻ advanced breast cancer [190]. The subsequent randomized, phase 3 trial, PALOMA-2, showed that as first-line therapy for ER α ⁺ HER2⁻ advanced breast cancer, palbociclib prolonged progression-free survival when given in combination with letrozole [171]. In the PALOMA-3 phase 3 trial, treatment with palbociclib and fulvestrant, prolonged progression-free survival among patients with ER α ⁺ positive, HER2⁻ advanced breast cancer who had disease progression after previous endocrine therapy compared with placebo and fulvestrant [191]. This was the first study with a CDK 4/6 inhibitor to report mature data for OS analysis and demonstrating that, in patients with endocrine-sensitive disease the use of combination regimen is associated with approximately 10 months improvement in OS [191].

Abemaciclib received FDA approval for the frontline treatment of postmenopausal women with ER α -positive, HER2⁻ advanced or metastatic breast cancer, based on results from the phase III MONARCH 3 trial, in which the addition of abemaciclib to anastrozole or letrozole reduced the risk of progression or death by 46% compared with a nonsteroidal aromatase inhibitor alone. Abemaciclib has previously received FDA approval as monotherapy, based on the MONARCH 1 trial, which demonstrated an objective response rate of 19.7%, with a median duration of response of 8.6 months. Importantly these studies showed benefit independent of hormonal therapy.

Ribociclib has, unlike its competitors, been specifically studied in premenopausal women in the frontline setting (in the MONALEESA-7 trial), and as in postmenopausal women, the results suggest an impressive improvement in PFS, with similar toxicity to that seen in the postmenopausal setting.

CDK5 has been considered as a therapeutic target for breast cancer because of the evidence above that CDK5 participates in augmenting pRB phosphorylation and the participation of CDK5 in the anti-tumor immune response [49]. Ahn and coworkers have reported three

CDK5/p25 inhibitors able to reduce *tau* phosphorylation at Ser396 and Ser404, namely Bellidin (14, IC50 = 0.2 μ M, ATP competitive), diazaphenanthrene 15 (IC50 = 2.0 μ M, substrate competitive), and 4-aminothiazole 16a (IC50 = 17 μ M, ATP competitive) [192,193].

11.5. Therapy resistance

Although the rationale for CDK inhibitors was based on a model in which pRB is the key substrate, subsequent studies revealed the biology is more complex. It has also been proposed that durable CDK inhibition [114] reflects the induction of a senescence phenotype [194]. Knockdown of pRB induced only partial resistance to CDK inhibition by palbociclib [195–197]. The combination of high p16^{ink4a} expression with loss of RB expression has emerged as one of the more commonly used approaches to exclude or include patients on clinical trials with CDK4/6 inhibitors. In preclinical models with genetic loss of p16^{ink4a} there is evidence for acquired resistance to CDK4/6 inhibitors [198,199]. However, in cancers that lose *CDKN2A* there is little evidence for a direct correlation between response and loss of the tumor suppressor [182]. pRB inactivation in breast cancer is primarily due to overexpression or amplification of the *cyclin D1* gene (*CCND1*) which is observed in as many as 50% of breast cancers, wherein it is believed to drive aberrant phosphorylation/inactivation of RB protein [200]. Inactivation of the CDK-inhibitor p16^{ink4a} (*CDKN2A*) contributes to the deregulation of RB phosphorylation, however, increased p16^{ink4a} is often found in human breast cancer and deletion of p16^{ink4a}/ARF did not affect MMTV-ErbB2 induced mammary tumors [201]. Expression rates of p16 and pRB differ according to the breast cancer molecular subgroup [202]. High p16^{INK4a} mRNA expression is associated with high tumor grade, is silenced in a fraction of breast cancer cases [203], Tumor samples overexpressing p16^{INK4a} were predominantly ER α negative [204]. reduced expression of p16^{ink4a} due to de novo *INK4a* methylation occurred in 24/120 in one study [204]. Lastly, loss of heterozygosity at the *RB* gene (*RBI*) locus has been defined in 20–30% of breast cancer and histological loss of RB protein has been documented with varying frequency up to 20% with a higher frequency in high-grade tumors [205].

Acquired resistance to CDK4/6 inhibition has implicated deregulated cyclin E expression in some [198,206], but not all studies [182]. *TP53* mutation was associated with poor clinical response to the CDK4/6 inhibitor abemaciclib in breast cancer patients [182] however this finding could be due to the association of TP53 mutations with triple-negative disease in the cohort analyzed [114]. Suppression of MDM2 was associated with improved response to CDK4/6 inhibition in a small collection of liposarcomas treated with palbociclib [207]. Therapy that interfering with CDK6 activity may also be associated with a higher risk of acquiring *TP53* mutations because inhibition of CDK6 kinase activity may provoke the outgrowth of p53-mutant clones from premalignant cells [122]. CDK6 expression levels correlate with the p53 pathway status in murine and human tumors and CDK6 binds to the promoters of genes governing the p53 antagonists *Prmt5*, *Ppm1d*, and *Mdm4* [122].

