CDK4/6 Inhibition Induces Epithelial Cell Cycle Arrest and Ameliorates Acute Kidney Injury

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Abstract

Acute kidney injury (AKI) is common and urgently requires new preventative therapies. Expression of a cyclin-dependent kinase (CDK) inhibitor transgene protects against acute kidney injury, suggesting that manipulating the tubular epithelial cell cycle may be a viable therapeutic strategy. Broad spectrum small molecule CDK inhibitors are protective in some kidney injury models, but these have toxicities and epithelial proliferation is eventually required for renal repair. Here, we tested a well-tolerated, novel and specific small molecule inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, PD 0332991, to investigate the effects of transient cell cycle inhibition on epithelial survival in vitro and kidney injury in vivo. We report that CDK4/6 inhibition induced G₀/G₁ cycle arrest in cultured human renal proximal tubule cells (hRPTC) at baseline and after injury. Induction of G₀/G₁ cycle arrest through CDK4/6 inhibition protected hRPTC from DNA damage and caspase 3/7 activation following exposure to the nephrotoxins cisplatin, etoposide, and antimycin A. In vivo, mice treated with PD 0332991 prior to IRI exhibited dramatically reduced epithelial progression through S-phase 24 hours after IRI. Despite reduced epithelial proliferation, PD 0332991 ameliorated kidney injury as reflected by improved serum creatinine and blood urea nitrogen levels 24 hours after injury. Inflammatory markers and macrophage infiltration were significantly decreased in injured kidneys three days following IRI. These results indicate that induction of proximal tubule cell cycle arrest with specific CDK4/6 inhibitors, or “pharmacological quiescence,” represents a novel strategy to prevent AKI.

Keywords: Cell cycle, cyclin-dependent kinase, acute kidney injury, proximal tubule, ischemia-reperfusion injury
Introduction

Acute kidney injury (AKI) is a potentially devastating, increasingly common, and costly syndrome characterized by sudden impairment of kidney function as a result of a toxic or ischemic insult (9, 23, 61, 67). Patients with AKI experience increased length of hospital stay, increased mortality, and increased risk of future development of chronic kidney disease and end stage renal disease (4, 7, 22, 28, 38, 66). Clinical outcomes are directly related to AKI severity, including even minor changes in serum creatinine (27). Even apparently reversible AKI resulting from contrast exposure or cardiothoracic surgery is associated with increased long-term risk of mortality (13, 18, 68). The combination of potentially severe and long-lasting outcomes related to AKI lead to high cumulative cost of care, with annual estimates of AKI associated costs at approximately $10 billion in the United States (7, 9). There are currently no approved therapeutics directly indicated to prevent or treat AKI and associated systemic maladies, and novel treatments are urgently needed.

Ischemia and toxins can cause AKI in humans (1, 30, 40, 41, 51, 54, 71) and bilateral ischemia reperfusion injury (Bi-IRI) is a widely accepted mouse model of human AKI (69). Within the first 24 hours following injury, tubular cells undergo apoptotic and necrotic cell death. In response, the surviving, normally quiescent proximal tubule epithelial cells rapidly proliferate, and approximately 70% of these cells enter the S-phase of the cell cycle (19, 20). Accompanying this robust proliferative response is DNA damage resulting from the post-ischemic, inflammatory environment (46). A very similar sequence of events can be induced by nephrotoxicants such as cisplatin (37, 47, 54).

Cyclin-dependent kinases 2/4/6 (CDK 2/4/6) mediate cell cycle checkpoint progression from G1 to S phase. Binding of their activating subunits, the D-type cyclins for CDK4/6 and the E- or A-type cyclins for CDK2, causes cell cycle progression into S phase via phosphorylation of the retinoblastoma protein (Rb) (35, 57). Significant recent work has shown cell types differ in their need for CDK4/6 vs. CDK2 activity for proliferation, with many cell types capable of briskly
dividing even in the absence of CDK4/6 or D-type cyclin activity (26, 32-34, 70). Of particular relevance, it is also now clear that some cell types in adult mammals (e.g. pancreatic beta cells, hematopoetic stem and progenitor cells) absolutely require CDK4/6 activity in order to traverse G1 (24, 48, 49).

While epithelial cell cycle reentry after injury has traditionally been viewed as an appropriate repair response to the loss of adjacent cells after an initial insult, recent evidence suggests a more complicated picture. Epithelial cell cycle inhibition in vitro, whether through overexpression of the CDK inhibitor p21CIP, or through broad spectrum small molecule CDK inhibitors, can actually protect against cisplatin-induced cell death (36, 45, 47). One of these broad spectrum CDK inhibitors, purvalanol, has been used in vivo to afford protection from cisplatin-induced AKI (47). The p21CIP CDK inhibitor is induced in response to DNA damage and other stresses, and mice lacking p21CIP exhibit exacerbated kidney ischemic injury, consistent with the hypothesis that cell cycle inhibition post-insult protects against AKI (36, 42). Importantly, small molecule inhibition of CDK2 does not induce a “clean” arrest in the G1 phase of the cell cycle, but instead leads to a G2/M block or an intra-S phase arrest, which is the outcome of treatment with various toxic chemotherapeutic agents that lead to cell death (45, 57). Also, while CDK2 inhibition offers protection in the short term, G2/M arrest would likely be an undesirable long-term effect as it has been recently reported that this induces progressive interstitial fibrosis in the kidney and increased cell apoptosis (69). Further, the currently available small molecule inhibitors of CDK2 are known to non-specifically inhibit various other kinases in the low micromolar range (2, 3). Finally, CDK2 is known to phosphorylate other non-Rb substrates that are involved in regulating transcription and DNA metabolism (57).

