

An overview on the prospective CDKs inhibitors as anti-cancer drugs: Review article

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Abstract: CDKs are members of serine/threonine kinase family and key enzymes in cell cycle progression and transcription and other major biological processes including neural differentiation and metabolism. Constitutive or deregulated hyperactivity of these kinases due to amplification, over expression or mutation of cyclins or CDK, contributes to proliferation of cancer cells, and irregular activity of these kinases has been reported in a wide variety of human cancers. Therefore, CDKs considered as attractive pharmacological targets for development of anticancer therapeutics. This review first summarizes the different functions of CDKs and the biological structure of CDKs, cell cycle and their role in cell cycle regulation. Then it focuses on CDKs inhibitors as anticancer and the different strategies in developing it, CDK2 inhibitors that inter clinical trials and CDK4/6 inhibitors that in the way of approval have been discussed. Future perspective of CDKs inhibitors as anticancer will be defined in the development of new target CDK1 and CDK9 inhibitors.

[Khulood H. Oudah, Nermin S. Abdou, Rabah A. T. Serya and Khaled A. M. Abouzid. **An overview on the prospective CDKs inhibitors as anti-cancer drugs: Review article.** *J Am Sci* 2017;13(4):6-23]. ISSN 1545-1003 (print); ISSN 2375-7264 (online). <http://www.jofamericanscience.org>. 2. doi:[10.7537/marsjas130417.02](https://doi.org/10.7537/marsjas130417.02).

Key words: CDKs, cell cycle, kinase inhibitors, CDK2, clinical trial, ATP competitive inhibitors, peptide, allosteric, CDK4/6 inhibitors, transcription, CDK1, CDK9.

1. Introduction

Cancer is a life threatening disease with remarkable impact on health (1). The severity of this disease can be estimated with the statistics that it is responsible for causing 12% of total deaths worldwide (2). There were many pieces of evidence to support that the inhibition of CDKs could play vital role in suppressing cancer (3). Frequent misregulation of CDKs in cancerous cell has made them to be striking targets for cancer therapy (4). The human protein kinases set (kinome), is constituted of 518 identified proteins, divided in seven families (5). Cyclin-Dependent Kinases (CDKs) are part of the CMGC family named after the members: Cyclin-dependent kinases (CDKs), Mitogen-activated protein kinases (MAPKs), Glycogensynthase kinases (GSKs) and CDK-like kinases (CLKs). For their discovery, Hartwell, Nurse and Hunt received the Nobel Prize in 2001 (6). The number of CDKs increased during development and was marked by a greater expansion of the cell-cycle related group; human cells have 20 CDKs and 29 cyclins (7). The crucial role of CDKs is that control of cell cycle, in spite of that only several of them have been shown to have direct role in the cell cycle progression (8). CDK1 is the ancestral mitotic kinase, and CDK2, CDK4 and CDK6 regulate progression through inter phase. CDK5 does not seem to regulate the cell cycle, but instead controls the development and function of neurons. CDK7, CDK8 and CDK9 regulate RNA polymerase II (RNA pol II) – dependent transcriptional initiation and elongation, and CDK11 is involved in mRNA splicing. The

function of CDK3, CDK10, CDK12 to CDK20 are not well known yet, but additional functions of these proteins in gene transcription (9), DNA damage repair (10), cell death (11), cell differentiation (12), metabolism (13), neural differentiation (14), development (15), and immune response (16) were investigated.

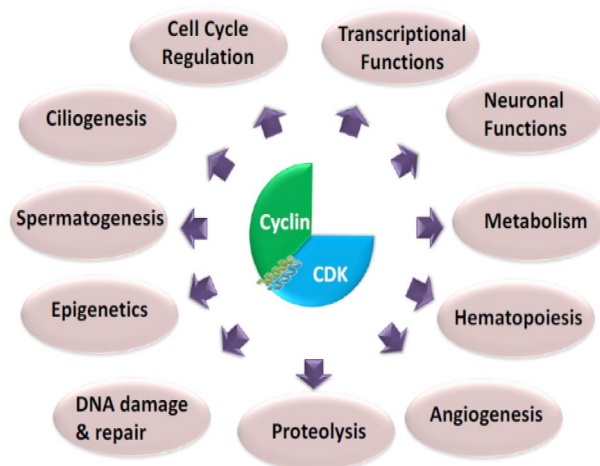


Figure 1. Functional diversity of Cyclin-dependent Kinases (16)

2. Structure and regulation of CDKs/Cyclins

In mammalian cells, monomeric CDKs are inactive and require association with a regulatory cyclin subunit to acquire a stable and active conformation (17). The spatiotemporal pattern of

expression and degradation of cyclins, named after their cyclical pattern of synthesis and degradation, together with their structural and molecular features, dictates their ability and availability to interact with a CDK, and thereby determines the orderly formation of different CDK/Cyclin complexes throughout the cell cycle or in specific functional pathways(18). Generally the amino acid of CDKs are highly conserved, taking CDK2 as a representative since its structure has been widely characterized, it shares 64%, 45%, 40%, 45% and 43% sequence identity with CDK1, CDK4, CDK5, CDK6, and CDK10, respectively(19,20). The structure consist of two lobes a small N-terminal lobe rich in β -sheet (N lobe), a large C-terminal lobe rich in α -helix (C lobe), and a deep cleft at the junction of the two lobes that is the site of ATP binding and catalysis (hinge region), also the N-lobe contains a glycine-rich inhibitory element (G-loop).

In the monomeric CDK2 structure, two regions differed from the canonical kinase structure and were predicted to function as regulatory elements. One is an

α -helix, present in other protein kinases, but having the unique sequences PSTAIRE only in cyclin dependent kinases; the other is the activation segment (T-loop), lies between two conserved motifs, D142FG and A173PE, in the C terminal lobe to contain the substrate binding site for the phosphorylation of the residue Thr 160 (21) (Figure 2) CDK2 monomeric is inactive and the binding with cyclin activates it,(22) cyclin interact directly with PSTAIRE helix segment and the T-loop from other side and parts of N and C lobes to interlock with them by van der Waals forces, hydrogen bonds, hydrophobic and polar interactions. Cyclin binding to a CDK induces major conformational change in the kinase subunit, the cyclin move the PSTAIRE helix into the catalytic cleft and rotate it by 90° , which in turn bring the glutamic acid side chain (Glu 51 carried on PSTAIRE helix) inside the catalytic cleft, where together with lysine residue, an aspartic acid residue and a magnesium ion it coordinate the ATP phosphate atoms and correctly orient them for catalysis (23).