Approaches to overcoming CDKI resistance were recently reviewed [114]. Additional secondary changes that occur upon treatment with CDK inhibitors that may contribute to both therapeutic efficacy and resistance include altered regulation of the Akt/mTORC

pathway, and altered gene expression a function of non-canonical signaling by cyclins [36,113,114]. Upstream mitogenic signaling is activated by CDK4/6 inhibition resulting in increased expression of cyclin D1. This, in turn, is associated with upregulation of Akt signaling [178], suggesting a role for PI3K/Akt inhibitors to overcome CDKI resistance [208]. Preclinical studies have suggested that resistance to CDK4/6 inhibitor can emerge through the selection for acquired mutation of *RBI*, amplification of *cyclin E*, amplification of *CDK6*, or suppression of CDK2 inhibitors (e.g. p27^{kip1} or p21^{cip1}). Resistant cells coordinately upregulated expression of cyclins A, E and D1, activated phospho-CDK2 and phospho-S477/T479 AKT. Additional findings in CDKI resistant cells, suggesting potential targets to overcome resistance include amplification of *cdk6* [209], increased Cdk4 phosphorylation status (CDK4 T172 phosphorylation) [210]. In addition, Increased androgen receptor (AR) function has been described, suggesting a role for androgen antagonists [211]. In identifying potential sensitizers, CDK4/6 Inhibitors were shown to sensitize PIK3CA mutant breast cancer to PI3K inhibitors [212]. Cyclin D1 overexpression, known to be associated with mitochondrial dysfunction (inhibition) [45] in breast cancer cells, was shown to correlate with dependence upon glutamine in an esophageal cancer cell line and CB-839 (glutaminase 1 inhibitor) plus metformin/phenformin reduced growth of palbociclib resistant esophageal cancer cell lines <https://doi.org/10.1038/s41467-019-09179-w>. Collectively these studies suggest the potential to overcome CDK inhibitor resistance with inhibitors of immune checkpoints, Akt, AR and glutaminase.

12. Expert opinion

Therapeutic targeting of CDKs for breast cancer has demonstrated improvement in outcomes. New CDK inhibitors with reduced toxicities are being explored. Although the CDKs have diverse substrates, cyclin D1 has been shown to be sufficient for the majority of the ‘hallmarks’ and ‘enabling characteristics’ of tumorigenesis, perhaps providing an explanation of the efficacy for these therapies. Of concern, patients continue to perish while CDK activity, assessed by kinase activity, is inhibited in their tumors. These findings suggest additional molecular mechanisms continue to drive therapeutic resistance, and or tumor growth through CDK-independent mechanisms. These mechanisms may relate to the growing number of kinase-independent functions of cyclins, including ER α agonist function, activation of chromosomal instability and recruitment of other enzyme including protein methylases [213].

There are several recent findings with important implications for targeting cyclin D1/CDK activity in breast cancer. Firstly, the findings that cyclin D1/CDK govern aspects of the anti-tumor immune response [61–64] suggest the opportunity to explore combination therapy between CDK inhibitors and checkpoint inhibitors. Secondly, the side effects of bone marrow suppression, as a limiting toxicity, warrant the development of additional CDK inhibitors with distinct toxicity profiles. In preliminary preclinical studies, GiT38 appears to have less neutropenia [214]. Thirdly, it will be important to develop efficient predictors of response to CDKs inhibitors with ctDNA appearing promising. Fourthly, because distinct domains of cyclin D1 conduct transcriptional activities that drive the process of mammary tumorigenesis [113], including chromosomal instability [14] in a kinase-independent manner [51,52,215], the notion of targeting additional domains of cyclin D1 appears warranted. A

growing body of evidence has demonstrated that cyclin D drives transcriptional programs in breast cancer, lymphoma [118] and neural cells [119].

Finally, and most importantly, women continue to die from metastatic breast cancer, including patients being treated with CDK inhibitors, despite the ability of the inhibitors to reduce CDK activity. Given the diverse additional functions of cyclin D1 to drive tumorigenesis, it will important to assess additional complementary approaches to inactivate the non-canonical functions of cyclin D1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work was supported in part by R01CA132115 (R.G.P.), and the Breakthrough, Breast Cancer Research Foundation [BCRF-16-197]. This project was funded in part from the Dr. Ralph and Marian C. Falk Medical Research Trust (R.G.P), and a grant from the Breast Cancer Research Foundation (R.G.P.) The Department specifically disclaims responsibility for analyses, interpretations or conclusions. Grant number for DOD Contract # W81XWH1810605.

