Chapter 14

Evaluating Chemical CDK Inhibitors as Cell Death Inducers

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Abstract

The cell cycle of eukaryotic cells is regulated by a family of protein kinases called cyclin-dependent kinases (Cdks). We have reported the identification and biological characterization of a highly potent, small-molecule pan-Cdk inhibitor, which inhibited Cdk1, 2, 4, 5, 6, and 9 with equal potency in the nM range. This compound inhibited multiple events in the cell cycle and induced cell death in human cancer cell lines as well as in peripheral blood or purified resting lymphocytes ex vivo. We describe the materials and methods to determine antitumor efficacy in vivo xenograft models. Pharmacodynamic marker assays that have been performed using tumors and normal tissues are explained. Moreover, we briefly describe methods for determining the effects of chemical Cdk inhibitors on peripheral blood cells or lymphocytes ex vivo.

Key words Antitumor efficacy, Cdk inhibitor, Immunohistochemistry (IHC) assay, In vivo xenograft model, In vivo pharmacodynamics (PD) assay, Lymphocytes, Peripheral blood cells

1 Introduction

The cell cycle of eukaryotic cells is regulated by a family of protein kinases called cyclin-dependent kinases (Cdks). In mammalian cells, multiple cyclin/Cdk complexes participate in the progression of the cell cycle. Namely, cyclin D-Cdk4, cyclin D-Cdk6, cyclin E-Cdk2, and cyclin A-Cdk2 regulate the progression from the G0/G1 phase through to the S phase. The progression from the G2 to the M phase is regulated by another type of Cdk, the cyclin B-Cdk1 (Cdc2) complex. Cyclin B-Cdc2 phosphorylates and activates key regulators of the M phase [1–6].

In addition to cell cycle regulation, Cdks participate in various cellular processes. Cdk7-cyclin H and Cdk9-cyclin T complexes are components of the transcription factors TFIIH and Positive Transcription Elongation Factor b (P-TEFb), respectively. These factors phosphorylate the carboxy-terminal domain (CTD) of RNA polymerase II, which is important for the elongation of transcription [7–9]. Cdk5/p35 or p39 complexes have numerous functions in the nervous system, including neuritis outgrowth and neuron migration,

Mar Orzáez et al. (eds.), *Cyclin-Dependent Kinase (CDK) Inhibitors: Methods and Protocols*, Methods in Molecular Biology, vol. 1336, DOI 10.1007/978-1-4939-2926-9_14,

and in the metabotropic glutamate receptor and dopamine signaling pathways [10]. In addition, Cdk5 appears to have a prominent role in promoting insulin secretion in pancreatic β-cells [11].

We have reported the identification and biological characterization of a highly potent, small-molecule pan-Cdk inhibitor, Compound M [12]. This compound inhibited Cdk1, 2, 4, 5, 6, and 9 with equal potency in the nM range and was selective against kinases other than Cdks. Compound M inhibited multiple events in the cell cycle in vitro, including retinoblastoma protein (pRb) phosphorylation, E2F-dependent transcription, DNA replication (determined using bromodeoxyuridine (BrdU) incorporation), and mitosis completion (assayed using flow cytometry) in the 10-nM range. Moreover, this compound induced cell death, as determined by the induction of the subG1 fraction, and activated caspase-3 and annexin V. In a nude rat xenograft tumor model, an 8-h constant infusion of Compound M inhibited pRb phosphorylation and induced apoptosis in HCT116 human colorectal cancer cells at ~30 nM, leading to the inhibition of tumor growth. In our studies, the suppression of pRb phosphorylation in tumor cells was clearly correlated with tumor cell growth inhibition and cell death both in vitro and in vivo. Compound M inhibited pRb phosphorylation in both tumor and gut crypt cells. Thus, pRb phosphorylation may be a suitable pharmacodynamics (PD) biomarker in both tumors and normal tissues for monitoring target engagement and predicting the efficacy of Compound M.

Interestingly, pan-Cdk inhibitors induced cell death in peripheral blood or purified resting (non-stimulated) lymphocytes ex vivo. Cell death was induced very rapidly (after 4 h of incubation), suggesting that the acute immunosuppression observed in rodents might be due, at least in part, to direct cytotoxic effects of pan-Cdk inhibitors on resting lymphocytes [13].

