Autosomal dominant polycystic kidney disease (ADPKD) is the most common monogenic nephropathy and the fourth leading cause of ESRD. It is characterized by progressive development of bilateral renal cysts, often accompanied by liver cysts and an increased risk for vascular abnormalities. ADPKD is caused by mutations to PKD1 or PKD2, encoding polycystin-1 or -2 (PC1/2).1,2 The polycystins are thought to form a complex that regulates Ca2+ influx in response to extracellular mechanical or chemical stimuli.3 Decreased intracellular Ca2+ levels result in downregulation of calcium-dependent phosphodiesterases and stimulation of calcium-inhibitable adenylyl cyclase 6 (AC6).4,6 As a consequence, elevated 3’,5’-cyclic AMP (cAMP) levels have been found in various murine PKD models.7–11 cAMP stimulates protein kinase A–mediated signaling and leads to increased fluid secretion, proliferation/dedifferentiation, and disrupted flow sensing/tubulogenesis—key features of cystogenesis.12–14 Hence, lowering intracellular cAMP levels has been a major focus in the development of therapeutic interventions for ADPKD.14

Multiple murine and human trials have indirectly targeted AC6, the AC isoform predominant in collecting duct principal cells, through the arginine vasopressin receptor 2 (AVPR2) or the somatostatin receptors (SSTR1–SSTR5).15,16 Binding of circulating vasopressin to AVPR2 stimulates AC6 by coupling through guanosine nucleotide–binding (G)-stimulatory proteins,17 and AVPR2 antagonists have alleviated cyst burden in preclinical8,18–21 and clinical22,23 trials. The most prominently used AVPR2 antagonist is tolvaptan, which has shown efficacy in multiple rodent models, and a long-term clinical trial of 1445 patients that reported a 2.8%/year increase in total kidney volume in the treated population compared with 5.5%/year in the placebo group.23 SSTRs inhibit AC6 activity through G-inhibitory proteins and are activated by the peptide hormone somatostatin.24,25 Because of the short half-life of somatostatin, more stable synthetic analogues have been tested in preclinical and clinical trials. Octreotide or lanreotide, which bind to SSTR2 and -3, have been tested in murine studies26,27 and small clinical trials, in which they slowed renal and hepatic cyst expansion.28–31 The recently developed pasireotide binds to SSTR1, -2, -3, and -5 and has shown enhanced efficacy over octreotide in the Pkd2−/WS25 mouse and PCK rat models.27 Despite the promising results, no treatment for ADPKD has been...
Figure 1. C57BL/6 Pkd1^{RC/RC} mice have milder PKD than the outbred model and respond to dDAVP treatment. (A) Masson’s trichrome–stained kidney cross-sections of 3-month-old or 6-month-old inbred and outbred animals. Outbred animals developed higher cyst burdens and larger kidneys compared with inbred mice. Scale bar: 500 μm. (B) Masson’s trichrome–stained liver sections of micro-hamartomas (small, dilated, irregularly shaped bile ducts surrounded by fibrosis). In outbred animals this abnormality was not found before...
approved to date. Here we performed a preclinical study to test whether tolvaptan and pasireotide combination therapy has enhanced efficacy over single drug treatments.

The preclinical study was performed in the homozygous Pkd1\[^{RC/RC}\] model, which closely mimics human ADPKD with slowly progressive PKD, but to minimize phenotypic variability the outbred 129S6; C57BL/6 sive PKD, but to minimize phenotypic variability human ADPKD with slowly progressive disease, an effect likely correlated to the C57BL/6 genetic background. In addition, inbred mice had a higher percentage of liver weight/extra-renal body weight (%LW/ERbody wt) and developed microhamartomas as early as 3 months, compared with 12 months in the outbred background (Figure 1, B and D), highlighting that genetic background effects can vary depending on the organ.

