CDK inhibitors R-roscovitine and S-CR8 effectively block renal and hepatic cystogenesis in an orthologous model of ADPKD

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Autosomal dominant polycystic kidney disease (ADPKD) and other forms of PKD are associated with dysregulated cell cycle and proliferation. Although no effective therapy for the treatment of PKD is currently available, possible mechanism-based approaches are beginning to emerge. A therapeutic intervention targeting aberrant cilia-cell cycle connection using CDK-inhibitor R-roscovitine showed effective arrest of PKD in *jck* and *cpk* models that are not orthologous to human ADPKD. To evaluate whether CDK inhibition approach will translate into efficacy in an orthologous model of ADPKD, we tested R-roscovitine and its derivative S-CR8 in a model with a conditionally inactivated *Pkd1* gene (*Pkd1* cKO). Similar to ADPKD, *Pkd1* cKO mice developed renal and hepatic cysts. Treatment of *Pkd1* cKO mice with R-roscovitine and its more potent and selective analog S-CR8 significantly reduced renal and hepatic cystogenesis and attenuated kidney function decline. Mechanism of action studies demonstrated effective blockade of cell cycle and proliferation and reduction of apoptosis. Together, these data validate CDK inhibition as a novel and effective approach for the treatment of ADPKD.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most common human-inherited disorder with an estimated incidence of 1:400–1:1,000. ADPKD is mainly characterized by formation and progressive enlargement of fluid-filled renal cysts that ultimately lead to end stage renal disease (reviewed in refs. 1–3). Cysts often form in liver, pancreas and other organs.[4] The disease is caused by mutations in either PKD1 or PKD2 genes, encoding the proteins polycystin-1 and polycystin-2, responsible for 85% and 15% of ADPKD cases, respectively.[4-6] Presently, there is no effective treatment for ADPKD, although significant progress has been made in recent years in identifying potential treatments.[7,8]

Cystic growth is associated with abnormalities in multiple cellular processes such as basement membrane thickening, increased proliferation and apoptosis, loss of cellular differentiation and polarity and acquisition of secretory phenotype by cyst-lining epithelia.[4,9-12] Several dysregulated molecular pathways that contribute to these cellular abnormalities were identified and exploited for targets of therapeutic intervention. Multiple compounds targeting cAMP accumulation, proliferation, fluid secretion and mTOR pathway have been tested in preclinical studies and entered clinical trials.[13-20] Unfortunately, completed trials with mTOR inhibitors were not successful, underscoring the importance of developing alternative therapeutic targets.

New insights into molecular mechanisms of cystogenesis transpired from the observation that polycystins and other proteins responsible for multiple forms of PKD localize to the primary cilium/centrosome.[21-23] The cilium is resorbed during cell division, and centrosomes can serve as organizers of the mitotic spindle. Disruption of proteins associated with cilia or centrosomes could lead to alterations in the cell cycle and proliferation as seen in cystic disease. It has been shown that polycystins can directly affect cell cycle progression and centrosome duplication.[24] Polycystin-1 was shown to directly regulate the cell cycle by inhibiting CDK2 activity through upregulation of p21^{waf1}, arresting cells in G$_0$/G$_1$ phase and controlling terminal
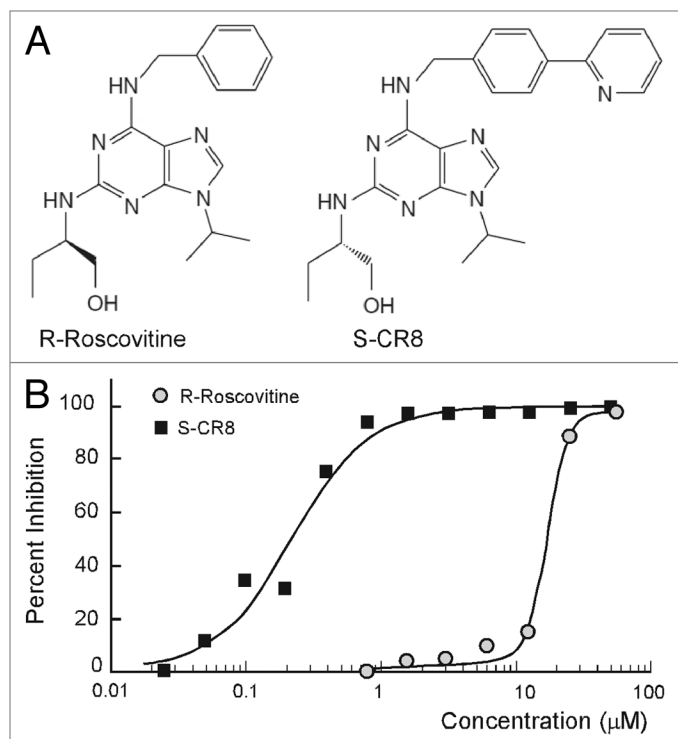
Figure 1. Comparative analysis of inhibitory activities of CDK inhibitors S-CR8 and R-roscovitine on cystogenesis in vitro. (**A**) Chemical structures of R-roscovitine and its derivative, S-CR8. (**B**) In vitro inhibition of cystic growth in MDCK 3D collagen-based assay. Values were measured in quadruplets in two independent experiments.