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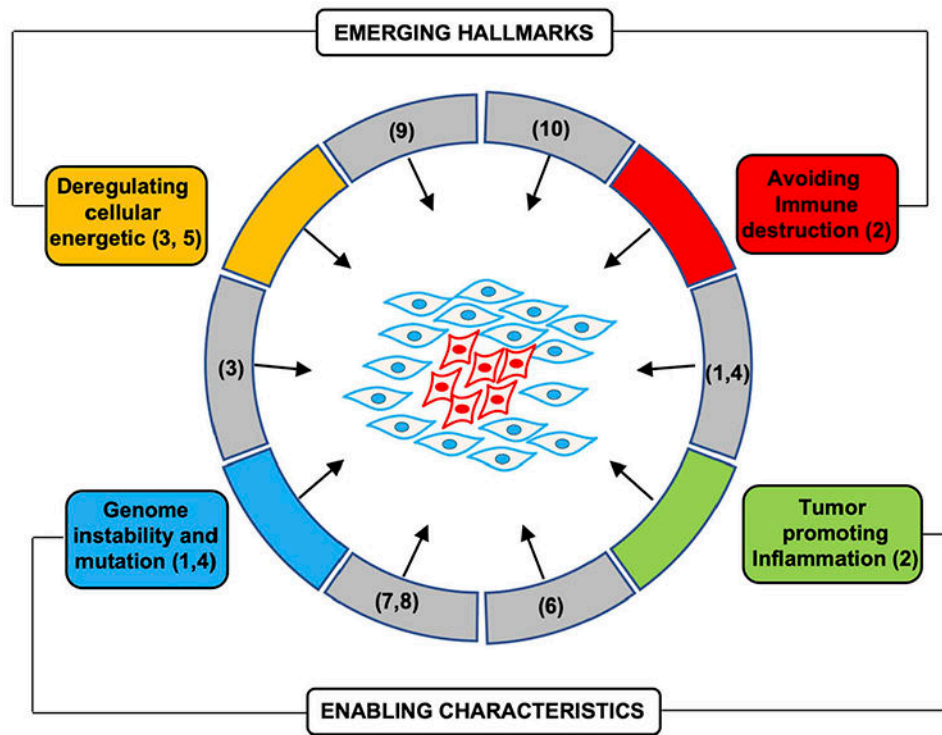
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Article highlights

- The cell cycle regulatory complex 'cyclin D1-CDK4/6' participates in the onset and maintenance of breast tumorigenesis.
- Cyclin D1 is sufficient for the hallmarks and enabling characteristics of tumorigenesis
- Breast cancer clinical studies have demonstrated the efficacy of targeting cyclin D/CDK activity, in human breast cancer. (an updated list and description of completed and ongoing clinical trials is provided)
- Many functions of cyclin D1 are Cdk-independent, including the induction of chromosomal instability which may promote tumor heterogeneity.
- Cyclin D1 and CDK inhibitors modulate anti-tumor immunity.



Ref #	Ref PubMed ID
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2	29137220
3	28522753
4	25940700
5	16864783
6	18180298
7	17047061
8	16899588
9	23864650
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Figure 1. Cyclin D1 participates in both the hallmarks and enabling characteristics of tumorigenesis. The references for studies demonstrating the function of cyclin D1 in the hallmark of cancer and enabling characteristic are shown.

