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Cyclin-Dependent Kinases (Cdk) as Targets for Cancer Therapy and Imaging

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1. Introduction

Aberration in proliferation and consequently in cell cycle control is a common aspect in carcinogenesis. As master cell cycle regulating proteins in all eukaryotic cells the Cyclin-dependent kinases (Cdk) were identified by Leland Hartwell, Paul Nurse, and Timothy Hunt in the 1970s and 1980s. Chronological activation of respective Cdk according to respective cell cycle phase G_1, S, G_2 or M is mediated through association with a regulatory Cyclin subunit, phosphorylation of Cdk and binding of endogenous activators and inhibitors, as well as subcellular localization (Shapiro, 2006).

In human cells four Cdk are essential components of the cell cycle machinery with key functions also in human cancer cells: Cdk1, Cdk2, Cdk4, and Cdk6 (Fig. 1) (Malumbres & Barbacid, 2009). First, Cyclin D-dependent kinases Cdk4 and Cdk6 are activated in human cell cycle in response to mitogenic signals to initiate G_1 phase progression and prepare DNA duplication in S phase (Malumbres & Barbacid, 2005). Cdk4-Cyclin D or Cdk6-Cyclin D and later also Cdk2-Cyclin E complexes sequentially phosphorylate retinoblastoma proteins (Rb) on different serine and threonine residues. Resulting Rb protein inactivation is required for the transcriptional activation of genes in G_1/S phase (Harbour & Dean, 2000). In G_1 phase endogenous inhibitors of monomeric Cdk4 and Cdk6 like INK4 and inhibitors of Cdk2/Cdk4/Cdk6-Cyclin complexes like Cip and Kip proteins exert important influence on Cdk catalytic activity (Blain, 2008; Sherr & Roberts, 1999). Once the cell irreversibly passed restriction point R at the end of G_1 phase, Cdk2-Cyclin A complex is formed, facilitating orderly execution of S phase events like DNA replication and centrosome cycle through phosphorylation of various proteins (Malumbres & Barbacid, 2005). Activation of Cdk1 by Cyclin A is required for DNA damage checkpoint control, later Cdk1-Cyclin B for G_2/ M phase transition and initiation of mitosis, especially chromosome condensation and microtubule dynamics (Malumbres & Barbacid, 2009). Therefore, active Cdk1-Cyclin complexes mediate phosphorylation of about 70 substrates, e. g., minichromosome maintenance (MCM), p53, lamins, and dyneins.

Initiation of cell re-entrance from G_0 to G_1 phase and early inactivation of Rb is assigned to Cdk3-Cyclin C (Ren & Rollins, 2004). Another Cyclin-dependent kinase, Cdk5, is involved in the regulation of neuronal function (Cruz & Tsai, 2004).

The second group of proteins belonging to Cdk family – Cdk7 to Cdk13 – are involved in the activation of cell cycle kinases and transcriptional regulation (Akoulitchev et al., 2000; Chen et al., 2006; Chen et al., 2007; Garriga & Grana, 2004; Hu et al., 2007; Kasten & Giordano, 2001). Cdk7 in complex with Cyclin H is given a special importance since it is the only Cdk activating kinase (CAK) in mammalian cells phosphorylating a threonine residue in the conserved T-loop of Cdk (Lolli & Johnson, 2005).

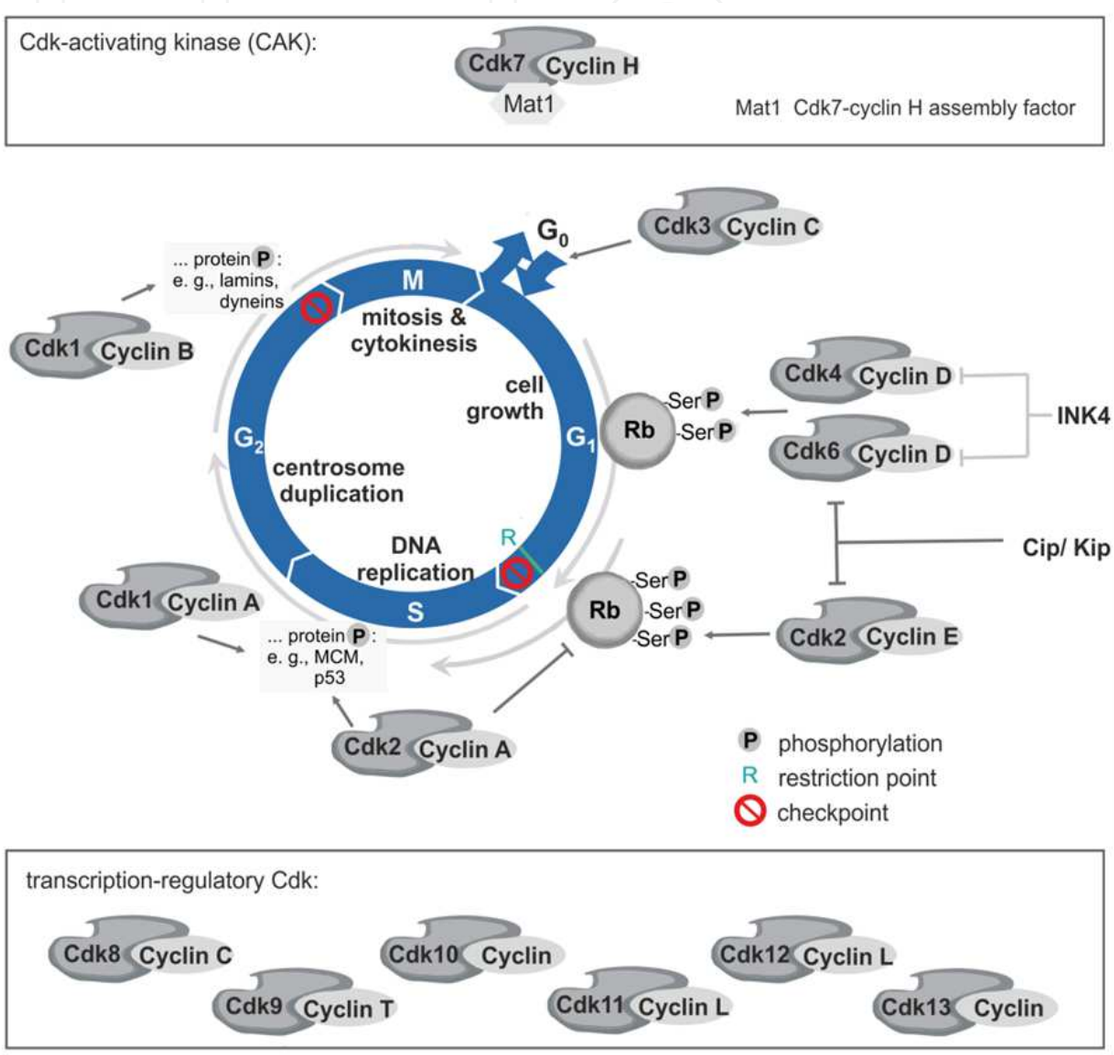


Fig. 1. Overview of human cell cycle activation and transcriptional regulation through Cdk-Cyclin complexes