Results

CDK inhibitor S-CR8 potently inhibits cystogenesis in vitro. To improve drug-like properties of R-roscovitine (metabolic stability, potency and selectivity), extensive medicinal chemistry studies identified a new and improved analog S-CR8, shown in **Figure 1A**.[33,34] We have used a standard assay of MDCK cystogenesis in vitro to assess potency of S-CR8 as described previously.[29,35] R-roscovitine was tested in parallel for comparison. MDCK cysts were grown in 96-well plates containing collagen gel with FBS-containing media for 4 d. Increasing concentrations of compounds were added to cysts and incubated for additional 4 d. Percent of inhibition of cystogenesis by each compound was measured by standard Alamar Blue assay (**Fig. 1B**) and confirmed by visual observation of cultured cysts under light microscope (not shown). The assay showed that both R-roscovitine and S-CR8 compounds reduce cyst formation in vitro in a dose-dependent manner with an IC_{50} of 16 μM and 0.2 μM, respectively. These data indicate that S-CR8 is approximately 80-fold more potent than R-roscovitine in cellular assay. This observation is in agreement with previously published data suggesting greater antitumor potency for S-CR8 compared with R-roscovitine in multiple cell lines (100-fold on the average of more than 65 cell lines).[33]

R-roscovitine and S-CR8 effectively inhibit renal cystic disease progression in _Pkd1_-conditional knockout mice. To determine whether CDK inhibition is an effective approach for the treatment of ADPKD, we sought to demonstrate the effect of R-roscovitine in an orthologous mouse model. This model has a germline _Pkd1_-null allele (_Pkd1_[tm1Gzbd]), a conditional knockout allele with lox sites flanking exons 21–23 (_Pkd1_[tm1Gztn]) and a tamoxifen-inducible _Cre_-gene.[32] As shown previously, conditional inactivation of the _Pkd1_ gene at day 5 results in a rapid onset PKD that is gender-independent.[32] Cysts in the liver are also observed in this model. Similar to other models with conditionally inactivated _Pkd1_ gene, the majority of cysts originate from distal nephron segments and collecting ducts.[17] In the current study, cystogenesis was induced with tamoxifen at postnatal day 5. Animals received daily injections of either R-roscovitine (100 mg/kg IP, once a day) or vehicle control from day 7–33 (**Fig. 2A**). The R-roscovitine-treated group showed a significant inhibition of PKD, evident by a decrease in kidney to body weight ratio, cystic volume and blood urea nitrogen (BUN) (**Fig. 2B and Table 1**). Effective reduction of cystic tissue in a representative R-roscovitine treated kidney is illustrated in **Figure 2C**.

In parallel, we also investigated the in vivo effect of S-CR8, a more potent and selective CDK inhibitor with a similar CDK inhibitory profile. Administration of S-CR8 (2.5 mg/kg IP, twice daily) was highly effective in inhibiting PKD progression in the conditional _Pkd1_-knockout model (**Fig. 2A–C**). Both compounds were equally well-tolerated in the course of treatment. As expected, S-CR8 was more dose-potent and showed greater PKD inhibition compared with R-roscovitine. Overall, these data support the conclusion that CDK inhibitors R-roscovitine and S-CR8 are effective in attenuating the progression of renal cystogenesis and improving kidney function in an orthologous _Pkd1_-linked mouse model of ADPKD.

differentiation of tubular epithelial cells.[25] Polycystin-2 is capable of binding helix-loop-helix protein Id2 and preventing its translocation to the nucleus, thus blocking cell cycle progression.[26] The translocation of Id2 in cells with mutated polycystins is linked to downregulation of p21 leading to increase of CDK2 activity and cell cycle progression.

