Cyclin-Dependent Kinase 4/6 (Cdk4/6) Inhibitors: Perspectives in Cancer Therapy and Imaging

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Abstract: Cyclin-dependent kinases 4 and 6 (Cdk4/6) are important components of cell cycle activation and control in early G_1 phase. Both enzymes and their regulators, e.g., cyclins, play critical roles in embryogenesis, homeostasis, and cancerogenesis. Cdk4/6 are attractive targets for cancer treatment. Recently, numerous selective small molecule inhibitors of Cdk4/6 have been developed. The potential of Cdk4/6 inhibitors, particularly, pyrido[2,3-*d*]pyrimidine derivatives, as both anti-cancer drugs and ^{124}I- and ^{18}F-radiolabeled tracers for cancer imaging using positron emission tomography is discussed.

Keywords: Cell cycle regulation, pyrido[2,3-*d*]pyrimidine, iodine-124, fluorine-18, radiolabeling, positron emission tomography.

INTRODUCTION

The first gap phase G_1 of the cell cycle comprises important preliminary steps for initiation of DNA replication and subsequent cell division in human embryogenesis, homeostasis and also in cancer development. Especially, the cyclin-dependent kinases 4 and 6 (Cdk4/6) function as critical activators of G_1 phase progression [1]. Resulting from high homology, identical substrate specificities and enzymatic activities Cdk4 and Cdk6 have overlapping functions in development [1], even though different tissue distribution or activation have been described [2, 3]. Cdk4 (formerly PSK-J3) and Cdk6 (formerly PLSTIRE) were discovered as possible cell cycle regulating proteins by cloning and amino acid sequence homology analyses in 1987 and 1992 [4, 5].

1. CDK4/6 IN HEALTH AND DISEASE

1.1. CDK4/6 in Cell Cycle Regulation

In the last decades the regulating role of Cdk4/6 activity and complex regulation mechanisms for their activation in the cell cycle were elucidated (reviewed in [1] and [6]). The catalytic activity of serine/ threonine protein kinases Cdk4/6 depends on the association with D-type cyclins, interaction with various regulatory proteins, activation by kinases and phosphatases, and their subcellular localization (Fig. (**1**)). In response to mitogenic signals synthesis of D-type cyclins (cyclin D1, D2, D3) and association with Cdk4/6 is initiated. Additionally, $p27^{Kip1}$, a member of the Cip/Kip family, appears to be a stabilizing factor for the formation of the Cdk4-cyclin D complex in proliferating cells and for the translocation to the nucleus [6]. Binding of $p21^{Cip1}$ and $p27^{Kip1}$ to Cdk4/6-cyclin D complexes in G_1 phase also serves to titrate these inhibitors from Cdk2-cyclin E and promote G_1 phase progression [7]. Furthermore, for catalytic activity phosphorylation of Cdk4/6-cyclin D complexes at Thr^{172} in the T-loop of Cdk4 by Cdk activating kinase complex (CAK) is essential [8]. Activated Cdk4/6-cyclin D complexes are transferred to the nucleus and phosphorylate various members of retinoblastoma (Rb) protein family, e.g., pRb, p107 and p130. Rb proteins exhibit multiple phosphorylation sites, for successive phosphorylation by Cdks preferring different sites. Cdk4/6 specific phosphorylation sites are pRb-Ser^{780}, pRb-Ser^{795} and p107-Ser^{842} [9-11]. Rb proteins are involved in the transcriptional control of genes *via* binding to transcription factors of E2F family. Cdk4/6-cyclin D mediated phosphorylation of pRb-Ser^{780} and pRb-Ser^{795} results in the release of pRb from E2F and, hence, promotion of transcription of genes required for subsequent cell cycle control, e.g. cyclin E, and for DNA replication in S phase, e.g., DNA polymerase α or proliferating cell nuclear antigen (PCNA) [12, 13]. Besides phosphorylation of Rb proteins phosphorylation of other substrates, like myristoylated alanine-rich C-kinase substrate (Marcks), replication licensing factor Cdt1, and antiproliferative transcription factor Smad3, by the Cdk4-cyclin D complex have been described demonstrating the more extensive regulatory function of Cdk4/6 in the cell cycle [14-16]. Activity of Cdk4/6-cyclin D is beyond from the short period in G_1 phase no longer required in the cell cycle. On the one hand, Cdk4/6 substrate phosphorylation is affected by nuclear export of Cdk4/6-cyclin D complexes or respectively single cyclin D [17]. On the other hand, Cdk4 activity is regulated through inhibitory phosphorylation of Tyr^{17} residue of Cdk4 by Src family kinases and activating dephosphorylation of this amino acid by Cdc25 phosphatases [18]. In addition, endogenous Cdk4/6 inhibitors interact with either Cdk4/6-cyclin D complexes (Cip/Kip proteins: $p21^{Cip1}$, $p27^{Kip1}$, $p57^{Kip2}$) or monomeric kinases (INK4 proteins: $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$) to negatively regulate G_1 progression. Besides its activating function as Cdk4/6-

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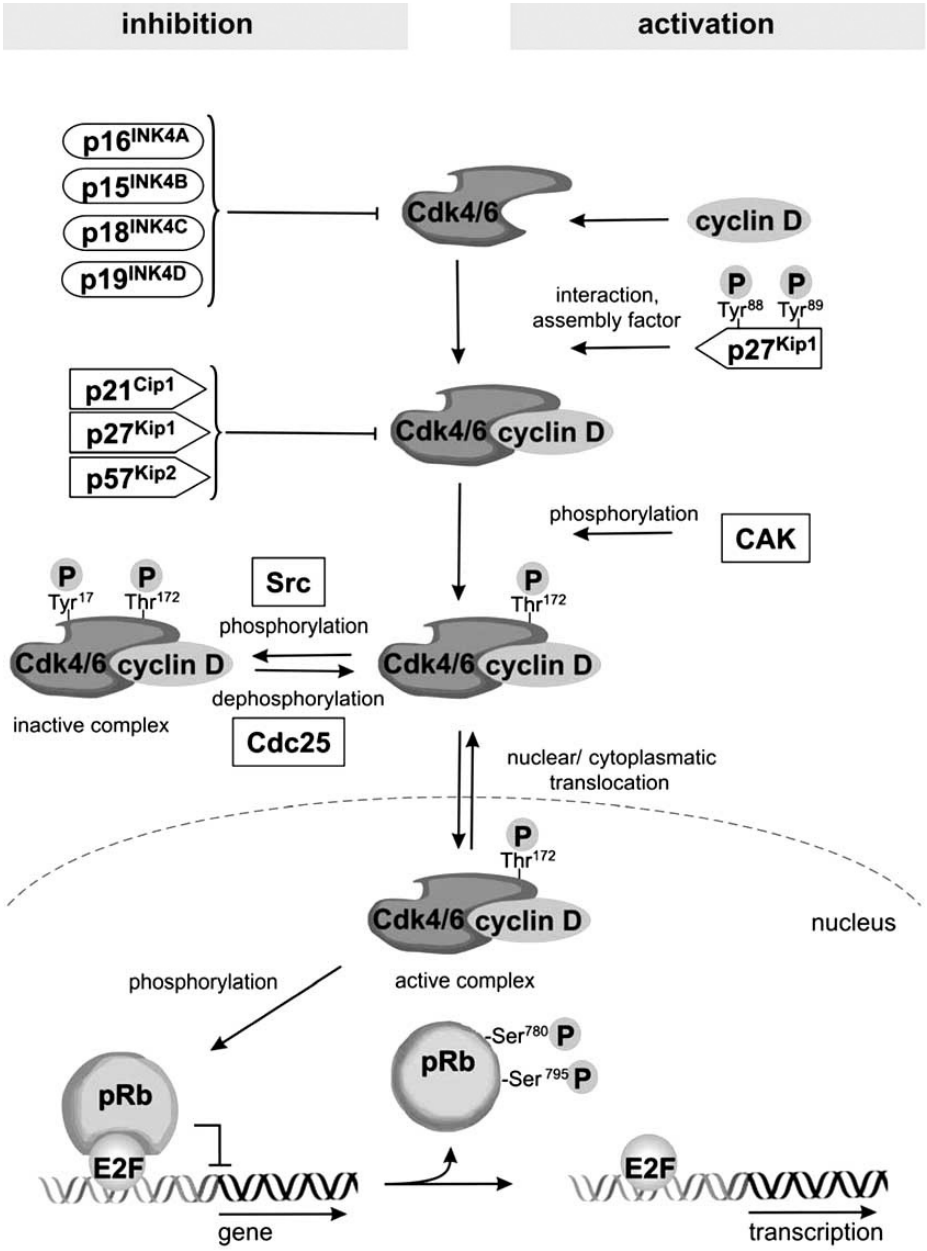

Fig. (1). The Cdk4/6-cyclin D/ pRb/ E2F pathway.