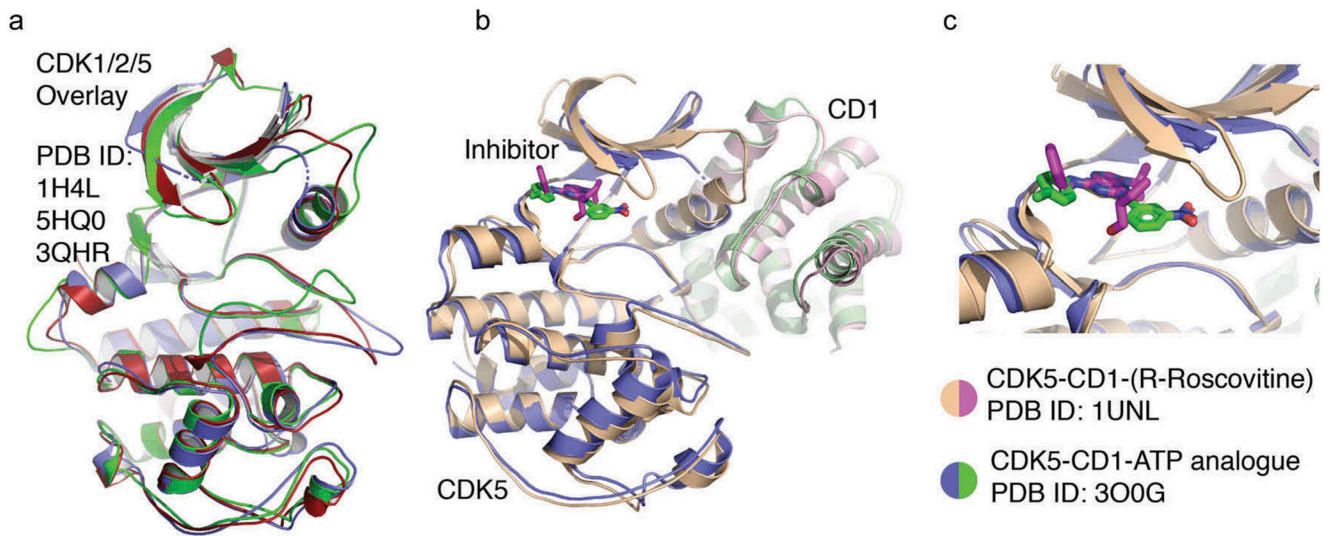


Figure 2.

Targeting of CDK5. (a). Structural overlay of CDK5 (1H4L) with its closest structural homologs CDK1 and CDK2. (b). Structural overlay of CDK5 with cyclin D1. (c). Structural overlay of CDK5 with the inhibitors R-roscovitine (1UNL) and ATP analogue, {4-Amino-2-[(4-chlorophenyl)amino]-1,3-thiazol-5-yl}(3-nitrophenyl) methanone (30OG). High magnification view is shown of the CDK5 active site in which R-roscovitine and the ATP analog bind.

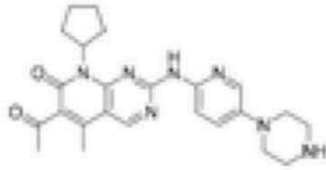
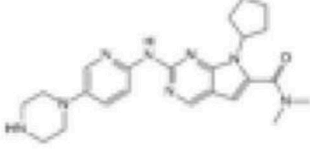
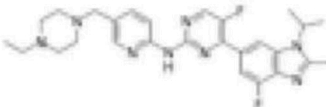
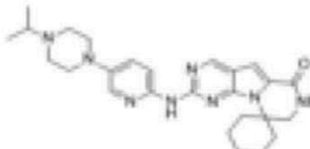
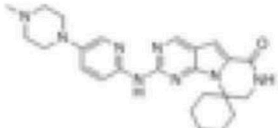
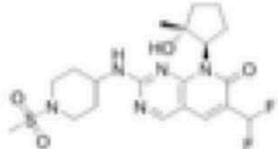
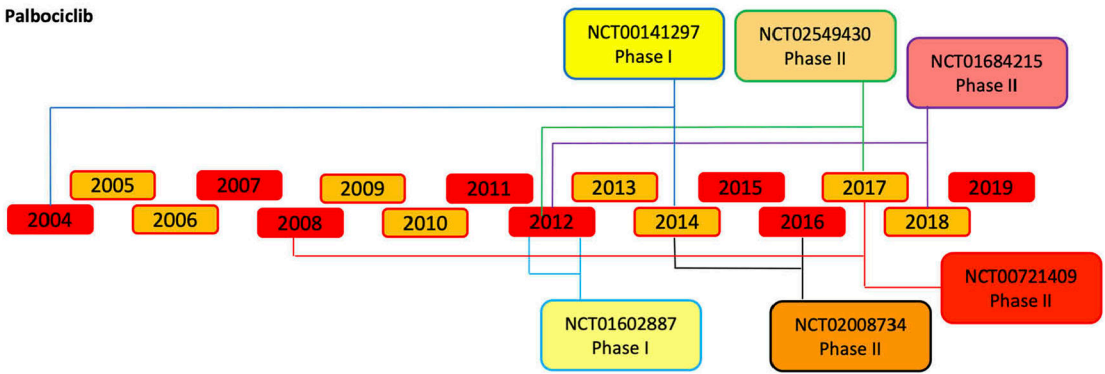
Compound	Chemical structure
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<u>Ribociclib</u> , KISQALI	
<u>Abemaciclib</u> , Verzenio	
G1T38	
G1T28, <u>Trilaciclib</u> , SHR6390	
PF-06873600	

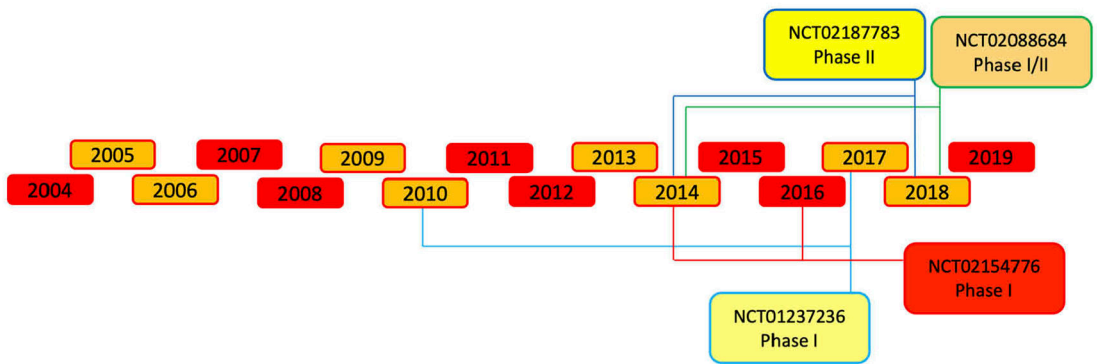
Figure 3.
Specific CDK4/6 inhibitors chemical structure.