2. Role of cyclin-dependent kinases in carcinogenesis

As important components of cell cycle activation and control the Cyclin-dependent kinase protein family contributes to tumor development and, in fact, an universal abnormal regulation of Cdk pathways has been described in human tumors induced by multiple mechanisms (Malumbres & Barbacid, 2009). Various genetic and epigenetic alterations in

human cancer including mutations and amplification of Cdk and positive regulatory Cyclin subunits, mutations or silencing of substrates (Rb) and endogenous Cdk inhibitors (INK4, Cip/ Kip proteins) lead to a hyperactivation of Cdk regulatory pathways (Table 1) (Deshpande et al., 2005; Malumbres & Barbacid, 2005). In consequence, critical cell cycle checkpoints are ignored resulting in abnormal cell proliferation and tumor progression.

Although tumor cells exhibit rather infrequent mutations of *cdk* genes – with the exception of G_1 kinases Cdk4 and Cdk6 – amplification, overexpression or hyperactivation of basic cell cycle regulators is a general feature of human tumors (Easton et al., 1998; Kim et al., 1999; Sotillo et al., 2001; Wolfel et al., 1995). Cdk hyperactivation is often affected by mutations of Cdk regulatory subunits. In consequence, overexpression of Cyclin A, Cyclin B, Cyclin E, and Cyclin D were reported in a wide spectrum of tumors, like leukemia or carcinomas and were associated with poor prognosis (Johansson & Persson, 2008; Ko et al., 2009). A common alteration in human tumors was demonstrated for tumor suppressor gene *rb*. Altered Rb proteins, momentous for transcriptional control, are insensitive to Cdk regulation and accelerate cell cycle progression (Nevins, 2001). Finally, abnormal regulation or inactivation of Cdk endogenous inhibitors $p15^{INK4B}$, $p16^{INK4A}$ and $p27^{Kip1}$ was described in numerous human tumors leading to enhanced Cdk activity (Ruas & Peters, 1998; Tsihlias et al., 1999).

	alteration	occurrence in cancer type
Cdk1	upregulation/ overexpression	hepatoma, carcinoma, leukemia
Cdk2	upregulation/ overexpression	hepatoma, carcinoma, leukemia
Cdk4	point mutation R24C amplification/ overexpression	melanoma, insulinoma, sarcoma carcinoma, glioma, sarcoma, …
Cdk6	analogous R24C mutation chromosomal translocations amplification/ overexpression	neuroblastoma lymphoma, leukemia carcinoma, glioma, sarcoma, …
Cyclins (A, B, E, D)	amplification/ overexpression chromosomal translocations	carcinoma, leukemia, … lymphoma, adenoma
Rb	mutation promoter methylation sequestration	retinoblastoma, osteosarcoma, carcinoma brain tumors carcinoma, melanoma, neuroblastoma
p15INK4B, p16INK4A	deletion promoter methylation	carcinoma, lymphoma, melanoma, … melanoma
p27Kip1	decreased transcription increased degradation	glioblastoma, carcinoma, melanoma, … carcinoma, lymphoma

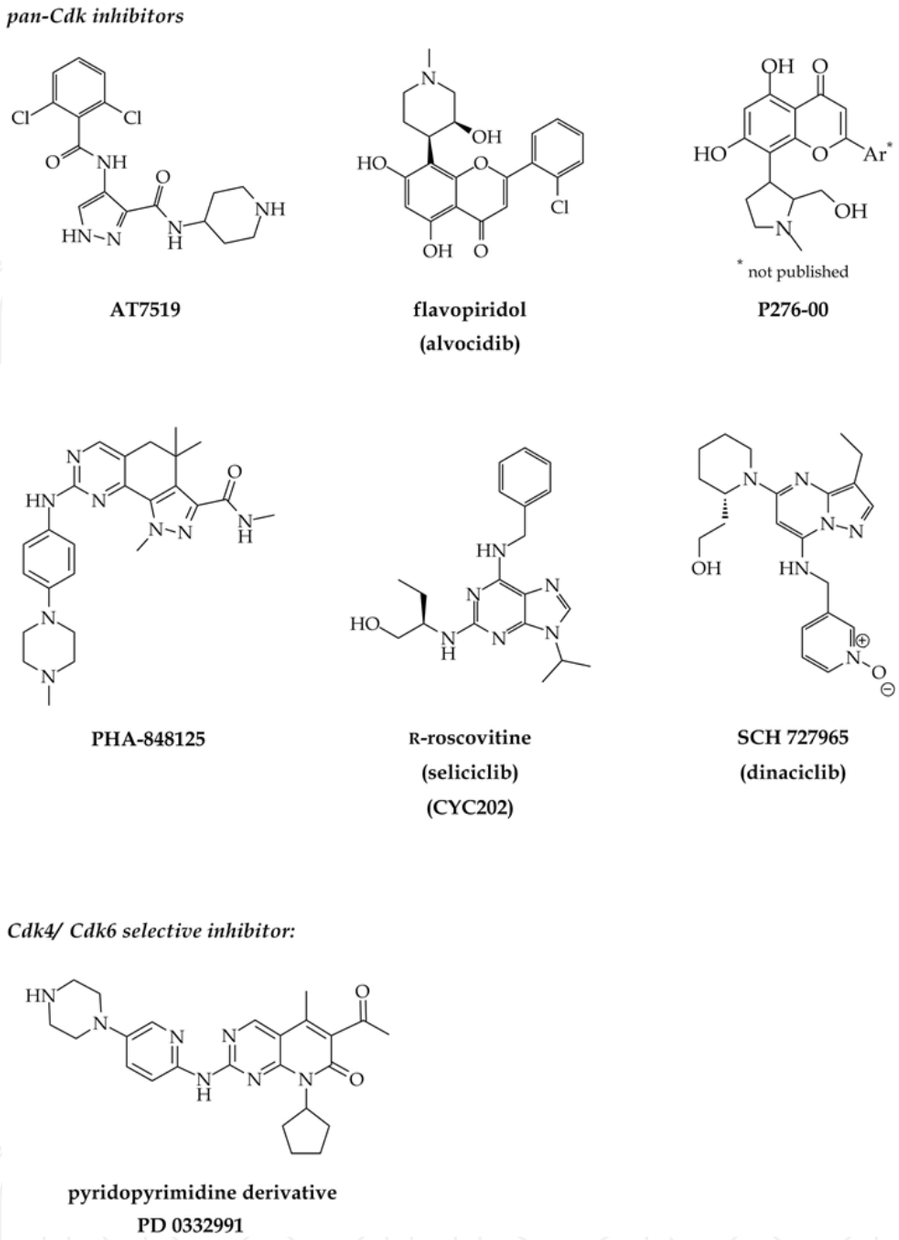
Table 1. Genetic and epigenetic alterations of Cdk pathway components in human cancer (Graf et al., 2010; Ortega et al., 2002; Weinberg, 2007)