Regulation of Cdk4/6 catalytic activity in cells – the phosphorylation of members of Rb family – by specific effectors (INK4, Cip/Kip proteins), kinases/phosphatases (Src, CAK/ Cdc25) and nucleocytoplasmatic translocation.

cyclin D assembly factor $p27^{Kip1}$, as well as $p21^{Cip1}$ and $p57^{Kip2}$, also inhibit Cdk4/6-cyclin D complexes depending on the growth state and conditions of cells [6]. All INK4 proteins, whose expression is induced in response to antimitogenic signals, are specific inhibitors of Cdk4/6 and prevent the association with D-type cyclins by competitive inhibition and formation of trimeric complexes with Cdk4/6 [19].

1.2. Role of Cdk4/6 in Embryogenesis

Progression of the cell cycle was considered to be driven by a number of different Cdks chronologically activated by association with specific cyclins in mammalians and important for exact embryogenesis. This classical model of cell cycle control was derived from biochemical evidence and discovery of 13 Cdk family members described in humans so far (reviewed in [20]). Thereby, Cdk4/6 and Cdk2 were described as most important checkpoint kinases in interphase (G_1, S, G_2) and Cdk1 in mitosis. Interestingly, in very early embryogenesis Cdk4/6 appear only to be required for subordinate cell cycle control, not for pRb inactivation and activation of E2F-dependent gene transcription (reviewed in [2]). In mouse oocytes amounts of Cdk4, Cdk6 as well as cyclin D are rapidly decreased [21]. Subsequently, early embryonic cells are deficient for pRb and Cdk4/6-cyclin D complexes are resistant to $p16^{INK4A}$ [22, 23]. Hence, this early phase of mouse embryogenesis is independent from activation by

Cdk4/6-cyclin D/ pRb/ E2F pathway. Genetic studies in mice questioned the classical model of cell cycle, the need for different Cdk-cyclin complexes in interphase, and also requirement of Cdk4/6 in embryogenesis. Mice with single disruption of *Cdk4* gene or respectively *Cdk6* gene in embryonic stem cells completed embryogenic development. However, loss of Cdk4 or respectively Cdk6 led to decreased growth (dwarfism), female infertility, and affected development of highly specialized cell types, e.g. pancreatic β-cells, lactotrophic cells of the pituitary, and erythroid cells [24-28]. Loss of both kinases, Cdk4 and Cdk6, resulted in late embryonic lethality caused by limited hematopoietic cell proliferation, but no defects in other tissues were observed in double mutant mouse embryos [28]. Similar effects were found in mice with concomitant ablation of all D-type cyclins [29]. Otherwise, complete development of embryos lacking Cdk4 and Cdk2 in the germ line was observed, indicating compensatory roles of Cdk4 and Cdk6 and requirement of only one kinase, either Cdk4 or Cdk6 [30]. These Cdk4 and Cdk2 targeted mice were born alive, but smaller than single targeted Cdk4 mice and with restricted numbers of cardiomyocytes responsible for early death of these animals. Another study by Berthet *et al.* revealed the death of $Cdk4^{-/-};Cdk2^{-/-}$ mouse embryos during gestation due to cardiac failure [31]. Similar phenotypes and effects on embryonic development were observed in $Cdk2^{-/-};Cdk4^{-/-};Rb^{-/-}$ triple mutant mice [32]. In contrast, $Cdk6^{-/-};Cdk2^{-/-}$ mice completed embryonic development and survived for up to 1.5 years, but also abnormalities in hematopoiesis similar to that observed in Cdk6 single mutants were obtained [28]. Knockout of all three interphase kinases, Cdk4, Cdk6 and Cdk2 clarified the pivotal role of Cdk1 in cell cycle, which has the ability to bind all types of cyclins and to promote the cell cycle of mice even in interphase [33]. Nevertheless, embryos lacking Cdk4, Cdk6 and Cdk2 had fatal hematopoietic and heart defects, certainly contributing to embryonic death at midgestation. In conclusion, Cdk4 and Cdk6 are not essential for early mouse embryogenesis, but all studies regarding germ line deletion of Cdk4 and/ or Cdk6 point out necessity of these kinases for late embryonic and complete mouse development, albeit only for some specialized cell types. For long-term and healthy survival of fertile mammalians, Cdk4 and Cdk6 are indispensable.

1.3. Role of Cdk4/6 in Homeostasis

In adult mammalians quiescence of cells is essential to obtain a *status quo* of tissues and cell populations, as well as for preservation of adult stem cells, which are later, if required, capable to generate proliferating progenitor cells. Homeostasis is characterized by balance of antiproliferative and mitogenic signals and in consequence Cdk-cyclin complexes and their endogenous regulators retain stem cells in quiescent G_0 phase of cell cycle. In adult stem cell populations cell cycle inhibitors of INK4, specific for Cdk4/6, and of Cip and Kip family are involved in repression of proliferation, but also in the self-renewal ability of progenitor cells (reviewed in [20]). Hence, due to modulation of Cdk4/6 by INK4, and Cip and Kip proteins, Cdk4/6 cooperate in these processes and control proliferative potential of stem cells and progenitor cells. During senescence of stem cells $p16^{INK4A}$ accumulates and induces G_1 phase arrest by association with Cdk4/6 [34]. Expression of *Cdk4* is repressed by transcription factor NFATc1 (nuclear factor of activated T cells c1), which is important for quiescence of hair follicle stem cells, otherwise precocious follicular growth is induced [35]. Reduction of Cdk4 mediated by siRNA in Cdk2-deficient cells showed decreased proliferation and self-renewal potential of neural progenitor cells and reduced neurosphere formation, suggesting an important regulatory and compensatory role of Cdk4 during postnatal brain development [36]. Other studies confirmed the critical requirement of Cdk4 activity for neuronal development, e. g. for Schwann cell proliferation in mice and also for short-term synaptic plasticity and metabotropic glutamate receptor-dependent long-term depression [37, 38]. Furthermore, Cdk4 is essential for postnatal development of specialized endocrine cell types. For instance, dysfunction of lactotrophic cell proliferation in the pituitary was found in Cdk4 targeted mice and reduction of β-islet pancreatic cells resulted in insulin-deficient diabetes of these mice during postnatal development [39, 40]. Other studies confirmed these results, but it has to be considered that Cdk4 is already ablated in germ line. Re-expression of Cdk4 in pancreatic β-cells and in the pituitary of Cdk4 *null* mice restored fertile and normoglycemic animals, but mice still exhibited dwarfism [40]. This indicates an extensive function of mammalian Cdk4 in establishing the homeostatic cell population. However, complete ablation of Cdk4 in germ line and (near) complete postnatal ablation of Cdk2 in tissues of adult mice exhibited, apart from Cdk4 associated defects described in the previous section, continuous generation of lymphoid and hematopoietic cells in spleen and thymus, and also hepatic regeneration in homeostasis [28]. Several studies corroborate the urgent requirement of Cdk4/6 for regular homeostasis in specialized tissues of high-capacity individuals, on the other hand abnormalities of Cdk4 activity favor neurodegenerative disorders like Alzheimer's disease or Parkinson's disease, and cancer development [41].