We have shown previously that pharmacological inhibition of cell cycle progression with the cyclin-dependent kinase (CDK) inhibitor R-roscovitine effectively attenuates cystogenesis in _jck_ and _cpk_ models of PKD.[27-30] Mechanistic studies demonstrated that roscovitine inhibited cystogenesis through cell cycle arrest, transcriptional regulation and inhibition of apoptosis. Importantly, roscovitine treatment suppressed cAMP and aquaporin 2 in the cystic kidneys, suggesting that CDK inhibition targets the most proximal step in cystogenesis.[31]

To further validate CDK inhibition as an approach to treat ADPKD, preclinical efficacy needs to be established in an orthologous model. The goals of this study were to confirm efficacy of R-roscovitine in an orthologous mouse model of ADPKD with a conditionally inactivated _Pkd1_ gene (_Pkd1_ cKO)[32] and to assess the efficacy of the second generation analog of roscovitine, S-CR8, a more potent and selective CDK inhibitor.[33] We demonstrate effective inhibition of both renal and hepatic cystogenesis with R-roscovitine and S-CR8 compounds. Mode of action studies demonstrate that both compounds act through blockade of cell cycle and proliferation and attenuation of apoptosis.

Assessment of CDK inhibitors effect on hepatic cystogenesis. Because our *Pkd1* cKO mice develop liver cysts in addition to PKD, we next examined the effect of CDK inhibition on hepatic cystogenesis. In contrast to kidney cystic disease, hepatic cystogenesis appears to be much less severe under conditions we used to induce *Pkd1* gene deletion (see **Fig. 2A**). At postnatal day 33, the sporadic surface cysts are usually visible in the vehicle-treated group with a cystic area accounting for less than 5% of the total hepatic area (**Table 1**). To assess the percentage of hepatic cysts, liver sections of animals treated with either vehicle or CDK inhibitors R-roscovitine and S-CR8 were H&E stained, scanned with light microscopy and digitized followed by Metamorph analysis. Percentage of cystic liver tissue in mice treated with R-roscovitine and S-CR8 was significantly decreased as compared with the vehicle-treated group (**Fig. 3A and B**; **Table 1**). Similar to the effects on kidney cysts, S-CR8 was more effective in reducing liver cyst growth relative to R-roscovitine.

CDK inhibitors target dysregulated cell cycle and apoptosis in cystic kidneys. To elucidate the mechanisms by which CDK inhibitors affect PKD in an orthologous mouse model, we probed key pathways of cystogenesis using western blotting of kidney lysates from *Pkd1* cKO mice treated with CDK inhibitors R-roscovitine and S-CR8. First, we examined the effects of CDK inhibitors on cell cycle progression. As shown in **Figure 4A**, Rb phosphorylation (p-Rb) was significantly increased in *Pkd1* cKO kidneys (indicating cell cycle activation) and decreased in samples treated with either R-roscovitine or S-CR8 (suggesting G_1/S cell cycle inhibition). We also observed significant upregulation of cyclinD3 (cycD3) expression and decreased cyclinD1 phosphorylation at Thr286 (p-cycD1) in vehicle-treated cystic kidneys, indicative of cell cycle activation and reversal of these patterns in samples treated with CDK inhibitors. Activation of Erk1/2, known to regulate cyclinD1, was effectively inhibited in treated samples. PCNA levels were decreased in treated samples, indicating inhibition of proliferation. Overall, S-CR8 showed more effective cell cycle blockade compared with R-roscovitine (**Fig. 4A**).

Next, we tested the effect of CDK inhibitors on apoptosis in treated kidneys. As shown in **Figure 4B**, we detected increased apoptosis in *Pkd1* cKO kidneys evident by induction of caspase 2, an initiator of the mitochondrial apoptotic pathway; caspase 8, an activator of extrinsic apoptotic pathway; as well as decreased Bcl-X$_L$ expression and increase in ApaF1, as compared with wild-type (WT) kidneys. Interestingly, aberrant expression of apoptotic markers was normalized in kidneys treated with R-roscovitine and to a lesser extent in kidneys treated with S-CR8.

