

Targetting Cdk in Cancer: An Overview and New Insights

Gousia Chashoo* and Ajit Kumar Saxena

Cancer Pharmacology Division, Indian Institute of Integrative Medicine, Jammu, India

Abstract

The master regulators, cyclin dependant kinases (CDKs), are the actual driving forces behind the progression of cell cycle in eukaryotic cells. The activity level of these kinases is maintained and controlled by the periodic synthesis and degradation of positive regulators, cyclins, negative regulators, cyclin kinase inhibitors (CKIs) and other reversible phosphorylation events. CDK/cyclin complexes regulate each phase of the cell cycle and the breakdown of this regulation in any phase results in uncontrolled growth and thus tumor formation. If not all, most of the cancers show direct or indirect deregulation of these kinases, therefore targeting CDKs is an important mode to develop new anticancer therapeutics. Promising preclinical data of many compounds led to the entry of a few of these compounds into clinical trials where excellent results have maintained the high hopes and the recent discovery of one of these compounds as a commercially available drug has further enriched this area of research. So far much has been said about these essential targets but there is a need to discuss their role, mechanism, avenues and progress timely for further understanding of CDKs as anticancer drug targets and to learn how best new CDK inhibitors could be put into clinically developed agents.

Keywords: CDKs; Cyclins; Cancer; Apoptosis; Targets; Drug discovery

Abbreviations: CDKs: Cyclin Dependent Kinases; Ckis: Cyclin Kinase Inhibitors; CAK: Cyclin Activating Kinase; PLK1: Polo Like Kinase 1; SCF: Skp1-Cullin-Box; APC/C: Anaphase Promoting Complex/Cyclosome; CDKL: CDK Like; CDC2L: CDC2 Like Kinase; CCRK: Cell Cycle Related Kinase; MPF: Maturation Promoting Factors

Introduction

Progression of cells through four sequential phases of cell cycle namely, G₁, S, G₂ and M phase is tightly controlled and monitored by checkpoints, the enzymatic complexes known as CDKs. Basically, CDKs are serine/threonine kinases consisting of a catalytic subunit (CDK) and a regulatory subunit (Cyclin). Genomic data base has revealed 21 genes encoding CDKs and five additional genes encoding a more distant group of proteins known as CDK like (CDKL) kinases. Generally, CDKs are sub-divided into two main categories including 11 classical CDKs (CDK1-11) and two newly proposed family members (CDK 12-13). Besides this there are some additional proteins whose names are either based on the presence of a cyclin-binding element such as (PFTAIRE and PCTAIRE proteins) or sequence relationship with the original CDKs, such as CDC2L (CDC2-like kinases) and CCRK (Cell cycle-related kinases) [1]. Each phase of cell cycle is regulated by a unique set of CDKs which are in turn positively regulated by onset of particular cyclin partners. So far 25 cyclin box-containing proteins have come into existence. In contrast to CDK protein levels, which remain stable throughout the cell cycle, the levels of activating cyclins differ in different stages of cycle [2]. Literature reveals that there exists a stringent balance between the *de novo* synthesis and targeted degradation of cyclins by the phosphorylation of specific residues, which prompt their recruitment and incorporation into multiprotein destruction complexes (Skp 1-Cullin-F-box protein (SCF) or anaphase-promoting complex/cyclosome (APC/C) and subsequent degradation within the proteasome [3]. The individual gene expression of cyclins is controlled by regulatory elements in their promoters. Besides cyclins, the activation of CDKs requires the presence of CDK-activating kinase (CAK). CAK phosphorylates the catalytic subunits of CDKs at threonine (Thr 160/161) residues and dephosphorylates threonine (Thr 14) and tyrosine (Tyr 15) residues (by CDC25 phosphatase) in the

activation (ATP binding) loop. The phosphorylation of Thr 14/Tyr 15 residues by proteins like Wee1 and Myt1 kinases negatively regulate the CDKs. In addition there are some endogenous protein inhibitors of CDK activity known as cyclin kinase inhibitors (CKIs) including the INK4 group such as p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d} and the CIP/KIP class such as p21^{CIP1/waf1}, p27^{kip1} and p57^{kip2} family members (Figure 1). Among the 13 identified CDKs three interphase CDKs (CDK 2, 4, 6 and their respective cyclins E/A and D) and one mitotic CDK (CDK 1 and cyclin A/B) are directly involved in regulating progression through the cell cycle. The transition through the G₁ phase is driven by CDK4/cyclin D or CDK6/cyclin D complex and into the S phase by CDK2/cyclin E. The transition through the S phase is regulated by CDK2/cyclin A complex and into the G₂/M phase by CDK1/cyclin B [4-7] (Figure 1). CDK3/ cyclin C have been found to play role in exit from cell cycle at G₀ phase [8].

CDKs are responsive to multiple signals (Figure 2). Besides playing important role in cell cycle progression emerging evidences reveal the role of CDKs and their regulatory partners in developmental processes including transcription (CDK7, cyclin H; CDK8, cyclin C; CDK9, cyclin T/K), epigenetic regulation (CDK2, cyclin E/A; CDK4, cyclin D; CDK8, cyclin C), stem cell self-renewal (CDK 1, cyclin A/B; CDK 2, cyclin A/E), proteolytic degradation (CDK2, cyclin E), metabolism (CDK 8, cyclin C), spermatogenesis (CDK 16, cyclin Y), neuronal functions (CDK 5, non cyclin proteins p35 and p39) and DNA damage and repair (CDK9, cyclin K; CDK12, cyclin K) [9]. Deregulated activity of any of these kinases result in alteration in normal cell maintenance and tissue homeostasis in a wide range of processes from embryonic development to tumourigenesis.