In this section, we describe the materials and methods used to determine antitumor efficacy by monitoring the tumor size. In addition, we explain the PD marker assays that have been performed using tumors and normal tissues in xenograft models. Moreover, we briefly describe methods for determining the effects of Cdk inhibitors on peripheral blood cells or lymphocytes ex vivo.

2 Materials

2.1 Animal Experiments

1. Animals: Nude mice, nude rats, SCID-mice, and NOD-SCID mice are immunodeficient rodents that are generally used in animal xenograft cancer models (*see* **Notes 1–3**). In general, the animals were bred under the following housing conditions: temperature, 23 ± 2 °C; relative humidity, 55 ± 15 %; illumination, 12 h; diet, CE-2; and housing method, ~ 4 rats/cage. In the case of nude rats, female 6–7-week-old F344 nude rats with 100–150 *g* of body weight were generally used.

2. Cannulation and intravenous (IV) dosing: isoflurane for anesthetization, custom infusion kits (INSTECH Co. Ltd., KVAH95T).
3. Tumor volume measurements: a digital caliper.
4. Blood cell count: potassium-EDTA coated syringe, automated hematology analyzer (SF-3000; Sysmex).

2.2 Determination of pRb Phosphorylation in Xenograft Tumor Samples Using a Western Blot

1. Glycerophosphate buffer: 50 mM glycerophosphate, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 % NP-40, 0.1 % Triton X-100, 10 mM sodium fluoride, 2 mM sodium vanadate, and proteinase inhibitors (5 μg/mL of leupeptin, pepstatin, antipain, chymostatin, and E64).
2. Protein assay: BCA protein assay (Pierce).
3. Antibodies and other reagents: Anti-Rb antibody; MK-15-1 from MBL, Protein G agarose, Anti-phospho Rb antibody (Ser780); #9307S; Cell Signaling Technology.
4. 5× SDS Buffer: 625 mM Tris–HCl [pH 7.5], 10 % sodium dodecyl sulfate (SDS), 25 % β-mercaptoethanol, 5 % glycerol, and bromophenol blue (BPB).
5. Polyvinylidene fluoride (PVDF) membrane.
6. Reagents for immunoblotting: PBS containing 1 % Tween 20, Blocking solution; 5 % skim milk in PBS containing 1 % Tween 20, horseradish peroxidase (HRP)-linked anti-rabbit IgG, ECL system.

2.3 Determination of BrdU Incorporation in Xenografted Tumor by Using Immunohistochemistry (IHC) Assays

1. BrdU.
2. Target Retrieval Solution (DAKO).
3. MOM Mouse IgG Blocking Reagent (Vector Laboratories), MOM immunodetection kit (Vector Laboratories).
4. Antibodies: Anti-BrdU antibody; Cat #347580; Beckton Dickinson, Anti-phosphor-Rb (Ser807/811) Ab; #9308; Cell Signaling Technology, Anti-activated caspase 3 Ab; #9661; Cell Signaling Technology.
5. PBS containing 10 % normal goat serum and 3 % bovine serum albumin.
6. Goat anti-rabbit IgG H&L-biotin conjugate, VECTASTAIN Elite ABC kit (Vector Laboratories).

2.4 Isolation of Lymphocytes for Ex Vivo Experiments Using Peripheral Blood or Isolated Lymphocytes

1. Balanced salt solution; 0.13 M NaCl, 0.01 % glucose, 5.0 mM $CaCl_2$, 98 mM $MgCl_2$, 0.54 mM KCl, and 15 mM Tris–HCl (pH 7.6).
2. Ficoll-Paque Plus.
3. IOTest 3 Lysing Solution (Beckman Coulter).
4. For stimulation; 10 ng/mL of phorbol 12-myristate 13-acetate (PMA) and 1 μM of Ionomycin.