To test the pathogenic role of cAMP in the inbred Pkd1\[^{RC/RC}\] model, mice were treated from 1 to 3 months with the synthetic vasopressin analogue desmopressin (dDAVP) to increase cAMP levels.\[^{20}\] Gross anatomy and Masson’s trichrome–stained kidney sections showed a higher cyst burden in dDAVP-treated mice (n=16) than in untreated controls (n=16) (Figure 1, E and F). This was further reflected in %KW/body wt, which was significantly elevated in the treated group (%KW/body wt: control, 1.74%±0.32%; dDAVP, 2.47%±0.45%; P=1.62×10\(^{-5}\)) (Figure 1G). Corresponding cystic and fibrotic volumes were also significantly increased in the dDAVP-treated mice (cystic volume: 47.00±34.86 μL in controls versus 88.64±46.49 μL in dDAVP mice [P=0.92×10\(^{-2}\)]; fibrotic volume: 2.19±2.00 μL versus 5.21±2.40 μL [P=0.81×10\(^{-3}\)]) (Figure 1, H and I) and cAMP levels were significantly higher (2.86±0.49 pmol/mg protein versus 3.24±0.47 pmol/mg protein; P=0.29×10\(^{-1}\)) (Figure 1J). The %LW/ERbody wt did not differ between the groups, and no sex difference was observed. Together, these results reemphasized the central role of cAMP in cyst progression/development in PKD1 and confirmed the suitability of the inbred Pkd1\[^{RC/RC}\] model for preclinical trials.

To evaluate the benefit of tolvaptan, pasireotide, or combination treatment in slowing PKD progression, inbred Pkd1\[^{RC/RC}\] mice were treated from 1 to 6 months. This long-term treatment was chosen to better mimic comparable treatment of patients with ADPKD, to adequately evaluate the efficacy of the combination therapy, and to assess potential adverse events. At the time of euthanasia, gross anatomic comparisons between kidneys of untreated control (n=22), tolvaptan-treated mice (n=21), pasireotide-treated mice (n=18), and tolvaptan plus pasireotide-treated mice (n=20) showed a clear reduction in kidney size of mice treated with either drug alone, with an even greater reduction (back to the size of wild-type [WT] kidneys) in combination-treated animals (Figure 2A). This observation correlated with the histologic analysis and %KW/body wt (Figure 2, B–D, Table 1, Supplemental Figure 2). Pairwise comparisons showed the greatest significance for the combined

12 months, but inbred mice developed similar-size microhamartomas as early as 3 months. Scale bar: 100 μm. (C and D) %KW/body wt and %LW/ERbody wt of inbred (green) and outbred (black) mice at 3 and 6 months, depicted as mean diamonds and SDs. (C) The %KW/body wt in inbred mice was less variable among non-littermates but overall was milder and more slowly progressive. Wild-type (WT) C57BL/6 mice also had lower %KW/body wt than WT outbred mice, while body weight remained constant, highlighting a clear background effect in kidney anatomy (%KW/body wt in 10 inbred mice and 4 outbred mice: 3 months, inbred, 1.35%±0.11%, outbred, 1.49%±0.20%; P=0.11); 6 months, inbred, 1.24%±0.05%, outbred, 1.66±0.24% (P=0.15×10\(^{-3}\)) (Supplemental Figure 1A). (D) The %LW/ERbody wt was elevated in inbred compared with outbred mice at 3 months (inbred, 5.39%±0.34%; outbred, 4.80%±0.30%; P=0.21×10\(^{-3}\)) and 6 months (inbred, 5.54%±0.40%; outbred, 4.97%±0.48%; P=0.34×10\(^{-3}\)). Gray dotted lines represent WT (C57BL/6 or outbred) mice. (E) Gross anatomy of representative kidneys from inbred WT mice, inbred Pkd1\[^{RC/RC}\] control mice, and dDAVP-treated mice (6 months of age) highlights increased kidney size after dDAVP treatment. Scale bar: 0.5 cm. (F) Masson’s trichrome–stained cross-sections of kidneys with mean±1×SD %KW/body wt. Cross-sections of dDAVP-treated mice showed more severe cystic disease with dilated tubules/ducts (inset, cortex) compared with untreated mice. Scale bar: 500 μm, 250 μm (inset). (G–J) %KW/body wt (G), renal cystic volume (H), renal fibrotic volume (I), and cAMP levels (J) of saline-treated inbred Pkd1\[^{RC/RC}\] control mice (C, green) and dDAVP-treated mice (D, purple), depicted as mean diamonds and SDs. Gray dotted lines represent WT values. dDAVP treatment significantly increased %KW/body wt (G), cystic volume (H), and fibrotic volume (I). As predicted, cAMP levels were elevated upon dDAVP treatment. %KW/BW, %KW/body wt. *P<0.05; ** P<0.01; ***P<0.001; ****P<0.0001. Data of outbred animals were obtained from reference 11.
Figure 2. Tolvaptan plus pasireotide treatment showed enhanced efficacy over single drug treatment. (A) Gross anatomy of representative kidneys from inbred WT and inbred Pkd1^{RC/RC} control mice, C; tolvaptan-treated mice, T; pasireotide-treated mice, P; and tolvaptan plus pasireotide–treated mice, B (6 months of age). Treatment with both drugs showed a clear additive effect, reducing kidney size back to WT range. Scale bar: 0.5 cm. (B) Masson’s trichrome–stained cross-sections of kidneys with mean ± 1SD %KW/body wt. Untreated animals showed multiple cysts and dilated tubules/ducts (inset, cortex) (Supplemental Figure 2). Cyst burden and dilations were reduced upon treatment with either drug alone and were nearly eliminated by combination therapy (Supplemental Figure 2). Scale bar: 500 μm, 250μm (inset). (C–F) %KW/body wt (C), renal cystic volume (D), renal fibrotic volume (E), and cAMP levels (F) of saline-treated Pkd1^{RC/RC}.
treatment with a marked additive effect over single treatments (Table 1). Masson’s trichrome–stained kidney sections showed a reduction in size/number of cysts and dilated tubules/ducts in tolvaptan- or pasireotide-treated mice and only a few cysts with mainly normal–appearing cortex and medulla in animals treated with both drugs (Figure 2B, inset, Supplemental Figure 2). This was further reflected in the overall cystic volume, which was significantly reduced upon single or combination treatment (Figure 2D, Table 1). In addition, treatment with tolvaptan significantly reduced fibrotic volume, which was even further decreased by treatment with pasireotide or both drugs (Figure 2E, Table 1). Consistent with the target of the treatments, CAMP levels were significantly reduced back to WT levels by the combination treatment (WT, 3.25±0.88 pmol/mg protein) (Figure 2F, Table 1). None of the treatments resulted in clear adverse events (see Concise Methods), even though body weight decreased slightly upon treatment with pasireotide alone or combined with tolvaptan (WT, 26.46±3.75 g) (Table 1). No significant difference was noted in BUN, which was in the WT range at 6 months of age (Supplemental Figure 1E), and no significant sex effects were observed.