Disruption of the G1 to S phase checkpoint in various human cancers has resulted in increased efforts to develop small molecules that selectively inhibit CDK4/6 (31). The pyridopyrimidine small molecule PD 0332991 has been shown to selectively inhibit CDK4/6 in the low nanomolar range and induce clean and dose-dependent G1 arrest in a variety of Rb-
positive cell lines (11, 24, 48, 62). Previous work has shown that PD 0332991 can induce G₁ arrest in hematopoietic progenitor cells and this pharmacological quiescence (PQ) was effective in mitigating radiation induced cellular apoptosis and toxicity (24). Additionally, the induction of G₁ arrest in hematopoietic progenitor cells affords significant protection from myelosuppression following treatment with the chemotherapeutic agent carboplatin (50). Ionizing radiation and DNA intercalating/adduct forming chemotherapeutics confer cytotoxicity through inducing extensive DNA damage. It is thought that the cytotoxic effects of these particular insults are cell cycle dependent.Traversal from G₁ to S phase in the setting of DNA damage is particularly damaging while early G₁ and late S phases are reported to be more resistant to genotoxic damage (24). Delaying this cell cycle progression after injury should also allow more time for DNA damage to be repaired prior to mitosis. Here we tested the hypothesis that inhibiting S phase traversal (“pharmacological quiescence”), with selective CDK4/6 inhibitors would protect renal proximal tubule cells from injury induced by DNA adduct forming chemotherapeutic agents in vitro and improve kidney function following ischemia reperfusion injury in vivo.

Methods

Compounds and dosing: PD0332991 was synthesized by the Center for Integrative Chemical Biology and Drug Discovery at the University of North Carolina (Chapel Hill, NC). Cisplatin (Teva, Inc, Irvine, CA), etoposide (Sigma-Aldrich, St. Louis, MO), and Antimycin A (Sigma-Aldrich, St. Louis, MO), were obtained from their respective manufacturers and handled according to the manufacturer’s recommendations. PD0332991 was dissolved in 50mM sodium lactate buffer (pH 4.0) to a final concentration of 15mg/ml. Mice were treated with a 150mg/kg dose of PD0332991, or vehicle, 1 hour prior to ischemia reperfusion surgery by oral gavage. 5-bromo-2'-deoxyuridine, BrdU, was dissolved in sodium chloride to 10mg/ml and mice were given I.P. injections at a dose of 100mg/kg.
Cell line: Primary human renal proximal tubule epithelial (hRPTC), were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in an incubator at 37°C in a humidified atmosphere of 5% CO₂ and cultured in Renal Epithelial Cell Basal Medium (ATCC) supplemented with accompanying Renal Epithelial Cell Growth Kit (ATCC) following manufacturer's recommendations.

Assessment of DNA Cell Cycle and Cell Proliferation: For the cell cycle flow cytometry assay, 44,000 cells were plated per well in 12-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Cells were then incubated with PD0332991 or dimethyl sulfoxide vehicle control for an additional 24 hours. hRPT cells were then harvested and fixed in ice cold methanol, and stored at -20°C. Cell pellets were spun at 1200rpm for 5 minutes, aspirated and washed in 3mls 1x PBS-CMF (Cellgro, Mediatech, Inc., Manassas, VA). Samples were respun, aspirated and resuspended in PBS-CMF/ 1% BSA Fraction V (Fisher, Fair Lawn, NJ) containing (100ug/ml) propidium iodide (Sigma-Aldrich, St. Louis, MO) and (150ug/ml) DNAse-free RNAse A (Sigma-Aldrich, St. Louis, MO). Samples are quantified using a CyAn™ ADP Analyzer (Beckman Coulter, Indianapolis, IN) and FlowJo analysis software (Version 7.2.2, TreeStar, Ashland, OR).