1.4. Role of Cdk4/6 in Cancerogenesis

Aberration in proliferation and consequently in cell cycle control is a common aspect in cancerogenesis. In fact, universal deregulation and hyperactivation of the Cdk4/6-cyclin D/ INK4/ Rb pathway has been described in human tumors provoked by multiple mechanisms [20]. In cancer development, hyperactivation of Cdk4/6 is often a result of overexpression of positive regulators (e.g., cyclin D), silencing of endogenous inhibitors (e.g., INK4, Cip and Kip proteins) or epigenetic alterations of their substrates (Rb) (reviewed in [1, 42]). Hyperactivity of Cdk4/6 was observed in different tumor types with preference for Cdk4 in epithelial malignancies (endocrine tissues and mucosa) and for Cdk6 in mesenchymal tumors (sarcomas and leukaemias) [20]. Disruption of Cdk4/6-associated cell cycle control is not only caused by regulators of Cdk4/6, but also directly by mutations and amplification of Cdk4/6. A miscoding point mutation of Cdk4 characterized by R24C substitution and resulting in an insensitivity to inhibitors of INK4 family was first described in patients with melanomas [43, 44]. The contribution of this mutation to tumor development has been further studied in $Cdk4^{R24C}$ knock-in mice and facilitated spontaneously occurrence of endocrine and epithelial hyperplasias, including insulinomas, sarcomas, pituitary, liver and breast tumors

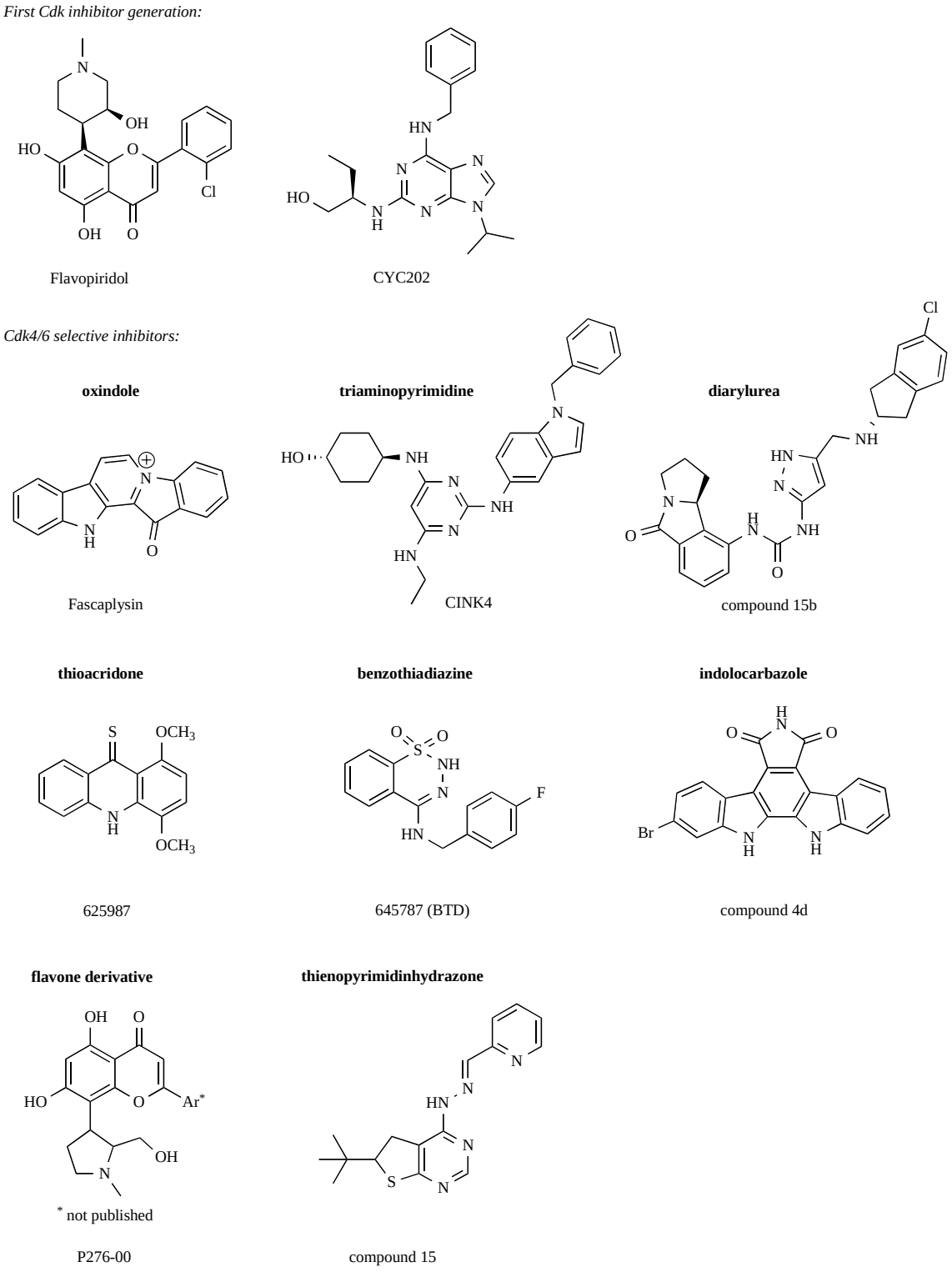

Fig. (2). Overview of Cdk4/6 inhibitors.

[45-47]. Furthermore, $Cdk4^{R24C}$ mutation resulted in enormous proliferation of carcinogen-induced primary melanomas and enhanced spontaneous metastasis in the lymph nodes and lung of mice [48]. An analogous point mutation in *Cdk6* gene preventing inhibition of Cdk6 by $p16^{INK4a}$ was described in a human neuroblastoma cell line [49]. Chromosomal translocations affecting the *Cdk6* promoter, led to Cdk6 overexpression, which contributed to the oncogenic properties of splenic marginal zone lymphoma and B-cell lymphocytic leukemias [50, 51]. In general, Cdk4/6 amplification or overexpression was observed in a wide spectrum of tumors, e. g. gliomas, sarcomas, lymphomas, melanomas, carcinomas of breast, squamous cell carcinomas and leukemias (reviewed in [52]). Recently, genetic studies in mice

supported the findings of Cdk4/6-associated tumor promotion and clarified some relations to common oncogenes. Thus, *cdk4* deletion in mice with deregulated Myc, typically developing skin neoplasias, prevented tumor development completely [53]. Also epidermal growth factor receptor ErbB-2 (HER2/neu)-induced, but not Wnt-1-induced breast tumor development in mice was inhibited by *cdk4* or respectively *cyclin D1* ablation [54, 55]. This corroborates and is in line with the findings, that Cdk4-cyclin D activation and nuclear accumulation is induced through oncogenic Ras signaling pathway even without mitogenic stimuli in human tumors [56]. In summary, the results suggest the essential and critical role of Cdk4/6 activity in tumor formation depending on incidence of oncogenic mutations. In consequence, Cdk4/6 turned out to be attractive targets for pharmacological anti-cancer drug development. For evaluation of anti-cancer drugs targeting Cdk4/6, the heterogeneity of Cdk4/6 kinase activity in different human tumor types has to be considered.