In addition to the alleviating effect of pasireotide on renal abnormalities, the drug also reduced urinary output, counteracting the tolvaptan-induced polyuria in animals treated with both drugs (Supplemental Table 1).23 While the mechanism for this antidiuretic effect of pasireotide is unclear, similar effects have been reported in patients treated with somatostatin.32,33 This may highlight a favorable effect of the combination treatment because polyuria is considered an adverse event of treatment with tolvaptan or pasireotide alone and even further decreased with use of the combination. (D and E) Similar trends were observed for cystic and fibrotic volumes. For these two parameters, pasireotide slightly outperformed tolvaptan. (F) cAMP levels were only significantly different (back to WT levels) in the combination treatment group, highlighting the importance of the additive effect. %KW/BW, %KW/body wt. *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

### Concise Methods

#### Inbreeding of the Pkd1RC/RC Model into the C57BL/6 Background

The animals were inbred into the C57BL/6 background using the IDEXX RADIL speed congenic service. First, the sex chromosomes were fixed, followed by microsatellite analysis of the autosomes. Animals were considered inbred when all of the microsatellite markers
matched the C57BL/6 strain. Thereafter, animals were maintained as homozygotes.

**Experimental Animals and Study Design**

The Institutional Animal Care and Utilization Committee approved the use of the C57BL/6 Pkd1<sup>RC/RC</sup> model, maintained at the animal facilities of the Mayo Clinic, Rochester, MN, and all experimental protocols described in this report.

**Desmopressin Treatment**

Inbred Pkd1<sup>RC/RC</sup> mice were divided into a control group and a treatment group at 3 weeks of age (n=32; eight animals per treatment group and sex). Littermates of the same sex were sorted into different treatment groups. From 1 to 3 months of age, animals received saline or dDAVP (30 ng/100 g body weight per hour) (V1005, Sigma-Aldrich) via osmotic minipump (Alzet model 1004) as described previously. The minipumps were replaced every 3 weeks, and all animals survived the trial period.