*Corresponding author: Gousia Chashoo, Cancer Pharmacology Division, IIM, Canal Road, Jammu, 180001, India, Tel: 09622700653; 09419080583; E-mail: chashoo.gousia@gmail.com

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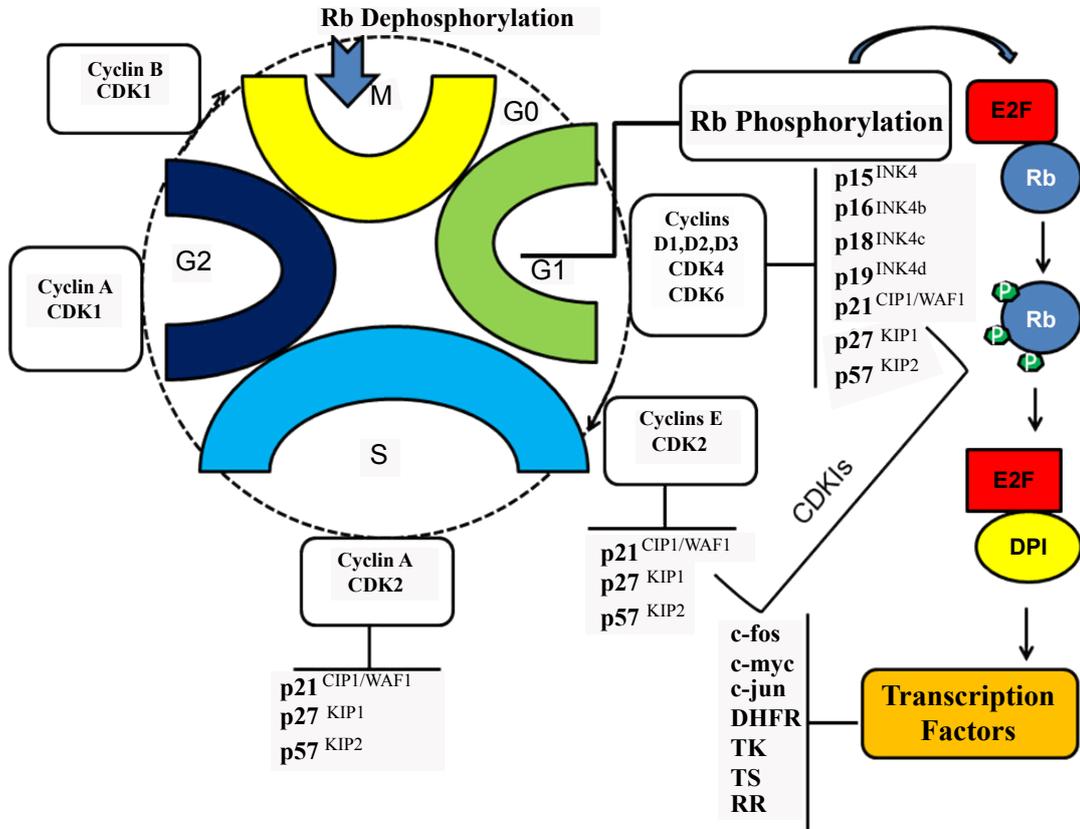


Figure 1: Regulation of cell cycle. Cell cycle is divided into four distinct phases (G1, S, G2, and M). Each phase of the cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs). CDKs are the key regulators of cell cycle which are in turn positively and negatively regulated by cyclins and CKIs, respectively. G₀ represents exit from the cell cycle. The restriction point governs the transition point beyond which progression through the cell cycle is independent of external stimuli. The entry into the synthetic phase i.e. S phase is governed by Retinoblastoma gene product (Rb). Hypophosphorylated Rb forms a complex with a group of transcription factors, E2F. When Rb is inactivated by CDK2-, CDK4, or CDK6-mediated phosphorylation, E2F transcription factors are released, resulting in progression into S phase and transcription of a range of targets involved in chemotherapy sensitivity.