For the Cell TiterGlo assay, 750 cells were plated per well in 96-well white wall clear bottom plates (Costar, Corning, NY). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Cells were then incubated at 37°C in a humidified atmosphere of 5% CO₂ with PD0332991 or dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) vehicle control for 16 hours. Cells were then treated with chemotherapy (cisplatin or etoposide) for 8hrs, followed by aspiration of medium and replenishment with fresh media. Plates were incubated for 7 days, removed from incubator and treated with CellTiterGlo® Assay System (Promega, Madison, WI) by following the manufacturer's instructions.
Assessment of DNA Damage and Apoptosis by γ-H2AX Flow Cytometry and Caspase Activation:

For the γ-H2AX assay, 44,000 cells were plated per well in 12-well plates. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Cells were then incubated with PD0332991 or dimethyl sulfoxide vehicle control for 16 hours. Cells were then treated with chemotherapy (cisplatin or etoposide) for 8 hours. hRPT cells were then fixed and stained using the H2A.X Phosphorylation Assay Kit (Flow Cytometry; Millipore, Temacula, CA) by the manufacturer’s instructions. γ-H2AX positive hRPT cells were then quantified using a CyAn™ ADP Analyzer (Beckman Coulter, Indianapolis, IN) and FlowJo analysis software (Version 7.2.2, TreeStar, Ashland, OR).

For the caspase 3/7 assay, 750 hRPTC cells were plated per well in 96-well white wall clear bottom plates (Costar, Corning, NY). Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ for 24 hours. Cells were then incubated with PD0332991 or dimethyl sulfoxide vehicle control for 16 hours. Cells were then treated with chemotherapy (cisplatin or etoposide) or Antimycin A for 24 hours. Antimycin A was used in normal glucose medium, and 2-deoxy-glucose was not added. Caspase 3/7 activation was measured directly in the 96-well plates using the Caspase-Glo® 3/7 Assay System (Promega, Madison, WI) by following the manufacturer’s instructions.

Ischemia Reperfusion Injury: All mouse experiments were performed according to the animal experimental guidelines issued by the Animal Care and Use Committee at Harvard University and University of North Carolina. For bilateral IRI, 8 week old male mice were anesthetized with pentobarbital sodium (60mg/kg body weight, intraperitoneally) prior to surgery. Body temperatures were controlled at 36.5°C-37.5°C throughout the procedure. Bilateral flank incisions into the peritoneum allowed access to both kidneys. Kidneys were exposed and
ischemia was induced by simultaneously clamping the renal pedicle of both kidneys with nontraumatic microaneurysm clamps (Roboz, Rockville, MD). Clamped kidneys were placed back into the peritoneal cavity for 28 minutes, and clamps were then removed resulting in reperfusion injury. The flank incisions were closed with wound clips and the mice were allowed to recover in their home cages. Unilateral IRI required one flank incision and clamping one kidney for 28 minutes. The tubular injury score was assessed exactly as described (21).

**Serum Creatinine and BUN measurements:** 24 hours after surgery, tail vein blood was collected in heparinized micro-hematocrit capillary tubes (Fisher, cat. #22-362-566) and centrifuged for 15 minutes at 5000 RPM. The supernatant was retained as serum. Serum creatinine was measured by the Beckman Creatinine Analyzer 2, which utilizes a 10mM picric acid solution mixed with a buffer containing 10mM sodium borate, 240mM sodium hydroxide, and 10mM SDS. Serum creatinine is measured against a creatinine standard and recorded as milligrams per deciliter. BUN measurements were made using 1µl of serum combined with 200µl of Infinity™ Urea solution (Fisher, Cat. TR12421). Six readings were made on a spectrophotometer at 340nm wavelength at 30 second intervals and compared to a standard solution at 30mg/dl. Serum creatinine and BUN measurements in figure 6 were both repeated in additional experiments with statistically significant outcomes.

**Immunofluorescence:** Mice were anesthetized with isofluorane and immediately perfused via the left ventricle with ice-cold PBS for 1 minute. Kidneys were fixed in 10% neutral buffered formalin solution overnight at 4°C and then switched to 70% ethanol. Kidneys were then embedded in paraffin wax and cut into 4 µm sections. For immunofluorescence studies sections were processed in xylene and an ethanol series and an antigen retrieval step was performed in sodium citrate buffer in a pressure cooker. Slides were then washed in 1X PBS, blocked in 10% normal goat serum (Vector Labs) and incubated with an anti-BrdU antibody made in rat (Abcam,
Results