Universal abnormal regulation of Cdk pathways especially in G_1/ S phase suggests involvement and importance of these kinases in carcinogenesis, despite of uncertain results concerning dependence of Cdk2, Cdk4 and Cdk6 on cell cycle progression in embryogenesis (Malumbres & Barbacid, 2009; Santamaria et al., 2007).
In consequence, cell cycle regulating Cdks are attractive molecular targets for new (radio)pharmaceutical strategies in both cancer therapy and diagnosis, considering the heterogeneity of Cdk activity in different human tumor types. Compounds directly inhibiting the cell cycle machinery hold promise in restoring missing cellular Cdk regulators and arrest of proliferating cells, thus providing a non-genotoxic therapy modality. Conspicuous amplification of Cdk in tumor cells provides an opportunity for visualization of tumors by means of positron emission tomography (PET).

3. Overview of small molecule Cdk inhibitors: Selectivity and mode of action

In the last decade, numerous of structurally diverse small molecules inhibitors of Cdk activity have been developed and evaluated *in vitro* and *in vivo*. Thereby, structural information of different Cdk, in complex with corresponding regulatory Cyclin subunits, and in association with inhibitors facilitated the development of new potent compounds with high specificity for Cdk versus other protein kinases (De Bondt et al., 1993; Jeffrey et al., 1995; Sridhar et al., 2006). Motivation of Cdk specific targeting was attributed to high cytotoxicities of first generation of unspecific kinase inhibitors, e. g., staurosporine targeting several protein kinase families, which limited clinical application (Sielecki et al., 2000). In addition, effort to the identification of Cdk-subtype selective inhibitors has been made to optimize therapeutic success and minimize side effects for cancer patients. In first preclinical and clinical trials of potent, pharmacological Cdk inhibitors only misleading results for tumor treatment were revealed due to non-specific and/or non-selective targeting (Shapiro, 2006). According to this, Cdk inhibitors were classified to their effects on Cdk family members as pan-Cdk or highly selective Cdk inhibitors. Nevertheless, the classification of Cdk inhibitors often reveals the subjective view of the authors, not least because of different experimental setup of Cdk affinity measurements, missing data of the selectivities to many kinases and diffuse boundaries between Cdk inhibitors with broad and narrow activity profile.
The major targets of pharmacological Cdk inhibitors are key enzymes regulating interphase (Cdk2, Cdk4, Cdk6) and mitotic Cdk1. But often also Cdk activating kinase Cdk7 and transcriptional Cdk, e. g., Cdk9 are affected.
All potent small molecule Cdk inhibitors interact with the catalytic active site of Cdk and compete with ATP or block ATP binding. Several review articles of the last decade well document the development and evaluation of dozens of Cdk inhibitors representing different chemical classes (Galons et al., 2010; Rizzolio et al., 2010; Senderowicz & Sausville, 2000; Sharma et al., 2008; Sridhar, 2006). About 25 of them reached clinical trials. However, several studies with Cdk inhibitors showing promising preclinical results – for example AG-024322, AZD5438, R547, SNS-032, and ZK 304709 – had to be terminated or were discontinued after phase I (Lapenna & Giordano, 2009) (www.clinical trials.gov).

This book chapter will focus on 9 promising compounds tested in clinical trials at the time: AT7519, BAY 1000394, flavopiridol, P1446A-05, P276-00, PD 0332991, PHA-848125, R-roscovitine, and SCH 727965 (Fig. 2, Table 2).



pan-Cdk inhibitors

Fig. 2. Molecular structures of selected Cdk inhibitors tested in clinical trials at the time

Most of the Cdk inhibitors are less selective and affect several Cdk family members, not only resulting in cell cycle arrest via blocking of Cdk1, Cdk2, and Cdk4, but also in manipulation of RNA synthesis through targeting of transcriptional kinases Cdk7 and Cdk9. Combination of cell cycle kinase inactivation and Cdk9 inhibition has been shown to trigger cell death via promotion of apoptosis in tumor cells (Cai et al., 2006). Otherwise, toxic effects of transcriptional manipulation in non-tumor cells via inhibition of Cdk7 and Cdk9 could be crucial for therapeutic application of single agent Cdk inhibitors. In addition, it has to be considered, that unselective targeting of transcriptional Cdk, e. g., Cdk10 and Cdk11 would

diminish therapeutic effects, due to their relevance for tumor suppression and apoptosis (Chandramouli et al., 2007; Iorns et al., 2008).

Among the first compounds described with Cdk specificity flavopiridol and R-roscovitine as derivatives of natural products were developed. All other Cdk inhibitors are mainly synthetic compounds.