2.5 Cell Cycle and Cell Death Assays of Whole Peripheral Blood Cells or Isolated Lymphocytes

1. CycleTEST PLUS DNA Reagent Kit (Becton Dickinson).
2. Apo-ONE Homogeneous Caspase-3/7 Assay kit (Promega).
3. Lysis buffer (CelLytic™ M; Sigma-Aldrich), protease inhibitor cocktail, Halt Phosphatase Inhibitor Cocktail.

3 Methods

3.1 Determination of Antitumor Efficacy Using Animal Models (see Notes 4–8)

All the animal experiments were performed in accordance with good animal practices, as defined by the International Animal Care and Used Committee (IACUC). The inoculation of human cancer cell lines or the dosing of compounds was started at least 1 week after purchase for quarantine purposes. In our experiments, human colorectal cancer cell line, HCT-116 was used.

1. Grow HCT116 cells to a semi-confluent cell density. Then treat the cells with trypsin, resuspend them in DMEM containing 10 % FBS and collect them by centrifugation.
2. Wash the cells with phosphate buffered saline (PBS), and finally suspend in PBS at 1×10^8 cells/mL.
3. Anesthetize nude rats via isoflurane inhalation. HCT116 cells are subcutaneously transplanted into both flanks of each nude rat using a 27-G needle and a 1-mL syringe (1×10^7 cells/100 μL). Once the tumor had reached a size of ~200 mm^3 L, start dosing with the compounds. For the HCT116 cells, 7–10 days are required before the tumor size reached ~200 mm^3 (*see* **Note 4**).
4. Anesthetize tumor-bearing nude rats via isoflurane inhalation and insert a cannula into the subclavian vein using custom infusion kits. Immediately after cannula insertion, the rat is placed in a free-movement cage and saline will be administered by continuous IV infusion using a syringe pump for around 24 h (0.1 mL/h) to avoid blood clotting (*see* **Note 5**).
5. Randomize rats according to tumor volumes and distribute into treatment groups of 4–8 rats each, with each treatment group having an approximately equivalent range of tumor volumes, on the day before the start of drug administration.
6. Dissolve the compound in the vehicle and administer it into tumor-bearing nude rats by continuous IV infusion for 8 h.
7. Measure the tumor diameter using a digital caliper twice a week (*see* **Note 7**). The statistical analysis can be performed using a repeated measure ANOVA followed by the Dunnett test.
8. Obtain approximately 0.15 mL of blood using a potassium-EDTA coated syringe to perform white blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts. Immunosuppression is a generally observed side effect of

antitumor agents (*see* **Note 3**). Collect the samples from the subclavian vein under isoflurane anesthesia on days 3 and 10. The cell counts can be determined using an automated hematology analyzer.

9. Measure the body weight and perform gross observations on each weekday during the experiment. Body weight changes (a decrease or suppression in the body weight increase) are a sign of general toxicity.
10. For measurement of drug concentrations, collect blood samples at the indicated times using a heparinized syringe via the subclavian vein. Prepare plasma samples from the blood samples using centrifugation and store at –80 °C until further assay. Measure the concentrations of the compounds using the LC/MS/MS method (*see* **Note 8**).

3.2 Determination of pRb Phosphorylation in Xenograft Tumor Samples Using a Western Blot (See Notes 9 and 10)

1. Dose compound M by a constant IV infusion in nude rats bearing an HCT116 xenograft tumor (*see* **Note 11**).
2. After 8 h, euthanize the animals and isolate the tumor tissues from the animals.
3. Suspend the tissues in glycerophosphate buffer and mince them using scissors.
4. Homogenize the tissue suspension in a Dounce homogenizer (ten strokes).
5. Centrifuge at 14,000 × *g* for 90 min at 4 °C, and store the resulting supernatant (cell lysate) at –80 °C.
6. Protein determination in the cell lysates can be performed using a BCA protein assay, according to the manufacturer's protocol.
7. Immunoprecipitate pRb in the supernatants with anti-Rb antibody. The cell lysates are incubated with the above mentioned anti-Rb antibody for 2 h at 4 °C.
8. Add protein G agarose and incubate the samples for additional 3 h at 4 °C with rotation.
9. Precipitate the bound pRb by centrifugation and elute in 5× SDS Buffer. Load and separate the proteins in 7.5 % SDS-PAGE and transfer to a PVDF membrane.
10. Block the membrane in blocking solution at room temperature for 1 h, and then probe with anti-phospho Rb antibody (Ser780) in blocking solution at 4 °C overnight.
11. Wash the membrane with PBS containing 1 % Tween 20 for three times.
12. Detect the bound antibody with HRP-linked anti-rabbit IgG, followed by the use of an ECL system.

