

Triptolide Reduces Cystogenesis in a Model of ADPKD

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ABSTRACT

Mutations in *PKD1* result in autosomal dominant polycystic kidney disease, which is characterized by increased proliferation of tubule cells leading to cyst initiation and subsequent expansion. Given the cell proliferation associated with cyst growth, an attractive therapeutic strategy has been to target the hyperproliferative nature of the disease. We previously demonstrated that the small molecule triptolide induces cellular calcium release through a polycystin-2–dependent pathway, arrests *Pkd1*^{-/-} cell growth, and reduces cystic burden in *Pkd1*^{-/-} embryonic mice. To assess cyst progression in neonates, we used the kidney-specific *Pkd1*^{lox/-}; *Ksp-Cre* mouse model of autosomal dominant polycystic kidney disease, in which the burden of cysts is negligible at birth but then progresses rapidly over days. The number, size, and proliferation rate of cysts were examined. Treatment with triptolide significantly improved renal function at postnatal day 8 by inhibition of the early phases of cyst growth. Because the proliferative index of kidney epithelium in neonates versus adults is significantly different, future studies will need to address whether triptolide delays or reduces cyst progression in the *Pkd1* adult model.

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Mutations in the gene encoding polycystin-1 (*PKD1*) account for approximately 85% of all cases of autosomal dominant polycystic kidney disease (ADPKD), with the remainder attributed to defects in the gene encoding polycystin-2 (*PKD2*). Disease progression is composed of new cyst formation over decades as well as subsequent cyst expansion and growth. ADPKD pathogenesis initiates with inappropriate epithelial cell proliferation¹ that may result from a defect in luminal flow sensing by the primary cilia.^{2,3} Mutations in either *PKD1* or *PKD2* will prevent calcium release mediated by ciliary bending,⁴ and this may be one of the initial signals for the more proliferative phenotype. As re-

cently demonstrated in a mouse model of ADPKD, disease progression may also be attributed to the underlying proliferative potential of the kidney.⁵ Cyst growth continues with eventual separation from the primary tubule followed by cyst expansion mediated by chloride-driven fluid secretion and epithelial cell stretching. Results from the Consortium of Radiologic Imaging Study of PKD (CRISP) have established that although the rate of cyst growth between adult patients with *PKD1* and *PKD2* is not different, patients with *PKD1* have a more severe disease as a result of apparent increased cyst initiation.⁶ Development of a therapeutic agent to target cyst initiation in *PKD1*

would therefore be of interest, because it would have a direct impact on the rate of disease progression and onset of ESRD.

Triptolide, a natural product isolated from the “Thunder God Vine,” has been used in traditional Chinese medicine to treat inflammatory and autoimmune disorders. In addition, triptolide has been shown to be a potent chemotherapeutic agent, because it has concentration-dependent antiproliferative or proapoptotic effects. We previously showed that triptolide acts as a polycystin-2 agonist, although this effect may be indirect, and can growth-arrest *Pkd1*^{-/-} cells via upregulation of p21.⁷ We originally hypothesized that restoring polycystin-2–mediated calcium release in a *Pkd1*^{-/-} embryonic murine model would reduce cyst formation. Although the early lethality of this model was a limitation, we observed a significant decrease in the cystic burden of the embryonic kidneys.⁷ Using a *Pkd1*-floxed mouse model system combined with a kidney-specific Cre, we now tested whether triptolide was acting to reduce cyst initiation or growth in the kidney. In this model, cyst progression is aggressive, because the

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Ksp-cadherin (cadherin 16) promoter driving Cre expression is activated mid-gestation^{8,9} in *Pkd1^{fllox/-};Ksp-Cre* progeny, although few macroscopic cysts are evident at birth (Figure 1A). Because cystic burden is negligible at birth but progresses rapidly within days, this model permitted us to examine the effect of triptolide on initial cyst formation as well as on progression and expansion.