Flavopiridol, an alkaloid derivative and member of the flavone group, displayed antiproliferative and cytotoxic effects on tumor cells at nanomolar concentrations (Sedlacek et al., 1996). This observation was associated with cell cycle arrest through inhibition of Cdk and induction of apoptosis in human hematopoietic cell lines, breast, lung, as well as head and neck squamous cell carcinoma (Kaur et al., 1992; Konig et al., 1997; Parker et al., 1998; Patel et al., 1998). In spite of inconsistent data of IC_{50} and K_d values for different Cdk, flavopiridol showed high affinity to all Cdk with IC_{50} values below 400 nM and exhibit higher selectivity to Cdk9 (IC_{50} < 10 nM) (Chao et al., 2000; Sedlacek, 2001; Sedlacek, 1996). Cdk9, as well as Cdk7 inhibition lead to profound influence on cellular transcription, e. g., on mRNA transcripts for cell cycle regulators Cyclin D, antiapoptotic proteins Bcl-2 and Mcl-2, and NFκB as well as p53 pathway (Lam et al., 2001; Lu et al., 2004). Independent from good antitumorigenic effects in preclinical studies, in 2008 only low specificity of flavopiridol for Cdk has been demonstrated due to nanomolar affinity to also 25 other protein kinases, like GSK3β and ERK (Karaman et al., 2008), maybe leading to discouraging results in some clinical trials (see next section).

Screening of about hundred compounds structurally related to flavopiridol identified **P276-00** as potent Cdk specific inhibitor with moderate selectivity for Cdk1, Cdk4, and Cdk9 (Joshi et al., 2007a). Similar to flavopiridol, P276-00 showed antiproliferative and proapoptotic activity in human breast, colon, lung, prostate carcinoma, and promyelocytic leukemia cell lines *in vitro* (Joshi et al., 2007b). Decreased Rb phosphorylation, G_1/ G_2 phase arrest, and caspase-dependent apoptosis could be observed in preclinical studies with multiple myeloma cells *in vitro* and *in vivo* (Manohar et al., 2011; Raje et al., 2009).

The purine derivative **R-roscovitine** inhibits Cdk1, Cdk2, Cdk5, Cdk7, and Cdk9 with selectivity to Cdk4 and Cdk6 (Meijer et al., 1997). In consequence, R-roscovitine lead to arrest of tumor cells in almost all cell cycle phases and affected cell proliferation, as it was demonstrated for over 60 human tumor cell lines (e. g., melanoma, lung, breast, colon carcinoma, leukemia). *In vivo* activity in mice bearing colorectal carcinoma xenografts, but also in hematopoietic progenitors (Mcclue et al., 2002; Raynaud et al., 2005; Song et al., 2007) and enhancement of antitumor effects of radiation and doxorubicin in combination with R-roscovitine was reported (Appleyard et al., 2009; Maggiorella et al., 2003). Like flavopiridol and other Cdk inhibitors with broad spectrum, several additional effects have been described for R-roscovitine *in vitro*: interruption of transcriptional elongation, interference with survival-associated pathways (IκB kinase inhibition), induction of p53 phosphorylation and apoptosis (Alvi et al., 2005; Dey et al., 2008; Hahntow et al., 2004).

A novel potent Cdk inhibitor with striking similarity to R-roscovitine regarding both chemical structure and cytotoxic properties is pyrazolo[1,5-*a*]pyrimidine derivative **SCH 727965** (Parry et al., 2010). SCH 727965 inhibits Cdk1, Cdk2, Cdk5 and Cdk9 activity *in vitro* in low nanomolar concentrations (IC_{50} < 5 nM) and exhibited antiproliferative effects due to complete suppression of Rb phosphorylation and apoptosis induction, respectively.

Fragment-based screening techniques identified pyrazole-3-carboxamide **AT7519** as potent Cdk2 inhibitor and antiproliferative agent (Wyatt et al., 2008). AT7519 caused cell cycle

arrest, growth inhibition, and apoptosis in a large spectrum of human solid tumor cells and colon carcinoma xenograft models (Squires et al., 2009). Influence on transcriptional regulating Cdk, as well as GSK3β, resulting in induction of apoptotic pathways was observed in multiple myeloma and leukemia cell lines (Santo et al., 2010; Squires et al., 2010).

Optimization of selectivity and physicochemical properties of pyrazolo[4,3-*h*]quinazoline-3-carboxamide derivatives identified **PHA-848125** as potent Cdk inhibitor (IC_{50} < 400 nM) with inhibitory effects on cell cycle progression and Rb phosphorylation *in vitro* in a wide range of tumor cell lines (Brasca et al., 2009; Caporali et al., 2010). Among a panel of 43 other serine-threonine and tyrosine kinases only tropomyosin receptor kinase A (TRKA), linked to cancer cell survival, was inhibited by PHA-848125 in the same nanomolar range (IC_{50} 53 nM). *In vivo* characterization of oral active compound PHA-848125 showed a dose-dependent inhibition of human A2780 ovarian carcinoma xenograft tumor growth on mice up to 91%. Significant tumor growth inhibition was also observed in a K-Ras mutant lung adenocarcinoma transgenic mouse model, carcinogen-induced tumors, and in disseminated primary leukemia models (Albanese et al., 2010; Degrassi et al., 2010).

Only a few details have been published for pan-Cdk inhibitor **BAY 1000394** with unknown structure targeting Cdk1, Cdk2, Cdk4 and Cdk9 with high affinity (IC_{50} < 10 nM) (Siemeister et al., 2010). Inhibition of cell proliferation was amongst others depicted for breast, cervical, and colorectal human tumor cell lines and described to be independent of Rb, p53 and tumor suppressor gene status. Suppression of Rb phosphorylation and growth inhibition after treatment with BAY 1000394 was observed in preclinical studies in a broad range of tumor xenografts.