3.3 Determination of BrdU Incorporation in Xenografted Tumor by Using IHC Assays (See Notes 9, 10, 12, 13)

1. Administered intravenously BrdU at 30 mg/kg into tumor-bearing nude rats via the tail vein.
2. Euthanize the animals 2 h after administration of the BrdU. Collect the target tissues, such as the tumor and small intestine.
3. Fix tissues in 10 % formalin for no more than 24 h and then embed in paraffin.
4. After deparaffinization, place the sections in Target Retrieval Solution and heat using a microwave for 20 min, then treat with 3 % H_2O_2 at room temperature for 10 min to inactivate the endogenous peroxidase.
5. For BrdU staining, block the sections with MOM Mouse IgG Blocking Reagent for 1 h.
6. Then, treat the sections with anti-BrdU antibody at 4 °C overnight.
7. The bound Abs are detected and developed using a MOM immunodetection kit according to the manufacturer's instructions.
8. For phospho-Rb and activated caspase 3 staining, maintain the sections at a sub-boiling temperature for 10 min. Then cool the slides on a bench top for 30 min.
9. Block the sections with PBS containing 10 % normal goat serum and 3 % bovine serum albumin.
10. Treat the sections with anti-phosphor-Rb (Ser807/811) Ab or anti-activated caspase 3 Ab at 4 °C overnight.
11. The bound Abs are detected using goat anti-rabbit IgG H&L-biotin conjugate and visualized using a VECTASTAIN Elite ABC kit according to the manufacturer's instructions.

3.4 Isolation of Lymphocytes for Ex Vivo Experiments Using Peripheral Blood or Isolated Lymphocytes

1. Dilute heparinized peripheral blood with an equal volume of balanced salt solution and layer on a Ficoll-Paque Plus in a centrifuge tube.
2. Centrifuge the tube at $400 \times g$ for 30 min at 20 °C.
3. Collect the cells in the middle layer and wash twice with balanced salt solution.
4. For isolation from the spleen, mince spleen cells isolated from the rat and filter the cells using a 100-μm nylon mesh.
5. Incubate the cells in IOTest 3 Lysing Solution for 10 min at room temperature to lyse contaminating erythrocytes and then wash with PBS.
6. Culture isolated splenocytes in RPMI 1640 containing 10 % heat-inactivated fetal calf serum (FCS), 100 U/mL penicillin, and 100 μg/mL streptomycin at $0.7–2.5 \times 10^6$ cells/mL.
7. For stimulation, add 10 ng/mL of PMA and 1 μM of Ionomycin.

3.5 Cell Cycle and Cell Death Assays of Whole Peripheral Blood Cells or Isolated Lymphocytes Treated with Cdk Inhibitors (See Note 14)