a

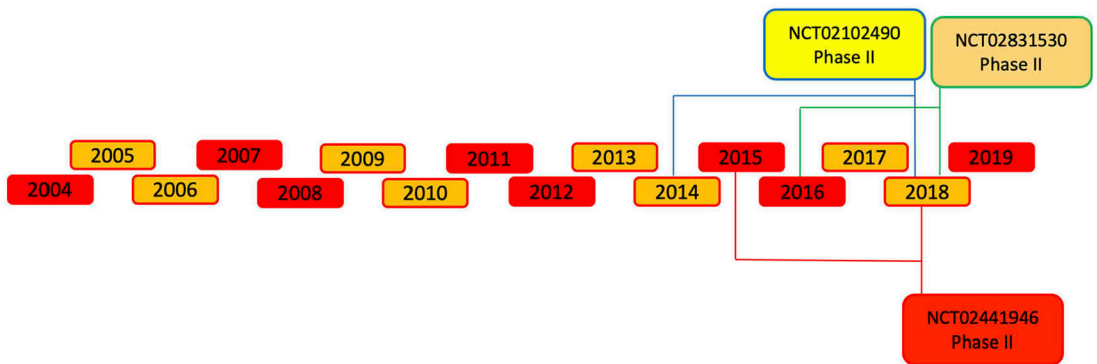
Palbociclib



Ribociclib

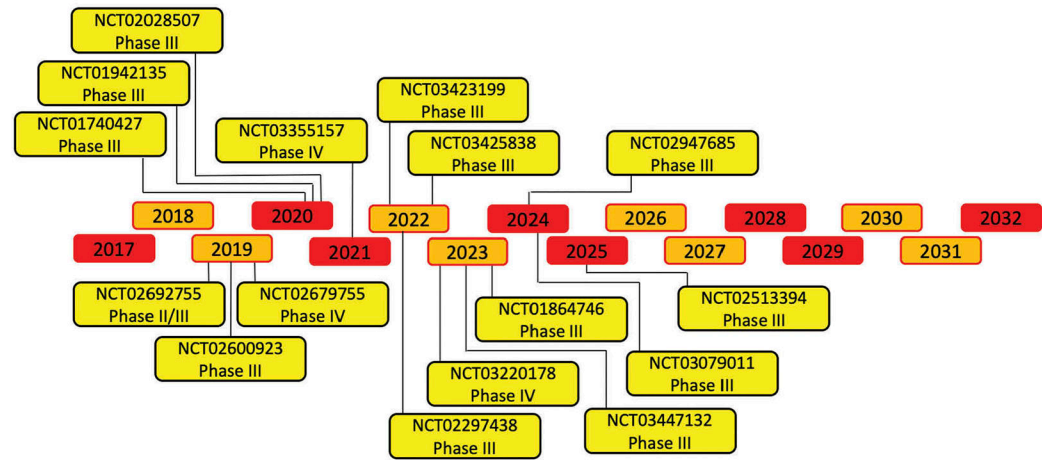


Abemaciclib

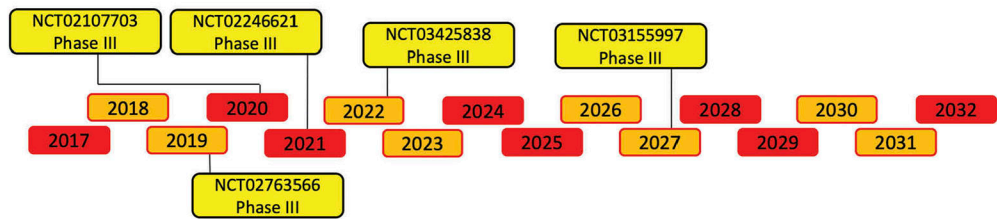


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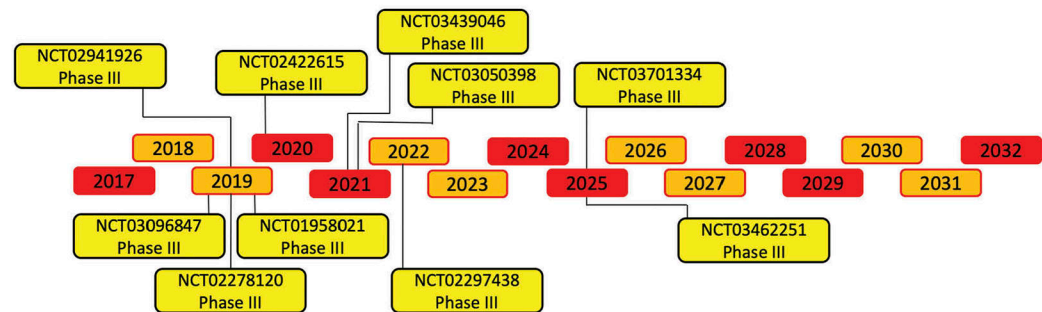