Triptolide (0.25 mg/kg) or DMSO as a control was delivered by intraperitoneal injection to lactating mothers beginning after birth (postnatal day 1 [P1]) and ending on P4. We confirmed that triptolide was effectively delivered through milk, because a higher concentration that was well tolerated by the mother (0.35 mg/kg) was toxic to neonates. Administration of triptolide during this early phase of cyst initiation resulted in a significant reduction in the number of

cysts (Figure 1, B and E), a decrease in kidney weight of cystic animals (Figure 1C), and a reduction in overall cystic burden (Figure 1D). Although kidney weight to body weight ratios were not different from control in noncystic animals (*i.e.*, all genotypes except *Pkd1^{fllox/-};Ksp-Cre*; Figure 1C), there was a minimal reduction in total body weight upon triptolide treatment (2.40 ± 0.05 versus 2.10 ± 0.05 g; $P < 0.0001$; DMSO $n = 33$ and triptolide $n = 34$, respectively), which may reflect the upper limit of our calculated maximum tolerated dosage in this model system. When we further examined cyst size distribution, indicative of newly formed *versus* mature cysts, we observed that triptolide reduced the percentage of the smallest cysts present within the kidney (Figure 1F). In contrast, triptolide did not reduce the percentage of cysts that were larger and at

varying phases of expansion (Figure 1F). We additionally confirmed that the origin of the nephron segments involved in cyst formation were not derived from the proximal tubules in this model as previously reported (data not shown).⁵ Because it has been demonstrated that early developing cysts normally exhibit increased proliferation,⁵ it seemed that triptolide reduced kidney enlargement by inhibition of the early proliferative phase of cyst initiation. To confirm the antiproliferative effect of triptolide on kidney tubule epithelial cells, we examined small cysts (circumference of seven to 15 cells) for expression of the proliferation marker Ki-67. Triptolide treatment resulted in a significant reduction in the number of proliferating cells present in small cysts (67.4 ± 1.5 versus $42.8 \pm 1.8\%$; $P < 0.0001$; DMSO $n = 440$ cysts and triptolide $n = 308$, respectively; Figure 1G).

Although cystogenesis in this *Pkd1* neonatal model is extremely aggressive, with lethality observed between 14 and 21 d, we continued to assess how long triptolide could delay disease progression. After 5 d of triptolide or DMSO delivered through lactation, pups were then administered 0.12 mg/kg per d triptolide or DMSO intraperitoneally beginning on P6 and ending on P8. Although kidney weight increased in both treatment groups, triptolide continued to have a mild inhibitory effect on cystic growth while having no adverse effect on kidney weight ratios of noncystic animals (Figure 2, A and B); however, total body weight of noncystic animals that were treated with triptolide showed a mild but statistically significant decrease, again indicating that we are at the maximum tolerated dosage for these neonates (4.60 ± 0.09 versus 4.00 ± 0.09 g; $P < 0.0001$; DMSO $n = 58$ and triptolide $n = 73$, respectively). Kidney weight to body weight ratios from the triptolide-treated group were mildly reduced compared with DMSO-treated controls, because histologic examination showed that cyst volume was increasing (Figure 2B). Because of experimental constraints at P4 (*i.e.*, pup size), we were unable to assess kidney function in those ani-