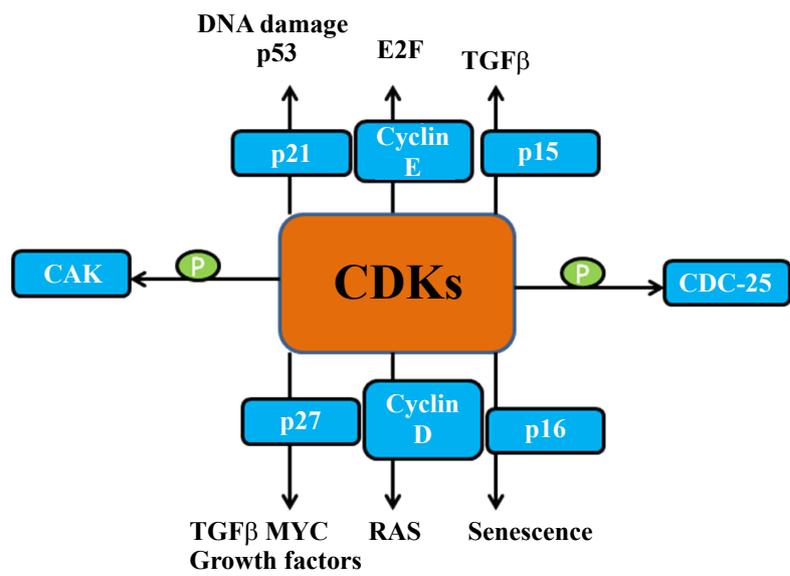


Figure 2: Cyclin dependent kinases are responsive to multiple signals. The genotoxic stresses such as DNA damage leads to the induction of p21^{cp1} through upregulation of p53. TGF-β mediated growth-inhibitory responses act on both p15^{INK4b} or p27^{KIP1}. Cyclin activating kinase (CAK) and phosphatases (CDC25) regulates CDKs via phosphorylation and dephosphorylation respectively. Growth factors and RAS signal CDKs through cyclin D and transcription factors (E2F) through cyclin E. p16^{INK4A} gets upregulated due to cellular ageing or senescence.

The cell cycle checkpoints stringently regulate each phase of cycle before the completion of whole process. Activation of these checkpoints induces cell cycle arrest through modulation of CDK activity which therefore allows the cells to repair most of their defects before their transmission to the resulting daughter cells. In case of excessive DNA damage or genetic defects in the repair machinery, cells either enter the senescence or undergo apoptosis. If howsoever, these genetic defects get accumulated, it leads to the genomic instability and ultimately to cell transformation and oncogenesis [10]. The emerging evidences suggest that constitutive and deregulated CDK activation may contribute not only to unscheduled proliferation that drives tumor cell cycles but also to genomic and chromosomal instability in cancer cells.

Regulation of CDKs

For the ordered execution of processes controlling cell growth, DNA replication and mitotic distribution of chromosomes to daughter cells there is a need for proper regulation of control mechanisms, which is monitored by a series of coordinated and sequential phase transitions of key regulators i.e., CDKs. CDKs are activated at specific points of cell cycle and their activity is tightly controlled by several complex mechanisms [11]. The catalytic activity of CDKs is upregulated primarily by cyclin binding and post-translational phosphorylation of conserved threonine residues by the CAK. The activated CDK-cyclin complex can be inhibited by phosphorylation of a conserved threonine-tyrosine pair or binding to CKIs. CDKs are closely related in size (35-40 KDa) and sequence (>40% identical). The typical CDK catalytic subunit contains a 300 amino acid catalytic core that is completely inactive when monomeric and unphosphorylated. *In silico* studies have revealed CDK2 apoenzyme is held in an inactive state by two major structural restraints: firstly, the substrate binding site is blocked by an extended loop termed the T loop and secondly, side chains in the ATP binding site are oriented so that the ATP phosphates are poorly positioned for efficient phosphate transfer. Cyclins possess a relatively conserved domain of 100 amino acids known as cyclin box, which has been found to be responsible for its potential to bind and activate CDKs [12,13]. Each CDK interacts with a specific subset of cyclins in varying numbers (Figure 1). The function of a particular cyclin is controlled by changes in the cyclin levels, which increase characteristically at specific cell cycle stages and are therefore categorized by the stage at which they are expressed. The levels of cyclin are tightly controlled by transcription and ubiquitin mediated degradation in a temporal manner. The binding of cyclin induces a conformational change in the T-loop, exposing the phosphorylation site (Thr 160/161 residue) on the T loop, setting the stage for the full activation of the kinase by CAK. CAK is a multisubunit enzyme whose catalytic subunit is a highly conserved CDK related protein kinase termed as CDK7 [14-16] and the regulatory subunit is a new cyclin termed cyclin H [16,17]. *In vitro* studies have revealed that, the addition of purified cyclin H to CDK7 reconstitutes the CAK activity, demonstrating that CAK, like its substrate, is a CDK-cyclin complex [16]. A single CAK can activate all the major CDK-cyclin substrates involved in the mammalian cell cycle control. Studies have shown that the phosphorylation of Thr 160/161 tends to rise and fall in parallel with the cyclin binding, revealing that the changes in the phosphorylation are probably not due to changes in CAK activity, but appear to reflect the ability of cyclin binding to stimulate CDK phosphorylation. CAK activity is therefore not rate limiting during normal cell proliferation, although its regulation may be important under some growth conditions. It can therefore be stated that CDK-cyclin complex can be inactivated by either removal of cyclin or dephosphorylation of Thr 160/161 residue. Although these are the two main ways to inactivate CDK-cyclin complex, however it