**PD 0332991 arrests human renal proximal tubule cells in G₀/G₁**

To determine if kidney epithelial cells were sensitive to CDK4/6 inhibition, cultured human renal proximal tubule cells (hRPTC) were treated with increasing doses of PD 0332991 and cell cycle stage was measured. PD 0332991 caused a potent and dose dependent increase in the percentage of cells in G₀/G₁ while decreasing the percentage of cells in the S phase and G₂/M phases of the cell cycle, indicating the induction of cell cycle arrest (Figure 1A-D). Mouse primary proximal tubule epithelial cells were also isolated and cultured to determine if PD 0332991 induced similar responses. Again, PD 0332991 treatment resulted in dose dependent increases in the fraction of cells in G₀/G₁ while reducing the number of cells in S and G₂/M cell cycle phases (Figure S1 A,B). Next, we tested if PD 0332991 could induce cell cycle arrest in hRPTCs treated with antimycin-A, cisplatin, or etoposide. Antimycin-A interrupts the electron transport chain, inhibits production of ATP, leads to production of superoxide and is known to be toxic to renal tubular epithelial cells (5). Treatment of hRPTCs with 31.25µM induced a reduction of the total fraction of cells in G₀/G₁ and increased cells in G₂/M phase. Pre-treatment of hRPTCs with PD 0332991 increased the proportion of cells in the G₀/G₁ phase of the cell cycle in a dose dependent manner, while decreasing cells traversing through the G₂/M phase (Figure 1B). Cisplatin is a cancer chemotherapeutic agent known to cause renal tubule cell damage and death *in vitro* and *in vivo* (47, 54). hRPTC treated with 25µM cisplatin and DMSO (vehicle for PD 0332991) displayed a stark reduction in the percentage of cells in the G₀/G₁ and an increase in cells at <2N, a sign of cell damage. Pre-treatment with PD 0332991 increased the percentage of cisplatin treated cells in G₀/G₁ while decreasing the percentage of cells that were <2N and in S phase in a dose dependent manner (Figure 1C). The hRPTCs responded similarly to treatment with 2.5µM etoposide, a chemotherapeutic agent that induces
DNA damage and apoptosis in dividing cells (64). PD 0332991 pretreatment resulted in a dose-dependent increase in G<sub>0</sub>/G<sub>1</sub> cell cycle arrest while decreasing the percentage of cells in S and G<sub>2</sub>/M phases of the cell cycle in cells exposed to etoposide (Figure 1D). Overall, these data indicate that PD 0332991 potently induces cell cycle arrest in cultured human and mouse renal tubule cells during baseline conditions and following treatment with either antimycin-A, cisplatin or etoposide in hRPTCs in vitro.

**PD 0332991 increases epithelial cell viability and prevents DNA damage induced by cytotoxic compounds**

Next we investigated whether treatment of hRPTC with PD 0332991 prior to exposure of etoposide would increase cell viability. As expected, 2.5µM etoposide treatment caused a substantial reduction in hRPTC cell number (CellTiter-Glo assay) (Figure 2A). However, PD 0332991 dose-dependently increased cell number at concentrations up to 100nM. Concentrations above 300nM caused a reduction in cell number in this assay, reflecting an increasing fraction of cycle-arrested cells with consequent reduction in total cell number, since we observed no apparent toxicity of PD 0332991 in this cell type (Figure 2A). Cisplatin causes DNA damage, triggers apoptosis of renal tubule cells in vitro and causes AKI in patients (39). Similar to results with etoposide, exposure of hRPTC to increasing doses of cisplatin (25, 50 and 100µM) reduced cell number compared to DMSO treated cells. Pretreatment of hRPTC with PD 0332991 prior to cisplatin exposure significantly increased cell viability in a dose-dependent manner (Figure 3A).

We also tested if PD 0332991 could rescue DNA damage in hRPTCs treated with cytotoxic compounds as indicated by expression of the DNA damage marker, γ-H2AX. hRPTCs treated with an increasing concentration of PD 0332991 did not result in substantial changes of γ-H2AX expression compared to cells treated with DMSO (Figure 2B). Etoposide is known to
induce DNA damage (64) and treatment of hRPTC with 2.5µM etoposide also caused a
dramatic increase in the percentage of hRPTC positive for the DNA damage marker γ-H2AX.

Pre-treatment with PD 0332991 significantly reduced the percentage of cells positive for γ-H2AX
as indicated by FACS analysis (Figure 2C). Cisplatin also increased γ-H2AX expression in
hRPTCs and pretreating cells with increasing doses of PD 0332991 reduced expression of the
DNA damage marker, although with a more subtle effect compared to the etoposide treated
cells (Figure 2D). Finally, PD 0332991 pre-treatment of cells exposed to antimycin-A blunted
increased expression of γ-H2AX (Figure 2E). Together, these results suggest that arresting
hRPTC in the G_1 phase of the cell cycle with PD 0332991 increases cell viability and mitigates
genotoxic injury.

**PD 0332991 prevents apoptosis of hRPTC exposed to cisplatin and antimycin-A**

Since cisplatin induces epithelial cell apoptosis, we next investigated whether PD
0332991 increased hRPTC viability after cisplatin exposure by inhibiting apoptosis. Renal cells
exposed to 25µM and 50µM cisplatin displayed a significant upregulation of caspase 3/7
activity, a readout of apoptotic activity, compared to cells treated with DMSO only. Pretreatment
with PD 0332991 reduced caspase 3/7 activity dose dependently at both cisplatin
concentrations (Figure 3B), suggesting that PD 0332991 increased hRPTC viability after
cisplatin exposure by inhibiting apoptosis. Increasing doses of antimycin-A (25, 55) also
activated caspase 3/7 in hRPTC. Pretreatment of hRPTC with PD 0332991 at doses of 300nM
and 1µM significantly reduced the level of caspase 3/7 activity resulting from antimycin-A
exposure (Figure 4A,B). There were differences in IC50 of PD 033291 between cisplatin,
etoposide and Antimycin A. We speculate that these reflect varying sensitivity of the cells to
these different toxicants, with more severe injuries inducing cell damage responses regardless
of cell cycle arrest. These data indicate that inducing pharmacological quiescence mitigates apoptosis induced by toxic drugs and common chemotherapeutic agents.