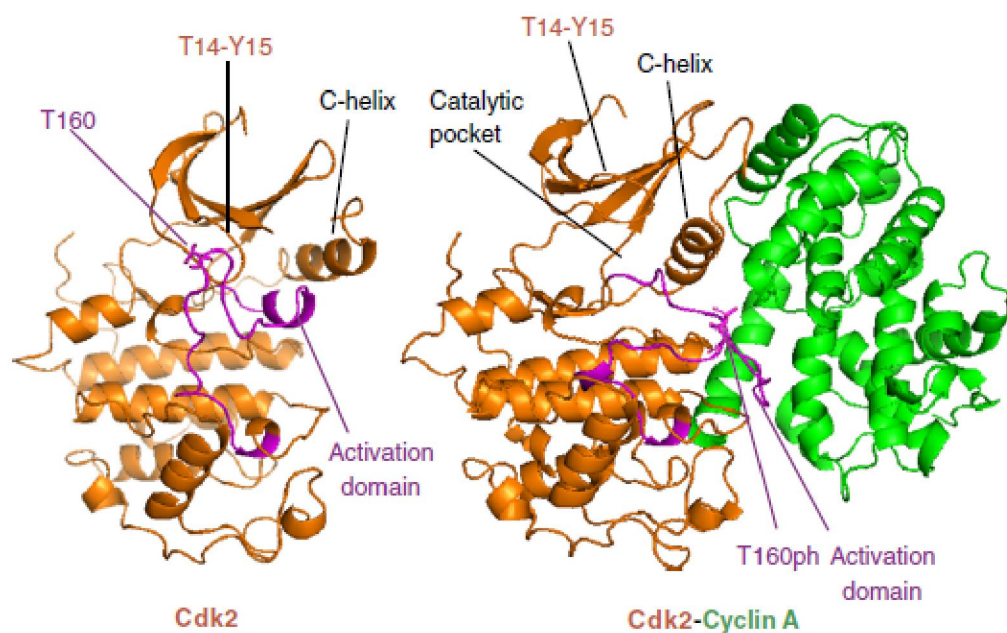


Figure 2. Structural biology of cyclin-dependent kinase and cyclin (take CDK2 and CDK2–cyclin A complex as examples). Left: Monomeric CDK2 (PDB code: 1HCL). Right: The complex between CDK2 and cyclin A (PDB code: 1JST) (23).

Furthermore, cyclin induce conformational switch of the activation segment (T-loop) of CDK2, thereby rendering the substrate binding cleft more accessible and exposed this loop for phosphorylation by the CDK-Activating Kinase CAK. When the T-loop becomes phosphorylated on Thr 160, it undergoes an additional conformational change which further stabilizes the T-loop, yielding a fully accessible substrate-binding site, and thereby fully

activation of enzyme (24, 25). The glycine-rich region (G-loop) in the N-lobe is an additional regulatory region as it contains residues (Thr14 and Tyr15) whose phosphorylation inhibits kinase activity. Phosphorylation of Thr14 and/or Tyr15 residues by Wee1 and Myt1 kinases inhibits several family members, preventing cell-cycle progression, for instance, in response to DNA damage (26). It has been confirmed that some delicate structural divergences

between CDKs occur in a few key regions such as loops β 3- α C, β 4- β 5, activation segment and N-terminal regions to ensure the recognition of specific cyclin and substrate (26). Additionally, recent structural dissection and topology analysis of CDKs have dissected the differences in CDKs ATP pocket, taking advantage of which could increase the inhibitory potency for a specific CDK (25, 27).

3. Role of Cyclin-Dependent Kinases in cell cycle

The cell cycle is a highly structured and regulated system, comprising of multiple regulatory, catalytic, and inhibitory proteins that act to direct normal mammalian cell proliferation and differentiation (28). It is not surprising, therefore, that the mechanisms that control normal cell division are frequently altered in many diseases, and aberrant cell-cycle control is a hallmark of most cancers (29). Cell division is divided into two distinct stages, mitosis (M), in which the cell prepares for and undergoes cell division (30), and interphase which is further divided into three subphases, G1, S, and G2 (Figure 3). All

phases of the cell cycle are controlled primarily through the cyclic expression of the regulatory cyclins and their catalytic partners, the CDKs, and inhibited by the CDK inhibitors (CKIs) (29, 31). Mitogenic signals stimulate the synthesis of D-type cyclins and activated CDK4/6 associates with the D-type cyclins (D1, D2, and D3) to regulate cycle progression in the G1 phase by phosphorylation of the tumor suppressor retinoblastoma (RB) (32). Partially phosphorylated (RB) preserves binding to the transcription factor E2F-DP which control the expression of genes including cyclin E gene. Similarly, the cyclin E/CDK2 complex regulates the late G1 phase and the induction of DNA synthesis in early S phase by further phosphorylation of RB, thereby completely releasing E2F-DP complex to transcribe genes required for G1-S transition (33). CDK2 also associates with cyclin A to control proper DNA replication and synthesis in the S phase (34). As the cell-cycle progresses, cyclin A then associates with CDK1 to promote cell entry into the M phase (35). All

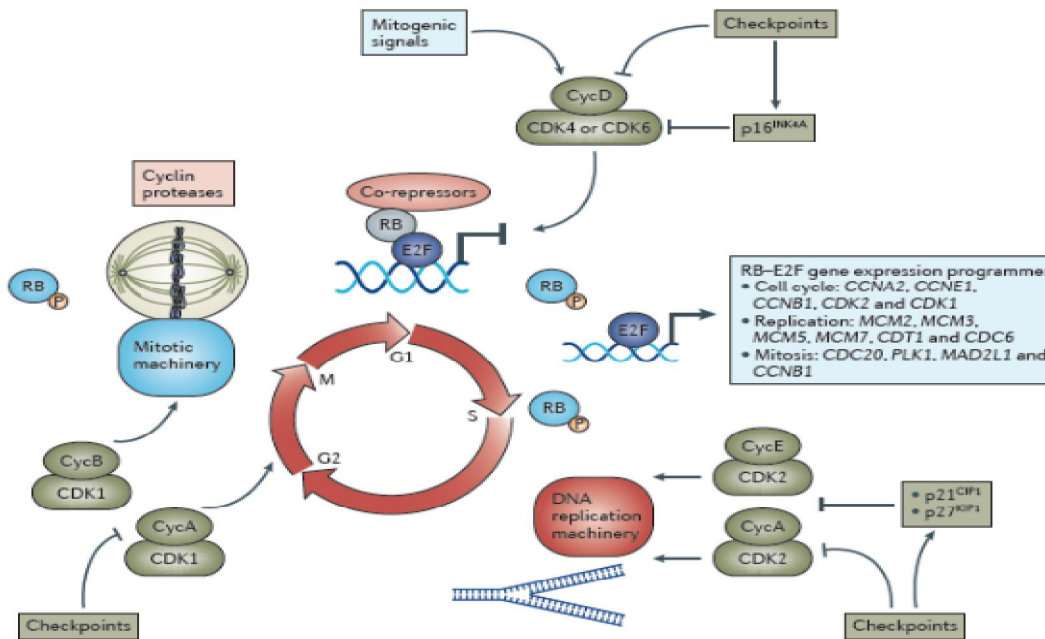


Figure 3. Cyclin-dependent kinase function in cell-cycle (34)

This function is also aided by the activity of CDK7 and cyclin H (36). CDKs include several families that differ on the basis of their structure and target (37). The INK4 family is composed of p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}, which specifically inhibit the catalytic subunits of CDK4 and CDK6 (38). Similarly, the CDK interacting protein/kinase inhibitory protein (CIP/KIP) family of CDKs (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) are potent inhibitors of the E- and A-type cyclins and their catalytic partners, CDK2 and

CDK1, and to a lesser extent of CDK1/cyclin B complex in G2 (37,39).

