CDK4/6 inhibition induces epithelial cell cycle arrest and ameliorates acute kidney injury

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1Renal Division, Brigham and Women’s Hospital, Boston, Massachusetts; 2Harvard Medical School, Boston, Massachusetts; 3Harvard Stem Cell Institute, Cambridge, Massachusetts; 4The Dana Farber Cancer Institute, Boston, Massachusetts; 5Department of Genetics, The University of North Carolina School of Medicine, Chapel Hill, North Carolina; 6Department of Medicine, The University of North Carolina School of Medicine, Chapel Hill, North Carolina; 7The Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina; and 8GI Therapeutics, Chapel Hill, North Carolina

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DiRocco DP, Bisi J, Roberts P, Strum J, Wong KK, Sharpless N, Humphreys BD. CDK4/6 inhibition induces epithelial cell cycle arrest and ameliorates acute kidney injury. Am J Physiol Renal Physiol 306: F379–F388, 2014. First published December 11, 2013; doi:10.1152/ajprenal.00475.2013.—Acute kidney injury (AKI) is a widely accepted mouse model of human AKI (69). Within the first 24 h following injury, tubular cells undergo apoptotic and necrotic cell death. In response, the surviving, normally quiescent proximal tubule epithelial cells rapidly proliferate, and ~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 postischemic, inflammatory environment (46). A very similar sequence of events can be induced by nephrotoxins 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 to traverse G1 (24, 48, 49).

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 (CKD) 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 leads to high cumulative cost of care, with annual estimates of AKI-associated costs at ~$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 h following injury, tubular cells undergo apoptotic and necrotic cell death. In response, the surviving, normally quiescent proximal tubule epithelial cells rapidly proliferate, and ~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 postischemic, inflammatory environment (46). A very similar sequence of events can be induced by nephrotoxins such as cisplatin (37, 47, 54).

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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
p21<sup>CIP</sup>, 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 p21<sup>CIP</sup> CDK inhibitor is induced in response to DNA damage and other stresses, and mice lacking p21<sup>CIP</sup> exhibit exacerbated kidney ischemic injury, consistent with the hypothesis that cell cycle inhibition postinsult protects against AKI (36, 42). Importantly, small molecule inhibition of CDK2 does not induce a "clean" arrest in the G<sub>1</sub> phase of the cell cycle, but instead it leads to a G<sub>S</sub>/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, G<sub>S</sub>/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). Furthermore, the currently available small molecule inhibitors of CDK2 are known to nonspecifically 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 G<sub>1</sub> 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 G<sub>1</sub> arrest in a variety of Rb-positive cell lines (11, 24, 48, 62). Previous work showed that PD 0332991 can induce G<sub>1</sub> 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 G<sub>1</sub> arrest in hematopoietic progenitor cells affords significant protection from myelosuppression following treatment with the chemotherapeutic agent carboplatin (50). Ionizing radiation and DNA intercalating/duct-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 G<sub>1</sub> to S phase in the setting of DNA damage is particularly damaging while early G<sub>1</sub> 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 before mitosis. Here, we tested the hypothesis that inhibiting S phase traversal (PQ) 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 IRI in vivo.

**METHODS**

**Compounds and dosing.** PD 0332991 was synthesized by the Center for Integrative Chemical Biology and Drug Discovery at the University of North Carolina (Chapel Hill, NC). Cisplatin (Teva, Irvine, CA), etoposide (Sigma, St. Louis, MO), and antimycin A (Sigma) were obtained from their respective manufacturers and handled according to the manufacturer’s recommendations. PD 0332991 was dissolved in 50 mM sodium lactate buffer (pH 4.0) to a final concentration of 15 mg/mL. Mice were treated with a 150-mg/kg dose of PD 0332991, or vehicle, 1 h before ischemia-reperfusion surgery by oral gavage. 5-Bromo-2′-deoxyuridine (BrdU) was dissolved in sodium chloride to 10 mg/ml and mice were given intraperitoneal injections at a dose of 100 mg/kg.