**PD 0332991 inhibits cell cycle activation after ischemia reperfusion injury in vivo**

We next asked whether PD 0332991 could induce renal epithelial cell cycle arrest in vivo. In contrast with cultured hRPTC that actively divide in vitro, tubule cells exhibit exceptionally low rates of cell cycle progression during homeostasis. After IRI, however, a rapid proliferative response results in the re-entry of up to 70% of tubular cells into the cell cycle 24 hours following injury (20). While it is known that D-type cyclins are expressed in kidney cells, it is not known if tubule epithelial cell proliferation is dependent on CDK4/6 (8, 65). To test this possibility, mice were treated with PD 0332991 during unilateral IRI using two different treatment schedules (Figure S2). In the first schedule mice were treated with a 150mg/kg dose of PD 0332991 (or sodium lactate vehicle) by oral gavage 1 hour prior to IRI. Mice were then injected intraperitoneally with a 100mg/kg dose of 5-bromo-2'-deoxyuridine (BrdU) 21 hours after IRI and sacrificed 3 hours later. Kidney sections were then stained with an anti-BrdU antibody and BrdU+ epithelial cells were quantified in uninjured contralateral (CLK) and IRI kidneys, in the vehicle treated and PD 0332991 treated groups. As expected, IRI significantly increased the number of BrdU+ epithelial cells per 20x field in the vehicle treated group (Figure 5A upper, and Figure 5B). However, injured kidneys from the mice treated with PD 0332991 had substantially reduced BrdU+ epithelial cells compared to injured kidneys from the vehicle treated group (Figure 5B).

In the second dosing schedule, mice were treated with 150mg/kg PD0332991 (or sodium lactate) by oral gavage 1 hour before IRI and 23 hours after IRI. BrdU was administered 21 hours and 45 hours after IRI and mice were sacrificed 48 hours after IRI. Following sacrifice, kidney tissue was sectioned and immunostained for BrdU detection and BrdU+ epithelial cells were quantified. While BrdU+ epithelial cells in injured kidney were increased overall in this
treatment protocol, kidneys in the IRI group that were treated with PD 0332991 again showed a significant decrease in BrdU+ cells (Figure 5A, lower, and Figure 5C).

We next sought to determine if the effects PD 0332991 on injured tubular epithelial cells were long-lasting or if the cell cycle deficit rebounded over time. To test this, mice were dosed with vehicle or PD 0332991 one hour prior to surgery and one group was given BrdU at 21 hours after surgery and sacrificed at 24 hours, while the second group was given BrdU at 69 hours after surgery and sacrificed at 72 hours. As expected, BrdU+ cells were significantly decreased at 24 hours in the PD 0332991 group compared to vehicle treated controls (Figure 5D, and Figure 5E). At 72 hours the number of BrdU+ cells in both groups were similar, indicating that the cells that had undergone pharmacological quiescence had re-entered the cell cycle at a rate similar to controls (Figure 5D, and 5E).

These results show that PD 0332991 can prevent S-phase entry in response to IRI in vivo, the responsive cells can recover from this cell cycle arrest, and that CDK4/6 are the critical CDKs regulating epithelial cell cycle after injury.

**PD 0332991 pre-treatment protects against ischemia reperfusion injury**

Preventing cell cycle traversal into S-phase via CDK4/6 inhibition has been shown to protect murine hematopoietic progenitors of the bone marrow following a high doses of total body irradiation or cytotoxic chemotherapy administration by reducing apoptosis (24, 50). We hypothesized that inducing PQ in kidney epithelial cells would afford protection in the bilateral ischemia reperfusion (BI-IRI) injury model. To test this hypothesis, mice were treated with 150mg/kg of PD 0332991 (or sodium lactate) via oral gavage 1 hour prior to bilateral BI-IRI. 24 hours after injury, mice treated with PD 0332991 showed a significant decrease in serum creatinine compared to mice treated with sodium lactate, 1.7 mg/dl ± 0.1 vs. 2.3 mg/dl ± 0.1 (Figure 6A). A statistically significant decrease in serum urea nitrogen concentration was also observed 24 hours after surgery in PD 0332991 treated mice compared to sodium lactate.
treated mice, 97.7 ± 4.3 vs. 116.9 ± 2.7 (Figure 6B). This treatment protocol did not reduce serum urea nitrogen levels in the PD 0332991 treated group 72 hours after surgery (Data not shown). Histological analysis of PAS stained kidney sections from mice treated with vehicle or PD 0332991 and analyzed 24 hours after IRI revealed a significant reduction in tubular injury score in mice pre-treated with PD 0332991 (Figure 6C,D). Overall, these data indicate that inducing pharmacological quiescence via CDK4/6 inhibition affords early protection in the setting of BI-IRI.