4. Cyclin-Dependent Kinases in Cancer

Cancer is characterized by uncontrolled tumor cell proliferation resulting from abnormal activity of various cell cycle proteins. Therefore, cell cycle regulators are considered attractive targets in cancer therapy. The catalytic subunits of CDK-cyclin complexes are infrequently mutated in cancer. Notable exceptions to this include mutations of CDK4 and CDK6, which have been described in distinct

subgroups of melanoma (CDK4) and other tumors (CDK4 and CDK6). More frequently observed in human malignancies are amplifications of the levels of the regulatory subunits of CDKs and cyclins, and mutations of endogenous CDK inhibitors. For example, cyclin D1 is over expressed in several tumors, such as parathyroid adenoma, leukemia, lymphomas and multiple myeloma, and colorectal, gastric, esophageal, lung, kidney and breast cancer, as a result of gene amplification, rearrangement or translocation (40). Cyclin D-dependent kinase activity might also be increased by other mechanisms, including inactivation of p16INK4A by gene deletion, point mutation or transcriptional silencing by methylation (40). Abnormal activation of CDK1 has been observed in a number of primary tumors (for example, breast, colon, prostate, oral, lung and esophageal carcinomas), most commonly owing to over expression of cyclin B1, and in some cases correlates with poor prognosis (41). CDK2 is deregulated in various malignancies, including lung carcinoma (cyclin A-CDK2), melanoma, osteosarcoma, ovarian carcinoma (cyclin E-CDK2), pancreatic neoplasia and sarcomas, most commonly owing to over expression of cyclin E and/or cyclin A, or inactivation of Cip-Kip inhibitors (42). The transcription kinase CDK9 is over expressed in myeloma, prostate cancer and lung cancer (43), suggesting that this kinase could be a potential target for cancer therapy. Abnormalities in transcriptional CDKs have also been reported in several human cancers. CDK8 expression has been reported in many types of cancer such as colorectal, gastric and breast cancers (44, 45). Also, CDK8 was found to be over expressed in gastric adenocarcinomas (46). CDK9-signaling pathways are involved in development of tumorigenesis and abnormal CDK9/cyclin T1 activity has been described in several human malignancies (47). CDK11 is an additional regulator of transcription, and recent evidence also indicates that it has a role in microtubule stabilization and in the control of sister chromatid cohesion (48, 49). The CDK11 gene (CDC2L) maps to a chromosome band region (1p36) which is frequently deleted in human cancers, and loss of one allele of Cdc2l facilitates skin carcinogenesis in mice (50). Other CDKs also have evidence in cancers not mentioned in this review.

5. Cyclin-Dependent Kinases inhibitors as anti-cancer and Strategies in developing it

Human cancers are characterized by altered cell cycle regulation. Dysfunctions in the mechanisms that coordinate cell cycle progression are intimately related to the characteristic features of cancer cells as defined by Hanahan and Weinberg (51). Owing to the central role of CDKs in the cell cycle, a large amount of CDKs inhibitors have been developed as potential

cancer therapeutics. Cyclin-dependent kinases constitute attractive pharmacological targets for the development of anticancer drugs and targeting CDKs has been pursued as a strategy for therapeutic intervention since the late 1980s (52-55). Efforts aimed at targeting cyclin-dependent kinases hyperactivity in human cancers began through purification of compounds from natural sources. These first generation ATP-competitive compounds served as templates for structure-guided, rational design of second generation drugs that bind the ATP-binding pocket of CDKs. Moreover this class of drugs was implemented by compounds identified in activity-based screens of chemical libraries.

Over the more recent years, alternative strategies have been sought to develop compounds targeting pockets and patches which are distinct from the ATP pocket, which are thought to offer greater promises of selectivity and therefore circumvent some of the undesired side-effects of ATP-competitive inhibitors (56-61). There are different strategies have been developed to target CDKs (Figure 4).

- ❖ **ATP-competitive inhibitors:** bind the ATP pocket and compete with ATP

- ❖ **Peptides targeting protein/ protein interfaces:** small peptides designed based on their similarity with endogenous CDKs inhibitors, such as p21, p27 and p57.

- ❖ **ATP- noncompetitive small molecules (Allosteric inhibitors):** its more selective inhibitors binding away from ATP-binding site.

6. CDK2 inhibitors are important target in cancer therapy

CDK2 is a member of protein kinase family. It play an important role in regulating various events of cell cycle (28). Over expression of CDK2 should cause the abnormal regulation of cell cycle, which would be directly associated with hyperproliferation in cancer cells. CDK2 over expression has been reported in types of cancers such as laryngeal squamous cell cancer, advanced melanoma and breast cancer (62, 63). Therefore, CDK2 was regarded as potentially therapeutic target for cancer therapy.

6.1. ATP-competitive inhibitors

ATP is essential for phosphorylation of CDKs and thus completes the activation of the enzyme, so inhibition of binding ATP to the kinases and thus inhibition of phosphorylation is the most successful strategy to develop powerful inhibition of CDKs concerned in the cell cycle (64).

ATP-binding pocket of CDK2 may be further divided into three sub sites (65).

- ❖ **Hinge region:** cleft located between N and C terminal lobes.

- ❖ **Phe80:** a shallow cavity located at the back of ATP binding cleft defined by the side chain of Phe80.

❖ Ribose/phosphate site: the ATP pocket formed in part by the flexible glycine loop. ribose/phosphate site is an extensive solvent-exposed

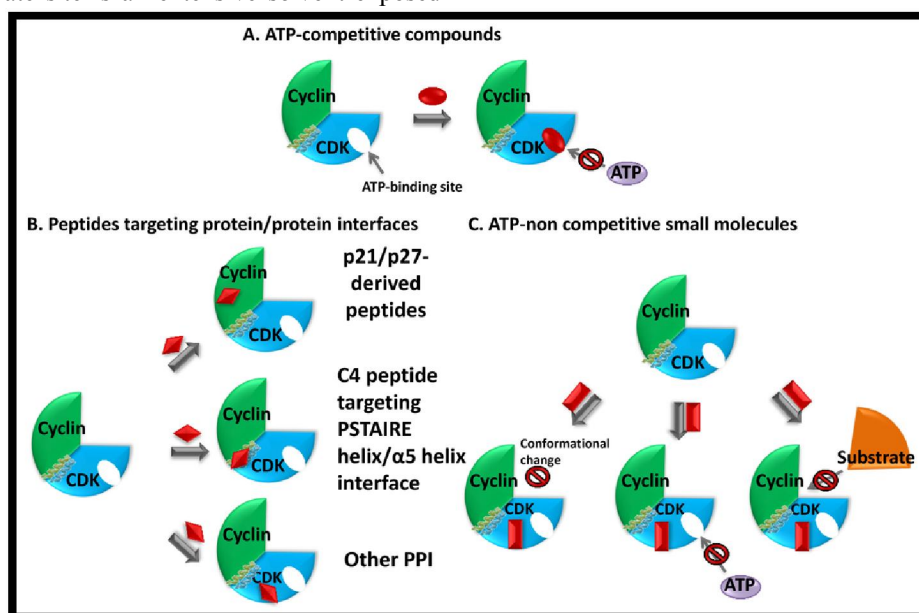


Figure 4. Strategies for targeting Cyclin-dependent kinases (61)

The ATP binding pocket would be most commonly targeted site for the majority of known potent CDK2 inhibitors, several chemically diverse small molecules from different chemical classes have been designed and evaluated and some of them enter the clinical trials, despite of the highly conserved nature of ATP binding cleft among kinases (66).

