Chapter 10

Evaluating the Effects of CDK Inhibitors in Ischemia–Reperfusion Injury Models

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Abstract

CDK inhibitors have been used to induce protection in various experimental models. Kidney ischemia–reperfusion (I/R) is a form of acute kidney injury resulting in a cascade of cellular events prompting rapid cellular damage and suppression of kidney function. I/R injury, an inevitable impairment during renal transplant surgery, remains one of the major causes of acute kidney injury and represents the most prominent factor leading to delayed graft function after transplantation. Understanding the molecular events responsible for tubule damage and recovery would help to develop new strategies for organ preservation. This chapter describes procedures to study the effect of CDK inhibitors in the cellular I/R model developed from an epithelial cell line deriving from pig kidney proximal tubule cells (LLC–PK1). We briefly describe methods for determining the protective effect of CDK inhibitors such as activation of caspase 3/7, western blot analysis, gene silencing, and immunoprecipitation.

Key words CDK inhibitors, Kidney injury, Ischemia–reperfusion, Renal preservation

1 Introduction

1.1 Pharmacological Inhibitors of CDK/Cyclin Complexes

Alterations in the activity of the CDKs are related to the proliferation of tumor cells and it is well established that their inhibition contributes to loss of proliferation and induction of apoptosis [1]. However, under certain physiological conditions, the cell cycle arrest during the use of CDK inhibitors can permit the initiation of cell repair mechanisms. In this sense, new research into inhibitors of CDK as potential therapeutic agents in the treatment of neurological damage has been developed. The administration of inhibitors of CDK that induce the inhibition of apoptosis with neuroprotective, anti-excitotoxicity and anti-inflammatory effects in models of cerebral and renal ischemia, and also neurodegenerative diseases, has been already described [2–5].

Chronic kidney disease (CKD) is an inflammatory disease that causes a progressive and irreversible loss of the kidney function characterized by a lower glomerular filtration rate below 50 % [6].

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_10,

There are two types of treatment available for patients with CKD: dialysis (hemodialysis or peritoneal dialysis) and renal transplantation. When transplantation is possible, the patients' half-life and quality of life is increased [6]. However, 25 % of all available kidneys are discarded as they are not suitable for transplantation. It is known that short periods of ischemia are sufficient to induce pathophysiological events in renal tubules that could compromise transplant viability. A kidney for transplantation will be subjected to a deprivation of oxygen, resulting in cellular hypoxia. The reperfusion process is also critical for the organ, mainly due to two processes. Firstly, there is oxidative damage caused from the increase in reactive oxygen species (ROS) during the reintroduction of oxygen to previously ischemic tissue. Secondly, there is an inflammatory response that initiates the infiltration of polymorphonuclear (PMN) cells that amplify cell damage. The reintroduction of oxygen reestablishes normal metabolic activity. This coincides with a peak in cell death caused by necrosis or apoptosis [7]. There are evidences showing that damage produced by PMN can be reduced by the use of immunosuppressants, for example cyclosporine [8], or fingolimod [9]. Similarly, compounds that inhibit CDK, for example flavopiridol or roscovitine [10] have been used as immunosuppressants and have shown to be protective against damages caused by the processes of I/R [11].

We have reported that the CDK inhibitors (roscovitine and TAT–NBI1) [5, 12] provide protection against cell death in a well established ischemia/reperfusion (I/R) model in porcine renal tubular cells (LLC–PK1) [13].

In this section, we describe the materials and methods used to evaluate the use of the CDK inhibitors for the prevention and treatment of damage produced in kidneys as a result of renal I/R injury.

2 Materials

2.1 Model for Renal Ischemia–Reperfusion (I/R) Injury in LLC-PK1

1. LLC-PK-1 Proximal tubular porcine LLC-PK-1 cells were obtained from ATCC (Rockville, MD). Cells were grown in M199 supplemented with 3 % FBS and were maintained at 37 °C in a 5 % carbon dioxide atmosphere in 20-cm tissue culture plates or T150 culture flasks. Cells were transferred two times per week.
2. M199 supplemented with 3 % (w/v) FBS.
3. 0.25 % trypsin–1 mM EDTA.
4. 20-cm tissue culture plates or T150 culture flasks.
5. 6-well tissue culture plates.
6. 40-μm cell strainers.

7. 37 °C tissue culture incubator, 5 % CO_2.
8. 37 °C tissue culture incubator, 18 % CO, 1.5 % O_2
9. Additional equipment and reagents for culturing cells, determining cell concentration with a hemacytometer.