**Cell line.** Primary human renal proximal tubule epithelial cells (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% CO<sub>2</sub> and cultured in renal epithelial cell basal medium (ATCC) supplemented with accompanying renal epithelial cell growth kit (ATCC) following the 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% CO<sub>2</sub> for 24 h. Cells were then incubated with PD 0332991 or dimethyl sulfoxide vehicle control for an additional 24 h. hRPTC cells were then harvested and fixed in ice-cold methanol, and stored at −20°C. Cell pellets were spun at 1,200 rpm for 5 min, aspirated, and washed in 3 ml 1× PBS-CMF (Cellogl, Mediatech, Manassas, VA). Samples were resuspended, as resuspended in PBS-CMF/1% BSA Fraction V (Fisher, Fair Lawn, NJ) containing (100 μg/ml) propidium iodide (Sigma) and (150 μg/ml) DNase-free RNase A (Sigma). 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% CO<sub>2</sub> for 24 h. Cells were then incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> with PD 0332991 or dimethyl sulfoxide (Sigma) vehicle control for 16 h. Cells were then treated with chemotherapy (cisplatin or etoposide) for 8 h, followed by aspiration of medium and replenishment with fresh media. Plates were incubated for 7 days, removed from the 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% CO<sub>2</sub> for 24 h. Cells were then incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> with PD 0332991 or dimethyl sulfoxide (Sigma) vehicle control for 16 h. Cells were then treated with chemotherapy (cisplatin or etoposide) for 8 h. hRPT cells were then fixed and stained using the H2A.X Phosphor-Flow 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) and FlowJo analysis software (Version 7.2.2, TreeStar).

For the caspase 3/7 assay, 750 hRPTC cells were plated per well in 96-well white wall clear bottom plates (Costar). Cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. Cells were then incubated with PD 0332991 or dimethyl sulfoxide vehicle control for 16 h. Cells were then treated with chemotherapy (cisplatin or etoposide) or antimycin A for 24 h. Antimycin A was used in normal glucose medium, and 2-deoxyglucose was not added. Caspase 3/7 activation was measured directly in the 96-well plates using the Caspase-Glo 3/7 Assay System (Promega) by following the manufacturer’s instructions.

**IRI.** 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. The animal protocol describing these experiments was reviewed and approved by the Harvard University Institutional Animal Care and Use Committee. For bilateral IRI, 8- wk-old male mice were anesthetized with pentobarbital sodium (60 mg/kg body wt ip) before surgery. Body temperatures were controlled at 36.5–37.5°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.
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 (Fig. 1, A–D). Mouse primary proximal tubule epithelial cells were also isolated and cultured to determine whether 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 (Fig. S1, A and B; the online version of this article contains supplemental data). Next, we tested whether 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 31.25 μM antimycin A induced a reduction of the total fraction of cells in G0/G1 and increased cells in G2/M phase. Pretreatment 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

RESULTS

PD 0332991 arrests hRPTC in G0/G1. To determine whether kidney epithelial cells were sensitive to CDK4/6 inhibition, cultured hRPTC were treated with increasing doses of PD 0332991 and cell cycle stage was measured. PD 0332991

with nontraumatic microaneurysm clamps (Roboz, Rockville, MD). Clamped kidneys were placed back into the peritoneal cavity for 28 min, 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 min. The tubular injury score was assessed exactly as described (21).

Serum creatinine and blood urea nitrogen measurements. Twenty-four hours after surgery, tail vein blood was collected in heparinized micro-hematocrit capillary tubes (Fisher, cat. no. 22-362-566) and centrifuged for 15 min at 5,000 rpm. The supernatant was retained as serum. Serum creatinine was measured by the Beckman Creatinine Analyzer 2, which utilizes a 10-mM picric acid solution mixed with a buffer containing 10 mM sodium borate, 240 mM sodium hydroxide, and 10 mM SDS. Serum creatinine is measured against a creatinine standard and recorded as milligrams per deciliter. Blood urea nitrogen (BUN) measurements were made using 1 μl of serum combined with 200 μl of Infinity Urea solution (Fisher, cat. no. TR12421). Six readings were made on a spectrophotometer at 340-nm wavelength at 30-s intervals and compared with a standard solution at 30 mg/dl. Serum creatinine and BUN measurements in Fig. 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 min. 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 1× PBS, blocked in 10% normal goat serum (Vector Labs), and incubated with an anti-BrdU antibody made in rat (Abcam, BU1/751/11003).