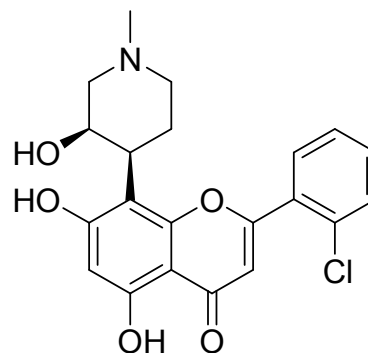
These molecules compete with ATP-binding site by forming one to three hydrogen bonds to the amino acids located in the hinge region of the target kinase, thereby mimicking the hydrogen bonds that are normally formed by the adenine ring of the ATP. Moreover, these inhibitors share similar properties, such as hydrophobicity, low molecular weight and heterocyclic flat structure(67). The structures of these molecules are chemically different and generally they are various heterocyclic families such as purines, pyrimidines, indoles, pyrazoles, thiazoles, or derived from natural product such as staurosporine or flavones.

We have chosen to present only the molecules which are currently in clinical trials. Nine compounds of CDK2 inhibitors now in clinical trials but none of them till have approved.

1. Flavopiridol

Flavopiridol (**1**) is a flavonoid derived from an Indian plant, rohitukine, with anti-inflammatory and immune-modulatory, as well as anticancer properties (68). Also known as alvocidib, at first it is identified as EGF receptor tyrosine kinase inhibitor, then it was found to be CDK2 inhibitor as a result of studying of crystal structure of flavopiridol complex with CDK2

Flavopiridol inhibit CDK1, CDK2, CDK4, CDK6, CDK7 and CDK9 also it displays antiproliferative efficacy in several solid tumor and sarcomas, as well as in leukemia, lymphoma and multiple myeloma (69, 70).



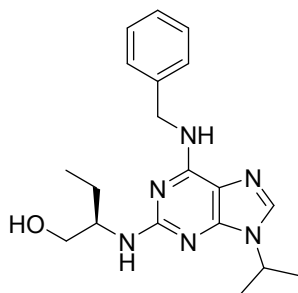
Flavopiridol (**1**)

Flavopiridol is the first CDK inhibitor selected for clinical trial (71), it is in phase II clinical trial for the treatment of acute myeloid leukemia and chronic lymphoid leukemia, and phase II clinical trial for multiple myeloma(72).

2. (R)-Roscovitine

Roscovitine (**2**) also known as **seliciclib (CYC202)**, it is a trisubstituted purine analogue that competes with ATP for binding to the active site on CDKs. It is highly potent selective inhibitor for

CDK1, CDK2 and CDK5 and to less extent showed low activity against CDK4 and CDK6. Seliciclib induce apoptosis from all phases of the cell cycle in cancer cell line. It inhibits CDKs with various IC50 value: CDK1 (2.7 μ M), CDK2 (0.1 μ M), CDK7 (0.5 μ M), CDK9 (0.8 μ M) (73, 74). The mechanism for R-roscovitine involves down regulation of RNA-polymerase II dependent transcription and enhanced expression of E2F-1(75).



(R)-Roscovitine (2)

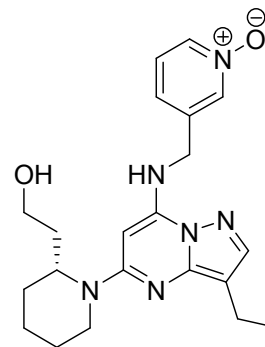
Roscovitine was entered into clinical trials in 2001 by Cyclacel pharmaceuticals, Inc. It is in phase II clinical trials and has been tested on lung cancer (non-small cell lung cancer, NSCLC), and phase II pituitary Crushing's disease(76). Also it has been tested in several phase I and II clinical trials both as monotherapy and combination therapy in several human cancers(77). It is the first drug in its class to be orally available (capsules). In 2015, it was licensed to ManRos Therapeutics by Cyclacel for the treatment of cystic fibrosis(78).

Due to the positive feedback of R-roscovitine as a potent cytotoxic agent, several bioisosteres were synthesized mainly focusing on redistribution of purine heteroatom in order to have similarity with roscovitine in high selectivity and low toxicity and enhance the disadvantage of short half-life of roscovitine in human (i.e. 2-5h) (79).

3. Dinaciclib

Dinaciclib (**3**), also called **SCH-727965**, it is pyrimidine derivative which is potentially inhibit of CDK1 (IC50:3nM), CDK2 and CDK5 (IC50:1nM), and CDK9 (IC50:4nM), and inhibit of transcription of apoptotic proteins, also inhibition of growth, migration and colony formation of human pancreatic cancer cells, and several other human cancers (80).

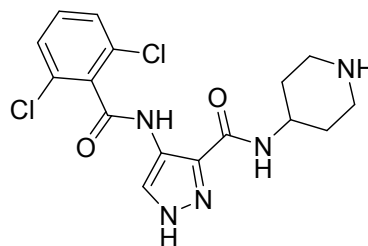
Dinaciclib has entered phase III clinical trials in 2012, for the treatment of chronic lymphocytic leukemia (81). Also it is in phase I clinical trials for advanced solid tumors; Metastatic triple-negative Breast Cancer; Melanoma; Multiple Myeloma(82).



Dinaciclib (3)

4. AT7519

AT7519 (**4**) is pyrazole-based inhibitor which shows inhibitory activity against CDK1 (IC50:190nM), CDK2 (IC50:44nM), CDK4 (IC50:67nM), CDK5 (IC50:18nM), and CDK9 (IC50:<10nM) (83). It affects cell cycle regulation, and also a potent inhibitor of RNA polymerase II-dependent transcription.



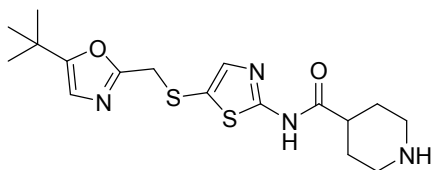
AT7519 (4)

AT7519 is in phase I clinical trials for treatment of refractory solid tumors and is given intravenously. Two separate phase II trials have also been underway since 2012 for mantle cell lymphoma and chronic lymphocytic leukemia(84), but no further data are available. Also it is in phase II clinical trials of chronic lymphocytic leukemia (85).

5. SNS032

SNS032 (**5**) is thiazoles derivative, selective inhibitor for CDK2 (IC50:38nM), CDK7 (IC50:62nM) and CDK9 (IC50:4nM) (86). Preclinical studies demonstrate that SNS032 was able to inhibit cell cycle activity along with transcription.

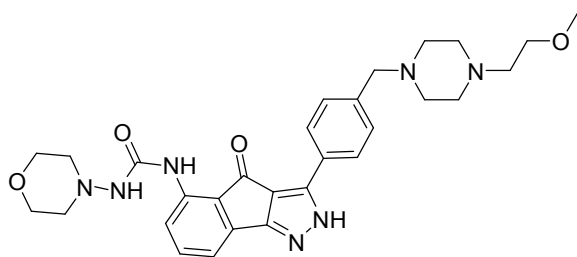
It is in phase I clinical trials for the treatment of chronic lymphoid leukemia along with multiple myeloma, and it give intravenous(86).



SNS032 (5)

6. RGB-286638

RGB-286638 (6) inhibit several CDKs in low nanomolar IC₅₀ range: CDK1 (IC₅₀:2nM), CDK2 (IC₅₀:3nM), CDK3 (IC₅₀:5nM), CDK4 (IC₅₀:4nM) and CDK9 (IC₅₀:1nM).