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Abemaciclib



Ribociclib



C

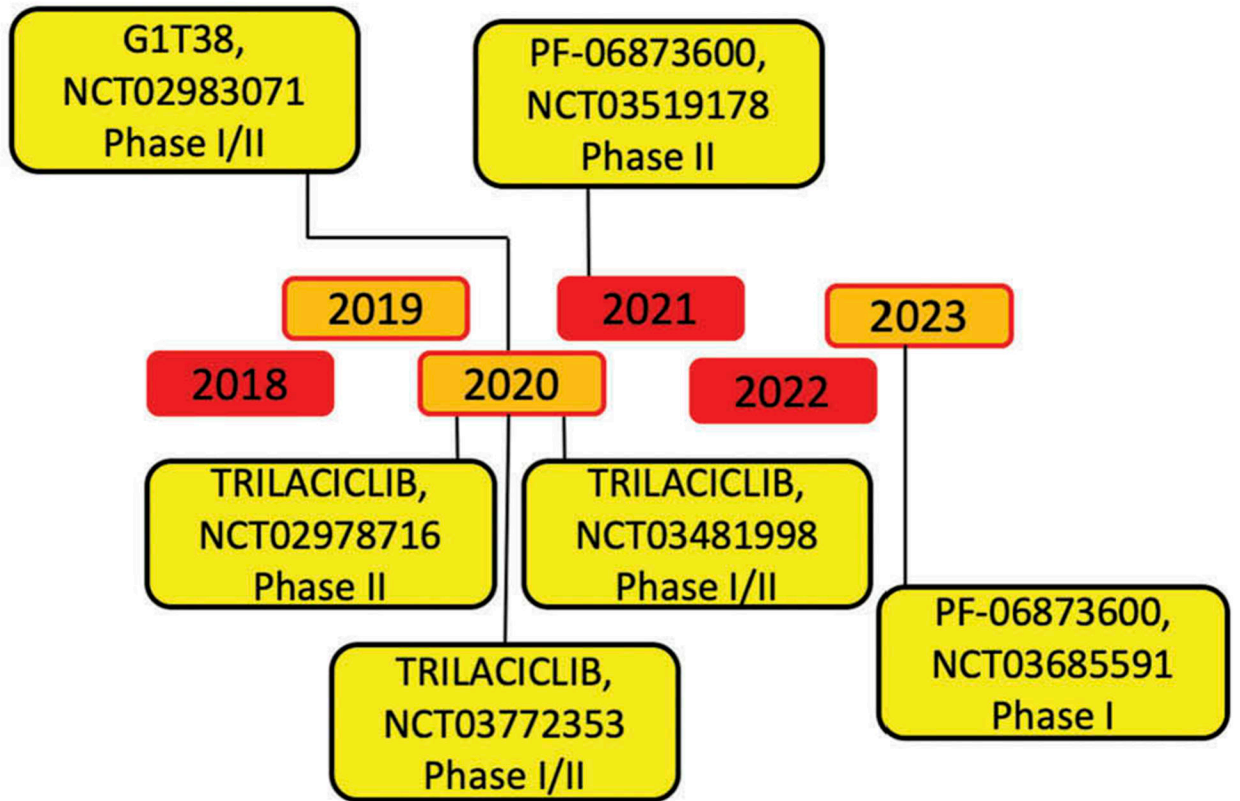


Figure 4.
Timeline of the principal CDK4/6 inhibitors clinical trials.

Table 1.

Completed clinical trials.