Fig. 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 (PD) 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 31.25 μM antimycin A, 25 μM cisplatin, and 2.5 μM etoposide.

Fig. 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 before 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 25 μM cisplatin increases γ-H2AX expression, and PD 0332991 reduces γ-H2AX expression. E: treating hRPTC with antimycin A (AA) increases γ-H2AX expression and PD 0332991 decreases γ-H2AX in cells treated with AA. Data analyzed by 1-way ANOVA followed by Tukey’s multiple comparison test in A. ***P < 0.0001, **p < 0.001.
cells traversing through the G2/M phase (Fig. 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 25 μM 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. Pretreatment 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 (Fig. 1C). The hRPTCs responded similarly to treatment with 2.5 μM etoposide, a chemotherapeutic agent that induces DNA damage and apoptosis in dividing cells (64). PD 0332991 pretreatment resulted in a dose-dependent increase in G0/G1 cell cycle arrest while decreasing the percentage of cells in S and G2/M phases of the cell cycle in cells exposed to etoposide (Fig. 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 before exposure of etoposide would increase cell viability. As expected, 2.5 μM etoposide treatment caused a substantial reduction in hRPTC cell number (CellTiter-Glo assay) (Fig. 2A). However, PD 0332991 dose-dependently increased cell number at concentrations up to 100 nM. Concentrations above 300 nM 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 (Fig. 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 100 μM) reduced cell number compared with DMSO-treated cells. Pretreatment of hRPTC with PD 0332991 before cisplatin exposure significantly increased cell viability in a dose-dependent manner (Fig. 3A).

We also tested whether 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 with cells treated with DMSO (Fig. 2B). Etoposide is known to induce DNA damage (64) and treatment of hRPTC with 2.5 μM etoposide also caused a dramatic increase in the percentage of hRPTC positive for the DNA damage marker γ-H2AX. Pretreatment 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 100 μM 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 50 μM cisplatin. Each data set analyzed by 1-way ANOVA followed by Tukey’s multiple comparison test in A and B. ***P < 0.0001, **P < 0.001, *P < 0.01.
significantly reduced the percentage of cells positive for γ-H2AX as indicated by FACS analysis (Fig. 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 with the etoposide-treated cells (Fig. 2D). Finally, PD 0332991 pretreatment of cells exposed to antimycin A blunted increased expression of γ-H2AX (Fig. 2E). Together, these results suggest that arresting hRPTC in the G1 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 25 and 50 μM cisplatin displayed a significant upregulation of caspase 3/7 activity, a readout of apoptotic activity, compared with cells treated with DMSO only. Pretreatment with PD 0332991 reduced caspase 3/7 activity dose-dependently at both cisplatin concentrations (Fig. 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 300 nM and 1 μM significantly reduced the level of caspase 3/7 activity resulting from antimycin A exposure (Fig. 4, A and B). There were differences in IC50 of PD 033291 between cisplatin, etoposide, and antimycin A. We speculate that these reflect a 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 PQ mitigates apoptosis induced by toxic drugs and common chemotherapeutic agents.

**PD 0332991 inhibits cell cycle activation after IRI 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 reentry of up to 70% of tubular cells into the cell cycle 24 h following injury (20). While it is known that D-type cyclins are expressed in kidney cells, it is not known whether 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 (Fig. S2). In the first schedule, mice were treated with a 150-mg/kg dose of PD 0332991 (or sodium lactate vehicle) by oral gavage 1 h before IRI. Mice were then injected intraperitoneally with a 100-mg/kg dose of BrdU 21 h after IRI and killed 3 h 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 20 × field in the vehicle-treated group (Fig. 5A, top, and B). However, injured kidneys from the mice treated with PD 0332991 had substantially reduced BrdU + epithelial cells compared with injured kidneys from the vehicle-treated group (Fig. 5B).