can also be inhibited by phosphorylation at two sites near the amino terminus (Thr 14 and Tyr 15). The side chains of these residues hang from the ceiling of the ATP-binding site and are certainly in a position to affect kinase activity when phosphorylated [12]. The mechanism of this inhibition is unknown, but it has been observed that the phosphorylation of Tyr 15 does not appear to inhibit ATP binding [18]. Phosphorylation of Thr 14 and Tyr 15 is particularly important in the control of CDK1 activation at mitosis. Like Thr 161, phosphorylation of Thr 14 and Tyr 15 roughly parallels the rise in cyclin B levels that occurs as cells approach mitosis [19,20]. CDK1/cyclin B complexes are thus maintained in an inactive state, until Thr14-Tyr15 dephosphorylation at the end of G₂ activates it. This abrupt dephosphorylation is brought about by the coordinated changes in the activities of kinases and phosphatases acting at these sites. Wee 1/Myt 1 is the major dual specificity kinase capable of phosphorylating both Thr 14 and Tyr 15 residues. Wee 1/Myt 1 activity declines during mitosis, contributing to the fall in inhibitory phosphorylation at this stage. The decreased activity during mitosis is due to the phosphorylation of Wee 1/ Myt 1. The dephosphorylation of both Thr 14 and Tyr 15 residues is carried out by CDC25, a dual specificity phosphatase. In mammalian cells there are three isoforms of CDC25 including CDC25A, CDC25B and CDC25C. All the three isoforms have been found to play a role in cell cycle phase transitions by regulating the activity of CDK1 and CDK2. CDC25A has been found to regulate CDK2/cyclin E and CDK2/cyclin A in G₁/S phase transition while as CDC25B and CDC25C regulate CDK1/cyclin E in G₁/M phase transition. CDC25B and CDC25C have also been found to play role in S-phase entry. A number of kinases including CDK1/cyclin B, Aurora A, Polo-like kinase 1 (PLK1) have been found to be responsible for phosphorylation of CDC25 [21]. Studies have revealed that there exists a positive feedback system as CDK1 stimulates CDC25, which in turn induces the abrupt mitotic dephosphorylation of CDK1. Further CDK1 stimulates the kinase that inactivates Wee1/ Myt 1 and inhibit the phosphatases that inactivates CDC25 and activates Wee1/ Myt 1 [22,23]. Another major mechanism for CDK regulation involves a diverse family of proteins known as CKIs. CKIs can shut down the fully active form of the enzyme. The four major CKIs belong to two classes including, p21 (CIP1/WAF1/CAP20/SDI1) [24,25] and p27 (KIP1) [26], which are related proteins with a preference for CDK2 and CDK4-cyclin complexes, whereas p16^{INK4A} and p15^{INK4B} are closely related CKIs specific for CDK4 and CDK6 cyclin complexes [26,27]. Although the CDK inhibitory mechanism of CKIs is largely unknown however it has been reported that CKIs bind tightly to the Thr160/161-phosphorylated CDK-cyclin complexes and directly inhibit the kinase activity. In many cases (FAR1, p40, p21), CKIs are phosphorylated by their CDK target suggesting an interaction with the protein substrate binding site [28,29]. In case of p21^{waf1}, it has been observed that the major mode of regulation is transcriptional. The transcription of p21 is induced by p53, a transcriptional regulator that mediates cell cycle arrest following DNA damage and in senescence. The p21^{waf1} expression is highly modulated during development under p53- independent control [30]. It has been proposed that the exit from cell cycle during terminal differentiation is mediated by p21^{waf1} in some tissues [30,31]. p21^{waf1} not only inhibits CDKs, but also proliferating cell nuclear antigen (PCNA) or E2F1 transcription factor [32,33]. P15^{INK4B} has also found to be regulated at transcriptional level, as its expression gets enhanced by treatment with negative growth factor TGFβ [27]. Likewise cyclins, CKIs are also regulated by stage specific degradation by the ubiquitin dependent proteolysis machinery.

Regulation of Cell Cycle by CDKs

The progression through cell cycle is a collective effort of the

sequential activation/inactivation of the control factors mentioned above. G_1 -S transition event is carried out by synthesis of D type cyclins (Cyclin D1, D2 and D3) that preferentially bind and activate CDK4 and CDK6 leading to the initiation of DNA synthesis. The central role in this event is taken up by retinoblastoma susceptibility protein, Rb [34]. Normally, Rb protein stays in a hypophosphorylated state in which it interacts with the transcription factor E2F, thereby preventing progression from G_1 to S. Here transcription is repressed by blocking the activation of E2F and recruitment of histone deacetylases to the promoters of the genes required for S-phase entry [35]. Cyclin D synthesized in this phase ensures proper phosphorylation i.e. inactivation of Rb protein which therefore releases E2F that are bound to DPI leading to the formation of transcriptionally active heterodimer E2F-DPI and also sequesters Cip/Kip proteins facilitating the expression of E-type cyclins (E1 and E2) which bind and activate CDK2 to complete the process of DNA synthesis (S-phase) (Figure 1). Progression of G_1 phase is also regulated by members of INK4 family which specifically inhibit CDKs 4 and 6. Accumulation of p16^{INK4a} induces G_1 arrest as it gets associated with CDKs 4/6 and releases D-type cyclins. The release of D-cyclins and association of CIP/KIP proteins with CDK2 culminates into G_1 arrest. Cyclin E-cdk2 allows the activation and transcription of genes necessary for S-phase entry and progression by further phosphorylating Rb and thus disrupting the binding of Rb to E2F. If howsoever the CDK activity gets inhibited during S phase, E2F remains there persistently leading to the S-phase delay and thus apoptosis. Here the induction of apoptosis has been found to occur via both p53-dependent and p53-independent mechanisms [36]. The completion of S phase is marked by the duplication of cell structures and separation of the chromosomes which is achieved by the activation of another checkpoint at the onset of G_2 before the initiation of mitosis. CDK 1 (CDC2) in complex with cyclin B are the key components of this checkpoint as it controls the centrosome cycle as well as mitotic onset (Figure 1). It has been found that the active CDK 1 cyclin complexes phosphorylate more than 70 substrates during G_2 and early mitosis which bring about the centrosome duplication, spindle assembly, chromosome condensation and so on. Collectively, the proteins involved in this checkpoint are said to be mitosis promoting factor (MPF), the activity of which initiates mitosis. Here the inhibition of CDK1 in the early mitotic phase result in cell cycle arrest in G_2 and the inhibition during mitosis results in exit from mitosis without cytokinesis [37]. As seen above, throughout the cell cycle, different cyclin proteins at different point of the cycle get rapidly degraded resulting in the loss of CDK activity. It is this loss of CDK activity which allows the transit from one phase of the cell cycle to the next. The obvious importance of CDKs in facilitating cell cycle progression serves CDK inhibition as a cell cycle checkpoint control mechanism.