CDK Inhibitor	NCT Number	Conditions	Interventions	Phases	Enrollment	Start Date	Comp. Date
Palbociclib		EBrCa	Palbociclib	Phase 2	132	01/01/14	01/01/16
		BrNeo	Palbociclib letrozole	Phase 2	61	10/19/12	10/25/18
		BrCa	Palbociclib Anastrozole Letrozole Exemestane Fulvestrant	Phase 2	115	10/01/12	02/09/17
Ribociclib		Healthy	Palbociclib	Phase 1	24	05/01/12	11/01/12
		BrCa	Palbociclib letrozole	Phase 2	177	09/15/08	12/20/17
		Neo	Palbociclib	Phase 1	74	09/01/04	12/01/14
Abemaciclib		BrCa	Ribociclib BYL719 Fulvestrant BKM120	Phase 1/2	70	05/19/14	04/17/18
		CDK4/6 Ac.	Ribociclib	Phase 2	106	08/25/14	01/17/18
		AST	Ribociclib	Phase 1	156	12/21/10	03/09/17
		MBC	Ribociclib Buparlisib Letrozole	Phase 1	13	06/27/14	10/26/16
Abemaciclib		BrCa	Abemaciclib	Phase 2	101	03/16/16	02/02/18
		MBC	Abemaciclib	Phase 2	132	06/10/14	10/22/18
		BrCa HR+	Abemaciclib Loperamide Anastrozole	Phase 2	224	08/01/15	02/12/18

BrCa: breast cancer; EBrCa: Early breast cancer; MBC: Metastatic breast cancer; CDK4/6 Ac.: CDK4/6 Activated pathway; BrCa ERα⁺: Breast cancer hormone receptor positive.

Table 2.

Ongoing clinical trials per each CDK inhibitor.

CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	ECom. Date
Palbociclib		Phase 2	366	01/07/19	01/07/31
		Phase 2	144	01/01/19	03/01/22
		Phase 1	85	12/08/18	12/01/20
		Phase 2	132	12/06/18	08/01/22
		Not Applicable	100	12/01/18	11/30/20
		Phase 2	42	12/01/18	12/31/19
		Phase 2	40	11/13/18	08/01/20
		Phase 1,Phase 2	62	10/15/18	10/15/25
		Phase 1	165	10/11/18	11/30/21
		Phase 2	24	10/08/18	12/31/21
		Phase 1	100	10/04/18	08/24/23
		Phase 2	76	09/15/18	10/01/22
		Phase 1	54	09/01/18	09/01/23
		Phase 1,Phase 2	54	08/20/18	12/31/19
		Phase 2	88	08/15/18	08/01/24
		Phase 2	100	07/20/18	07/01/22
		Phase 2	54	06/27/18	06/01/22
		Phase 1	57	03/18/18	10/01/20
		Phase 4	260	03/01/18	12/31/21
		Phase 3	180	02/09/18	02/01/22
	Phase 2	160	02/01/18	03/01/22	
	Phase 3	400	01/01/18	09/01/23	
	Phase 1,Phase 2	36	12/20/17	11/01/19	
	Phase 1	150	11/27/17	07/31/21	
	Phase 1,Phase 2	40	11/13/17	10/01/20	
	Phase 3	1050	11/09/17	10/01/22	
	Phase 1	120	10/31/17	10/01/23	

CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	E Com. Date
		Not Applicable	100	10/24/17	10/31/22
		Phase 2	196	10/24/17	10/31/21
		Phase 1,Phase 2	111	09/30/17	10/07/22
		Phase 1,Phase 2	156	09/20/17	10/21/20
		Phase 2	220	08/09/17	12/31/24
		Phase 1,Phase 2	102	08/02/17	08/02/21
		Phase 2	150	08/02/17	07/01/22
		Phase 4	960	07/24/17	12/31/23
		Phase 2	100	07/05/17	03/01/20
		Phase 2	48	06/30/17	11/30/24
		Phase 2	33	06/28/17	03/01/19
		Phase 3	496	06/21/17	08/31/24
		Phase 2	48	06/15/17	06/30/21
		Phase 3	800	03/22/17	04/15/24
		Phase 1	196	12/13/16	02/29/20
		Phase 2	40	10/26/16	10/01/22
		Phase 1	90	10/01/16	12/01/21
		Phase 2	22	09/30/16	02/01/19
		Phase 2	360	09/06/16	12/01/22
		Phase 2,Phase 3	35	09/01/16	12/01/19
		Phase 2	15	09/01/16	11/01/19
		Phase 1,Phase 2	32	08/24/16	07/01/21
		Phase 2	100	08/01/16	01/01/20
		Phase 1	148	06/14/16	10/10/19
		Phase 2	182	06/01/16	06/01/21
		Phase 2	71	06/01/16	06/01/19
		Phase 2	180	05/24/16	11/01/19
		Phase 2	120	05/01/16	01/01/20
		Phase 2	37	05/01/16	null
		Phase 2	33	05/01/16	02/01/20
		Phase 3	130	04/15/16	03/29/19

CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	E Com. Date
		Phase 2	200	04/15/16	06/01/30
		Phase 4	300	03/09/16	12/31/19
		Phase 2	190	02/01/16	12/01/22
		Phase 1	18	01/01/16	03/01/19
		Phase 2	180	12/16/15	02/01/19
		Phase 1,Phase 2	54	12/07/15	08/14/19
		Phase 1,Phase 2	51	11/11/15	11/01/20
		Phase 2	70	10/01/15	08/01/20
		Phase 2	200	10/01/15	12/01/24
		Phase 1	26	09/11/15	01/26/20
		Phase 2	6452	08/12/15	null
		Phase 3	5600	08/01/15	09/01/25
		Phase 2	486	08/01/15	01/01/19
		Phase 1,Phase 2	36	07/01/15	04/01/22
		Phase 2	138	07/01/15	12/01/19
		Phase 2	102	05/01/15	09/01/19
		Phase 3	339	03/23/15	12/02/22
		Phase 2	200	02/01/15	06/01/24
		Phase 2	306	01/01/15	03/01/19
		Phase 2	125	01/01/15	04/01/19
		Phase 2	152	12/29/14	03/28/19
		Phase 3	596	03/01/14	07/01/20
		Phase 1	17	01/24/14	12/01/20
		Phase 2	160	01/01/14	06/01/19
		Phase 3	1250	11/01/13	11/01/23
		Phase 3	521	09/26/13	01/02/20
		Phase 2	87	04/10/13	04/30/25
		Phase 3	666	02/22/13	06/30/20
		Phase 1	20	03/01/11	null
		Phase 2	205	10/01/09	null

Abemaciclib

CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	E Com. Date
		Phase 2	60	11/28/18	02/20/21
		Phase 3	1050	11/09/17	10/01/22
		Phase 1, Phase 2	111	09/30/17	10/07/22
		Phase 2	150	08/02/17	07/01/22
		Phase 3	4580	07/12/17	06/10/27
		Phase 2	37	05/26/17	11/30/24
		Phase 1	148	05/04/17	11/08/21
		Phase 3	450	12/05/16	11/27/19
		Phase 1	100	11/14/16	06/30/21
		Phase 1	163	11/04/16	01/31/19
		Phase 2	225	09/14/16	05/15/19
		Phase 1	215	06/29/16	07/31/20
		Phase 2	225	05/23/16	02/26/21
		Phase 2	247	04/20/15	11/08/19
		Phase 3	493	11/01/14	07/01/21
		Phase 3	669	07/22/14	02/28/20
		Phase 1	198	03/10/14	12/16/20
		Phase 1	12	12/18/13	12/23/18
		Phase 1	130	07/31/12	03/01/21
		Phase 1	220	12/07/09	10/03/19
		Phase 3	4000	12/27/18	12/26/25
		Phase 2	150	10/30/18	12/28/18
		Phase 2	315	08/29/18	10/17/22
		Phase 2	40	06/14/18	06/14/20
		Phase 3	160	05/24/18	06/30/25
		Phase 2	100	05/01/18	02/01/25
		Phase 2	150	03/15/18	10/01/19
		Phase 3	350	02/02/18	10/31/21
		Phase 2	120	12/12/17	02/28/25
		Phase 3	1050	11/09/17	10/01/22
		Phase 1	60	10/18/17	01/01/20

CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	E Com. Date
G1T38		Phase 1 Phase 2	111	09/30/17	10/07/22
		Phase 2	150	08/02/17	07/01/22
		Phase 2	94	07/13/17	05/01/19
		Phase 3	140	06/30/17	05/14/21
		Phase 2	52	06/20/17	03/25/20
		Phase 1 Phase 2	58	03/02/17	09/01/19
		Phase 1	52	01/01/17	11/01/18
		Phase 3	3775	11/29/16	11/22/19
		Phase 3	500	10/24/16	09/30/19
		Phase 1 Phase 2	107	06/14/16	07/31/20
		Phase 1	312	06/14/16	10/30/20
		Phase 1 Phase 2	86	03/09/16	02/01/23
		Phase 2	132	03/01/16	12/01/21
		Phase 1	7	02/15/16	12/01/18
		Phase 2	120	02/01/16	04/01/23
	Trilaciclib		Phase 1	28	01/01/16
		Phase 1	20	11/01/15	11/01/20
		Phase 3	780	06/09/15	02/19/20
		Phase 1	88	02/04/15	10/26/20
		Phase 3	672	11/20/14	09/27/19
		Phase 3	670	12/17/13	07/26/19
		Phase 1	256	10/22/13	12/31/19
		Phase 1	132	09/06/13	09/16/19
		Phase 1 Phase 2	102	01/01/17	12/01/20
		Phase 2	102	02/07/17	02/01/20
PF-06873600		Phase 1 Phase 2	32	12/12/18	11/12/20
		Phase 1 Phase 2	106	03/27/18	12/30/20
		Phase 2	220	03/07/18	12/12/21

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CDK Inhibitor	NCT Number	Phases	Enrollment	Start Date	E.Com. Date
PF-06952229		Phase 1	100	10/04/18	08/24/23