**PD 0332991 treatment reduces renal inflammation following ischemic kidney injury**

While serum biomarkers of renal injury were decreased following PD 0332991 treatment at 24 hours after injury we were interested in evidence of protection after cell cycling had resumed. To this end, mice were treated with PD 0332991 one hour prior to unilateral IRI and expression of pro-inflammatory markers were examined by quantitative PCR in kidneys on day 0, day 1 and day 3 after surgery. Interestingly, changes were not noted on day 1 after injury (cell cycle arrest) however, there were significant decreases in the inflammatory markers tumor necrosis factor-alpha (TNF) and monocyte chemoattractant protein-1 (MCP-1) three days after injury (Figure 7B, C). MCP-1 is known to attract monocytes and macrophages to injured kidney, is induced by TNF signaling, and has been thought of as a diagnostic marker and potential therapeutic target in chronic kidney diseases such as diabetic nephropathy (29, 60). Due to the role of MCP-1 in macrophage attraction we analyzed kidneys for expression of the macrophage marker F4/80. This marker was also significantly decreased on day 3 after surgery (Figure 7A). To confirm the reduction of macrophage infiltration in injured kidney tissue, sections of kidney of vehicle and PD 0332991 treated mice were taken 3 days after surgery. Sections were stained with F4/80 and the percentage of kidney area occupied by macrophages was quantified (Figure 8A, B). This analysis revealed a significant reduction in renal macrophage infiltration in the PD
0332991 treated group 3 days after injury, suggesting possible latent/long term beneficial effects of G₀/G₁ cell cycle arrest during acute kidney injury.

Discussion

In this study, we provide evidence that a small molecule inhibitor of CDK4/6 effectively promotes G₀/G₁ arrest in renal epithelial cells, protects these cells from DNA damage and apoptosis as a result of exposure to cytotoxic chemotherapeutic agents *in vitro*, and ameliorates kidney damage following AKI *in vivo*. This cell cycle inhibitor has been previously reported to induce G₁ phase cell cycle arrest in other cell types, however this is the first report describing the effects of this small molecule in primary renal epithelial cells and this is the first report showing CDK4/6 inhibition leading to epithelial cell arrest following AKI (24, 48, 50). CDK4/6 are essential in mediating cell cycle progression from G₁ to S phase and treatment of renal cells with PD 03332991 induced cell cycle arrest. Previous evidence showed that PD 0332991 protects cells from DNA damage induced by ionizing radiation *in vitro* and ameliorates myelosuppression induced by total body irradiation or carboplatin, leading to our hypothesis that induced cell cycle arrest would protect renal epithelial cells from genotoxic stresses. Our results therefore extend the potential uses of CDK4/6 inhibition from use in oncology into nephrology.

Cell cycle inhibitors have been investigated in the treatment of renal diseases, however overall results from clinical trials with these therapeutics have been largely unsuccessful (17, 43, 56). Previous clinical and preclinical studies had primarily tested CDK2 inhibitors in polycystic kidney disease, mesangial proliferative glomerulonephritis, crescentic glomerulonephritis and collapsing glomerulopathy (6, 12, 14, 44). The CDK2 inhibitor roscovitine had been used in a phase 2 trial to treat IgA nephropathy, however this trial was halted due to the development of severe adverse events (43). By contrast, CDK4/6 inhibitors are well tolerated in humans and PD
0332991 recently received “breakthrough therapy” designation by the FDA, intended to expedite development and review of potential new medicines that have the potential to substantially improve over currently available therapies. While CDK2 inhibitors have shown promising results in reducing cisplatin toxicity of renal epithelial cells \textit{in vitro} and in a cisplatin model of AKI \textit{in vivo} (45-47) existing CDK2 inhibitors have important off-target inhibitory activities, including cSrc, which is known to be activated in renal epithelial cells after IRI (15, 16, 59). They also inhibit other CDK's (e.g. CDK7 and CDK9) involved in transcription, DNA damage response and DNA metabolism (57). These compounds primarily induce G\textsubscript{2}/M block or an intra-S phase arrest, promoting apoptosis (45, 57). Accordingly, these relatively “dirty” CDK2 inhibitors fail to maintain kidney cells in G\textsubscript{0}/G\textsubscript{1}, which is well tolerated, but instead induce cell cycle states associated with checkpoint activation and apoptotic cell death.