CDKs in Cancer

Because of the frequent perturbations in human malignancy and the observation that cell cycle arrest by CDK inhibition could induce apoptosis, targeting CDKs is a major concern for anticancer therapy. It has been well defined that, in contrast to normal cells, tumor cells are unable to stop at predetermined points of the cell cycle because of the loss of checkpoint integrity, which in turn can be due to the inactivation of certain CDKs, or to overexpression of CDKs and cyclins (Figure 3).

Interphase CDKs

Interphase CDKs (mostly CDK 4 and CDK 6) and their regulators have frequently been found to be mutated in human cancers (Figure 3) [1,38,39]. CDK4 has been found to be altered in a small set of melanoma

patients by a miscoding mutation (Arg24Cys) that blocks binding of INK4 inhibitors. CDK6 is known to get overexpressed in some leukemias as a consequence of nearby translocations. Cdk4 and Cdk6 are also amplified or overexpressed in several malignancies (including sarcoma, glioma, breast tumours, lymphoma and melanoma). Even though we are well aware of the alteration of these CDKs in different malignancies, however the casual role of these alterations in tumor development is still difficult to assess. It has been found that CDK4 is co-amplified with Mdm2 in most of the tumors [1]. In certain other cases misregulation of D-type cyclins and INK4 inhibitors has been a common feature [38,39]. These observations reveal that CDK4 and CDK6 kinases are hyperactive in human cancer with preference for CDK6 in mesenchymal tumours (leukemias and sarcomas), and CDK4 in epithelial malignancies (in endocrine tissues and mucosae) and in some sarcomas. Although CDK2 has not been found to be frequently mutated in human cancer. However, the overexpression of E-type cyclins and frequent silencing of p21 and p27 inhibitors during tumour development suggests a potential involvement of CDK2 in human cancer [38].

Experimental evidence indicates that there is a selective dependence on interphase CDKs as far as human cell lines are concerned. For instance, colon carcinoma cell lines have been found to efficiently proliferate in the absence of CDK2, however, there occurs an inhibition in the proliferation of glioblastomas and osteosarcomas cell lines once this kinase is inhibited or downregulated [40,41]. Another observation in mice shows that, although the proliferation of brain or connective tissue is independent of CDK2, the neoplastic process in these cell line demands the requirement of this kinase. Investigations using gene targeted mouse tumor models have shown the development of skin tumors in Cdk4-null mice induced by Myc. No, such tumor formation has been observed in their wild counterparts [42]. Further, Cdk4-deficient mice have been found to be resistant to mammary tumors expressing Erbb2 and Hras under the control of the mouse mammary tumour virus promoter [43] as such the expression of CDK 4 is not essential for the development of mammary glands. Similarly, mice lacking cyclin D1 or expressing a cyclin D1 mutant that does not activate CDK4 are resistant to breast tumours induced by ErBB2 [44,45]. However, lack of cyclin D1 has no effect on breast tumour development induced by Myc or Wnt1 [45]. These observations indicate that active CDK4-cyclin D1 complexes are required for skin or breast tumour development, depending on the nature of the oncogenic insult. Thus, CDK4 inhibition by small molecules may have therapeutic value in treating ErBB2-positive breast tumours [46]. Similar reports have emerged that an immediate senescence is observed in lung cells expressing endogenous K-Ras oncogene by inhibiting CDK 4 without altering the expression of CDK2 or CDK6 [47], suggesting that a robust and selective pharmacological inhibition of Cdk4 may provide therapeutic benefit for NSCLC patients carrying K-RAS oncogenes. In spite of all these excellent reports, the question whether CDK inhibition could have therapeutic value in the treatment of selective malignancies based on their acquired and/or innate dependency of interphase CDKs still persists and there exists an interesting possibility that deserves to be explored.