Cdk4/ Cdk6 selective inhibitors

Several compounds, particularly, members of chemical classes of benzothiadiazines, diarylureas, indolocarbazoles, oxindoles, pyrido[2,3-*d*]pyrimidines, thienopyrimidinhy drazones, thioacridones, and triaminopyrimidines were developed in the last decade and described with preferential selectivity to Cdk4 and Cdk6 (Graf, 2010; Lee & Sicinski, 2006). Cdk4 and Cdk6 reveal due to their high homology and identical substrate specificities comprehensive activities in the cell cycle. According to the occurrence in different tissues Cdk4 and Cdk6 compensate each other in function (Ciemerych & Sicinski, 2005; Meyerson & Harlow, 1994). **P1446A-05** and pyrido[2,3-*d*]pyrimidine **PD 0332991** seem to be the most promising compounds due to their inclusion in clinical evaluation. Unfortunately, no information about chemical structure and mechanism of Cdk4 selective inhibition is provided in the literature for P1446A-05. However, potency of PD 0332991 to inhibit Cdk4 and Cdk6 pathway and tumor cell progression has been extensively studied *in vitro*, as well as *in vivo* in mouse xenograft models (Baughn et al., 2006; Fry et al., 2004; Menu et al., 2008; Saab et al., 2006). In all studies an antiproliferative response to PD 0332991 treatment as a result of G_1 phase arrest after inhibition of Cdk4- and Cdk6-mediated Rb phosphorylation was observed in lymphoma, myeloma, sarcoma, breast, lung, and colon carcinoma. Though, treatment efficiency of Cdk4/ Cdk6 selective inhibitors like PD 0332991 is limited to tumors with wild-type *rb* gene status. Furthermore, PD 0332991 does not induce apoptosis and seems to be ineffective in the majority of quiescent G_0/ G_1 arrested leukemic cells with primarily defects in apoptosis pathway, as it was demonstrated for chronic lymphocytic leukemia (Wesierska-Gadek et al., 2011).

4. Cdk inhibitors for cancer therapy

Despite of promising preclinical data and numerous potent Cdk inhibitors in clinical trials, none of these molecules has already been approved as drug for cancer therapy. Nevertheless, evaluation of various Cdk inhibitors contributed to important information about bioavailability, pharmacokinetics, as well as, competing toxicities and lead to alterations in dosing schedule to reach a maximum in response. To date, monotherapy did not demonstrate convincing utility of a certain Cdk inhibitor for cancer treatment, although clinical trials of several hematologic malignancies have shown stable disease. Encouraging results depicting antitumor activity of Cdk inhibitors also in solid tumors have been obtained in various combination studies with cytostatic or cytotoxic agents. However, beneficial effects of Cdk inhibition alone and in combination with other chemotherapeutic agents to tumor treatment must be demonstrated in randomized clinical trials in the future.
Information about current clinical trials of Cdk inhibitors were achieved from the webpage *www.clinicaltrials.gov* and a summary is given in Table 2.

Flavopiridol was the first Cdk inhibitor entering clinical trials. Since the early 1990s flavopiridol was extensively studied in patients with refractory neoplasms, a variety of solid tumors and hematopoietic malignancies. Clinical trials with single flavopiridol were completed for, e. g., advanced gastric cancer, endometrial adenocarcinoma, metastatic melanoma, multiple myeloma, and non-small cell lung cancer in phase II, but only unsatisfying results concerning antitumor activity and toxicities like myelosuppression, diarrhea as well as thromboses were observed (Burdette-Radoux et al., 2004; Dispenzieri et al., 2006; Grendys et al., 2005; Schwartz et al., 2001; Shapiro et al., 2001). Also, no response to flavopiridol administered as 24 hour continuous infusion in chronic lymphocytic leukemia patients was observed (Flinn et al., 2005). Detection of high serum protein binding of flavopiridol and collection of pharmacokinetic data contributed to the adjustment of treatment-schedule of flavopiridol. In consequence, hematopoietic malignancies showed encouraging responses to flavopiridol. Weekly application of flavopiridol as a single agent for 6 weeks in a phase I study of chronic lymphocytic leukemia patients achieved a median progression-free survival of 12 month (Byrd et al., 2007; Phelps et al., 2009). Addition of prophylactic corticosteroid dexamethasone to flavopiridol treatment improved tolerability in chronic lymphocytic leukemia patients (Lin et al., 2009). In combination with cytostatic drugs cytosine arabinoside and mitoxantrone in phase II clinical trials a 75% response rate of patients with acute myelogenous leukemia was observed (Karp et al., 2007). Combined treatment of flavopiridol with chemotherapeutic agents cisplatin, carboplatin, docetaxel, gemcitabine, irinotecan or paclitaxel, respectively, have shown response or at least stable disease in phase I trials (Bible et al., 2005; Fekrazad et al., 2010; Fornier et al., 2007; George et al., 2008). However, subsequent phase II clinical trial with flavopiridol in combination with docetaxel resulted again in disappointing activity and significant toxicity in patients with metastatic pancreatic adenocarcinoma (Carvajal et al., 2009). Currently ongoing and recruiting clinical studies focus on evaluation of flavopiridol (combined with chemotherapeutic agents like bortezomib, oxaliplatin, or respectively, lenalidomide) in patients with B-cell neoplasms, lymphoma, multiple myeloma, and germ cell tumors.

P276-00 is currently in phase I/II clinical trials to determine anticancer activity in patients with advanced Cyclin D1 positive malignant melanoma, mantle cell lymphoma, squamous cell head and neck carcinoma, and multiple myeloma. A phase I study in patients with advanced refractory neoplasms has already been completed. No results have been provided in the literature yet. Combination studies currently recruiting patients are initiated for

P276-00 administered along with radiation in head and neck squamous cell carcinoma patients, and in combination with gemcitabine in patients with advanced pancreatic cancer.

A phase I clinical trial of **R-roscovitine** has been performed in 22 patients with advanced cancer utilizing several schedules resulting in only minor therapeutical success with hypokalemia, rash and fatigue as dose limiting side-effects (Benson et al., 2007). Further evaluation of R-roscovitine in patients with non-small cell lung cancer was described: R-roscovitine in combination with cisplatin and gemcitabine in phase I showed similar adverse events, e. g., hypokalemia, liver γ-glutamyltranspeptidase elevation, vomiting, and a 42.9% response rate in 14 patients (Siegel-Lakhai et al., 2005). A phase II study of oral R-roscovitine to treat non-small cell lung cancer has been terminated without any reports on available data. Antitumor activity and pharmacodynamic effects of R-roscovitine sequentially administered with sapacitabine was initiated in 2009 in patients with advanced solid tumors.