**Urine Collection/Analysis**

One week before euthanasia, all animals in each treatment group and the control group were placed in a metabolic cage from 6 pm to 6 am (in each group, two to four mice were combined in one metabolic cage). Urine volumes were recorded the next morning and osmolality was measured using pHOx Ultra (Nova Biomedical).

**Tissue and Blood Harvest/Analysis**

The animals were euthanized by CO₂ exposure, and the body weight of each animal was recorded. Blood was then collected via cardiac puncture, and kidneys, liver, spleen, and heart were harvested and weighed. The left kidney and small pieces of each liver lobe were flash frozen, and the right kidney plus all other organs were fixed in 4% paraformaldehyde. For the tolvaptan/pasireotide trial, total blood was used for a complete chemistry and electrolyte analysis measuring 14 variables (Abaxis, VetScanVS2, Comprehensive Diagnostic Panel). This panel included BUN and a liver function test quantifying aspartate aminotransferase, alanineaminotransferase, and alkaline phosphatase. All BUN values were in the range of WT values. The aspartate aminotransferase and alanine aminotransferase values did not significantly differ from WT values, and alkaline phosphatase values in the control and tolvaptan-treated mice were slightly lower than WT values but within normal range (normal ranges provided by VetScanVS2 profiles). BUN levels of animals beyond 6 months of age were measured using pHOx Ultra.

**Histomorphometric Analysis**

Cystic volumes and fibrotic volumes were calculated as previously described<sup>20</sup> using three cross-sections per kidney. In short, the cystic volume was calculated by measuring the percentage cystic area of the three cross-sections, adjusted to kidney weight

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**Table 1. Summary of measurements for the combination treatment preclinical trial**

<table>
<thead>
<tr>
<th>Group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Measurements±SD</th>
<th>P Values&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C (n=22)</td>
<td>T (n=21)</td>
</tr>
<tr>
<td>%KW/body wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>1.87±0.23</td>
<td>–</td>
</tr>
<tr>
<td>T</td>
<td>1.69±0.20</td>
<td>0.0101&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P</td>
<td>1.61±0.14</td>
<td>0.0023&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>B</td>
<td>1.43±0.16</td>
<td>5.51E-10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>%LW/ER/body wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>5.54±0.40</td>
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<td>T</td>
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<tr>
<td>P</td>
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<td>1.23E-06&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>B</td>
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<td>4.91E-07&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Cystic volume (μl)</td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>91.31±36.73</td>
<td>–</td>
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<tr>
<td>T</td>
<td>71.85±34.19</td>
<td>0.1453</td>
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<td>P</td>
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<td>B</td>
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<td>5.16E-08&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Fibrotic volume (μl)</td>
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<td></td>
</tr>
<tr>
<td>C</td>
<td>5.18±1.98</td>
<td>–</td>
</tr>
<tr>
<td>T</td>
<td>3.72±1.85</td>
<td>0.0263&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>P</td>
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</tr>
<tr>
<td>B</td>
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<td>7.26E-08&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>cAMP (pmol/μg protein)</td>
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<td></td>
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<tr>
<td>C</td>
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</tr>
<tr>
<td>T</td>
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</tr>
<tr>
<td>P</td>
<td>3.92±1.37</td>
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</tr>
<tr>
<td>B</td>
<td>3.28±0.59</td>
<td>0.01671&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>26.32±3.17</td>
<td>–</td>
</tr>
<tr>
<td>T</td>
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<tr>
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<tr>
<td>B</td>
<td>21.37±2.23</td>
<td>4.32E-10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

C, control (Pkd1<sup>RC/RC</sup>, untreated); T, tolvaptan; P, pasireotide; B, both (tolvaptan plus pasireotide).

<sup>a</sup>Six-month-old inbred animals.