2.2 Caspase 3/7 Activity Assays

1. 5 mM EDTA in PBS.
2. Extraction Buffer: 50 mM PIPES, 50 mM KCl, 5 mM EDTA, 2 mM $MgCl_2$, 2 mM DTT, supplemented with protease inhibitors.
3. Caspase assay buffer: PBS, 10 %, glycerol, 0.1 mM EDTA, 2 mM DTT, and 20 μM of Ac-DEVD-AFC (caspase-3 substrate).
4. 96-well microplates: Tissue culture microplate with black wall is recommended.
5. Liquid nitrogen.
6. A fluorescence microplate reader: Capable of monitoring fluorescence intensity at Ex/Em = 390/510 nm.
7. Kit for quantification of protein: Pierce BCA Protein Assay Kit (Pierce) is recommended.

2.3 Western Blot Analysis

1. Equipment for SDS-PAGE and Immunoblotting (Vertical acrylamide electrophoresis unit and Electroblotting unit-fully submerged, Bio-Rad Mini-PROTEAN series recommended).
2. Kit for quantification of protein: Pierce BCA Protein Assay Kit (Pierce) is recommended.
3. Nitrocellulose and chromatography paper.
4. Buffer lysis: 25 mM Tris–HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 % SDS, plus protease and phosphatase inhibitors.
5. TBS solution: 10 mM Tris–HCl, pH 8.0, 150 mM NaCl.
6. Buffer transfer: 25 mM Tris–HCl pH 7.5, 192 mM glycine, and 20 % methanol.
7. Nonfat milk powder.
8. Primary antibody.
9. Antibody-HRP.
10. Substrate for detection (ECL system).

2.4 Gene Silencing with Small Interfering RNA

1. siRNA of interest. The siRNA for CDK5 mRNA (#6216) and the negative control siRNA (#6568) are from Cell Signaling. The siRNAs for p35 and Cyclin I are from Santa Cruz Biotechnology (sc-36153 and sc-35141, respectively).
2. Lipofectamine 2000 (store at +4 °C until use).
3. Opti-MEM I Reduced Serum Medium (prewarmed).
4. 6-well tissue culture plates.

5. Equipment for culturing cells.
6. Solution A: Dilute 50–100 nM of siRNA transfection into 100 µl of Opti-MEM® I Reduced Serum Medium without serum.
7. Solution B: Mix Lipofectamine™ 2000 gently before use, then dilute the appropriate amount (50 pmol) in 100 µl of Opti-MEM® I Medium without serum.
8. SDS-PAGE and Immunoblotting equipment and reagents.

2.5 Immunoprecipitation Assay

1. The Co-IP kit Pierce Direct IP Kit #26148 is recommended and is used with the following solutions:
   - Coupling Buffer: 0.01 M sodium phosphate, 0.15 M sodium chloride; pH 7.2
   - IP Lysis/Wash Buffer: 0.025 M Tris–HCl, 0.15 M NaCl, 0.001 M EDTA, 1 % NP-40, 5 % glycerol, pH 7.4
   - 100× Conditioning Buffer: neutral pH buffer and Elution Buffer: pH 2.8, containing primary amine.
   - Lane Marker Sample Buffer 5×, 0.3 M Tris–HCl, 5 % SDS, 50 % glycerol, 100 mM DTT, pH 6.8.
2. Kit for quantification of protein: Pierce BCA Protein Assay Kit (Pierce) is recommended.
3. The antibodies Cyclin I (sc-5547) and p35/p35 (sc-820) are obtained from Santa Cruz Biotechnology and Tubulin (T8203) are from Sigma Aldrich.
4. Rocking platform or rotator.
5. SDS-PAGE and Immunoblotting equipment and reagents.

3 Methods

These methods describe some techniques which allow the study of the effect of CDKI in the cellular I/R model developed from an epithelial cell line deriving from pig kidney proximal tubule cells (LLC–PK1). LLC–PK1 cells are subjected to Hx–Hp/Nx conditions in the presence and absence of the CDK/cyclin inhibitors (*see* Subheading 3.1). In the vehicle control-treated cells, Hx–Hp/Nx conditions induce cell death, which is characterized by increased caspase-3/-7 activity (*see* Subheading 3.2). Also, comparison between the expression levels of different proteins under normoxic and Hx–Hp/Nx conditions are characterized by Western blot analysis (*see* Subheading 3.3). The molecular mechanism of cell death protection provided by treatment with CDK/cyclin inhibitors is obtained from gene silencing (*see* Subheading 3.4) and immunoprecipitation studies (*see* Subheading 3.5).