Mitotic CDKs

CDK1, in complex with A or B type cyclins, is one of the master regulators of mitosis. The loss of function of CDK1 has been found to be associated with human lung cancers [48]. Overexpression of CDK1 has been observed in ovarian cancers [49]. In a case study CDK1 has been found to be overexpressed in patients suffering from Oral squamous

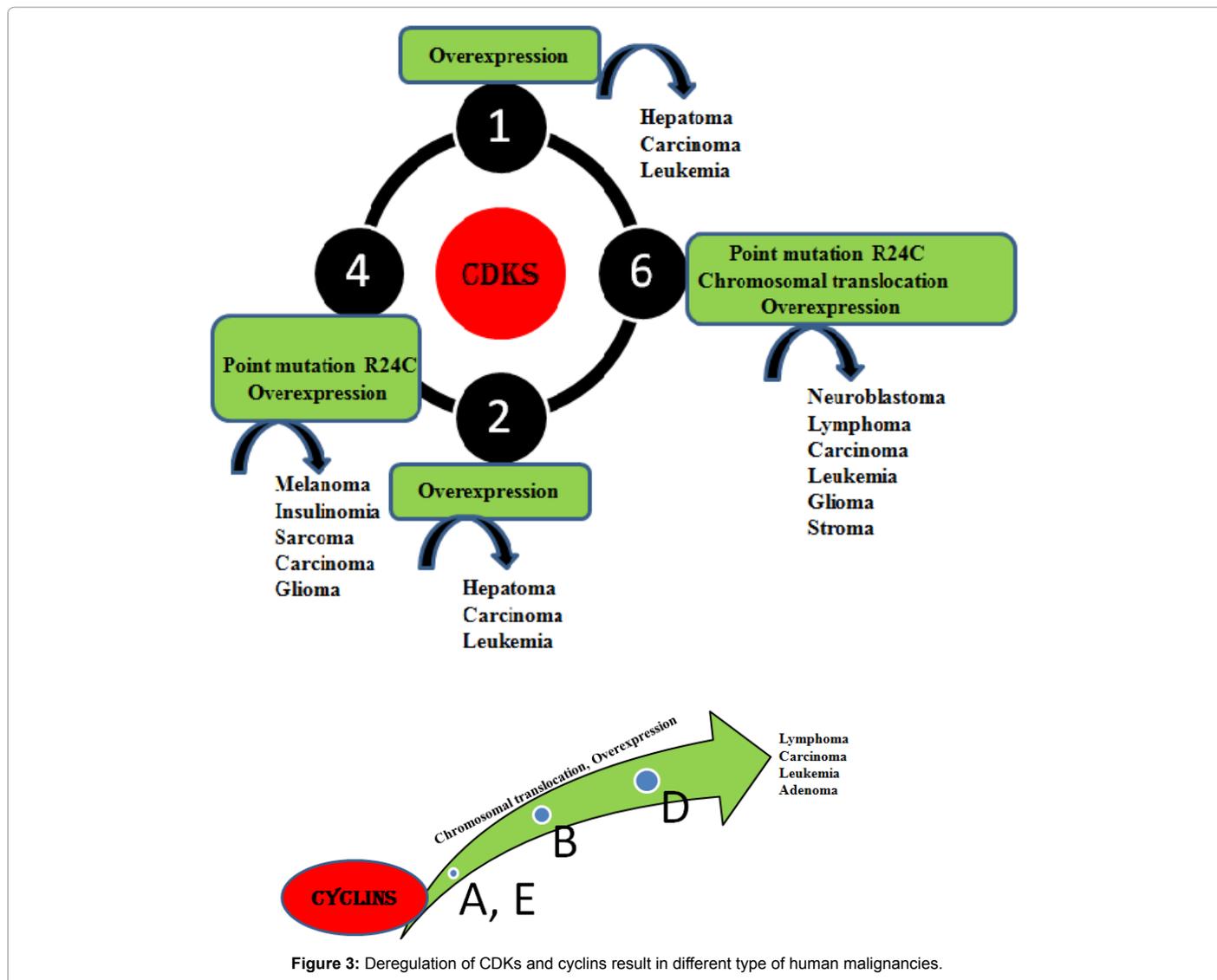


Figure 3: Deregulation of CDKs and cyclins result in different type of human malignancies.

cell carcinoma [50]. Studies have shown that CDK1 inhibition represents a plausible strategy for expanding the utility of PARP inhibitors to BRCA-proficient breast cancers [51]. Phosphorylation of Ezh2 (enhancer of Zeste 2), an H3K27 histone methyl transferase by CDK1 leads to enhanced cellular proliferation in various human cancers [52]. CDK1 plays an important role in enhancing cellular proliferation by influencing genetic network of cell cycle (e.g. p53, p21, p16, p27 and so on). Targeting CDK1 by potential inhibitors, but preventing the detrimental side effects resulting from unintentionally interfering with the essential functions of Cdk1 in proliferative tissues may aid in development of more efficacious chemotherapy. Besides CDK1, other kinases namely Polo like kinases (Plks), Aurora and Nek kinases play crucial roles in regulating the centrosome cycle and formation of the mitotic spindle [53,54]. Overexpression of the genes encoding these kinases correlates with poor clinical outcome in tumors with chromosomal instability [55,56].