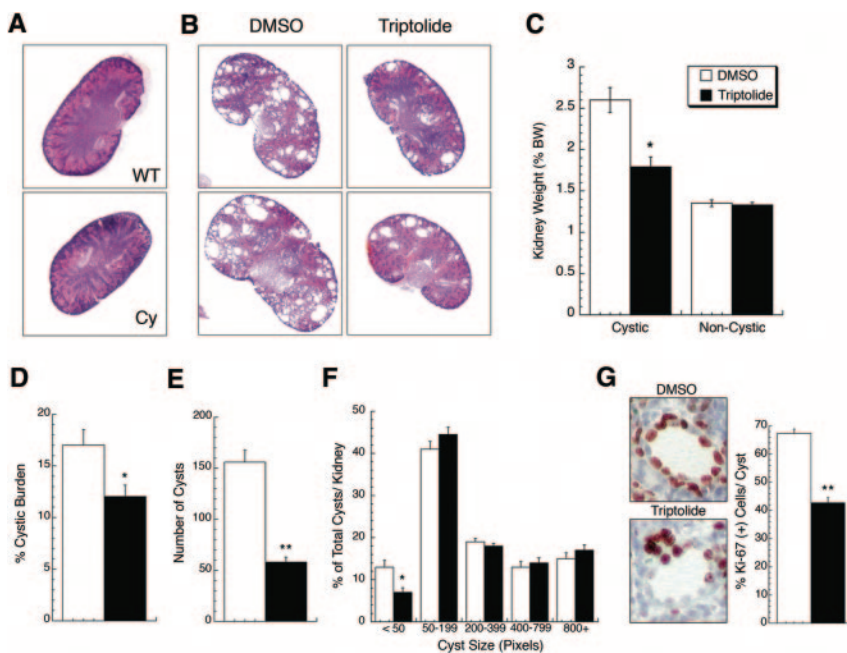


Figure 1. Triptolide reduces cyst formation in *Pkd1^{fllox/-};Ksp-Cre* P4 neonates. (A) Histologic examination of P1 kidneys by light microscopy and hematoxylin and eosin (H&E) staining from wild-type (WT) *Pkd1^{fllox/+}* or cystic (Cy) *Pkd1^{fllox/-};Ksp-Cre* mice. (B) H&E staining of representative P4 kidneys from cystic mice treated with DMSO or triptolide through lactation. (C) P4 kidney weight as a percentage of body weight (BW). Data are means \pm SEM; $n = 10$ (cystic, DMSO), $n = 11$ (cystic, triptolide), $n = 33$ (noncystic, DMSO), $n = 34$ (noncystic, triptolide). (D through G) Comparison of DMSO-*versus* triptolide-treated P4 neonates. The percentage of cystic burden (D), number of total cysts per kidney (E), cyst size as percentage of total cysts per kidney (F), and the percentage of positive Ki-67 cells per cyst (G) are shown. Data are means \pm SEM; $n = 18$ for DMSO and $n = 21$ for triptolide (D through F) and $n = 440$ for DMSO and $n = 308$ for triptolide (G). * $P < 0.01$; ** $P < 0.0001$.

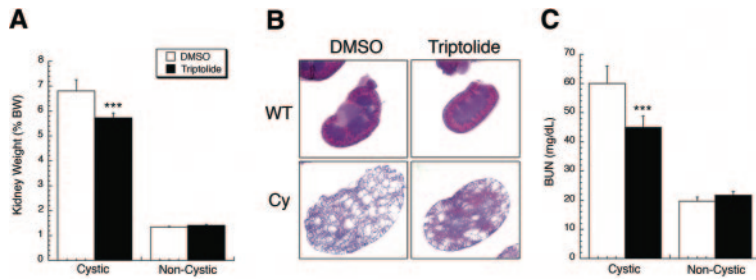


Figure 2. Triptolide preserves renal function. (A) P8 kidney weight as a percentage of body weight (BW). Data are means \pm SEM; $n = 16$ (cystic, DMSO), $n = 25$ (cystic, triptolide), $n = 58$ (noncystic, DMSO), $n = 71$ (noncystic, triptolide). (B) H&E staining of representative P8 kidneys from wild-type (WT) or cystic (Cy) neonates treated with DMSO or triptolide. (C) BUN levels from P8 neonates treated with DMSO or triptolide. Data are means \pm SEM; $n = 11$ (cystic, DMSO), $n = 20$ (cystic, triptolide), $n = 35$ (noncystic, DMSO), $n = 47$ (noncystic, triptolide). *** $P < 0.05$.

mals and therefore wanted to determine blood urea nitrogen (BUN) levels at P8. Although cyst expansion was readily progressing, BUN levels indicated that renal function in triptolide-treated animals had not deteriorated as quickly as in DMSO control animals (Figure 2C).

We completed an additional triptolide treatment experiment in which neonates received a direct intraperitoneal injection from P4 until P12. This time course allowed us to assess whether triptolide could reduce cyst number even after early stages of cystogenesis had begun (*i.e.*, P1 through P3). Noncystic neonates that were treated with triptolide during this time course did not show a reduction in body weight as compared with P4 and P8 animals (6.20 ± 0.22 versus 6.99 ± 0.20 g; $P = 0.01$; DMSO $n = 29$ and triptolide $n = 42$, respectively), and kidney weight ratios were unaffected as well. The percentage of cystic burden at P12 was identical in both triptolide and DMSO groups, and limited analysis of BUN levels at P10 showed that the early benefits of triptolide administration on preserving renal function had been lost (71 ± 4 versus 75 ± 1 mg/dl; $P = 0.278$; DMSO $n = 5$ and triptolide $n = 4$, respectively); however, even though cystic burden was no different between the two experimental groups, we did observe a significant decrease in the number of cysts at P12 when triptolide was administered (320 ± 10 versus 245 ± 9 ; $P < 0.0001$; DMSO

$n = 30$ and triptolide $n = 20$, respectively). Although this decrease in cyst number was not as pronounced as the difference at P4, we believe that triptolide inhibits the early phases of cyst growth, even through the course of disease progression. Triptolide does not attenuate cyst expansion in this neonatal model, and the overall cystic area of the kidney progresses to the same level over time. This result was not surprising because aggressive cystic progression is a limitation in this model and it is unknown whether triptolide penetrates large isolated cysts or can be filtered and delivered through the kidney as renal function declines.