2. CDK4/6 AS TARGETS FOR CANCER THERAPY

2.1. Novel Small Molecule Cdk4/6 Inhibitors

The aim of the specific therapeutic Cdk4/6 targeting in cancer cells is to stop cell proliferation and restore cell cycle arrest in G_1 phase, certainly minimizing or avoiding adverse side-effects for cancer patients. Several strategies for inhibition of Cdk4/6 activity could be realized: indirect approaches targeting upstream regulatory pathways and direct targeting of Cdk4/6 subunits to decrease catalytic activity. This review focuses on direct approach of Cdk4/6 modulation by small molecule inhibitors. First generations of pharmacological Cdk inhibitors in clinical trials, e.g., flavopiridol and *R*-Roscovitine (CYC202) were potent Cdk inhibitors (Fig. (**2**)), but non-specific and non-selective compounds inhibiting multiple kinases and thus resulted in misleading information concerning advantages for tumor treatment (reviewed in [57]). From the actual point of view, general side-effects and toxicities because of unintentional targeting of Cdk1 and Cdk7 is not surprising. In the last years also relevance of Cdk10 and Cdk11 for tumor suppression were discovered, and in consequence unselective targeting of Cdk10 and Cdk11 would limit therapeutic effects of Cdk inhbitors [58, 59]. Design and screening of next generation Cdk inhibitors beside potency also considered high specificity to Cdks. Some of these compounds described over the last decade have preferential and selective affinity to Cdk4/6 and are almost planar heterocycles targeting the ATP binding site of Cdk4/6-cyclin D complexes. Structures and IC_{50} values of potent Cdk4/6 selective compounds, members of chemical classes of oxindoles, triaminopyrimidines, diarylureas, thioacridones, benzothiadiazines, indolocarbazoles, and pyrido[2,3-*d*]pyrimidines (Fig. (**2**)) were summarized by Lee & Sicinski in 2006 [60]. In the last three years only a few novel compounds were added to the pool of Cdk4/6 inhibitors, including thienopyrimidinhydrazone derivatives and the flavone derivative P276-00 (Fig. (**2**)), but these new compounds have no advantages over known Cdk4/6 inhibitors concerning Cdk4/6 inhibitory potency and selectivity against other Cdks [61, 62].

So far, no Cdk4/6 inhibitor is applied in commercial therapeutic use. Two Cdk4/6 selective compounds PD 0332991 and P1446A-05 are undergoing phase I clinical trials for cancer therapy (read up on: http://clinicaltrials.gov). No information about P1446A-05 has been provided in the literature. Otherwise, the pyrido[2,3-*d*]pyrimidine derivative PD 0332991 (Fig. (**3**)) is of particular interest in the literature and has been extensively studied *in vitro* in tumor cells, as well as *in vivo* in mouse xenograft models of colorectal cancer and disseminated myeloma, demonstrating effective responses [63-66]. Already in 2001, another derivative of pyrido[2,3-*d*]pyrimidine class PD 0183812 has been evaluated and showed high potency in human tumor cell treatment [67]. Series of compounds based on a pyrido[2,3-*d*]pyrimidine scaffold were designed and screened concerning their Cdk4/6 inhibitory effect and selectivity [68, 69]. Several of these derivatives were shown to be highly potent Cdk4/6 inhibitors (IC_{50} < 20 nM) with selectivity against Cdk1, Cdk2 and other kinases. Beside PD 0332991 (IC_{50}(Cdk4/6-cyclin D) = 9-15 nM), the iodine-containing analog (compound 30, [69]) is a very potent Cdk4-cyclin D inhibitor (IC_{50} = 5 nM) and selective against Cdk1-cyclin B, Cdk2-cyclin A, and Cdk2-cyclin E (IC_{50} > 365 nM) causing inhibition of human tumor cell proliferation. In our opinion, these potent and selective Cdk4/6 inhibitors are not only suitable for cancer therapy, but also for imaging of human tumors with the non-invasive imaging technique positron-emission-tomography (PET). Therefore, positron-emitting Cdk4/6 inhibitors and the nonradioactive analogs based on the known pyrido[2,3-*d*]pyrimidines compound 30 (CKIA) and PD 0332991 were designed, synthesized and characterized by our group (Fig. (**3**)).

Pyrido[2,3-*d*]pyrimidine derivatives CKIA, CKIB, CKIC, CKID, and CKIE were evaluated concerning their cellular and molecular effects in human tumor cell lines. Iodine-containing compounds CKIA and CKIB showed significant and specific inhibition of tumor cell proliferation and G_1 phase arrest by targeting of Cdk4/6-cyclin D/ pRb/ E2F signaling pathway [70]. Also fluorine-containing compounds CKIC, CKID and CKIE were evaluated in this context. A comparison of selected results for all compounds in human tumor cell lines HT-29 and FaDu are given in the next section.

2.2. Pharmacological Effects of Pyrido[2,3-*d*]pyrimidine-based Cdk4/6 Inhibitors

Cell Cycle

Flow cytometry DNA analysis of cells exhibited effects of pyrido[2,3-*d*]pyrimidine derivatives on cell cycle distribution of human tumor cells HT-29 and FaDu. Because of considerable limited viability of tumor cells at concentrations higher than 1 μmol/L CKIA and CKIB, only experiments up to this concentration could be performed for these compounds. Already 24 h after incubation a significant concentration-dependent increase of tumor cells in G_1 phase up to 85-90%, and a decrease in S and G_2/M phase were observed (Fig. (**4**)). In both cell lines effects of CKIA, CKIB and CKIE on cell cycle were considerably higher than for CKIC and CKID. Cell cycle arrest (> 85% in G_1 phase) was observed at 24 h incubation with 1 μmol/L CKIA, CKIB and