CDK Inhibitors in Cancer Therapy

CDK activity is needed for the cell division cycle and the tumors hyperactivate CDKs. CDKs have therefore long back been proposed

as good targets. However, the importance of CDKs in normal cellular growth may underlie the observed narrow therapeutic window. The drug discovery and lead optimisation efforts have provided a wealth of potential drug candidate molecules capable of inhibiting CDKs over the last decade, however, uptill now only few CDK inhibitors have been approved for commercial use. Among the panel of inhibitors Flavopiridol (NSC 649890, L86-8275 or HMR 1275) a semisynthetic small molecular derivative of rohitukine, an alkaloid isolated from *dysoxylum binectariferum* is the first CDK inhibitor to undergo clinical evaluation in humans. It is considered as a first generation CDK inhibitor capable of inhibiting most of the CDKs (pan-CDK Inhibitor). Flavopiridol has been found to inhibit CDK1/cyclin B (IC₅₀, 30-40 nM), CDK2/cyclin A, CDK2/cyclin E (IC₅₀, 100 nM), CDK4/cyclin D (IC₅₀, 20-40 nM), CDK6/cyclin D (IC₅₀, 60 nM) and CDK7/cyclin H (IC₅₀, 110-300 nM) [57]. Flavopiridol inhibits activity of most of the CDKs by directly occupying the ATP binding site. Inhibition of CDKs 1, 2 and 4 by flavopiridol has been found to directly arrest cell cycle at the G₁/S and G₂/M phase transitions, and also leads to delay in S phase progression [58,59]. Further, literature reveals that tumour cells lacking CDK4, show G₁ arrest by inhibiting CDK6 after treatment with flavopiridol [60], suggesting that the patterns of flavopiridol induced

cell-cycle arrest (G_1/S and/or G_2/M arrest) appear to be cell type-specific. Several phase I clinical trials have shown that flavopiridol as single agent has an antitumor effect in patients with renal, prostate, colon, metastatic gastric cancer and non-Hodgkin's lymphoma [60-63]. Some previous and very recent studies have demonstrated Flavopiridol treatment as an active therapeutic approach for the treatment of refractory or relapsed chronic lymphocytic leukemia [64,65]. In spite of all these successful preclinical stories, clinical efficacy of Flavopiridol has been found to be limited due to many adverse effects like, secretory diarrhea, neutropenia, nausea, vomiting and pro inflammatory syndrome [60-63]. Besides flavopiridol other first generation CDK inhibitors include Olomoucine, Roscovitine (CY-202), R- Roscovitine (Seliciclib), Kenpaullone (NSC 664704, 9-Bromopaullone), SNS-032 (BMS-387032), AT7519, AG-024322, (S)-Roscovitine, R547 (Ro-4584820) [66,67]. These inhibitors target different series of CDKs and are commercially available. Based on their preclinical studies, these have either not entered the clinics e.g. Olomoucine and Kenpaullone or have failed in the clinical trials due to adverse effects like nausea, vomiting, asthenia and hypokalemia (in case of Roscovitine) [68,69], myelosuppression (in case of SNS-032) [70], fatigue and mucositis (in case of AT7519) [71] and some other reasons like inability of the compound to effectively discriminate from other treatment modalities as in case of AG-024322 [72]. Besides all these failures most of these compounds are actively used as research tools.

Unlike first generation CDKIs, second generation CDKIs are more selective and possess more potent activity against their targets. The second generation inhibitors include Fascaplysin (CDK4), Ryuvudine (CDK4), Purvalanol A (CDK2), NU2058 (CDK2), BML-259 (CDK5), SU 9516 (CDK2), PD-0332991 (CDK4 and CDK6), P276-00 (CDK2), AT7519M (CDK1, CDK2, CDK4 and CDK5), BAY 1000394 (CDK1, CDK2, CDK3, CDK4, CDK7 and CDK9) [73]. Most of these inhibitors except some are used for research purpose and have not entered the clinics yet. PD-0332991 (Palbociclib) an oral and selective inhibitor of CDK4 and CDK6 has undergone several phase I/II clinical studies for advanced solid tumors (excluding SCLC and retinoblastoma) or follicular and diffuse large-cell non-Hodgkin's lymphoma [74]. Thrombocytopenia and neutropenia have been observed to be the most common adverse effects. Recently PD-0332991 has received US FDA for potential treatment of patients with oestrogen receptor (ER)-positive breast cancer [75]. Phase I/II studies of P276-00 along with radiation therapy have been done for head and neck cancers, fatigue, hypotension, nausea, sweating and dry mouth were the major adverse effects being observed [76]. AT7519M is currently in Phase II clinical study for relapsed and/or refractory chronic lymphocytic leukemia [77]. BAY 1000394 is currently in Phase-I clinical trials for advanced malignancies [78]. MK-7965 (dinaciclib) CDK2, CDK5 and CDK9 inhibitor is in Phase II trials for acute lymphoblastic leukemia, acute myeloid leukemia, breast cancer, melanoma and non-small cell lung carcinoma [79]. Dinaciclib has advanced to Phase III clinical trials for the treatment of refractory chronic lymphocytic leukemia (CLL) [80]. Two new inhibitors LEE 011 and LY2835219, CDK 4/6 specific inhibitors have entered clinics after showing robust anti-tumor activity. LEE 011 has entered phase III clinical trials for breast cancer and LY2835219 (Abemaciclib) is in phase II trials for mantle cell lymphoma [79]. CDK inhibitors continue to hold much promise as a new modality in the treatment of cancer. The development of CDK inhibitors has been complicated by a lack of efficacy in solid tumors, toxicity issues and challenges with dosing schedules. A lack of good biomarkers to predict the response of tumors to CDK inhibitors is also thought to contribute to their failures so far.