Safety and tolerability of **SCH 727965** was demonstrated in phase I clinical trials for multiple malignant indications including solid tumors, non-Hodgkin's lymphoma, multiple myeloma, and chronic lymphocytic leukemia (Shapiro et al., 2008). The drug was administered once every 21 days as a 2 hour intravenous infusion and dose limiting toxicity was neutropenia. To improve treatment success a phase I study currently recruiting patients is initiated in patients with advanced cancer for schedule adjustment (3 infusions ever 7 days in a 28 day cycle). First results in previously treated patients with chronic lymphocytic leukemia showed common toxicities, like fatigue, nausea, and diarrhea, but also evidence of therapeutic benefit of SCH 727965 (Flynn et al., 2009). In an active phase II clinical trial activity of SCH 727965 in patients with advanced breast cancer, non-small cell lung cancer, mantle cell lymphoma, or B-cell chronic lymphocytic leukemia each in comparison to standard treatment (capecitabine, erlotinib, bortezomib, or alemtuzumab) is determined. Furthermore, phase I/II studies including patients with malignant melanoma and multiple myeloma, as well as plasma cell neoplasms will be performed.

Two phase I/II studies with **AT7519**, currently recruiting patients are under evaluation. Single AT7519 treatment of patients with advanced/metastatic solid tumors or refractory non-Hodgkin's lymphoma will provide data to applicable dose, pharmacokinetics, pharmacodynamics and side effects. First results of this study suggested evidence of clinical activity of AT7519, due to reduction of PCNA, a protein required for DNA replication, as a result of Cdk inhibition and an increase of apoptosis markers (Mahadevan et al., 2011). Mucositis, neutropenia, and reversible thrombocytopenia were identified as dose limiting toxicities. In another ongoing clinical trial, efficacy of either AT7519 alone or AT7519 in combination with proteasome inhibitor bortezomib is determined in patients with previously treated multiple myeloma.

PHA-848125 was first evaluated in a multi center phase I study to investigate the safety and pharmacokinetics in patients with advanced/ metastatic solid tumors. Oral administration of PHA-848125 in different treatment schedules showed good tolerability with ataxia and tremors as dose-limiting side-effects and occasional hematological toxicities (Benouaich-Amiel et al., 2010; Cresta et al., 2010; Tibes et al., 2008). Similar results were found in a phase I study of PHA-848125 in combination with gemcitabine (Bahleda et al., 2010). Antitumor activity of PHA-848125 is currently assessed in phase II clinical trials in patients with recurrent or metastatic thymic carcinoma previously treated with chemotherapeutic agents.

Clinical studies of pan-Cdk inhibitor **BAY 1000394** and Cdk4 selective inhibitor **P1446A-05** are in the beginning and thus less well documented. Both compounds are tested in first clinical trials at the moment to identify tolerated dose of the drug and to determine both limiting side effects and efficacy in patients with advanced solid tumors. Evaluation of P1446A-05 includes also hematologic malignancies.

PD 0332991 was first tested in a phase I dose escalation trial in 57 patients with Rb positive advanced cancer (e. g., breast, colorectal cancer, melanoma) (O'dwyer et al., 2007). Doses from 25 mg to 150 mg of single agent PD 0332991 were administered orally for 21 days in a 28 days cycle. Stable disease was manifested for 9 patients and dose limiting toxicity was myelosuppression. Another phase I study assessing mechanism of action, safety, and pharmacodynamic effects of PD 0332991 was performed in patients with mantle cell lymphoma using fluorine-18-labeled fluorodeoxyglucose ([^{18}F]FDG) and fluorothymidine ([^{18}F]FLT) positron emission tomography imaging (Leonard et al., 2008). Numerous phase II clinical trials of PD 0332991 are ongoing and currently recruiting patients: with advanced non-small cell lung cancer, with refractory solid tumors, with recurrent Rb positive glioblastoma, and with metastatic liposarcoma. Evaluation of PD 0332991 in combination with letrozole for treatment of hormone-receptor positive advanced breast cancer, in combination with bortezomib for mantle cell lymphoma, as well as in combination with velcade and dexamethasone in patients with multiple myeloma is ongoing after encouraging antitumor activity was revealed in phase I clinical trials (Niesvizky et al., 2009; Niesvizky et al., 2010; Slamon et al., 2010).

5. Specific aspects regarding kinase inhibitor resistance

An important problem for new approaches in cancer therapy, also resulting in significant limitations of the potential use of kinase inhibitors, is the resistance of tumor cells to cytotoxic anticancer drugs acquired during therapy. As a relevant example, resistance to kinase inhibitor imatinib selective for Bcl-Abl was observed in chronic myelogeneous leukemia and gastrointestinal stromal tumor patients (Bixby & Talpaz, 2011; Wang et al., 2011). Development of kinase inhibitor resistance is accomplished by increased expression and even more common by specific point mutations of Bcl-Abl oncogene. In consequence, association of inhibitor with kinase is prevented without rigorous effects on enzyme activity. Despite of no reported appearance of Cdk inhibitor resistance in clinical trials so far, essential side chains for Cdk inhibitor specificity and selectivity, but not for ATP binding were predicted. An aromatic amino acid in the conserved Cdk domain, e. g., phenyl-alanine-80 in Cdk2, provides a hydrophobic site for essential *van der Waals* contacts with the Cdk inhibitor (for example isopropyl of R-roscovitine and acetyl of PD 0332991) (Krystof & Uldrijan, 2010). In parallel to mutation-based steric hindrance of a aurora B inhibitor causing kinase resistance (Girdler et al., 2008), histidine residues in Cdk4 and Cdk6 have been predicted to contribute to selectivity of PD 0332991 and discriminate Cdk1 and Cdk2 (Lu & Schulze-Gahmen, 2006). Point mutations modifying these side chains could directly result in the loss of Cdk inhibitor binding. However, findings of sufficiency of Cdk1 to drive the mammalian cell cycle in mouse embryogenesis raise the questions if Cdk1 could substitute all the other interphase Cdks in human tumor cells anyway and whether a point mutation is likely to cause resistance to a pharmacological Cdk inhibitor (Santamaria, 2007).