In some ways, the induction of epithelial cell cycle arrest at the time of acute kidney injury seems a counterintuitive strategy to mitigate AKI based on the paradigm of kidney repair and regeneration. Following injury, it is historically held that surviving epithelial cells must dedifferentiate, proliferate, and then redifferentiate to restore tubule structure and function (58). Preventing epithelial cell proliferation would therefore appear to hinder a key step in renal repair. As has been noted by others (46, 53), however, transiently inducing cell cycle arrest in epithelial cells can prevent apoptosis and potentially avoid other maladaptive epithelial responses to injury, such as pro-fibrotic cytokine secretion (69). Additionally, it is possible that maintaining epithelial cells for an extended time in G\textsubscript{1} would allow for more time to repair DNA damage and thus prevent proliferation of cells with genotoxic insults. Our results suggest the possibility that epithelial cells may die through mitotic catastrophe after IRI, a form of delayed cell death linked to mitosis in the setting of premature or inappropriate entry of cells into mitosis (63). Importantly, CDK4/6 inhibition with reversible inhibitors such as PD 0332991 only transiently prevents S phase traversal, with cells re-entering the cell cycle 36-48 hours after treatment (Figure 5) (50). The transient nature of cell cycle arrest may be a critical determinant of the usefulness of PD
0332991 to prevent early cell death but still allow for normal repopulation of injured tubules through subsequent proliferation.

Intriguingly, the beneficial effects of CDK4/6 inhibition were seen after the re-initiation of cell cycle. Treatment with PD 0332991 significantly reduced the expression of the inflammatory markers TNF and MCP-1, 72 hours after surgery. Activation of this inflammatory signaling pathway is considered to be a critical factor in exacerbating renal disease, possibly through the recruitment of monocytes and macrophages to injured tissue. We were able to show that macrophage infiltration in injured kidney tissue was significantly decreased in PD 0332991 treated mice 72 hours after surgery. Previous work has shown that roscovitine treatment could resolve inflammation in multiple injury models through induction of neutrophil apoptosis (52). We did not observe PD 0332991 induced macrophage cell death, and further work is needed to determine the precise mechanism responsible for the reduction in macrophages following IRI. Given the known association between AKI and development of future CKD, it will be interesting to determine whether kidney protection through CDK4/6 inhibition will also reduce the risk of future CKD. Since most clinically important AKI is diagnosed after the injury, it will also be important to determine whether CDK4/6 inhibition administered after the onset of AKI is therapeutic.

In summary, our in vitro and in vivo results show that CDK4/6 inhibition protects renal cells from toxic insults. While cell cycle modulation as therapy for renal injury has shown promise previously, prior results have relied on toxic compounds that non-selectively inhibit CDK2 and related kinases, and such strategies have caused adverse events over the long term. In contrast, selective CDK4/6 inhibitors such as PD 0332991 induce a clean G1 arrest, and appear to be well tolerated in humans according to results from recent phase I clinical trials (10). CDK4/6 inhibition is therefore a promising strategy to protect against AKI.
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Disclosures
NES and KKW are co-founders of G-Zero Therapeutics, which holds license to PQ and related technology. JB, PR and JCS are employees of G-Zero Therapeutics. All authors are inventors on filed patents related to this work.

References


Figure Legends

**Figure 1.** Response of cultured human renal proximal tubule cells (hRPTC) to CDK4/6 inhibition at baseline and after exposure to cisplatin and etoposide. FACS analysis of cultured hRPTC indicates that PD 0332991 causes a dose dependent arrest in the G0/G1 phase of the cell cycle. (A-D) Cell cycle arrest occurs under baseline conditions and during exposure to 31.25µM antimycin-A, 25µM cisplatin and 2.5µM etoposide.

**Figure 2.** PD 0332991 significantly increases cell viability and decreases DNA damage of hRPTC exposed to etoposide. (A) Etoposide typically decreases cell viability, however PD 0332991 dose dependently increases cell viability of hRPTC that are exposed to this DNA damaging agent. (B) Treatment of hRPTC with PD 0332991 alone does not alter expression of γ-H2AX. (C) Treatment of hRPTC with etoposide increases γ-H2AX expression, while pretreatment with PD 0332991 prior to exposure to etoposide, dose dependently decreases the percentage of cells positive for the DNA damage marker γ-H2AX, as indicated by FACS. (D) Treatment of hRPTC with 25µM cisplatin increases γ-H2AX expression, and PD 0332991 reduces γ-H2AX expression. (E) Treating hRPTCs with antimycin-A increases γ-H2AX expression and PD 0332991 decreases γ-H2AX in cells treated with antimycin-A. Data analyzed by one-way ANOVA followed by Tukey’s multiple comparison test in (A). ***p < 0.0001, **p < 0.001.
Figure 3. Treatment of hRPTC with PD 0332991 significantly improves cell viability and decreases caspase 3/7 activity as a result of cisplatin exposure. (A) Doses of cisplatin at 25, 50, and 100µM result in decreased cell viability. Pretreating cells with PD 0332991 significantly increases hRPTC viability at all tested doses of cisplatin exposure. (B) PD 0332991 pretreatment significantly decreases caspase 3/7 activity in a dose dependent manner in hRPTC treated with 25 and 50µM cisplatin. Each data set analyzed by one-way ANOVA followed by Tukey’s multiple comparison test in A and B. ***p < 0.0001, **p < 0.001, *p < 0.01.