1. For flow cytometric analysis, dilute whole peripheral blood cells or isolated lymphocytes with RPMI 1640 containing 10 % heat-inactivated FCS, 100 U/mL penicillin, and 100 μg/mL streptomycin and expose to Cdk inhibitor with/without PMA and Ionomycin.
2. After 0–48 h (the incubation time depends on the experiments), collect the cells, fix and stain their nuclei with propidium iodide (PI) using the CycleTEST PLUS DNA Reagent Kit.
3. Collect the data in a fluorescence-activated cell sorter (FACS) Calibur flow cytometer and analyze using CellQuest software (Becton Dickinson). The S-phase population are calculated using ModFit LT software (Verity Software House).
4. For trypan blue exclusion assay, plate isolated lymphocytes at a density of 0.7×10^6 cells/mL in 96-well plates with various concentrations of Cdk inhibitor.
5. After 24 h, determine cell viability using trypan blue exclusion. Perform the assays in triplicate, and express the results as the mean plus or minus standard deviation.
6. For caspase 3/7 activation assay, plate isolated lymphocytes as **step 4**. After 8 h of exposure to inhibitors, caspase-3/7 activity is detected using the Apo-ONE Homogeneous Caspase-3/7 Assay kit according to the manufacturer's instructions.
7. For western blot analysis, culture isolated lymphocytes with inhibitors in the presence or absence of PMA-Ionomycin and resuspend in lysis buffer containing a protease inhibitor cocktail and the Halt Phosphatase Inhibitor Cocktail.
8. Sample tubes are kept at –80 °C for 15 min. Collect the supernatant after centrifugation at $15{,}000 \times g$ for 30 min.
9. Determine protein concentration using the BCA Protein Assay Kit. Load equal amounts of protein (10–20 μg) and separate in 7.5 % SDS-PAGE, then transfer to Immobilon-P membranes (Millipore).
10. Block the membranes were blocked with PBS containing 0.2 % Tween 20 and 5 % skim milk, and incubate with primary antibodies.
11. The bound antibodies are detected with anti-mouse/rabbit IgG-HRP and visualized using an ECL system.

4 Notes

1. The selection of the animal is, of course, dependent on the aim of the study. In general, not all human cancer cell lines are transplantable. The transplantability of cancer cells depends on the type of immunodeficiency in the animal model. For example, HCT116 is transplantable in both nude mice and rats, but

Colo205 is only transplantable in mice. We recommend testing to see whether a particular cell line of interest can grow within the planned animal model at the beginning of the study.

2. When testing small molecular compounds in vivo, the animal body weight is another important factor to consider when choosing between mice and rats. In general, as mice are smaller than rats, the total amounts of the required test compound will be smaller for mice than for rats. Moreover, nude mice are, in general, cheaper than nude rats.
3. In our studies, nude rats were used for two reasons: (1) we wanted to collect blood at several time points from a single animal during the course of the study, and (2) we wanted to dose the compound using an intravenous (IV) constant infusion. Inserting a cannula into mice is difficult because of the animal's relatively smaller size. Of note, a subcutaneously (SC) constant infusion can be performed in mice using an osmotic infusion pump.
4. For molecular targeted drug research, a specific human cancer cell line might be needed for in vivo studies. If such cells cannot be grown in animals, an appropriate model for performing in vivo experiments might be difficult to establish. To improve the transplantability or growth efficiency of cell lines, Matrigel (BD Biosciences) is often used. Matrigel is a solubilized basement membrane preparation extracted from the EHS mouse sarcoma. We suspended cells in 50 % Matrigel and 50 % PBS and inoculated animals. Co-inoculation with Matrigel improved the transplantability and growth efficiency of some cell lines.
5. In our experiment with Cdk inhibitor, Compound M, we chose a constant IV infusion to avoid high Cmax effects and to maintain a constant exposure level at a specific concentration. In this experiment, we used 0.1 % ascorbic acid-30 % polyethylene glycol (PEG) as the vehicle, which enables to dose up to around 8–10 h. If the test compound dissolves, 5 % glucose or saline are highly safe vehicles for continuous IV dosing over a period of several months.
6. Before the start of in vivo PD or efficacy experiments, the "target" plasma concentration (biological effective concentration) and treatment time for efficacy should be estimated. We estimated the in vivo target concentration based on the potency of the compound during in vitro cell-based experiments in addition to the serum protein binding ability of the compound. In the case of Compound M, we first performed in vitro PD (inhibition of pRb phosphorylation) and cell growth inhibition/cell death induction experiments to determine the effective concentrations in vitro. The serum protein binding of the

compound is also important for estimating these parameters. We performed an in vitro cell-based experiment in a medium containing 10 and 50 % serum and tested how the IC_{50} value shifts in the presence of high serum concentrations. Then, the infusion dose of the compound needs to be estimated; in other words, the dose of compound required for the infusion to enable the target concentration to be reached in animals must be determined. Before the start of PD and efficacy studies, we performed constant IV infusion dosing experiments to determine the correlation between the dose of the compound and the plasma concentration level in animals.