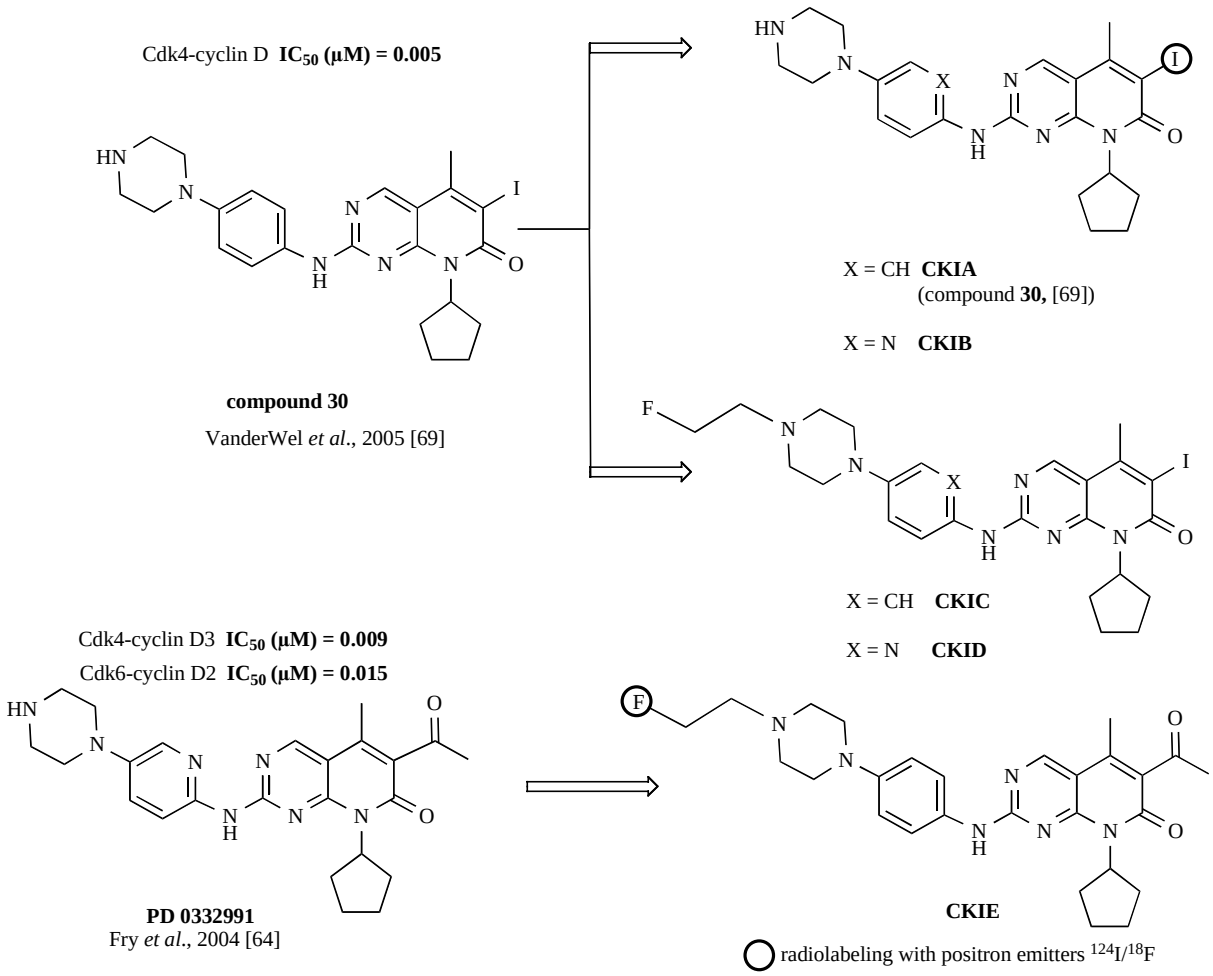

Fig. (3). Overview of novel pyrido[2,3-*d*]pyrimidine compounds.

Pyrido[2,3-*d*]pyrimidine derivatives are derived from known Cdk4/6 inhibitors, designed for targeting of Cdk4/6 by PET after radiolabeling with positron emitters ^{124}I or ^{18}F, respectively.

CKIE in HT-29 cells. FaDu cells also showed G_1 phase arrest, although lower alterations of cell cycle distribution with respective compounds were detected. Only at high concentrations 5 μmol/L and 10 μmol/L CKIC and CKID showed potent increment of tumor cells in G_1 phase.

pRb Phosphorylation

The influence of pyrido[2,3-*d*]pyrimidine derivatives on Cdk4/6-cyclin D specific phosphorylation of pRb on amino acid Ser^{780} and thus the interaction with Cdk4/6 was characterized in whole cell lysates by chemiluminescent detection of specific antibodies after SDS-PAGE and Western Blot analyses. Also total amount of pRb was determined; β-actin served as internal control. In both tumor cell lines HT-29 and FaDu a definite concentration dependent hypophosphorylation of pRb at Ser^{780} was found after incubation with 0.1 μmol/L, 1 μmol/L and 10 μmol/L CKIA and CKIE, respectively (Fig. (**5**)). Results of pRb-Ser^{780} also showed a down-regulation after treatment with CKIB, CKIC and CKID, but only at higher concentrations (1 μmol/L CKIB and 10 μmol/L CKIC/CKID). Incubation with CKIA and CKIE additionally showed a clear decrease of total pRb amounts in HT-29 and FaDu cells.

Summary

Novel pyrido[2,3-*d*]pyrimidine derivatives (Fig. (**3**)) derived from potent and selective Cdk4/6 inhibitors clearly showed inhibitory effects on Cdk4/6 dependent cell cycle regulation of human tumor cells in pharmacological concentrations. CKIA, CKIB and CKIE are the most promising compounds causing a G_1 phase arrest in the tumor cells provoked by the specific inhibition of Cdk4/6-cyclin D/pRb pathway. CKIC and CKID showed minor effects on the cell cycle distribution and Cdk4/6 inhibition and lower potency against tumor cell proliferation. In conclusion, pyrido[2,3-*d*]pyrimidines CKIA, CKIB and CKIE with high potential for tumor growth inhibition are promising compounds for the pharmacological cancer treatment. In consideration of the success of clinical trials with Cdk4/6 inhibitor PD 0332991, also evaluation of other selective Cdk4/6 inhibitors should be taken into account for tumor therapy. In addition, the high potency and selectivity of CKIA and CKIE for Cdk4/6 inhibition is a prerequisite for further studies focused on the radiopharmacological evaluation of potent radiolabeled derivatives [^{124}I]CKIA and [^{18}F]CKIE as suitable radiotracers for Cdk4/6 imaging *in vivo* by PET.

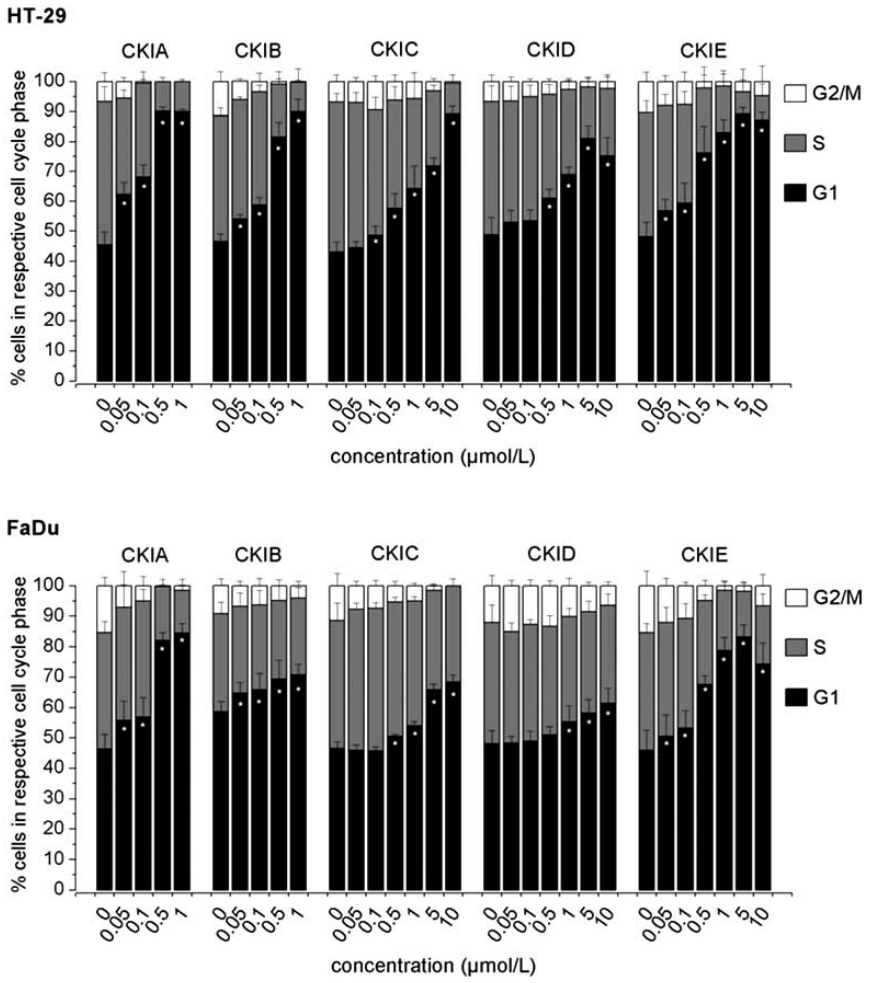

Fig. (4). Inhibition of G_1 phase progression.

The figure shows the cell cycle distribution of HT-29 and FaDu tumor cells at 24 h after treatment with different concentrations of pyrido[2,3-*d*]pyrimidine derivatives (mean ± standard deviation, $n \geq 8$, * $p < 0.05$ compared to control 0 µmol/L).