In the second dosing schedule, mice were treated with 150 mg/kg PD 0332991 (or sodium lactate) by oral gavage 1 h before IRI and 23 h after IRI. BrdU was administered 21 and 45 h after IRI and mice were killed 48 h after IRI. Following death, kidney tissue was sectioned and immunostained for BrdU detection and BrdU + epithelial cells were quantified. While BrdU + epithelial cells in the 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 (Fig. 5A, bottom, and C).

We next sought to determine whether the effects PD 0332991 on injured tubular epithelial cells were long-lasting or whether the cell cycle deficit rebounded over time. To test this, mice were dosed with vehicle or PD 0332991 1 h before surgery and one group was given BrdU at 21 h after surgery and killed at 24 h, while the second group was given BrdU at 69 h after surgery and killed at 72 h. As expected, BrdU + cells were significantly decreased at 24 h in the PD 0332991 group compared with vehicle-treated controls (Fig. 5, D and E). At 72 h the number of BrdU + cells in both groups was similar, indicating that the cells that had undergone PQ had reentered the cell cycle at a rate similar to controls (Fig. 5, D and E).

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 pretreatment protects against IRI.** Preventing cell cycle traversal into S phase via CDK4/6 inhibition has been shown to protect murine hematopoietic progenitors of the bone marrow following 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 Bi-IRI injury model. To test this hypothesis, mice were treated with 150 mg/kg of PD 0332991 (or sodium lactate) via oral gavage 1 h before bilateral BI-IRI. Twenty-four hours after injury, mice treated with PD 0332991 showed a significant decrease in serum creatinine compared with mice treated with sodium lactate, 1.7 ± 0.1 vs. 2.3 ± 0.1 mg/dl (Fig. 6A). A statistically significant decrease in serum urea nitrogen concentration was also observed 24 h after surgery in PD 0332991-treated mice compared with sodium lactate-treated mice, 97.7 ± 4.3 vs. 116.9 ± 2.7 (Fig. 6B). This treatment protocol did not reduce serum urea nitrogen levels in...
the PD 0332991-treated group 72 h after surgery (data not shown). Histological analysis of periodic acid Schiff-stained kidney sections from mice treated with vehicle or PD 0332991 and analyzed 24 h after IRI revealed a significant reduction in tubular injury score in mice pretreated with PD 0332991 (Fig. 6, C and D). Overall, these data indicate that inducing PQ 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 h after injury, we were interested in evidence of protection after cell cycling had resumed. To this end, mice were treated with PD 0332991 1 h before unilateral IRI and expression of proinflammatory markers was 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-α (TNF) and monocyte chemoattractant protein-1 (MCP-1) 3 days after injury (Fig. 7, A and B). MCP-1 is known to attract monocytes and macrophages to the injured kidney, is induced by TNF signaling, and has been thought of as a diagnostic marker and potential therapeutic target in CKDs 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 injury (Fig. 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 (Fig. 8, A and 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 AKI.

**DISCUSSION**

In this study, we provide evidence that a small molecule inhibitor of CDK4/6 effectively promotes transient 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 0332991-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 currently available therapies. While CDK2 inhibitors have shown promising results in reducing cisplatin toxicity of renal epithelial cells in vitro and in a cisplatin model of AKI 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 CDKs (e.g., CDK7 and CDK9) involved in transcription, DNA damage response, and DNA metabolism (57). These compounds primarily induce G2/M block or an intra-S phase arrest, promoting apoptosis (45, 57). Accord-ingly, these relatively “dirty” CDK2 inhibitors fail to maintain kidney cells in G0/G1, which is well-tolerated, but instead they 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 AKI 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, transitively inducing cell cycle arrest in epithelial cells can prevent apoptosis and potentially avoid other maladaptive epithelial responses to injury, such as profibrotic cytokine secretion (69). Additionally, it is possible that maintaining epithelial cells for an extended time in G1 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 reentering the cell cycle 36–48 h after treatment (Fig. 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 reinitiation of cell cycle. Treatment with PD 0332991 significantly reduced the expression of the inflammatory markers TNF and MCP-1, 72 h 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 h after surgery. Previous work showed 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 nonselectively 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 G1 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.

AUTHOR CONTRIBUTIONS
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REFERENCES