We also examined the effect of R-roscovitine and S-CR8 on the level of RNA polymerase II-dependent transcription, as shown in **Figure 4C**. Both compounds decreased CDK7 expression levels and effectively inhibited RNA pol II phosphorylation in treated kidneys.

Discussion

A significant progress in understanding the molecular mechanisms responsible for the development and progression of
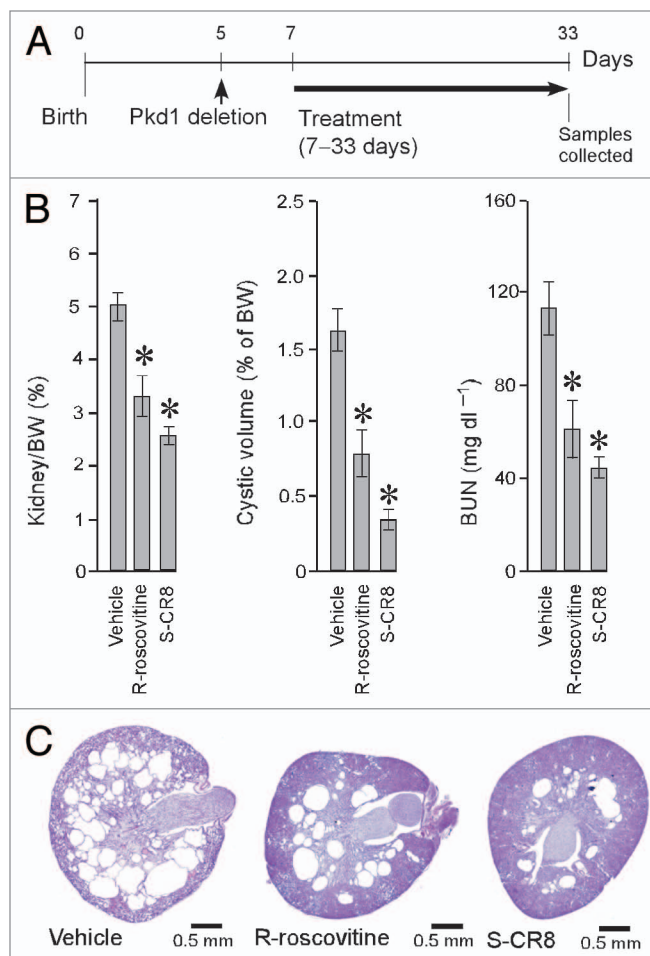

Figure 2. CDK inhibitors R-roscovitine and S-CR8 inhibit renal cystogenesis in *Pkd1*-conditional knockout mice. (**A**) Time frame of induction of *Pkd1* deletion with tamoxifen and schedule of treatment with R-roscovitine and S-CR8. (**B**) Quantitative analysis of effect of R-roscovitine and S-CR8 on cystogenesis in kidney measured as kidney/body weight (BW) ratio, cystic volume and blood urea nitrogen (BUN); * p < 0.05 compared with vehicle control. Error bars indicate SEM; (**C**) Representative kidney sections (H&E staining) from treated mice and vehicle control suggest preservation of kidney parenchyma in animals treated with CDK inhibitors as compared with vehicle-treated group.

ADPKD has been made in recent years, providing the foundation for translation of preclinical therapeutic approaches into clinical development.[7,36] Of particular interest is a recent discovery that proteins disrupted in ADPKD and other forms of cystic diseases map to a common site: primary cilia.[21-23] There is a direct link between cilia, centrosomes and cell cycle dysregulation in PKD as reviewed in refs. 37 and 38. Through use of the CDK inhibitor R-roscovitine, we have recently shown that treatment of *jck* and *cpk* mice with slowly progressive and aggressive forms of PKD, respectively, resulted in a striking inhibition of cystic disease and improvement of renal function.[29] Because these models do not carry mutations in *Pkd1* or *Pkd2* genes that are responsible for human ADPKD, therapeutic benefits of targeting the dysregulated cell cycle need to be validated in orthologous models. In fact, it has been previously shown that polycystins directly arrest

Table 1. Anti-cystic effect of CDK inhibitors R-roscovitine and S-CR8 in *Pkd1* cKO mice