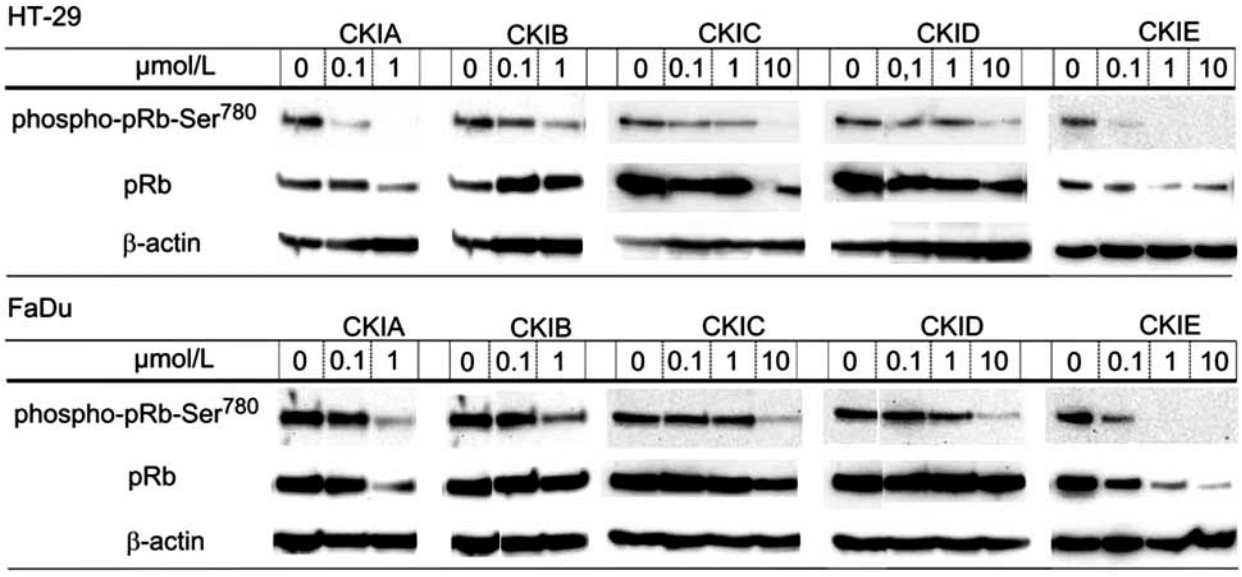

Fig. (5). Inhibition of pRb phosphorylation.

The figure shows representative Western Blots of HT-29 and FaDu whole cell lysates characterizing the phosphorylation of pRb at Ser^{780} and total amount of pRb after incubation with pyrido[2,3-*d*]pyrimidine derivatives. As internal control also β-actin is illustrated.

3. CDK4/6 AS TARGETS FOR CANCER IMAGING

3.1. Novel ^{124}I- and ^{18}F-radiolabeled Pyrido[2,3-*d*]pyrimidine Derivatives

The application of potent radiolabeled Cdk4/6 inhibitors in PET is a novel approach of functional imaging of human tumors. Non-invasive imaging technique PET affords the opportunity of functional imaging of cellular and molecular mechanisms *in vivo*. Thereby, assessment of Cdk4/6 protein status in tumors and other tissues would be of particular interest. Additionally, physiological processes can be displayed three-dimensionally by PET, which would provide pharmacological data and metabolic pathways of radiolabeled Cdk4/6 inhibitors, to further understand their mode of action *in vivo* and availability in tumor therapy. For the first time, radiolabeling of potent Cdk4/6 inhibitors (CKIA, CKIB and CKIE) with positron-emitters was accomplished by our group. Synthesis and iodine-124-radiolabeling of CKIA and CKIB were already described [70, 71]. Radiolabeling of a novel fluorine-18-containing compound, [^{18}F]CKIE, was performed in a two step synthesis by preparation of the fluoroethylated prosthetic group 1-bromo-2-[^{18}F]fluoroethane and subsequent introduction into the inhibitor molecule *via N*-alkylation (Fig. (**6**)).

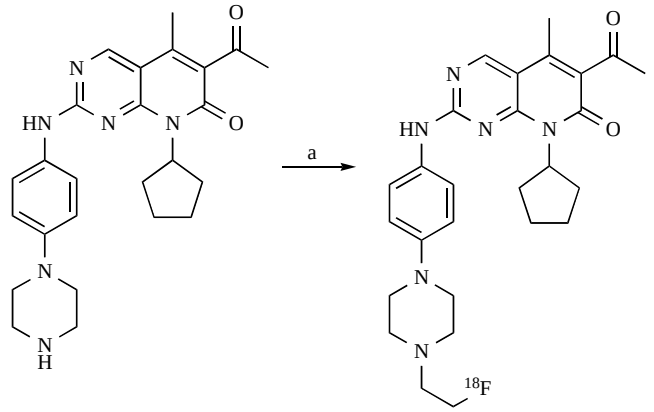

Fig. (6). Radiosynthesis of [^{18}F]CKIE.

a: DMF, NaOH, NaI, 1-bromo-2-[^{18}F]fluoroethane, 130°C, 20min.

Starting from 2.1 GBq 1-bromo-2-[^{18}F]fluoroethane 273 MBq [^{18}F]CKIE were obtained in 86 min with a radiochemical yield of 22% (decay-corrected). The radiochemical purity was >98% and the specific activity was 22.4 ± 3.5 GBq/μmol.

The iodine substituent of CKIA and CKIB represents an attractive site for an isotopic substitution with radioiodine isotope iodine-124 (^{124}I). Its half-life of 4.18 d affords extended radiopharmacological evaluation and imaging studies using PET. Nevertheless, high positron energy and minor positron emission (26%) are disadvantages, especially for resolution of PET images. Fluorine-18 (^{18}F), with a half-life of 109.8 min is of outstanding importance as PET nuclide with nearly 97% positron emission. ^{124}I-labeled Cdk4/6 inhibitors [^{124}I]CKIA and [^{124}I]CKIB were evaluated concerning their radiopharmacological properties in cellular radiotracer uptake studies, biodistribution studies and small animal PET studies in NMRI *nu/nu* mice bearing the human FaDu (head and neck squamous cell carcinoma) tumor [70, 71]. Important results of characterization of [^{124}I]CKIA and a novel ^{18}F-labeled derivative [^{18}F]CKIE as potential radiotracers for imaging Cdk4/6 *in vivo* are summarized below.

3.2. Radiopharmacological Properties of Pyrido[2,3-*d*]pyrimidine-based Cdk4/6 Inhibitors

Radiotracer Uptake in Tumor Cells

Radiotracer uptake studies in human tumor cell lines HT-29 (human colorectal adenocarcinoma) and FaDu showed fast and substantial uptake of [^{124}I]CKIA up to approximately 1000%ID/ mg protein after 2 h at 37°C (Fig. (**7**), see also: [70]). Cellular uptake of [^{18}F]CKIE was considerable lower at 37°C (approximately 50% ID/ mg protein after 2 h). The time-dependent uptake was similar in both cell lines for respective compound. Whereas [^{124}I]CKIA uptake was stead-

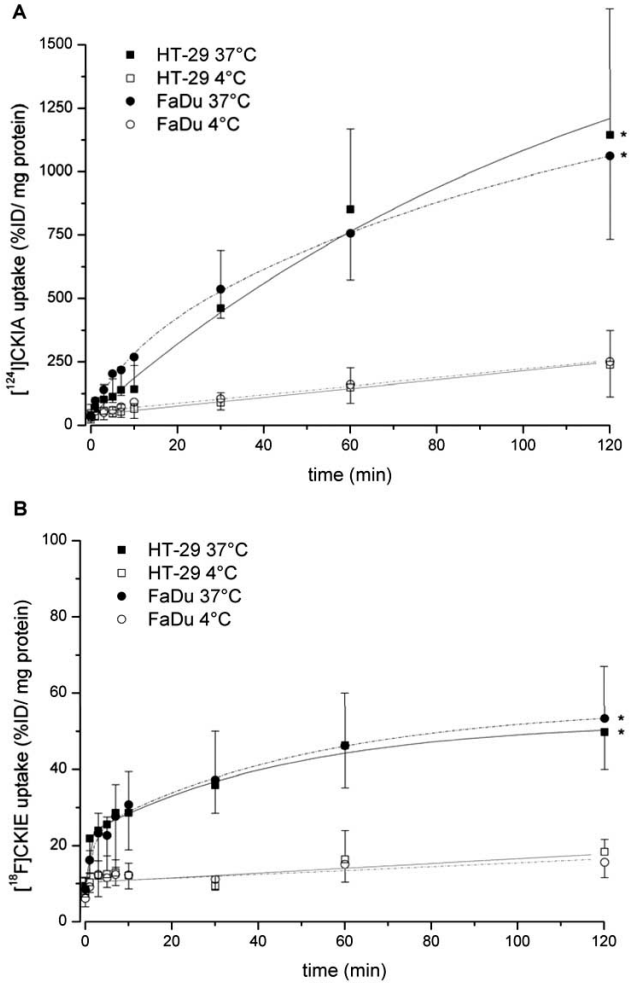

Fig. (7). Cellular radiotracer uptake of [^{124}I]CKIA (A) and [^{18}F]CKIE (B).