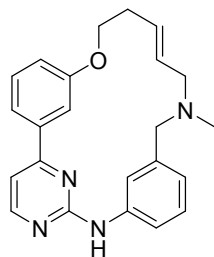


RGB-286638 (6)

It has completed phase I clinical trial for the treatment of multiple myeloma and solid tumors (87), but the planned phase I clinical trial for relapsed or refractory hematological malignancies was withdrawn in 2012 prior to enrollment (88). It was announced on May 2013 its dissolution and that the liquidation should take place in 2014, so no clear data are available (89).

7. TG02

TG02 (7) is pyrimidine- based derivative, it induce G1cell cycle arrest and apoptosis in a broad range of tumor cell lines. It inhibit CDK1 (IC₅₀:9nM), CDK2 (IC₅₀:5nM), CDK3 (IC₅₀:8nM), CDK5 (IC₅₀:4nM) and CDK9 (IC₅₀:3nM) (90).

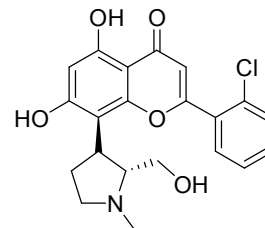


TG02 (7)

TG02 was selected for phase I clinical trials for the treatment of chronic lymphocytic leukemia and advanced hematological malignancies(91).

8. P276-00

P276-00 (8) is analog of flavopiridol where the piperidine moiety has been changed for a pyrrolidine. It inhibit CDK1 (IC₅₀:79nM), CDK2 (IC₅₀:224nM), CDK4 (IC₅₀:63nM) and CDK9 (IC₅₀:20nM) (92).

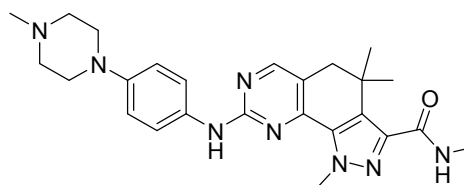


P276-00 (8)

It is in phase II clinical trials for various types of tumors(93).

9. PHA-848125

PHA-848125 (9) is quinazoline-based inhibitor; also called milciclib, it is potent and selective CDKs inhibitor along with significant antitumor activity (94). It inhibit CDK1 (IC₅₀:2nM), CDK2 (IC₅₀:3nM), CDK4 (IC₅₀:5Nm), CDK5 (IC₅₀:4nM), It induce cell cycle arrest at G1 phase of cell division cycle, accompanied by inhibition of pRb phosphorylation on cell contact. It is in phase II clinical trial for the treatment of thymic carcinoma(95).



PHA-848125 (9)

6.2. Peptides targeting protein/ protein interfaces

The activity of the complex of CDK2 with cyclin A/E is normally counteract by the action of CDKI family Cip/Kip, any deletions, mutations and/or proteasome-mediated proteolysis of these tumor suppressor gene products, may lead to deregulation of cell cycle, since inactivation of these endogenous inhibitors would induce over expression of CDK2 and then enhance cell proliferation potential(96).

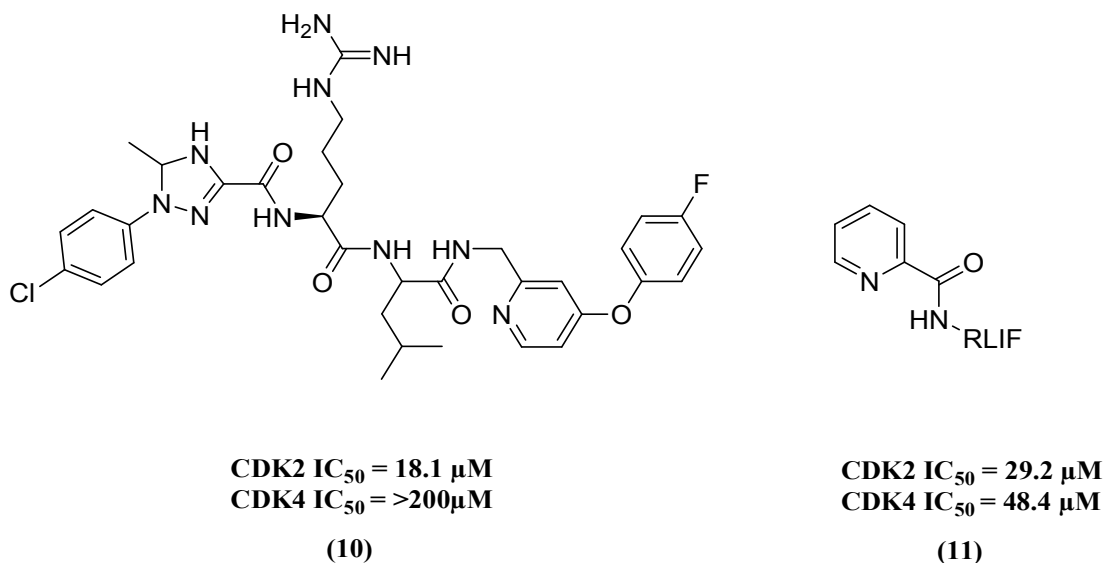


Figure 5. Peptidomimetics as ATP noncompetitive inhibitors of CDK2/cyclin A

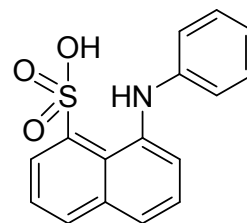
Kaelin group, during their studies of the interaction of CDK2/cyclin complex with several cell cycle regulators, they found that there are a common recognition motif for these regulators (including the endogenous CDKs inhibitors: p21, p27 and p53)(97). This eight-residue peptide motif exhibit highly conserved amino-acid sequence and the peptides containing this sequence are able to inhibit the activity of CDK2/cyclin complex. Therefore, peptidomimetics molecules have been designed to mimic endogenous CDK inhibitors (p16, p21, p27) or endogenous substrates (EF2, P53, PRb, P107) to interfere with the interface between the CDK and cyclin partner or to interrupt conformational changes required for activation of the CDK-cyclin complexes(98). McInnes *et al.* applied the REPLACE -mediated exchange of the known octapeptide cyclin groove inhibitor (HAKRRLIF) into N- and C-terminal di-capped or N-terminal mono-capped peptides to develop non-ATP competitive CDK2/ cyclin A inhibitors (99, 100), some recent examples are compounds (10) and (11) (figure 5).

6.3. ATP- noncompetitive small molecules (Allosteric inhibitors)

CDK2 was regarded as potential target for cancer therapy, so several CDK2 inhibitors have been designed and most of them were ATP competitive inhibitors, but there were some difficulties in design selective inhibitor due to highly conserved nature of the ATP- binding pocket among the CDK-subfamily members (101). Hence, it is essential to find new binding pockets which provide more selectivity for CDK2 inhibitors other than catalytic site. The introducing of allosteric inhibitors would solved this

problem, these inhibitors link CDK2 away from their ATP-binding site.

ANS (12) (1-AnilinoNaphthalene-8-Sulfonic acid) is the first example for this family, which discovered by the team of schönbrunn (102a), two molecules of ANS were able to interact with CDK2 in a different site from the ATP-binding site, once the ANS molecules are linked to CDK2, a change of conformational of the PSTAIRE helix occurred, thus rendering prevention of binding of CDK2 with cyclin.