7. The tumor volume (mm^3) was calculated using the following formula:

$$\text{Tumor volume}\left(\text{mm}^3\right) = \text{length} \times \left(\text{width}\right)^2 \times 0.5.$$

The relative tumor volume was calculated using the following formula:

$$\text{Relative tumor volume} = V \,/\, V_0,$$

where V_0 equals the tumor volume on day 0 and V equals the tumor volume on different observation days (for example, days 0, 3, 6, 10 and 13). Also, the following parameters were also determined:

$$\%T \,/\, C = 100 \times \Delta T \,/\, \quad C \qquad if \;\; \Delta T > 0,$$

or

$$\%T \,/\, C = 100 \times \Delta T \,/\, \text{Ti} \qquad if \;\; \Delta T < 0,$$

where, ΔT equals the change in the mean relative tumor volume compared with the initial relative tumor volume for the treatment group, ΔC equals the change in the mean relative tumor volume compared with the initial relative tumor volume for the vehicle control group, and Ti equals the initial relative tumor volumes for the treatment group. Low positive $\%T/C$ values reflect the control of tumor growth, while negative values indicate tumor regression. According to the National Cancer Institute (NCI) guidelines, $\%T/C \leq 42$ % is considered to indicate significant antitumor activity, while $\%T/C < 10$ % is indicative of a highly active agent.

8. The biological consequences of dosing compounds in animals should be discussed with regard to not only the amount of the dosed compound, but also the actual exposure of the compound. Thus, determining the plasma or tissue concentrations of the compound is important for understanding the results.

9. In our studies, we used three PD markers: the phosphorylation of pRb, BrdU incorporation, and the induction of activated caspase-3. As the Rb protein is a direct substrate of Cdks, we can determine the inhibition of Cdk in cells by a decrease in its phosphorylation. BrdU incorporation and the activation of caspase-3 are markers of cell proliferation (cell cycle progression from G1 to S phase) and cell death, respectively. Thus, by monitoring these markers, we can determine the cellular biological consequences of Cdk inhibition: the inhibition of cell proliferation, and the induction of cell death. In our experiments, the inhibition of pRb phosphorylation and BrdU incorporation occurred within the same range of Cdk inhibitor plasma concentrations. Moreover, our Cdk inhibitor showed an antitumor efficacy at a dose that inhibited pRb phosphorylation. The suppression of pRb phosphorylation in tumor cells is clearly correlated with tumor cell growth and cell death in this model [12].
10. In general, biomarkers should ideally be tested for target engagement and biological effects. For example, in the case of receptor tyrosine kinases (RTKs), the autophosphorylation of RTK could be used to test for target engagement, while markers involved in the Ras/MAPK or PI3K pathway could be used to test for biological effects. These markers are important for understanding the mode of action and the mechanism of the biological effects of the test compounds.
11. Before starting the PD studies, we suggest performing a few experiments to optimize the assay conditions, i.e., to test the expression of the PD marker protein/mRNA in the tumor samples. The expression of PD markers at a quantitatively measurable level is essential for PD experiments. If the expression is insufficient, another PD marker or cell lines with a higher expression level of the marker protein should be considered. The stability of the PD markers is another important factor, because in some cases, the marker proteins are very unstable after the isolation of tumors from animals. For example, some phosphorylated substrate proteins are unstable and rapidly dephosphorylated or degraded after isolation. For this reason, we added proteinase inhibitor and phosphatase inhibitor cocktails to our tissue lysis buffer. Moreover, we performed experiments such as (1) the isolation of tumors from animals and incubation on ice or at 4 °C for different periods, followed by (2) the detection of PD markers at each time point to test stability. This strategy might be useful for determining the optimum assay condition. If the protein of interest is unstable, the protein should be solubilized from the tissues immediately after isolation.