3.1 Model Renal Ischemia–Reperfusion (I/R) Injury in LLC-PK1

1. Culture the cells until they are 60–80 % confluent (*see* **Note 1**).
2. Aspirate the cell culture medium and wash the cells twice with PBS, then add 5 ml of 0.05 % Trypsin–EDTA.
3. Incubate at 37 °C for 8–10 min.
4. Add 10 ml of cell culture medium containing FBS per flask.
5. Using a 1000 μl tip, pipette the medium across the wells to detach the colonies. Pipette up and down five times to break up cell colonies into a single cell suspension.
6. Transfer the cell suspension to a 15 or 50 ml conical tube and centrifuge at $300 \times g$ for 5 min at room temperature.
7. Aspirate the supernatant and loosen the cell pellet by tapping the bottom of the tube.
8. Resuspend with 2–5 ml of cell culture medium.
9. Place a 40 μm cell strainer into a 50 ml conical tube and add the cell suspension to the cell strainer (Clumps of cells will be retained while single cells and medium will pass into the conical tube). When all of the suspension has passed through, remove the cell strainer and discard.
10. Remove a sample of the single cell suspension to perform a cell count, determine the cell number and calculate the amount of the medium needed to achieve the desired cell density. Grow the cells to form a confluent monolayer in a 6-well plate format at a cellular density of 4×10^5 cells/well. Prepare two identical experiments (Nx group and Hx-Hp/Hx group).
11. Incubate both groups at 37 °C, 21 % O_2; 5 % CO_2 overnight.
12. The next day, change the Hx-Hp/Hx group to hypoxia-hypercapnia conditions (Hx-Hp). Incubate the cells at 37 °C and 1.5 % O_2; 18 % CO_2 overnight (*see* **Note 2**).
13. Maintain the Nx group at 37 °C, 21 % O_2; 5 % CO_2 overnight.
14. Treat the cells with the CDK inhibitors and incubate both groups at 37 °C, 21 % O_2; 5 % CO_2 for an additional 24-h period (*see* **Note 3**).
15. Evaluate the hypoxic induction through caspase 3/7 activity.

3.2 Evaluation of Hypoxia: Caspase 3/7 Activity Measurements

Subjecting the cells to hypoxia and hypercapnia and afterwards reoxygenating will cause cellular damage and cell loss attributable to apoptosis (*see* **Note 4**). The method to measure the induction of Hx-Hp/Nx is through the activation of caspase 3/7 (*see* **Note 5**).

1. Carefully remove the culture medium and cells in suspension into a labeled 15 ml conical centrifuge tube for the appropriate condition. Keep on ice throughout the remainder of the protocol.
2. Wash the well two times in cold PBS for 5 min at room temperature and keep the washings.

3. Harvest cells by adding a solution of 5 mM EDTA in PBS and incubate at 37 °C for 10–15 min (This procedure may take longer than normal trypsinization).
4. Collect the cells and centrifuge the cell suspension at 300 × *g* for 5 min to pellet the cells. Discard the supernatant.
5. Add cold extraction buffer to the cell pellet, use 30 μl of extraction buffer per 2 wells of a 6-well plate and incubate the cells on ice for 5 min. This hypotonic buffer allows the liberation of the cell contents without completely breaking the cells.
6. Transfer the cell lysate into new microcentrifuge tube. Make sure the tubes are closed. Drop the tube into liquid nitrogen to freeze. Thaw sample on cold water (thawing takes a few minutes). Vortex briefly.
7. Repeat freeze-thaw cycle two more times.
8. Thaw the cell lysate. Collect the supernatant by centrifugation at 16000 × *g* for 7 min at 4 °C.
9. Measure the protein concentration.
10. For 50 μg of cellular lysate (*see* **Note 6**), add 200 μl of caspase assay buffer.
11. The DVDase activity is continuously monitored following the release of fluorescent AFC at 37 °C with a spectrofluorometer such as Wallac 1420 Workstation (λexc 390 nm; λem 510 nm).
12. The DEVDase activity is expressed with an increase in caspase-3 activity compared to a positive control (cells under Hx–Hp/Nx conditions without treatment) and measured by the relative fluorescence or as percentage of the initial fluorescence signal value.