ANS (12)

Rastelli described in 2014 (102b) the first examples of truly type III allosteric ligands of CDK2 (figure 6). Through virtual screening of commercially available compounds in the allosteric pocket of the CDK2-ANS binary complex, and using a combination of docking (Auto Dock) and post docking, the authors identified new core Inhibitors. Competition experiments performed in the presence of staurosporine confirmed the allosteric mode of action. These compounds (13) and (14) (figure 7) inhibit the growth of breast cancer cells in the micro molar range (103).

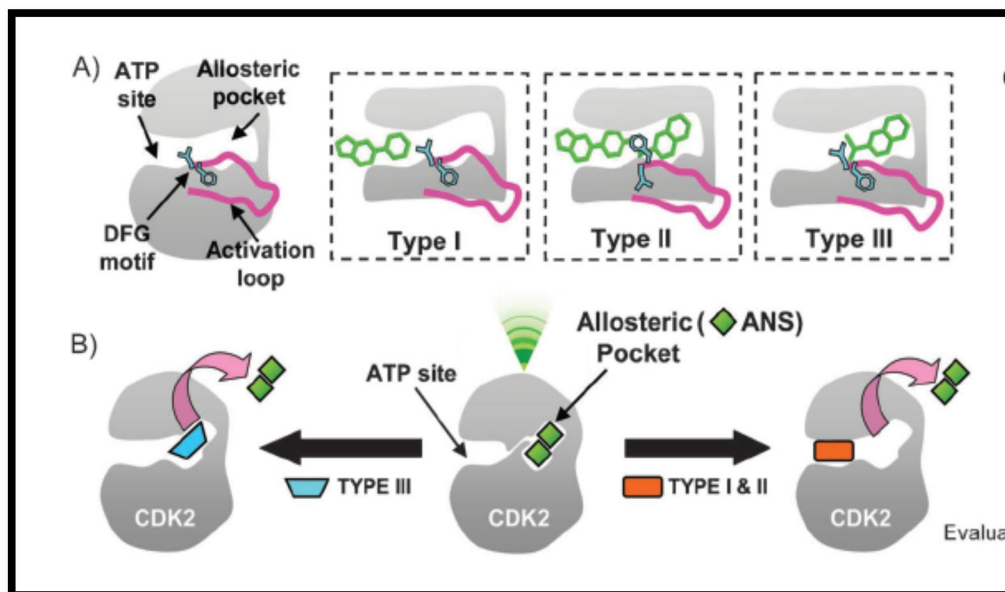


Figure 6. Exploiting the CDK2–ANS interaction to identify small-molecule ligands. A) Type I kinase inhibitors bind to the ATP site, type II inhibitors extend from the ATP site into the neighboring allosteric pocket, and type III inhibitors bind in a purely allosteric manner. The activation loop is shown in magenta, the DFG motif in cyan, and inhibitors in green. B) The allosteric site in CDK2 accommodates two ANS molecules, which are displaced upon binding of ligands by direct competition (type III) or indirectly through conformational changes caused by binding of certain type I or type II ligands to the ATP site. (102a)

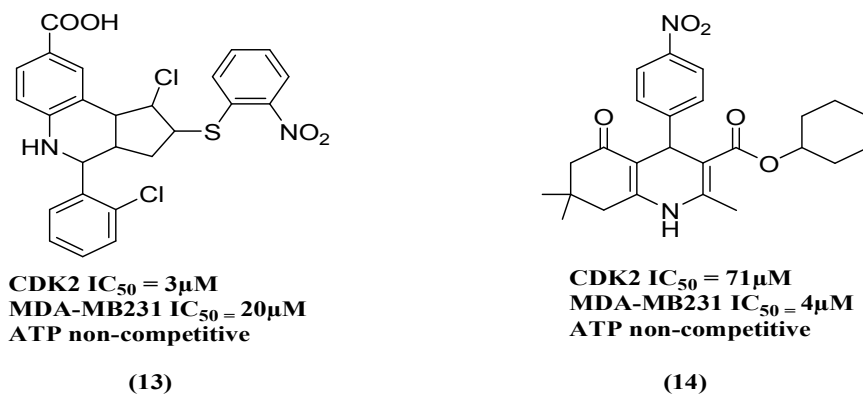


Figure 7. ATPnon competitive inhibitors of CDK2/cyclinA

7. Cyclin-Dependent Kinase 4/6 inhibitors

The cyclin D-CDK4/6-INK4-Rb pathway is important for cell cycle entry, and alterations in this pathway are a hallmark of cancer (104). The cyclin D-CDK4/6-INK4-Rb pathway is activated by restricting the function of the tumor suppressor protein Rb (**Figure 3**). Rb regulates the activity of the E2F family of transcription factors, which induce G1-to-S phase transition of the cell cycle by transcribing genes involved in DNA replication and cell cycle regulation (105). Deregulation of the cyclin D-CDK4/6-INK4-Rb pathway is observed in many types of cancer such as squamous cell carcinomas (SCCs)(106), and breast

cancer (107), mantle cell lymphoma, melanoma and lung cancer(108).

A new class of second-generation CDK inhibitors with greater specificity has emerged recently. These compounds are Adenosine Triphosphate (ATP)-competitive inhibitors with greater selectivity for CDK4 and CDK6 than for other CDKs and are currently being tested in a variety of cancers. The role of these CDK4/6 inhibitors in combination with other agents has been investigated in breast cancers, and their efficacy attributed to alterations in the cyclin D1-CDK4/6-INK4-Rb pathway (109).

Three highly specific CDK4/6 inhibitors (Palbociclib, Bemaciclib and Ribociclib) are either approved by the FDA or in late-stage development, while MM-D37K and G1T28-1 are now under clinical evaluation.

1. Palbociclib

Palbociclib (**15**) (**figure 8**) also known as (*PD0332991* and *IBRANCE*), it is pyridopyrimidin derivative, it's the first CDK4/6 inhibitor that exhibit efficacy in multiple cancers. Palbociclib specifically inhibits the cyclin D-CDK4/6 complex activities at concentrations of 11 nM (cyclin D1-CDK4) and 15 nM (cyclin D1/2/3-CDK6) (110). In advanced breast cancer cases, the progression free survival interval doubled from 10.2 months in patients receiving letrozole alone to 20.2 months in patients receiving a combination of letrozole and Palbociclib (107). Treatment with Palbociclib is associated with reversible neutropenia, nausea, fatigue, diarrhea, stomatitis, and asthenia (107, 111). In February 2015, FDA granted accelerated approval for palbociclib in combination with letrozole for the treatment of HR-positive, HER2-negative advanced breast cancer as initial endocrine based therapy in postmenopausal women. On February 19, 2016, the U. S. Food and Drug Administration approved palbociclib (IBRANCE Capsules, Pfizer, Inc.) in combination with fulvestrant for the treatment of women with hormone receptor (HR)-positive, human epidermal growth factor receptor 2 (HER2)-negative advanced or metastatic breast cancer with disease progression following endocrine therapy (112, 113).