12. There are pros and cons to both a western blot and an IHC assay. For a western blot, the protein of interest should be solubilized from the tumor tissues. However, xenograft tumors of human cancer cell lines are often inhomogeneous and often contain necrotic areas, which cause variations among samples. The formation of necrotic areas is dependent on the cancer cell line (xenograft tumors of some cancer cell lines tend to form a necrotic area, but others do not) and tumor size (larger tumors tend to contain larger necrotic areas). The selection of adequate cell lines and tumor sizes is important for western blot analyses. For immunohistochemistry, this point is not a concern, as necrotic areas can be visually distinguished from the tumors.
13. However, IHC is not suitable for quantitative or throughput analyses. A western blot has the advantage of allowing ~15 or 20 samples to be analyzed at once. However, for quantification, the band intensity must be scanned. If the PD marker can be detected using an ELISA, instead of a western blot, quantitative analyses with a good throughput are feasible.
14. Pan-Cdk inhibitors exhibited an antitumor efficacy while rapidly causing immunosuppression in a rodent tumor model at only 8 h after administration in mice. To understand why Cdk inhibitors caused such rapid immunosuppression, we tested the direct effects of inhibitors on isolated blood cells or lymphocytes in ex vivo experiments. Interestingly, pan-Cdk inhibitors induced cell death in peripheral blood or purified resting (non-stimulated) lymphocytes very rapidly (after 4 h of incubation), suggesting that the acute immunosuppression observed in rodents might be, at least in part, caused by direct cytotoxic effects of pan-Cdk inhibitors on resting lymphocytes [13]. Cell cycle related Cdks were not activated in resting lymphocytes; the phosphorylation of pRb was observed only after stimulation by PMA and Ionomycin. Instead, CTD of the largest subunit of RNA polymerase II is phosphorylated, indicating that Cdk7 or Cdk9 (which phosphorylate this domain) are activated in resting lymphocytes. Indeed a pan-Cdk inhibitor suppressed CTD phosphorylation in resting cells at a dose required for cell death induction. A Cdk4/6 inhibitor with selectivity against Cdk7 and 9 [14] did not induce cell death in resting lymphocytes. These results suggest that CTD phosphorylating activity, possibly arising from Cdk7 or 9, might be important for the survival of resting lymphocytes.

References

1. Sherr CJ (1996) Cancer cell cycles. Science 274:1672–1677
2. Sherr CJ (2000) The Pezcoller lecture: cancer cell cycles revised. Cancer Res 60:3689–3695
3. Pines J (1995) Cyclins and cyclin-dependent kinases: theme and variations. Adv Cancer Res 66:181–212
4. Sherr CJ, Roberts JM (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 13:1501–1512
5. Pavletich NP (1999) Mechanism of cyclin-dependent kinase regulation: structures of cdks, their cyclin activators, and CIP and INK4 inhibitors. J Mol Biol 287:821–828
6. Koepp DM, Haper JW, Elledge SJ (1999) How the cyclin became a cyclin: regulated proteolysis in the cell cycle. Cell 97:431–434
7. Falco GD, Giordan A (1998) CDK9 (PITALRE): a multifunctional cdc2-related kinase. J Cell Physiol 177:501–506
8. Palancade B, Bensaude O (2003) Investigating RNA polymerase II carboxyl-terminal domain (CTD) phosphorylation. Eur J Biochem 270: 3859–3870
9. Price DH (2000) P-TEFb, a cyclin-dependent kinase controlling elongation by RNA polymerase II. Mol Cell Biol 20:2629–2634
10. Dhavan R, Tsai L-H (2001) A decade of CDK5. Nat Rev Mol Cell Biol 2:749–759
11. Lilja L, Yang S, Webb D et al (2001) Cyclin-dependent kinase 5 promotes insulin exocytosis. J Biol Chem 276:34199–34205
12. Hirai H, Takahashi-Suzuki I, Shimomura T et al (2011) Potent anti-tumor activity of a macrocycle-quinoxalinone class pan-Cdk inhibitor in vitro and in vivo. Invest New Drugs 29:534–543
13. Kobayashi M, Takahashi-Suzuki I, Shimomura T et al (2011) Cell death induction in resting lymphocytes by pan-Cdk inhibitor, but not by Cdk4/6 selective inhibitor. Invest New Drugs 29:921–931
14. Hirai H, Shimomura T, Kobayashi M et al (2010) Biological characterization of 2-aminothiazole-derived Cdk4/6 selective inhibitor in vitro and in vivo. Cell Cycle 9:1590–1600