inhibitor	targets (IC50)	clinical trials in patients with
AT7519	Cdk2, Cdk5, Cdk9 (< 50 nM) Cdk1, Cdk4, Cdk6 (≤ 210 nM) GSK3β (89 nM) (Squires, 2009)	- advanced, metastatic solid tumors, non-Hodgkin's lymphoma (phase I) - multiple myeloma (alone and in combination with bortezomib (phase I)
BAY 1000394	Cdk1, Cdk2, Cdk4, Cdk9 (≤ 11 nM) (Siemeister, 2010)	- advanced solid tumors (phase I)
flavopiridol (alvocidib)	Cdk4, Cdk9 (< 50 nM) Cdk1, Cdk2, Cdk6, Cdk7 (≤ 400 nM) GSK3β (450 nM) (Sedlacek, 2001)	- lymphoma, multiple myeloma, leukemia, sarcoma, various advanced solid tumors (alone and in combination with cytotoxic agents) (phase I, II)
P1446A-05	Cdk4 (n. a.) (www.piramallifesciences.com)	- advanced refractory malignancies (hematologic or solid tumors) (phase I)
P276-00	Cdk1, Cdk4, Cdk9 (< 100 nM) Cdk2, Cdk6 (≤ 400 nM) Cdk7, GSK3β (2.8 μM) other protein kinases (> 10 μM) (Joshi, 2007a)	- head and neck squamous cell carcinoma, multiple myeloma, mantle cell lymphoma, melanoma (phase I, II) - head and neck squamous cell carcinoma (in combination with radiation) (phase I, II) - advanced pancreatic cancer (in combination with gemcitabine) (phase I, II)
PD 0332991	Cdk4, Cdk6 (≤ 15 nM) Cdk1, Cdk2, Cdk5, other protein kinases (> 10 μM) (Fry, 2004)	- mantle cell lymphoma (alone and in combination with bortezomib) (phase I) - advanced cancer (phase I) - refractory solid tumors, non-small cell lung cancer, glioblastoma, liposarcoma (phase II) - hormone-receptor positive breast cancer (alone and in combination with letrozole) (phase I, II) - multiple myeloma (in combination with velcade and dexamethasone) (phase I, II)
PHA-848125	Cdk2 (45/363 nM) Cdk1, Cdk4, Cdk5, Cdk7 (< 400 nM) TRKA (53 nM) (Brasca, 2009)	- advanced/ metastatic solid tumors (phase I) - thymic carcinoma (phase II)
R-roscovitine (seliciclib) (CYC202)	Cdk1, Cdk2, Cdk5, Cdk7, Cdk9 (≤ 800 nM) Cdk4, Cdk6, other protein kinases (≥ 14 μM) (Meijer, 1997; Popowycz et al., 2009)	- non-small cell lung cancer (phase II) - advanced solid tumors (in combination with sapacitabine) (phase I)
SCH 727965 (dinaciclib)	Cdk1, Cdk2, Cdk5, Cdk9 (< 5 nM) (Parry, 2010)	- advanced solid tumors, lymphoma, leukemia (phase I) - melanoma, multiple myeloma (phase I, II) - advanced breast and lung cancer (phase II) - lymphoma, leukemia (phase II)

Table 2. Cdk inhibitors currently under clinical evaluation. Information to clinical trials were obtained from *www.clinical trials.gov.* (GSK3β: glycogen synthase kinase 3β; TRKA: tropomyosin receptor kinase A)

Referring to this, a critical view on the advantages and disadvantages of Cdk inhibitor selectivity for the success of therapeutic approaches should be given. Is development of exclusively selective Cdk inhibitors challenging tumor therapy?

First of all, due to high homology in Cdk active sites development of monospecific Cdk inhibitor is nearly impossible. This prediction becomes apparent by the given IC_{50} values in the list of potent Cdk inhibitors in clinical trials (Table 2). Only PD 0332991 exhibits high selectivity to Cdk4 and Cdk6 *in vitro*. But at certain pharmacological concentrations, there is no exclusion of influence on various members of Cdk family.

Cdk inhibitors with improved selectivity promise minimization of undesired side effects, since no effects on global transcriptional machinery (Cdk7, Cdk9) would be expected in healthy cells as it was described for pan-Cdk inhibitors. Certainly, selective inactivation of any Cdk would not be as efficient as inhibition of multiple Cdk, including Cdk1, and involves the risk of easy development of Cdk inhibitor resistance, especially by alterations in the primarily targeted pathway. The response on Cdk4 and Cdk6 selective inhibitor PD 0332991 is significantly linked to functional Rb, transcriptional repression through E2F and the ability to attenuate Cdk2 activity, otherwise resistance to PD 0332991 acquired in tumor cell lines (Dean et al., 2010). In contrast, high efficiency of treatment in Cdk4/ Cdk6-dependent tumors and even reversal of resistance to estrogen receptor pathway targeting with tamoxifen by combined therapy with PD 0332991 could be achieved (Finn et al., 2009).

Pan-Cdk inhibitors affect multiple pathways in tumor cells and attack heterogeneous malignant cells (e. g., chronic lymphocytic leukemia, multiple myeloma), hindering Cdk inhibitor resistance. But, one has to consider the consequences of pan-Cdk inhibitors on healthy proliferating cells, like hematopoietic and dermal cells *in vivo*, which are much more sensitive to blockage of global cell cycle machinery and regulators.

To emulate and characterize Cdk inhibitor resistance *in vitro*, ambitions to generate cellular models were described. An increased activity due to increased protein synthesis of Cyclin E and simple overexpression of Cdk1 was observed in ovarian and colorectal carcinoma cell lines with acquired resistance to flavopiridol (Bible et al., 2000; Smith et al., 2001). But overexpression of Cyclin E in the cells is not mandatory associated with decreased sensitivity to flavopiridol (Smith, 2001). Also no evidence to mutational modification of Cdk was given in the studies. In fact, resistance to flavopiridol and another Cdk inhibitor was related to increased expression or activity of ATP-binding cassette transporter ABCG2 minimizing accumulation of the inhibitors and responsible for multidrug resistance in certain cancer cells (Robey et al., 2001; Seamon et al., 2006). Thus, mechanisms of resistance to Cdk inhibitors described to date seem to be inconsistent in dependence of the genetic status of different cell lines. Decreased sensitivity to a certain drug is often a result of multiple alterations in the tumor cells and further studies will elucidate the appearance of Cdk-associated genetic changes as a consequence of long-term treatment with Cdk inhibitor.

A hopeful therapeutic strategy to overcome development of inhibitor resistance is the combination of Cdk inhibitors with common chemotherapeutic agents for fast and effective elimination of malignant cells. Recent clinical studies with combined therapeutic regimes will clarify the benefit for the patients.