No	No of animals	K/BW ratio (%)			Kidney cystic volume (%BW)			BUN (mg dl⁻¹)			Liver cystic tissue (%)		
1	Vehicle	27	4.98	±	0.26	1.63	±	0.14	113	±	11	4.62	±	0.53
2	R-roscovitine	14	3.29	±	0.39*	0.80	±	0.16*	61	±	12*	2.25	±	0.25*
3	S-CR8	19	2.55	±	0.19*	0.34	±	0.07*	45	±	4*	1.48	±	0.19*

* indicates p < 0.05 for reduction in drug-treated vs. vehicle-treated animals. Values expressed as mean ± SEM.
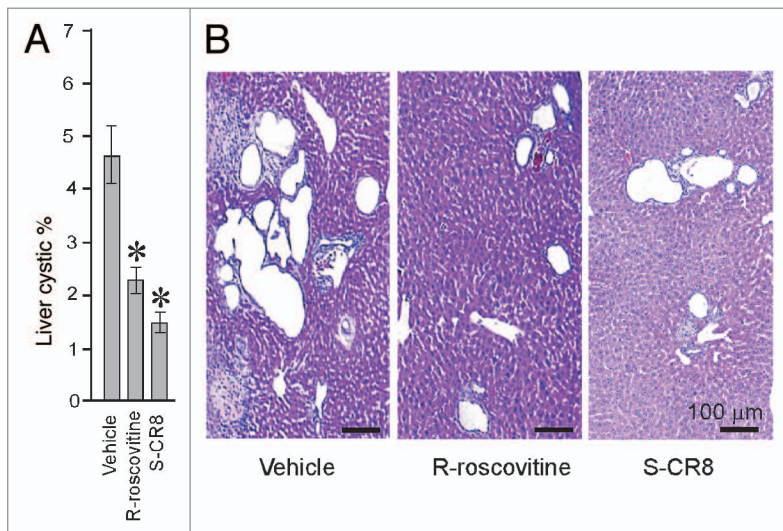

Figure 3. CDK inhibitors R-roscovitine and S-CR8 effectively inhibit hepatic cystogenesis in *Pkd1* cKO mice. (**A**) Quantitative assessment of effects of R-roscovitine and S-CR8 on percentage of liver cysts. * p < 0.05 compared with vehicle control. Hepatic cystic areas are decreased in R-roscovitine and S-CR8-treated animals. Data are expressed as means ± SEM. (**B**) Representative light microscopic (H&E staining) images of liver sections from animals treated with R-roscovitine, S-CR8 and vehicle. Treatment with R-roscovitine and S-CR8 reduced hepatic cystogenesis. Scale bars, 100 μm.

cell cycle progression in G_1 through mechanisms that converge on the induction of p21 and CDK2 inhibition and are important in maintaining centrosome integrity.[24-26] Herein, we demonstrate effective blockade of PKD in an orthologous model of ADPKD with a conditionally inactivated *Pkd1* gene using the CDK inhibitors R-roscovitine and S-CR8, a more potent and selective analog. Unlike many of the first generation CDK inhibitors that lack specificities and target multiple families of kinases, roscovitine is a highly selective, orally bioavailable compound targeting a small subset of CDKs that have been tested in clinical settings.[39,40] The need to further improve its relatively low potency and metabolic stability has led to the generation of an improved, highly potent derivative, S-CR8.[33]

Mechanistic studies showed effective blockade of the cell cycle, attenuation of apoptosis and transcriptional inhibition with both R-roscovitine and S-CR8. Notably, both compounds displayed similar protein kinase selectivity profiles characterized by targeting CDK2/cyclinA, CDK2/cyclinE, CDK5/p25, CDK7/cyclinH and CDK9/cyclinT with approximately 2–3-fold greater potency of S-CR8 for each individual target.[33] On the other hand, S-CR8 showed ~80-fold greater potency over R-roscovitine in cellular assay. This remarkably potent effect of S-CR8 in cellular cystic assay parallels previous findings on the survival of several tumor cell lines.[34] It is possible that cellular effects of S-CR8 represent cumulative or synergistic effects on a subset of specific CDKs. In addition, target accessibility within a cell, permeability and subcellular compartment localization may also contribute to the observed differences in cellular assays.