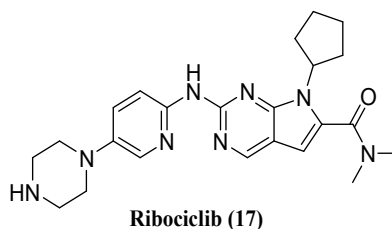
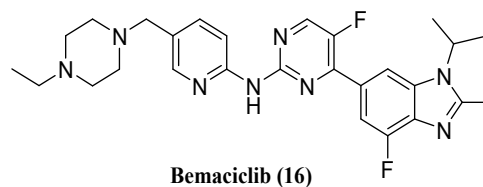
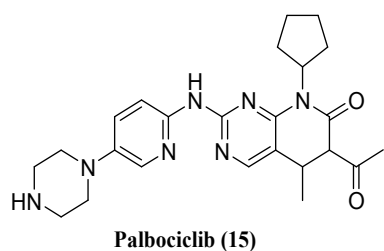


Figure 8. CDK4/6 inhibitors

2. Bemaciclib

Its pyrimidine-benzimidazole derivative also known as (LY2835219) (**16**) (**figure 8**). Abemaciclib is blocking cells at G1 phase due to the inhibition the phosphorylation of pRb, it is orally bioavailable and effective at inhibiting CDK4/6 activity at nanomolar doses. It displays anti-proliferative activity *in vitro* and *in vivo* at clinically achievable plasma concentrations. The (IC₅₀) values for abemaciclib are 2 nM for cyclin D1-CDK4 and 10 nM for cyclin D1/2/3-CDK6 (114). In a recently completed phase I study in patients with solid tumors, the safety, pharmacokinetic, and pharmacodynamics profiles of abemaciclib were evaluated, it was recently approved as a single agent for heavily treated patients with advanced hormone receptor (HR)-positive breast cancer, clinical phase III studies in patients with mBC (including patients with brain metastases) and non-small cell lung cancer (NSCLC) are currently ongoing (115).

3. Ribociclib

LEE-011 is pyrolo-pyrimidine derivative (**17**) (**figure 8**) similarly to palbociclib; it blocks RB phosphorylation and causes cell cycle arrest of tumor cells (99). Furthermore, Ribociclib showed antitumor activity in xenografts of neuroblastoma (including senescence induction) (99), liposarcoma (116), rhabdomyosarcoma (117) and Ewing sarcoma (118). Although it has not yet been approved by the FDA, Ribociclib shows increased CDK4/6 specificity with (IC₅₀) values of 10 nM for cyclin D1-CDK4 and 39 nM for cyclin D1/2/3-CDK6 is currently in phase III studies for breast cancer (NCT02712723) (40).

4. MM-D37K

Recently, in 2014, new selective CDK4 and CDK6 inhibitor, entered in clinical development. **MM-D37K** a new chimeric peptide that act as a replacement of the endogenous p16/ink4a inhibitor

consists of p16/ink4a derived short sequence and cell penetrating peptide (CPP)-Antp (Penetratin). Its mechanism of action is blocking cell cycle at G1 phase and induce apoptosis in tumor cell lines, also *in vivo* it show anti-tumor effect in lung and colon xenografts

models. This is the first chimeric peptide entering in clinical development (119).

5. G1T28-1

G1T28-1 is another selective CDK4 and CDK6 inhibitors (120). It is found to overcome myelosuppression that occur by all selective CDK4/6 inhibitors (121), and preserve bone marrow from chemotherapy (122). However, neither structures nor SAR studies have been revealed for **MM-D37K** and **G1T28-1** yet.

8. Transcriptional CDKs inhibitors as future perspective as anticancer drugs

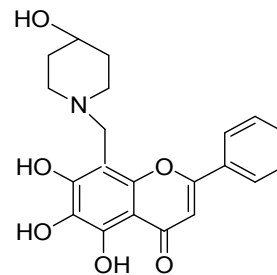
8.1. CDK1

The protein encoded by this gene is a member of the Ser/Thr protein kinase family. This protein is a catalytic subunit of the highly conserved protein kinase complex known as M-phase promoting factor (MPF), which is essential for G1/S and G2/M phase transitions of eukaryotic cell cycle. Mitotic cyclins stably associate with this protein and function as regulatory subunits. The kinase activity of this protein is controlled by cyclin A2 and B. Upon entry into mitosis, cyclin A2 is degraded and CDK1 activity is maintained in complexes with B type cyclins. The phosphorylation and dephosphorylation of this protein also play important regulatory roles in cell cycle control. The expression of CDK1 and associated cyclins (cyclin A2 and cyclin B1) is coordinated through the activity of E2F-assembled complexes (123, 124). These include the canonical E2F and RB constituents, as well as the transcription factor FOXM1 and the DREAM (dimerization partner, RB-like, E2F and multivulval class B) complex, which are particularly relevant for the coordination of transcripts involved in mitotic progression (125, 126). CDK1 activity is inhibited by phosphorylation at Thr14 and Tyr15, mediated by the kinases membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (MYT1; also known as PKMYT1) (127) and WEE1 (128), respectively; this phosphorylation is relieved by CDC25 phosphatases (128).

CDK1 activity is rarely deregulated in cancer, one of the few examples being *CCNB3* gene amplifications in neuroendocrine prostate cancer (129) in contrast to the genetic deregulation of the CDKs that coordinate the S phase. Furthermore, CDK1 has been shown to be required for tumor formation and progression. Interestingly, inhibition of CDK1 triggered apoptosis of MYC-driven mouse lymphomas and liver tumors (130), in addition to human basal-like triple-negative breast cancer cells (131). CDK1 overexpression has been documented in lymphoma, advanced melanoma and lung cancer, and loss of cytoplasmic CDK1 predicts poor survival and confers chemotherapeutic resistance in the latter (132, 133, 134).

Therefore, attempts have been made to discover selective inhibitors for CDK1; although most of pan-CDKs inhibitors also inhibit CDK1 in a less extent, no selective inhibitors for CDK1 was discovered.

BA-J (8-Hydroxypiperidinomethyl-baicalin) (**18**) is a novel selective CDK1 inhibitor, its flavonoid derived from *Scutellaria baicalensis*; a perennial herb whose dried root is commonly used in traditional Chinese medicine for the treatment of hyperlipomia, hypertension, hyperglycemia, inflammation, allergic reactions, viral infections, cancer and so on (135).



BA-J (18)

BA-J is natural flavonoids selective CDK1 inhibitors, has broad spectrum anti-cancer activity (IC₅₀ 12.3 μM). Because of the differential mechanisms controlling redox-states in normal and cancer cells, BA-j can capture oxygen free radicals (O₂⁻) and selectively increase the level of H₂O₂ in cancer cells, thereby specifically oxidize and activate the intrinsic apoptosis pathway bypassing the extrinsic death receptor pathway, thus inducing apoptosis in cancer cells rather than in normal cells. BA-j is different from cytotoxic anticancer drugs which can activate both the intrinsic apoptosis pathway and the extrinsic death receptor pathway, and therefore harm normal cells while killing cancer cells. The molecular and biochemical mechanisms of reactive oxygen species (ROS) regulation suggest that BA-j may be developed into a novel anticancer agent (136, 137, and 138).