The *Pkd1*^{fllox}; *Ksp-Cre* neonatal murine model of cystogenesis offers a unique opportunity to study kidney-specific deletion of *Pkd1*. Using this model, we have shown that triptolide is capable of decreasing cyst number at the earliest stages of initiation but does not affect cyst expansion and fluid secretion. We observed the most dramatic effect on cyst number at P4, where cellular Ki-67 expression was decreased in the smallest cysts from triptolide-treated animals. This trend in cyst number reduction was observed even when triptolide was administered directly to neonates from P4 through P12. Renal function was improved at P8 as compared with DMSO control animals; however, continual cyst expansion in both experimental groups led to no difference in cystic burden by P12. Current therapeutic strategies have fo-

cused on attenuation of cAMP generation^{10–12} or mammalian target of rapamycin inhibition,^{13–15} thereby decreasing cyst volume and renal enlargement. Similar to roscovitine, also shown to ameliorate cyst formation in other models,^{16,17} triptolide increases p21 expression in cultured *Pkd1*^{-/-} kidney epithelial cells.⁷ Our data here demonstrate a novel *in vivo* antiproliferative effect of triptolide on cyst epithelium, where the delay in disease progression is attributed to an inhibition of the early phases of cyst growth. It may therefore be possible to address multiple mechanisms of ADPKD disease progression through combination therapies targeting both cyst initiation and expansion.

Recently, a developmental switch was identified in *Pkd1*-mediated cyst progression whereby *Pkd1* deletion in neonatal mice up to P12 rapidly results in cystic disease, but *Pkd1* inactivation in adults leads to cyst formation only after many months.^{18,19} In addition, it was found that cyst epithelium specifically shows an increase in proliferation even after whole-kidney proliferation (P24) has ceased.⁵ These data highlight the importance of the model system where small effects of a therapeutic agent in a rapidly progressive model of ADPKD may translate into a robust and sustained benefit in a more indolent model. Because the proliferative index of kidney epithelium in neonates *versus* adults is significantly different, it will be of future importance to determine whether triptolide can delay or reduce cyst progression in the more slowly developing *Pkd1* adult model.

CONCISE METHODS

Pkd1^{fllox/fllox} and *Ksp-Cre* Mouse Lines

Pkd1-floxed and *Ksp-Cre* mice were generated as described previously.^{5,8,9} *Pkd1*^{fllox/fllox} females and *Pkd1*^{+/-}; *Ksp-Cre* males were mated for all experiments. For P1 through P4 studies, lactating mothers were given intraperitoneal injections once daily of 0.25 mg/kg triptolide or an equivalent volume of DMSO delivered in sterile PBS. P1 through P8 studies incorporated the lactation model (P1

through P5) plus direct daily intraperitoneal injections (P6 through P7) of each neonate with 0.12 mg/kg triptolide or DMSO. P4 through P12 studies involved only direct intraperitoneal injection of 0.12 mg/kg per d triptolide or DMSO. At the end of the study (P4, P8, or P12), all neonates were killed, and the kidneys were harvested and weighed. All approved animal protocols were conducted in accordance with Yale Animal Resources Center and Institutional Animal Care and Use Committee regulations.

Histologic Preparation and Analysis

Kidneys were fixed in 4% paraformaldehyde/PBS, embedded in paraffin, and prepared by sagittal cross-sectioning. Sections were stained with hematoxylin and eosin and examined by light microscopy. All kidneys were photographed under the same magnification, and cystic burden was computed using Image J analysis software (National Institutes of Health, Bethesda, MD). The number of cysts was counted, and the cystic burden (area of cysts within the total area of the kidney) was calculated. Ki-67 immunohistochemistry was performed by the Yale Pathology Core Tissue Services. Multiple fields of each kidney were photographed using a $\times 40$ objective, and blinded copies of small cysts were counted for the percentage of positive Ki-67 cells. A total of 440 cysts (4505 cells) were counted for DMSO-treated animals, and 308 cysts (3196 cells) were counted for triptolide-treated animals. The average number of cells per cyst (in circumference) for both DMSO and triptolide samples was 10. Statistical analysis was performed using *t* test. Data are reported as means \pm SEM, and significance was defined as $P < 0.05$.

BUN Measurement

In accordance with Institutional Animal Care and Use Committee regulations, all P8 neonates were anesthetized with isoflurane preceding cardiac puncture. Blood samples were centrifuged in heparinized BD Microtainer plasma separator tubes (BD Biosciences, Franklin Lakes, NJ). The serum was collected, and BUN levels were analyzed by the Yale Mouse Metabolic Phenotyping Center (National Institutes of Health grant U24 DK59635).

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DISCLOSURES

None.

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