3.3 Western Blot Analysis

We studied the molecular mechanism and found that the CDK5 was responsible for the protective effect in the I/R model [14–16]. In order to analyze the role of CDK5 in cell survival it is necessary to evaluate the protein expression levels of CDK5, the regulatory proteins and their substrates in LLC–PK1 renal tubular cells under normoxic and Hx–Hp/Nx conditions. This is measured in the absence or presence of CDK/cyclin inhibitors using western blot analysis (*see* **Note 7**).

1. Obtain whole cell extracts by lysing the cells in a buffer lysis. Determine the protein concentration by the BCA protein assay.
2. Load the cell extracts and separate in 10–12 % SDS-PAGE and then transfer to a nitrocellulose membrane.
3. Block the membrane with 5 % nonfat dried milk in TBS containing 0.1 % Tween 20 at room temperature with shaking for 1 h.
4. Incubate the membrane with primary antibody in the blocking solution at 4 °C overnight by shaking.

5. Wash the membranes in TBS containing 0.1 % Tween 20 four times for at least 10 min each time with extensive agitation.
6. Incubate with appropriate horseradish peroxidase secondary antibodies with 5 % nonfat dried milk in TBS containing 0.1 % Tween 20 at room temperature for 90 min with shaking. Visualize the proteins using enhanced chemiluminescence technology.

3.4 Gene Silencing with Small Interfering RNA

CDK5 plays a key regulatory role in cell death and survival in the cellular pathways related to I/R-induced damage. Silencing of CDK5 and their regulatory proteins is needed to demonstrate the involvement of these proteins in the recovery process (*see* **Note 8**).

1. Grow the LLC-PK1 cells to form a confluent monolayer in a 6-well plate format at a cellular density of 3×10^5 cells/well. Prepare two identical experiments (Random group and siRNA group). Incubate both groups at 37 °C, 21 % O_2; 5 % CO_2 overnight.
2. The next day, the cells will be 30–50 % confluent at the time of transfection (*see* **Note 9**). Transfect the siRNA oligonucleotides in Opti-MEM at the recommended concentrations using Lipofectamine 2000 according to the manufacturer's instructions (*see* **Note 10**).
3. Remove the growth medium by aspiration and wash the cells with PBS. Add 800 μl of Opti-MEM to the plate and incubate at 37 °C in a CO_2 incubator whilst preparing the solutions.
4. Prepare the solutions A and B (*see* **Note 11**), mix gently and incubate for 5 min at room temperature.
5. After the 5 min incubation period at room temperature, combine solution A with solution B. Mix gently and incubate for 20 min at room temperature.
6. Add 200 μl of the solution to each well containing cells and medium. Mix gently by rocking the plate back and forth.
7. Incubate the transfected cells for 4 h at 37 °C in a CO_2 incubator (*see* **Note 12**)
8. Remove the transfection mixture and replace with normal growth medium and incubate the cells for an additional 24 h at 37 °C, 21 % O_2; 5 % CO_2.
9. At 24 h post-transfection, incubate the cells under Hx–Hp conditions (37 °C and 1.5 % O_2; 18 % CO_2) for 24 h. Treat with the CDK inhibitors and maintain under Nx conditions for a further 24 h.
10. Check the efficacy of the silencing by Western blot.

3.5 Immunoprecipitation Studies

Evaluation of the influence of CDK inhibitors on the regulation of CDK5 and its regulatory subunits by co-immunoprecipitation experiments is described (*see* **Note 13**).