8.2. Cyclin-Dependent Kinase9 inhibitors

8.2.1. An overview on CDK9

This kinase was found to be a component of the multiprotein complex TAK/P-TEFb (positive transcription elongation factor b), which is an elongation factor for RNA polymerase II-directed transcription and functions by phosphorylating the C-terminal domain (CTD) of the largest subunit of RNA polymerase II. This protein forms a complex with and is regulated by its regulatory subunit cyclin T or cyclin K (139, 140) Peng *et al.* are the ones that described (P-TEFb) as a novel CDK/cyclin pair, calling their subunits CDK9 and Cyclin T for the first time (141). After that, Cyclin T1, T2a, and T2b were identified. Each binds CDK9 and possesses (P-TEFb) activity,

although 80% of CDK9 binds Cyclin T1, 10% binds T2a, and 10% binds T2b (142). A year later, Cyclin K was also found to interact with CDK9 *in vivo* (143).

8.2.2. Mechanism of action of CDK9

CDK9 is not a typical Cdc2-like kinase. It does not act in cell-cycle regulation processes; rather, it acts in differentiation processes (144). It is the catalytic subunit of P-TEFb that, in association with Cyclin T, has the ability to phosphorylate the CTD substrate of RNA polymerase II and reach the RNA transcription elongation (143). Although there are other cyclin-dependent kinases that are capable of phosphorylating the CTD, the only one that activates gene expression in a catalyst manner is CDK9. Therefore, Cyclin T/CDK9 is a dedicated kinase functioning in transcription, with CTD being the major functional target of the complex *in vivo* (145).

8.2.3. CDK9 inhibitors as anticancer

The CDK9–cyclin T complex stabilizes the elongation of nascent mRNA transcripts and has a crucial role in several biological processes, such as cell growth, proliferation and differentiation (43). Deregulation of CDK9/cyclin T1 activity is essentially associated with its over expression in several B and T-cell lymphomas, as well as in neuroblastoma, primary neuroectodermal tumor, rhabdomyosarcoma and prostate cancer (146-149), classical Hodgkin's lymphoma (145), Burkitt's lymphoma, follicular lymphoma (145), neuroblastoma (146), myeloid leukemia (150).

Several ATP- competitive nonspecific CDK inhibitors are highly active against CDK9 and possibly other transcription-related CDKs. Inactivation of these CDKs has a greater effect on cellular transcription, affecting primarily the accumulation of mRNA transcripts that have a rapid turnover, such as those that encode cell cycle regulators, nuclear factor- κ B- and p53-responsive gene transcripts, and anti-

apoptotic factors. Thus, chemicals that inhibit transcriptional CDKs could possess anti-cancer activity by enhancing apoptotic responses in cancer cells, particularly tumorous hematopoietic cells as their survival depends on the continuous expression of anti-apoptotic proteins (55).

However, all the broad-range CDKs inhibitors display activity against CDK9 and some of them are even at nanomolar concentrations. Nevertheless, primary mechanisms responsible for the observed antitumor activity of a large proportion of CDKs inhibitors such as flavopiridol, (R)-roscovitin and Dinaciclib predominantly appear to be the down regulation of antiapoptotic proteins through transcriptional CDK inhibition (151-153). For this reason, the hypothesis that inhibition of transcriptional CDKs might be an effective anticancer strategy has gained broad consideration from medicinal chemists.

There are some examples for the effort of the researcher to introduce selective CDK9 inhibitor over other kinases;

1. DRB (5,6-dichlorobenzimidazole-1- β -D-ribofuranoside) (**19**) (**figure 9**) is one of the most widespread inhibitor of transcription elongation, which is selective for CDK9 (IC₅₀ = 340 nM) over other CDKs (CDK1 & CDK2 IC₅₀ >10 μ M) (154). It induces apoptosis with independence of p53 (155). The binding mode of **DRB** was described in 2010 by Baumli *et al.*, it inhibits ATP binding site of CDK9 by change conformation of the glycine-rich loop and the β 3- α C loop through chlorine-hydrogen bond.

2. CAN508 (4-arylazo-3,5-diamino-1H-pyrazole) (**20**) (**figure 9**), it was discovered through a routine screening of small-molecule compound collection (156, 157), it is not only CDK9 inhibitor but also it inhibits endothelial cell migration as well as angiogenesis and reduces expression of VEGF by several human cancer cell lines (158).

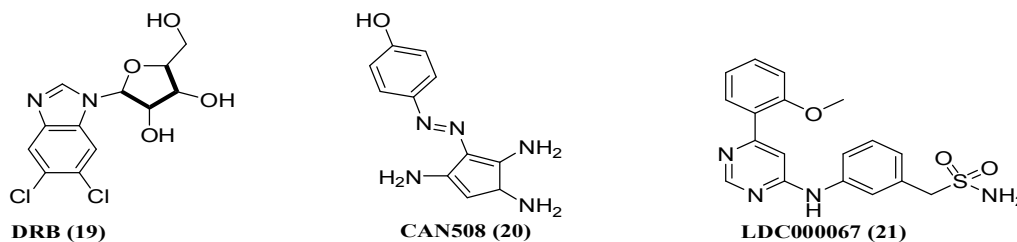


Figure 9. CDK9 inhibitors

3. LDC000067 (4-aniline-6-phenylpyrimidine) (**21**) (**figure 9**), is a new CDK9 inhibitor, it was initiated from a small molecule screen of a chemical library (159). Its selectivity for CDK9 (IC₅₀ = 44 nM) over other CDKs was in the range of 55-fold (vs CDK2) to over 230-fold (vs CDK6 and CDK7). It was used as a

biomedical probe to interview gene control mechanisms mediated by CDK9 by Albert *et al.* in 2014 (160).

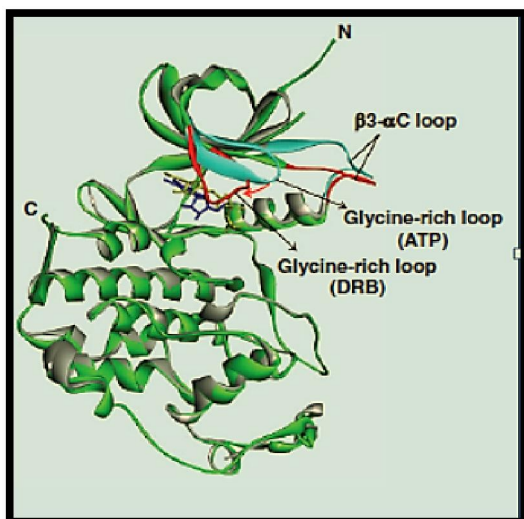


Figure 10. selective CDK9 inhibitors DRB, cocrystal structures of CDK9/cyclin T/DRB(3MY1) (161)

9. Conclusions

In summary, CDK complexes have critical roles in multiple aspects of biology, including proliferation control and transcription. After the generally disappointing results seen in clinical trials with non-selective CDK inhibitors, the importance of selectivity of compounds for specific CDKs and of patient selection is now widely accepted. CDK inhibitors finally appear to be self-confident to have a clinical influence, and this has been made possible through the development of more selective and potent ATP-competitive CDK inhibitors. This opportunity will likely yield new and useful drugs for the treatment of cancer and other proliferative diseases. Additional CDK-selective agents may complement these ATP-competitive inhibitors based on their ability to disrupt substrate binding to cyclins, to block the binding of CDKs to their cyclin partners, or to abrogate ATP or protein substrate binding to the CDK subunit in an allosteric manner. These novel approaches for the identification of CDK inhibitors designed based on CDK2 structural information can potentially be implemented in the development of non-ATP-competitive agents targeting CDK1, CDK4, CDK5, CDK6, CDK7, CDK8, CDK9, and other CDKs deemed important therapeutic targets in the treatment of cancer.

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