Most of the inhibitors mentioned above are ATP competitive CDK inhibitors. The major shortcoming of these inhibitors is lack of selectivity and toxicity due to their high homology with ATP binding sites on CDKs. In order to develop more specific inhibitors different approaches including the identification of small molecules and peptides that can mimic endogenous CDK inhibitors such as P21, P27, PRB family i.e they can bind to the CDKs *via* protein-protein interactions need to be developed [81,82]. The preclinical optimization of many of these inhibitors is going on and some of them are showing good results in *in vitro* studies against human cancer lines e.g. 3-Amino thioacridine (3 ATA) has been found to inhibit cancer cell proliferation in Osteosarcoma, esophageal carcinoma, mesothelioma and head and neck squamous carcinoma by inhibiting the activity of CDK4/cyclin D in an ATP non-competitive manner [83]. SU9516 and Compound 1 are other ATP non-competitive inhibitors of CDK4/cyclin D and are active against colon carcinoma [84] and melanoma [85] respectively. Spa 310, NBI 1, Peptide C4 and CYC103 inhibit CDK2/cyclin A non-competitively where in Spa10 showed antiproliferative potential against human lung alveolar adenocarcinoma [86], NBI 1 against colorectal, colon, adenocarcinoma, glioblastoma and ovarian carcinoma [87] and Peptide C4 was active against breast cancer, leukemia and hepatocellular carcinoma [88]. The antiproliferative activity of CYC103 is still unknown. Some other ATP non-competitive inhibitors include p21 and p107 derived peptides. These have been found to inhibit CDK2/CyclinA, CDK2/CyclinE and CDK4/CyclinD in a non-competitive manner, but the antiproliferative activity in cancer cell types is still unknown. In spite of all these known facts, the development of such ATP non-competitive inhibitors is itself a challenge [82] and this can be confirmed by the fact that till date none of such inhibitors have entered the clinical trials.

Combination Therapy

The results from the first clinical trials investigating the utility of CDK inhibitors in combination with existing chemotherapy permit a cautiously optimistic outlook. This is further enhanced by a plethora of biological mechanistic indications why CDK inhibitors would be expected to synergise with various chemotherapy agents in tumor cell killing. CDK inhibitors improve the efficacy of chemotherapy. Most of chemotherapy drugs have been found to work during S/G_2 phase, so arresting cells in this phase with CDK1/CDK2 inhibitors (e.g. dinaciclib) may lead to greater cytotoxic effects. However CDK4/CDK6 inhibitors (e.g. PD0332991) should not be used in combination with chemotherapy, as the cells will be arrested in the G_1 , and then not remain sensitive to chemotherapy that selectively kills dividing cells in the S or G_2/M phase. These inhibitors can rather be used in combination with targeted agents for example inhibitors of HER2, mTOR and so on.

Sequential Phase I clinical study of paclitaxel and flavopiridol in esophagus, lung and prostate cancer patients revealed a comparatively better clinical activity than paclitaxel alone [89]. Besides this, flavopiridol in combination with many other known anticancer chemotherapeutic agents like docetaxel, gemcitabine, irinotecan, vorinostat, oxaliplatin, Xurouracil/leucovorin, paclitaxel, carboplatin, 1-beta-D-arabinofuranosylcytosine, mitoxantrone and cytosine arabinoside [90-97] had shown promising results in phase I clinical trials. Many of these combinations e.g. flavopiridol and docetaxel for pancreatic cancer however, failed in Phase II study [98]. Preclinical models have demonstrated synergistic activity of UCN-01 with a number of cytotoxic drugs, more often with topoisomerase inhibiting agents. Several phase I studies have been conducted with UCN-01 in combination with cytotoxic chemotherapy [99]. UCN-01 has been

evaluated in combination with topotecan in relapsed ovarian cancer, demonstrating no significant clinical activity [100]. A phase II study of UCN-01 in combination with irinotecan has also been carried out in patients with metastatic triple negative breast cancer, the clinical activity was however found to be unimpressive [101]. Phase II clinical studies of PD-0332991 with aromatase inhibitor letrozole in ER-positive breast cancer has been found to show encouraging results compared to the letrozole alone [102]. A phase II study of AT7519M in combination with Bortezomib in patients with previously treated multiple myeloma is being carried out [103]. LEE 011 in combination with letrozole is undergoing phase III clinical study (namely MONALEESA-2) among women with ER-positive, HER-2 negative advanced breast cancer [104].

Outcomes and what next

Tremendous research from almost a decade has come out with an optimistic outlook over CDKs as cancer targets. Recent U.S. FDA approval of Palbociclib a CDK 4/6 inhibitor for breast cancer treatment has increased the enthusiasm of many research groups worldwide for evaluating more and more CDK inhibitors in pre-clinical and clinical studies. Although, from therapeutic point of view there is a considerable progress in this hot area of research, yet there is much more to explore. Many questions are still unanswered. What are the actual consequences that drive cells to undergo cell cycle from a quiescent state? What type of role such events play in tumor formation? What is the role of interphase CDKs in maintaining progenitor cell and thus immortal cancer cells? Whether the alteration in the expression of CDKs and their regulators play a crucial role in determining the proliferation status and thus affect the cell reprogramming efficiency? CDKs are important targets in cancer research and much of the work and discussions is essential in this context for exploring their utilities in cancer therapy.

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