1. Prepare cellular extracts from the LLC–PK1 control cells or from those cells subjected to Hx–Hp/Nx conditions in the presence or absence of the CDK/cyclin inhibitor.
2. Use the Co-IP kit for the immunoprecipitation studies according to the manufacturer's instructions (*see* **Note 14**).
3. Obtain 1 mg of protein, seed and treat 2 plates for the treatment (100 × 100 mm).
4. Aspirate media and rinse cells once with Coupling Buffer.
5. Remove PBS and add 1 ml of ice-cold IP Lysis/Wash Buffer to each plate and incubate the plates on ice for 5 min with periodic mixing.
6. Transfer the lysate to a microcentrifuge tube and centrifuge at ~13,000 × *g* for 10 min to pellet the cell debris.
7. Transfer supernatant to a new tube for protein concentration determination. Measure the protein concentration in the cell lysates using a BCA protein assay, according to the manufacturer's protocol. Use bovine serum albumin (BSA) as a standard.
8. Dilute the cell extract in 400 μl of IP Lysis/Wash Buffer.
9. Add the sample to the 10 μg of antibody-coupled resin in the spin column and incubate the column with gentle end-over-end mixing or shaking for 1 h to overnight at 4 °C (*see* **Note 15**).
10. Remove the bottom plug, loosen the screw cap and place the column in a collection tube. Centrifuge the column and save the flow-through. Do not discard the flow-through until confirming that the IP was successful.
11. Place the column into a new collection tube, add 200 μl of IP Lysis/Wash Buffer and centrifuge. Wash the sample three times.
12. Wash the sample once with 100 μl of 1× Conditioning Buffer.
13. Place the spin column into a new collection tube, add 25 μl of Elution Buffer and centrifuge.
14. Keep the column in the tube and add 75 μl of Elution Buffer. Incubate for 10 min at room temperature.
15. Centrifuge the tube and collect the flow-through. Perform additional elutions as needed.
16. Add 5× Sample Buffer to the sample to make a 1× final solution (i.e., add 5 μl of 5× Sample Buffer to 20 μl of sample).
17. Heat the sample at 95–100 °C for ~5 min. Analyze the eluate to ensure that the antigen has completely eluted.
18. Analyze by western blot to reveal that the immunoprecipitation of CDK5 and cyclin I has been effective. Separate the co-immunoprecipitation by SDS-PAGE and transfer to a nitrocellulose membrane. Incubate the membrane in 5 % nonfat milk powder overnight at 4 °C with primary antibodies, fol-

lowed by incubation with appropriate horseradish peroxidase secondary antibodies. Visualize the signals with the ECL system.

4 Notes

1. All solutions and equipment must be sterile, and aseptic technique should be used accordingly.
2. The door of the hypoxia incubator should remain closed for the incubation period of 24 h.
3. It is important to leave some wells without treatment as a control for hypoxia or normoxia.
4. LLC–PK1 cells were subjected to Hx–Hp/Nx conditions in the presence and absence of the CDK/cyclin inhibitors. In the vehicle control treated cells, Hx–Hp/Nx conditions induced cell death, which was characterized by increased caspase-3/-7 activity. The presence of CDK/cyclin inhibitors enhanced cell viability under Hx–Hp/Nx conditions, together with diminished caspase-3/7 activity.
5. For the determination of the caspase-3/7 activity a fluorescence assay was used [17]. The caspases are cysteine-proteases characterized by the presence of a cysteine residue that breaks other proteins on an aspartic residue. In this assay, the caspase-3/7 from the cell lysate hydrolyses the substrate: Acetyl-Asp-Glu-Val-Asp-7-amino-trifluoromethyl-coumarin (Ac-DEVD-AFC), liberating the AFC, which is a fluorescent marker. The liberation of AFC as a function of time allows for the evaluation of the activity of caspase-3/7 in each sample.
6. For the protocol for caspase-3/7 activity determination it is necessary to have 2 wells per treatment to obtain a sufficient quantity of protein.
7. The expression of CDK5, p35/p25, cyclin I as well as phosphorylation levels of ERK1/2, H2A.x, and Chk1 in total cell extracts was analyzed by western blot in the presence of CDK inhibitors (TAT-NBI1 or roscovitine). The results showed that the treatment with CDK inhibitors restores the normoxic phenotype in the LLC–PK1 Hx–Hp/Nx model [5].
8. The silencing of CDK5 and cyclin I hinders the protective action of CDK inhibitors, but, in contrast, p35 silencing had a protective effect itself in Hx–Hp/Nx renal tubular cell.
9. Subconfluent cells are healthy and are required for successful transfection experiments. It is recommended to ensure cell viability 1 day prior to transfection.
10. Do not add antibiotics to media during transfection as this will cause cell death.

11. The siRNA and Lipofectamine calculations are made to have a final volume of 1 ml per well.
12. Prolonged serum starvation may result in unwanted cell detachment or death.
13. Under Nx conditions, CDK5 maintains a binding equilibrium between both partners p35/p25 and cyclin I signaling to cell survival. Hx–Hp/Nx conditions would produce an increase in the p35 protein levels promoting cell death and under Hx–p/Nx conditions, treatment with the CDK5 inhibitors would affect the CDK5/p35 complex without affecting the activity of the pro-survival CDK5/cyclin I complex. This differential inhibition would promote the degradation of p35, the stabilization of the CDK5/cyclin I complex, and the engagement of a cell survival program that would protect cells from Hx–Hp/Nx-induced damage.
14. The Co-IP kit (Pierce Direct IP Kit #26148) is useful for the immunoprecipitation of a target protein whose molecular weight is similar to the heavy or light chain antibody fragment.
15. Immunoprecipitation is achieved using less than 10 μg of antibody. After the antibody is coupled to the AminoLink Resin, the antigen sample is incubated with the immobilized antibody to form the immune complex. The complex is washed to remove non-bound material, and a low pH elution buffer is used to dissociate the bound antigen from the antibody.