The uptake of the radiotracers are shown in human tumor cell lines HT-29 and FaDu over a period of 2 h at 37°C and 4°C. Symbols represent observed data (means ± standard deviations, n = 8-12, * p < 0.05 37°C versus 4°C of respective cell line). Lines represent computer-derived fits.

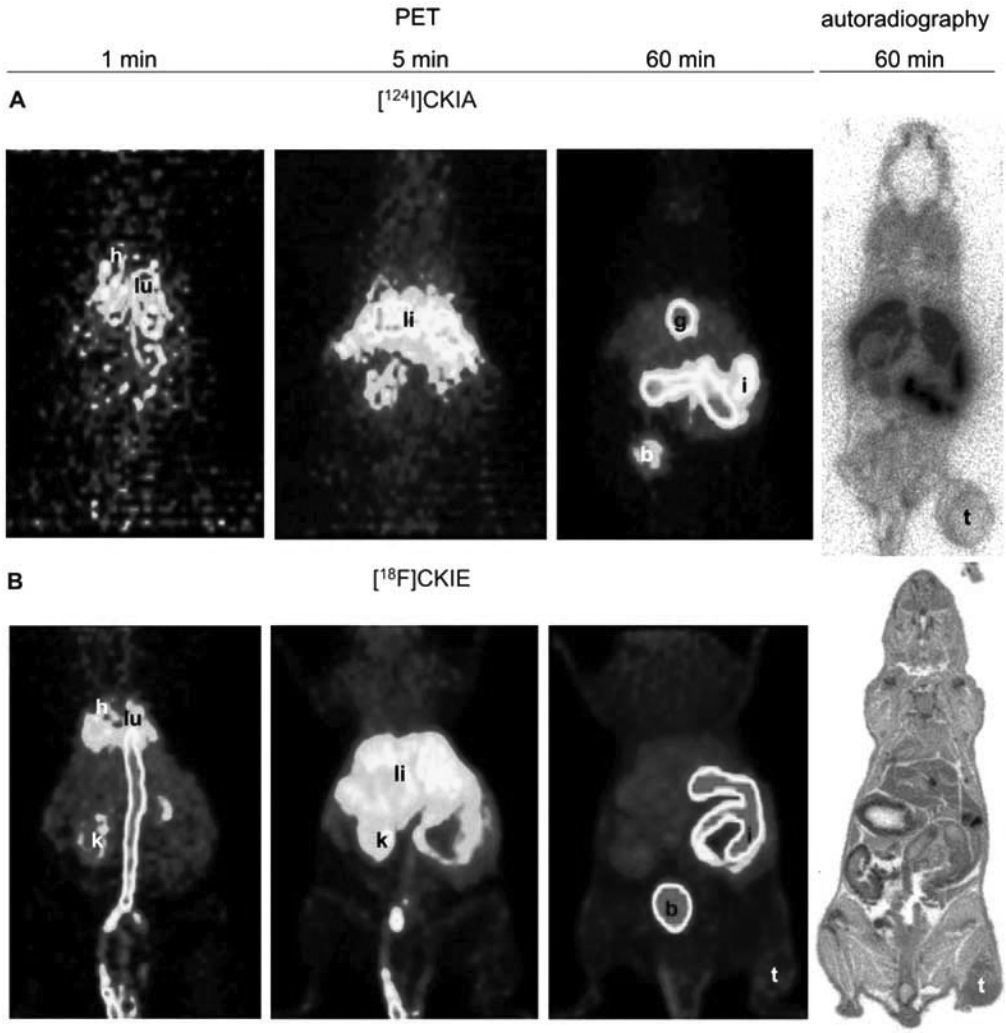

Fig. (8). PET studies and autoradiography with [^{124}I]CKIA (A) and [^{18}F]CKIE (B) in FaDu tumor bearing mice.

Approximately 10 MBq of [^{124}I]CKIA and [^{18}F]CKIE were administered intravenously within 15 s in a 0.3 mL volume into the tail vein. The figure shows the maximum intensity projections at 1 min, 5 min and 60 min after single intravenous injections of the radiotracers (h: heart, lu: lung, li: liver, k: kidney, g: gall bladder, i: intestine, b: bladder, t: tumor). The autoradiograms on the right side demonstrate radioactivity distribution in a coronal section 60 min after injection.

ily increased with time, [^{18}F]CKIE showed only marginal increase after 30 min at 37°C. Interestingly, cellular radiotracer uptake was significantly lower at 4°C for both compounds [^{124}I]CKIA (100%ID/ mg protein after 2 h) and [^{18}F]CKIE (10%ID/ mg protein after 2 h), indicating a specific uptake mechanism.

Small Animal PET and Whole-Body Autoradiography

The radiopharmacological evaluation of [^{124}I]CKIA and [^{18}F]CKIE also involved small animal PET studies and subsequent whole-body autoradiography of female NMRI *nu/nu* mice bearing xenotransplanted FaDu tumor using established protocols as described elsewhere [71-73]. Both radiotracers showed a similar distribution pattern (Fig. (**8**)). In the first minute after intravenous injection [^{124}I]CKIA and [^{18}F]CKIE were distributed with the blood stream. Radioactivity was predominantly detected in the heart (h) and lung (lu) region. After 5 min radioactivity was already concentrated in the liver (li) and to a lower extent in the kidneys (k). Between 5 min and 60 min further uptake of radioactivity in the abdomen was detected, especially in the gall bladder (g), intestines (i), and bladder (b). Predominantly, radiotracers were hepatobiliary eliminated, but partly also by renal excretion.

Time activity curves after injection of the radiotracers [^{124}I]CKIA and [^{18}F]CKIE demonstrated a rapid clearance of the radioactivity from the blood, and primary accumulation in the liver (Fig. (**9**)). Thereby, region of interests (ROIs) over the heart represent the radioactivity concentration in the blood. The half-life of radioactivity elimination from the blood was calculated to be 7.2 min for [^{124}I]CKIA and 7.9 min for [^{18}F]CKIE, respectively, using a monoexponential model. In addition, stability analyses in the blood showed a rapid metabolization of the radiotracers [^{124}I]CKIA and [^{18}F]CKIE *in vivo*. After 1 min more than 90%, after 30 min less than 20%, and after 60 min only about 5% of intact parent compounds were measured in the blood. Metabolites were not identified. Radiotracer kinetics in the liver were different for [^{124}I]CKIA and [^{18}F]CKIE (Fig. (**9B**)). Whereas

radioactivity concentration was nearly constant between 5 min and 60 min after injection of [^{124}I]CKIA, a decrement of activity in the liver was observed in this period after injection of [^{18}F]CKIE (Fig. (**9**)).