Unlike tumorigenesis, cystogenesis is accompanied by an increase in apoptosis shown to be causally linked to cystic transformation: deletion of anti-apoptotic Bcl-2 and AP-2β genes in mice results in cystic disease.[41,42] Also, in vitro formation of cysts by MDCK cells grown in collagen matrix is accompanied by increased apoptosis.[43] Direct inhibition of apoptosis in vivo was shown to ameliorate PKD in mouse models.[44] It has been proposed that an imbalance between pro-apoptotic and pro-proliferative factors plays a critical role in the development of cystic kidney disease.[10] Our data suggest that anti-proliferative mechanistic effects of R-roscovitine and S-CR8 combined with their anti-apoptotic effects seen in *Pkd1* cKO-treated kidneys are responsible for therapeutic efficacy.

Cystic epithelial cells in multiple forms of PKD are characterized by increased rates of both proliferation and apoptosis. Given the structural similarity between R-roscovitine and S-CR8 and similar kinase-inhibitory profiles, it is expected that they induce similar molecular responses in treated cells. Indeed, both compounds affected cell cycle and apoptosis in treated samples. At the same time, we observed some subtle differences between the two compounds. Specifically, S-CR8 showed a mechanistically stronger effect on cell cycle machinery, but a somewhat milder effect on attenuation of apoptosis relative to R-roscovitine. Notably, S-CR8 demonstrated better in vivo efficacy than R-roscovitine in reducing cystogenesis and preserving renal function, suggesting that dysregulated cell cycle and proliferation rather than apoptosis may play a pivotal role in promoting cystogenesis in the *Pkd1* cKO model.

The relationship between proliferation and apoptosis in ADPKD is complex and not completely understood. While caspase inhibition slowed cystic disease progression in Han:SPRD rats, effective rapamycin treatment of the *orpk*-rescue mouse model and *Pkd1* cKO model was associated with increased apoptosis.[17,44,45] It has been shown recently that Cdc25A plays an important role in driving renal and hepatic cystogenesis in several animal models, providing further support for a key role of cell

cycle dysregulation and proliferation in polycystic kidney and liver diseases.[46] The authors showed elevated expression of Cdc25A phosphatase, a regulator of the cell cycle in cystic cholangiocytes of PKD patients, PCK rats and *Pkd2^ws25/-* mice. Also, genetic and pharmacological inhibition of Cdc25A suppressed renal and hepatic cystogenesis in animal models.[46]

In summary, we have established efficacy of the CDK inhibitor R-roscovitine and its more potent derivative, S-CR8, in an orthologous model of ADPKD. Importantly, both CDK inhibitors significantly suppressed kidney cystic disease progression and functional decline as well as liver cystogenesis. Mechanistic studies showed effective inhibition of cell cycle, proliferation and apoptosis in treated kidneys. Taken together, our study provides further experimental support for the use of potent and selective CDK inhibitors for the treatment of ADPKD.

Materials and Methods

Mouse colony handling and treatment. Mice were maintained and treated in accordance with Genzyme Institutional Animal Care and Use Committees (IACUC) guidelines. The generation and genotyping of *Pkd1* cKO mice was done as described previously.[32] Briefly, we crossed females homozygous for a *Pkd1*-conditional knockout allele (*Pkd1^tm1Gztn^*) with males heterozygous for a *Pkd1* germline mutation (*Pkd1^tm1Gzbd^*)[47] and homozygous for a tamoxifen-inducible *Cre* allele.[48] Resulting animals heterozygous for the *Cre* allele and the *Pkd1^tm1Gztn^*-conditional allele were either heterozygous for *Pkd1^tm1Gzbd^* germline allele (mutants) or carried wild-type *Pkd1* allele (wild-type controls). Disease was induced by intraperitoneal (IP) injection of nursing females with tamoxifen (250 mg/kg of body weight) to deliver it to pups with milk at day 5 after birth. R-roscovitine and S-CR8 were synthesized as previously described.[49,50] Treatment with R-roscovitine was performed by IP injection of 100 mg/kg once daily from day 7–33 after birth. Treatment with S-CR8 was performed twice daily by IP injection with 2.5 mg/kg starting from day 7–33 after birth. Two and a half percent propylene glycol/10% Cerestar in water was used as a vehicle. Mice were euthanized by CO_2 asphyxiation, and tissues were harvested for histological examination. Blood urea nitrogen levels were measured using a VetACE™ analyzer (Alfa Wasserman).