References

1. Orzaez M, Guevara T, Sancho M, Perez-Paya E (2012) Intrinsic caspase-8 activation mediates sensitization of erlotinib-resistant tumor cells to erlotinib/cell-cycle inhibitors combination treatment. Cell Death Dis 3:e415
2. Iyirhiaro GO, Brust TB, Rashidian J, Galehdar Z, Osman A, Phillips M, Slack RS, Macvicar BA, Park DS (2008) Delayed combinatorial treatment with flavopiridol and minocycline provides longer term protection for neuronal soma but not dendrites following global ischemia. J Neurochem 105(3):703–713
3. Hilton GD, Stoica BA, Byrnes KR, Faden AI (2008) Roscovitine reduces neuronal loss, glial activation, and neurologic deficits after brain trauma. J Cereb Blood Flow Metab 28(11): 1845–1859
4. Menn B, Bach S, Blevins TL, Campbell M, Meijer L, Timsit S (2010) Delayed treatment with systemic (S)-roscovitine provides neuroprotection and inhibits in vivo CDK5 activity increase in animal stroke models. PLoS One 5(8):e12117
5. Guevara T, Sancho M, Perez-Paya E, Orzaez M (2014) Role of CDK5/cyclin complexes in ischemia-induced death and survival of renal tubular cells. Cell Cycle 13(10):1617–1626
6. Bagshaw SM, Mortis G, Godinez-Luna T, Doig CJ, Laupland KB (2006) Renal recovery after severe acute renal failure. Int J Artif Organs 29(11):1023–1030
7. Kosieradzki M, Rowinski W (2008) Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplant Proc 40(10):3279–3288
8. Singh D, Chander V, Chopra K (2005) Cyclosporine protects against ischemia/reperfusion injury in rat kidneys. Toxicology 207(3): 339–347
9. Delbridge MS, Shrestha BM, Raftery AT, El Nahas AM, Haylor JL (2007) Reduction of ischemia-reperfusion injury in the rat kidney by FTY720, a synthetic derivative of sphingosine. Transplantation 84(2):187–195
10. Aydemir A, Abbasoglu O, Topaloglu S, Ertoy D, Ayhan A, Kilinc K, Karabulut E, Sayek I

(2002) Protective effect of roscovitine on renal ischemia-reperfusion injury. Transplant Proc 34(6):2027–2028

11. Osuga H, Osuga S, Wang F, Fetni R, Hogan MJ, Slack RS, Hakim AM, Ikeda JE, Park DS (2000) Cyclin-dependent kinases as a therapeutic target for stroke. Proc Natl Acad Sci U S A 97(18):10254–10259
12. Canela N, Orzaez M, Fucho R, Mateo F, Gutierrez R, Pineda-Lucena A, Bachs O, Perez-Paya E (2006) Identification of an hexapeptide that binds to a surface pocket in cyclin A and inhibits the catalytic activity of the complex cyclin-dependent kinase 2-cyclin A. J Biol Chem 281(47):35942–35953
13. Hotter G, Palacios L, Sola A (2004) Low O2 and high CO2 in LLC-PK1 cells culture mimics renal ischemia-induced apoptosis. Lab Invest 84(2):213–220
14. Taniguchi Y, Pippin JW, Hagmann H, Krofft RD, Chang AM, Zhang J, Terada Y, Brinkkoetter P, Shankland SJ (2012) Both cyclin I and p35 are required for maximal survival benefit of cyclin-dependent kinase 5 in kidney podocytes. Am J Physiol Renal Physiol 302(9):F1161–F1171
15. Brinkkoetter PT, Olivier P, Wu JS, Henderson S, Krofft RD, Pippin JW, Hockenbery D, Roberts JM, Shankland SJ (2009) Cyclin I activates Cdk5 and regulates expression of Bcl-2 and Bcl-XL in postmitotic mouse cells. J Clin Invest 119:3089
16. Brinkkoetter PT, Pippin JW, Shankland SJ (2010) Cyclin I-Cdk5 governs survival in postmitotic cells. Cell Cycle 9(9):1729–1731
17. Fearnhead HO (2001) Cell-free systems to study apoptosis. Methods Cell Biol 66: 167–185