Figure 4. PD 0332991 significantly decreases caspase 3/7 activity in hRPTC treated with Antimycin A. (A, B) Marked caspase 3/7 activation occurs in hRPTC treated with 31.25µM Antimycin A. Treating cells with 300nM and 1µM PD 0332991 prior to Antimycin A exposure significantly decreases caspase 3/7 activity.

Figure 5. PD 0332991 attenuates tubular epithelial cell division after IRI and cell division can recover 3 days after injury. (A) Confocal micrographs of kidney treated with Vehicle or PD and sac’d 24 hours (upper) or 48 hours (lower) after IRI. (B) 24 hours after surgery BrdU+ tubular epithelial cells were significantly decreased in mice treated with PD one hour before surgery. (C) Mice treated with vehicle or PD 1 hour prior to surgery and 23 hours after surgery had significantly less BrdU incorporation in tubular epithelial cells when examined 48 hours after IRI surgery. (D) Representative images of BrdU+ cells at indicated timepoints after IRI in mice treated with Vehicle or PD 1 hour prior to surgery. (E) While PD significantly reduces BrdU+ cells one day after IRI, cells
incorporating BrdU rebound on day 3. In (B, C) N=5 in vehicle treated group and N=6 in PD treated group. in (E) N=4 in each group. Analyzed by two-way ANOVA and Bonferroni’s posttest. ***p < 0.001 and **p < 0.01.

Figure 6. Serum creatinine and urea nitrogen levels are significantly decreased 24 hours after bilateral ischemia reperfusion injury in mice treated with PD 0332991. (A) Serum creatinine levels increased to 2.27 ± 0.073 mg/dl in 8 vehicle treated mice 24 hours after bilateral ischemic reperfusion injury and 1.74 ± 0.143 mg/dl in 9 PD 0332991 treated mice. (B) Serum urea nitrogen levels in 6 vehicle treated mice increased to 116.916 ± 2.74 mg/dl and 6 PD 0332991 treated mice had significantly lower urea nitrogen levels measured at 97.71 ± 4.31 mg/dl, 24 hours after injury. (C) Tubular injury in mice that received vehicle or PD033291. In vehicle treated mice, outer medullary tubules are characterized by necrosis (asterisks), hyaline casts and evidence of epithelial damage in the majority of tubules. In PD033291-treated mouse kidney, damage was still present (asterisks), but a substantial number of less damaged tubules were evident (arrowheads) where only mild tubular dilation is visible, without necrosis or cast formation. (D) Quantification of tubular injury score in vehicle (n = 6) or PD033291 (n = 7) groups. Data analyzed by two-way ANOVA followed by Bonferroni’s post-test (A,B) or by one-way ANOVA (D). *** p < 0.001, * p < 0.05.

Figure 7. One dose of PD 0332991 significantly reduces markers of inflammation 3 days after ischemic reperfusion injury. (A-C) Expression of macrophage marker F4/80 and inflammatory genes TNF and MCP-1 is significantly decreased on day 3 after IRI.
One dose of PD 0332991 was given one hour prior to surgery. N = 4 for each group.

Data analyzed by two-way ANOVA followed by Bonferroni’s post test. ** p < 0.01, *** p < 0.001.

Figure 8. Macrophage infiltration is significantly decreased on day 3 of IRI following treatment with PD 0332991. (A) Confocal micrographs of control kidney and injured kidney on day 3 of IRI treated with Vehicle or PD 0332991 1 hour before surgery. Macrophage marker F4/80 in green. (B) Percentage of medullary kidney positive for F4/80 is significantly decreased in subjects treated with PD 0332991. Analyzed by two-way ANOVA followed by bonferroni’s posttest. ***p < 0.001.
Figure 1

Fraction of Total Population

A. <2N  G0-G1  G2-M  S
B. +31.25µM AA
C. +25µM Cisplatin
D. +2.5µM Etoposide
Figure 4

A. 

Caspase 3/7 Activity (arbitrary units)

<table>
<thead>
<tr>
<th>DMSO</th>
<th>PD0332991 300nM</th>
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</thead>
<tbody>
<tr>
<td>3.9μM AA</td>
<td>7.81μM AA</td>
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</table>

B. 

Caspase 3/7 Activity (arbitrary units)

<table>
<thead>
<tr>
<th>DMSO</th>
<th>PD0332991 1μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.9μM AA</td>
<td>7.81μM AA</td>
</tr>
</tbody>
</table>
Figure 5

A. 24 hours after injury

B. 1x Vehicle  1x PD0332991

C. 2x Vehicle  2x PD0332991

D. Day 0  Day 1  Day 3

E. Vehicle  PD0332991 15mg/kg

BrdU+ Epithelial Cells per HPF

Day 0  Day 1  Day 3
Figure 8

A. Laminin/F4/80/DAPI

Vehicle  PD 0332991

CLK

Day 3 IRI

B. Percentage F4/80+ Medulla

Vehicle

[^3]

CLK

Day 3 IRI