Cell culture. MDCK kidney epithelial cells (ATCC) were grown in MEM/10% FBS. To test drug potency in vitro, the standard MDCK cyst assay was used. MDCK cysts were grown in 3D collagen I gel as previously described.[35] Briefly, cysts from MDCK cells were grown in collagen I gel in 96-well plates for 4–5 d until cystic lumens were fully formed. Serial dilutions of drugs were added to cysts in culture followed by incubation for 4 d. Inhibition of cystic growth was quantified using Alamar Blue assay (BioSource). Fluorescence was measured with the microplate reader Spectra Max Gemini (Molecular Devices). Values were measured in quadruplets in two independent experiments.
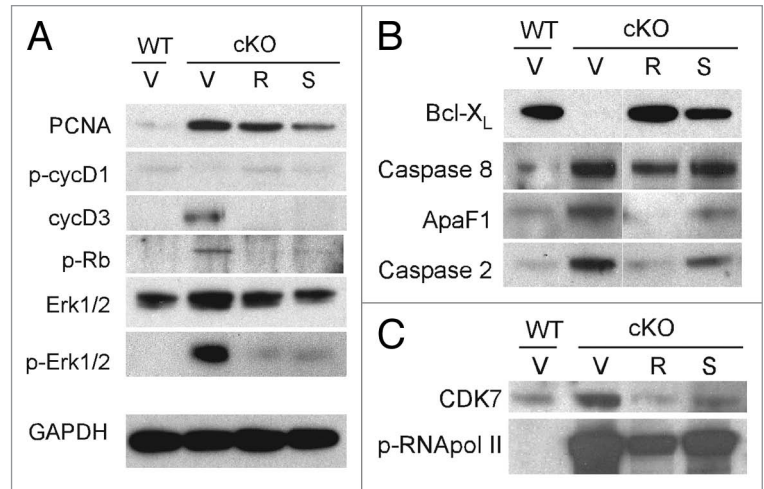

Figure 4. Molecular pathways of cystogenesis affected by CDK inhibitors R-roscovitine and S-CR8 in kidneys from *Pkd1* cKO mice. Representative immunoblots show expression of markers in total kidney extracts from wild-type controls (WT) and *Pkd1* cKO animals (cKO) treated with vehicle (V), R-roscovitine (R) or S-CR8 (S). Shown are effects of CDK inhibitors on cell cycle markers (**A**), apoptotic pathway (**B**) and transcriptional inhibition (**C**).

Histology and quantitative analysis of cystogenesis. Percentage of cystic tissue was quantified as described previously.[29] Briefly, longitudinal and cross kidney or liver sections (4 μm) were stained with hematoxylin and eosin (H&E) with a Tissue Tek® 2000 processor (Sakura-Finetek). Slides were scanned and digitized with an ACIS® system (Clarient) followed by processing with the Metamorph Imaging Series® software (Molecular Devices Corp.). Cystic volume was measured as cystic percentage (ratio of cystic area to a total section area) normalized by body weight.

Western blot analysis. Total kidney lysates were prepared in RIPA buffer containing 1 mM DTT, 5 mM EDTA, 2 mM NaF, 1 mM Na_3VO_4, with Pefablock™ and complete protease-inhibitor cocktail (Roche Applied Science). Protein concentration was determined with BCA assay (Pierce). Proteins (40 μg) were resolved by SDS-PAGE using 4–12% NuPage gradient gels (Invitrogen) and blotted with semi-dry apparatus (Genomic Solutions) as previously described.[29] Membranes were blocked with 5% non-fat milk in PBS and incubated overnight with primary antibodies at 4°C. Membranes were washed with Tris-buffered saline (TBS) containing 0.1% of Tween-20. Secondary antibodies (Promega) conjugated with horseradish peroxidase were used at 1:5,000 dilution. Immunoreactive proteins were detected by enhanced chemiluminescence (GE Healthcare). Equal loading of protein was controlled by anti-GAPDH staining. We used primary antibodies: GAPDH, PCNA, CDK7 (US Biological), ApaF1 (BD Pharmingen), Bcl-X_L (BD Biosciences), caspase 2 (Chemicon), caspase 8, p-Rb, p-cycD1 (Cell Signaling), cycD3 (BD Transduction), p-RNApol II (Abcam).

Statistics. Data are expressed as means ± SEM. Comparisons were made by two-tailed t-test with GraphPad Prism software (GraphPad Software, Inc.), and $p < 0.05$ was considered statistically significant.

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