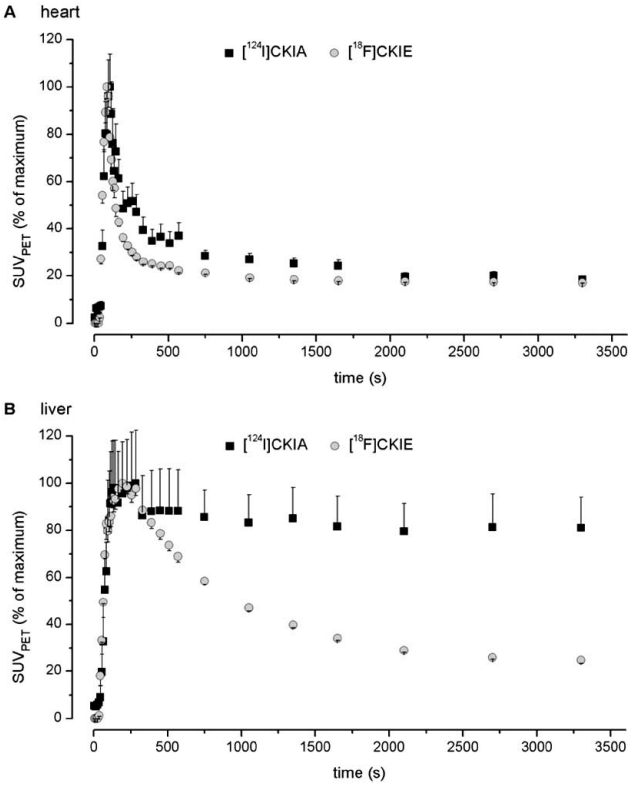

Fig. (9). ***In vivo*** **kinetics of [^{124}I]CKIA or [^{18}F]CKIE.**

The time activity curves are derived from ROIs over the heart (**A**) and liver (**B**) of FaDu tumor bearing mice after single intravenous injection of the radiotracers. As an index of radioactivity uptake *in vivo*, standardized uptake values (SUV_{PET}) were calculated in the ROI for each PET measurement. The SUV_{PET} expressed as percentage of maximum (means ± standard deviations, [^{124}I]CKIA: n = 3, [^{18}F]CKIE: n = 6) defines the radioactivity concentration in relation to the injected radioactivity normalized to body weight of the mouse.

PET images and autoradiograms only showed marginal uptake of radioactivity in the FaDu tumor after injection of [^{124}I]CKIA (Fig. (**8**)). In contrast, the tumor was conspicuously pictured on the right thight of the mice 60 min after injection of [^{18}F]CKIE. In particular, radioactivity in the peripheral region of the tumor could be detected (Fig. (**10A**)). Calculation of the radioactivity concentration in the tumor region on the right leg compared to the radioactivity concentration in the muscle of the left leg allows the assessment of specific tumor uptake. Thereby, a precise differentiation of the tumor mass is not possible because of the limited spatial resolution (1.8 to 2.3 mm) of the PET scanner used [71-73]. Nevertheless, a low and constant tumor-to-muscle ratio of 1.5 could be calculated from the PET data (Fig. (**10B**)).

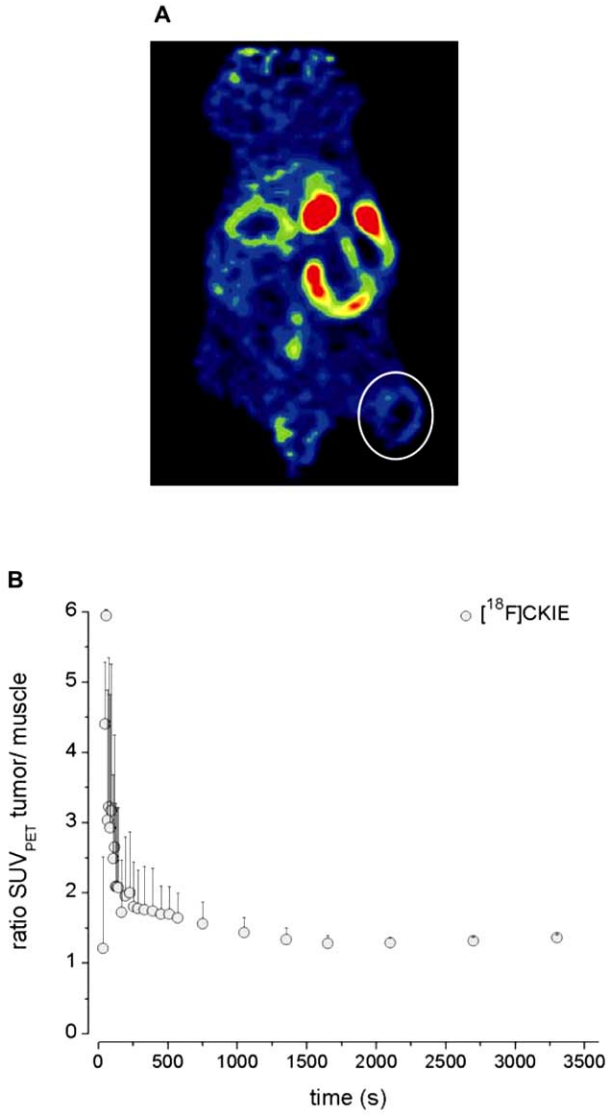

Fig. (10). Characterization of radioactivity uptake in the tumor after intravenous injection of [^{18}F]CKIE.

A The coronal section of a representative FaDu tumor bearing mouse at midframe time 55 min shows the uptake in the peripheral region of the tumor.

B The figure shows the time-dependent ratio of the radioactivity concentration (SUV_{PET}) in the tumor and muscle (means ± standard deviations, n = 6).

Summary

The radiolabeling of potent pyrido[2,3-*d*]pyrimidines (Fig. (**3**)) for the first time allowed the quantification of cellular uptake and image representation as well as cognition of metabolic processes of Cdk4/6 inhibitors *in vivo*. Despite of quantitative differences in the uptake of [^{124}I]CKIA and [^{18}F]CKIE a significant accumulation of both radiotracers in the tumor cells could be determined. The fast and specific uptake of these compounds was an important prerequisite for the characterization *in vivo*. The identification of transport mechanism in future studies would contribute to the better understanding of effective activity of pyrido[2,3-*d*]pyrimidines in the cells. The PET studies identified a rapid

clearance and metabolization of [^{124}I]CKIA and [^{18}F]CKIE in the blood and primary hepatobiliary excretion of radioactiviy as basic metabolism characteristics of the radiotracers *in vivo*. The short biological half-life and elimination of [^{124}I]CKIA and [^{18}F]CKIE from the blood have to be considered as limiting properties for the specific uptake in the tumor. Nevertheless, in case of [^{18}F]CKIE radioactivity uptake in the peripheral proliferative region of the FaDu tumor could be assumed. However, the ubiquitous distribution of a probable metabolite of [^{18}F]CKIE and the constant ratio of radioactivity concentration in the tumor and muscle indicate an unspecific tumor uptake.

CONCLUDING REMARKS

Because of their critical contribution to the tumor development cell cycle regulating kinases Cdk4/6 are attractive targets for new developments in tumor therapy and diagnosis. On the one hand, results of the evaluation of novel members of pyrido[2,3-*d*]pyrimidines support the suitability of CKIA and CKIE concerning specific and selective inhibition of Cdk4/6-mediated cell cycle progression and tumor cell proliferation. Further studies *in vivo* are needed to characterize the effectiveness of the Cdk4/6 inhibitors for tumor therapy. On the other hand, radiopharmacological analyses of radiolabeled pyrido[2,3-*d*]pyrimidines entail a critical assessment of the advantage of [^{124}I]CKIA and [^{18}F]CKIE for the clinical application in tumor diagnosis. Nevertheless, evaluation of other radiolabeled Cdk4/6 inhibitors with optimized properties *in vivo* are still of outstanding interest for the prospective characterization of Cdk4/6 in tumors by means of PET.

ABBREVIATIONS

CAK	=	Cdk activating kinase complex
Cdk	=	Cyclin-dependent kinase
E2F	=	E2 promoter binding factor
PCNA	=	Proliferating cell nuclear antigen
PET	=	Positron emission tomography
pRb	=	Retinoblastoma protein

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