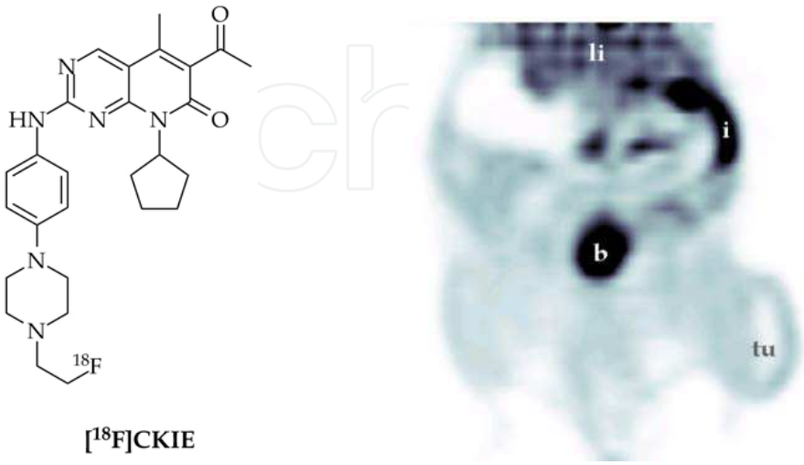
6. Cdk inhibitors as molecular probes for cancer imaging

The development of potent radiolabeled Cdk inhibitors, as radiotracers for tumor visualization using positron emission tomography (PET) is a novel approach allowing

functional, non-invasive characterization and imaging of Cdk in human tumors. Particularly, radiolabeled Cdk4/Cdk6 inhibitors are of interest for the assessment of Cdk4/Cdk6 protein status and activity in human tumors in current translational cancer research. PET affords the opportunity of three-dimensional imaging and quantitation of physiological processes *in vivo*. Additionally, PET provides pharmacological data of radiolabeled Cdk4/Cdk6 inhibitors, which may help to understand mechanism of action *in vivo* and estimate the applicability of the compounds for tumor therapy.

In this regard, new Cdk4/Cdk6 inhibitors derived from pyrido[2,3-*d*]pyrimidine lead structure (Vanderwel et al., 2005) were designed, synthesized and characterized in our institute for the first time. Evaluation of five compounds – CKIA, CKIB, CKIC, CKID, and CKIE – showed specific and selective inhibition of Cdk4/ Cdk6-mediated pathway, induction of G_1 phase arrest and blocking of tumor cell proliferation in pharmacological concentrations (Graf et al., 2009; Graf, 2010). Most potent Cdk4/ Cdk6 inhibitors CKIA, CKIB, and CKIE were radiolabeled with positron-emitters iodine-124 [^{124}I] or fluorine-18 [^{18}F], respectively, and characterized concerning their radiopharmacological properties in cellular radiotracer uptake studies, biodistribution and, small animal PET studies (Graf, 2009; Graf, 2010; Koehler et al., 2010). Iodine-124 with a half-life of 4.18 days allows extended radiopharmacological evaluation. Nevertheless, minor positron emission (26%) and high positron energy are disadvantages leading to low resolution PET images. Most frequently used PET nuclide fluorine-18 exhibits nearly 97% positron emission with a half-life of 109.8 minutes.

In vitro radiotracer uptake studies using [^{124}I]CKIA, [^{124}I]CKIB, and [^{18}F]CKIE demonstrated substantial tumor cell uptake, an important prerequisite for PET studies, in NMRI nu/nu mice bearing the human squamous cell carcinoma tumor FaDu. Dynamic small animal PET studies demonstrated rapid clearance of [^{124}I]CKIA, [^{124}I]CKIB, and [^{18}F]CKIE from the blood and fast hepatobiliary excretion. The half-life of radiotracer elimination from the blood was calculated to between 7.2 and 7.9 min. Only marginal tumor uptake of radiotracers [^{124}I]CKIA and [^{124}I]CKIB was observed. In the case of [^{18}F]CKIE higher uptake was detected in the peripheral cell-cycle active region of the tumor one hour after intravenous injection (Fig. 3). However, the constant tumor-to-muscle ratio of 1.5 suggests a non-Cdk4- or non-Cdk6-mediated association of [^{18}F]CKIE in human tumor xenografts in mice.



[^{18}F]CKIE

li, liver, i, intestine, b, bladder, tu, human squamous cell carcinoma.

Fig. 3. PET studies with fluorine-18 radiolabeled pyrido[2,3-*d*]pyrimidine derivative CKIE

In conclusion, the short biological half-life in the blood and low tumor uptake of the studied radiolabeled pyrido[2,3-*d*]pyrimidines limit the clinical application of these Cdk4/ Cdk6 inhibitors as radiotracers for the characterization of Cdk4/Cdk6 in tumors by means of PET. Nevertheless, further development and evaluation of suitable radiolabeled Cdk inhibitors with optimized properties *in vivo* are still of outstanding interest for the prospective functional characterization of Cdk in tumors by means of PET.

7. Conclusion

Critical contribution of cell cycle regulating kinases Cdk to carcinogenesis provides a promising target for diagnostic characterization of malignancies and development of novel therapeutic interventions. Numerous compounds directly inhibiting Cdk and, as a consequence, cell proliferation have been developed, and 9 of them are currently under clinical evaluation (phase I and II) as antitumor agents. Most of the candidates are pan-Cdk inhibitors affecting several Cdk family members with advantages in efficiency of tumor treatment due to not only inhibition of cell proliferation but also apoptosis induction. Only one inhibitor – pyrido[2,3-*d*]pyrimidine PD 0332991 – has been comprehensible described with preferential selectivity for Cdk4 and Cdk6 applicable for Rb positive tumors with primarily defects in Cdk4/ Cdk6 pathway. Application of Cdk inhibitors to patients with advanced cancers resulted in stabilization of disease. Combination with classical chemotherapeutic agents and adjustment of therapeutic schedules may also cause tumor regression and contribute to prevention of drug resistance. More detailed preclinical evaluation using suitable tumor models and focused clinical trials will give valuable implications for new mechanism-based approaches and Cdk drug developments as well as tumor specific treatment.

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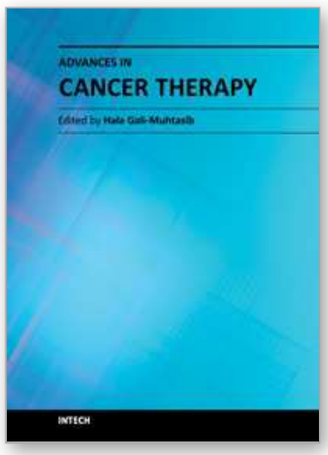

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