<sup>b</sup>Significant at P<0.05.
Figure 3. Hepatic hypertrophy of Pkd1RC/RC mice can be corrected by pasireotide treatment. (A) %LW/ERbody wt of saline treated, inbred Pkd1RC/RC control mice (C, green), tolvaptan-treated mice (T, blue), pasireotide-treated mice (P, yellow), and tolvaptan plus pasireotide–treated mice (B, red) (6 months of age) depicted as mean diamonds and SD. Gray dotted line represents C57BL/6 WT value. The %LW/ERbody wt returned to WT level in animals treated with pasireotide. (B) Number of DAPI-positive nuclei per 0.01 mm² in liver cross-sections of Pkd1RC/RC controls (green), pasireotide-treated mice (yellow), and WT mice (black), depicted as mean diamonds and SD. Per given area, untreated animals had fewer nuclei compared with pasireotide-treated or WT animals, indicative of larger cells (hypertrophy). (C) Representative image of part B. Scale bar: 50 μm. Hepatic hypertrophy has not previously been reported as a characteristic of ADPKD. Because both PC1 and PC2 are expressed in hepatocytes (Supplemental Figure 3), reduced expression or function of PC1 may be the cause for the observed higher %LW/ERbody wt. %LW/ERBW, %LW/ERbody wt. *P<0.05; ****P<0.0001.

Evaluation of Adverse Events of Tolvaptan/Pasireotide-Treated Animals

Because the trial required numerous pump replacements, five animals died immediately after surgery or within the following 2 days (one in the tolvaptan group, three in the pasireotide group, and one in the combination treatment group). These deaths were probably not drug-related, so death was not considered an adverse event. At the time of euthanasia, every animal was inspected for gross health issues. Body weight had decreased slightly in animals treated with pasireotide and the drug combination (Table 1). However, no notable differences in feeding/drinking habits were observed; thus, the underlying cause and significance of this observation remains unknown. In addition, two animals presented with one severely dysplastic kidney (one in the pasireotide group and one in the combination treatment group) and hence were excluded from the analysis. No other gross abnormal abnormalities were observed, and no abnormalities were noted from the histologic analysis. The blood panel (Abaxis, VetScanVS2, Comprehensive Diagnostic Panel) was used to evaluate potential ion imbalances as well as liver toxicity. No significant differences were noted upon treatment with either drug alone or the combination. Consequently, no definite adverse events were noted within our study.

Immunofluorescence Labeling/Analysis

Tissue were prepared for immunofluorescence labeling as previously described.11 Biotinylated-LTA (1:500; Vector Laboratories) and Aqp2 (1:100; Santa Cruz Biotechnology) were used as proximal tubule and collecting duct markers, respectively. Staining against Tamm-Horsfall protein (1:200; Santa Cruz Biotechnology) was also performed but not quantified because <2 dilations/cyst per animal stained positive for the maker. Quantification was performed using ImageJ software, and cysts/tubular dilations were counted if their diameter exceeded 50 μm.

cAMP Analysis

Pieces from the flash-frozen left kidney were ground to fine powder and used for the cAMP assay as previously described following the manufacture’s protocol (Enzo Life Sciences).11

Crude Membrane Preparation and Western Blotting

Huh7 and renal cortical tubular epithelial cells were cultured in DMEM media containing 10% FBS, penicillin/streptomycin, and glucose. Cells grown to confluence were washed three times with cold PBS, scraped and eluted in low-ionic-strength buffer (10 mM Tris HCL [pH, 7.4], 2.5 μl MgCl2, 1 mM EDTA) containing protease inhibitor (Roche), and incubated for 30 minutes. Cells were then homogenized using a 26.5-gauge needle and nuclei were spun out at 2500 g for 5 minutes (4°C). The supernatant was collected and membrane fragments were pelleted at 20,000 g for 30 minutes (4°C). Pelleted membranes were eluted in low-ionic-strength buffer, and protein content was quantified using a bicinchoninic acid assay.
(Thermo Fisher Scientific). For SDS PAGE, 25 µg (input ratio of 1) of membrane preparation was loaded onto a 3%-8% Tris-acetate gel (150 V for 2.5 hours). The gel was transferred to a polyvinylidene fluoride membrane and probed for PC1 (7e12, 1:1000) or PC2 (Yce2, 1:2000; Santa Cruz).

Statistical Analyses
All analyses were performed using JMP Pro 9. Comparisons between three or more groups were performed by 2-way ANOVA and least-squares means Tukey honest significant difference post hoc test (analyses of Figures 2 and 3, Supplemental Figure 1). Comparisons between two groups were performed by 2-way ANOVA and least-squares means t post hoc test (analyses of Figure 1). Linear regression was used to test for potential synergism of tolvaptan and pasireotide, but the analysis was not significant (P=0.98). A P value below the α level of 0.05 was considered to represent a statistically significant difference. All data are represented as mean ± SD.

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DISCLOSURES
None.

REFERENCES


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