Src Inhibition Ameliorates Polycystic Kidney Disease

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ABSTRACT

Despite identification of the genes responsible for autosomal dominant polycystic kidney disease (PKD) and autosomal recessive PKD (ARPKD), the precise functions of their cystoprotein products remain unknown. Recent data suggested that multimeric cystoprotein complexes initiate aberrant signaling cascades in PKD, and common components of these signaling pathways may be therapeutic targets. This study identified c-Src (pp60c-Src) as one such common signaling intermediate and sought to determine whether Src activity plays a role in cyst formation. With the use of the nonorthologous BPK murine model and the orthologous PCK rat model of ARPKD, greater Src activity was found to correlate with disease progression. Inhibition of Src activity with the pharmacologic inhibitor SKI-606 resulted in amelioration of renal cyst formation and biliary ductal abnormalities in both models. Furthermore, the effects of Src inhibition in PCK kidneys suggest that the ErbB2 and B-Raf/MEK/ERK pathways are involved in Src-mediated signaling in ARPKD and that this occurs without reducing elevated cAMP. These data suggest that Src inhibition may provide therapeutic benefit in PKD.


Polycystic kidney disease (PKD) can be transmitted as a dominant or recessive disorder. Autosomal dominant PKD (ADPKD; MIM 173900; 173910) is caused by mutations in one of two genes, PKD1 or PKD2.1 Autosomal recessive PKD (ARPKD) is a developmental disorder caused by mutations in a single gene. The ARPKD disease-causing gene, PKHD1 (polycystic kidney and hepatic disease 1), is a large gene located on chromosome 6p21.1-p12.2,3 It is predicted to yield a novel 4074–amino acid, multidomain, integral membrane protein called fibrocystin,2 or polyductin,3 of unknown function. Principal manifestations of ARPKD involve the fusiform dilation of renal collecting tubules (CT) or ducts and biliary dysgenesis as a result of ductal plate abnormalities.4 The precise function(s) of the protein products (cystoproteins) remain unknown; however, studies of orthologous and nonorthologous animal models of PKD have provided new insights into the complex cellular pathophysiology common to many cystic diseases.5–8

Numerous studies have demonstrated that cystoproteins exist in multimeric protein complexes at various sites within the cells, thereby implicating a number of signaling pathways in PKD.9–12 Aberrant integration of complex signaling events results in increased cellular proliferation, secretory and matrix abnormalities, and epithelial de-differentiation, leading to cyst formation. Pathway analysis demonstrates that these signaling cascades exhibit complex, multiple points of cross-talk and amalgamation.12

Previous work in our laboratory demonstrated that the combination of therapies inhibiting EGF receptor (EGFR) autophosphorylation in addition to
decreasing EGFR ligand availability provide a novel example of effective combination therapy in murine PKD.13 Extending this concept, we reasoned that identification of signaling intermediates that regulate multiple steps in a single pathophysiologic pathway in addition to acting as an integration point for multiple signaling cascades may provide even greater benefit than EGFR combination therapy. Analysis of multiple pathways implicated in PKD and the potential cross-talk of these pathways allowed us to identify intermediate signaling molecules that could provide such potential therapeutic targets.14–18

We focused on signaling intermediates that have multiple interactions with the EGFR axis and also play critical roles in multiple signaling cascades implicated in PKD.12,16,17 We identified c-Src (pp60c-Src) as a common intermediary in multiple PKD pathways, as well as a critical mediator and co-factor in the activation and amplification of the EGFR axis.19–23

In this study, we sought to evaluate the potential of Src inhibition as a therapeutic target in ARPKD. We initially examined Src activity in the BPK nonorthologous murine model of ARPKD. After successful use of Src inhibition in the BPK mouse, we sought to determine whether increased Src activity contributed to renal and biliary cyst formation and progression in an orthologous model of human ARPKD, the PCK rat.

This study demonstrates that (1) increasing Src activity (pY418) correlates with disease progression in both the BPK and PCK rodent models of ARPKD; (2) in both models, highly specific pharmacologic inhibition of Src activity with SKI-606 ameliorates the renal and biliary lesions characteristic of human ARPKD; (3) in BPK kidneys, Src inhibition correlates with decreased EGFR (ErbB1) activity and; (4) in PCK kidneys, ErbB2 (rather than ErbB1) is overexpressed and Src inhibition decreases ErbB2 activity. Src also inhibits the B-Raf/MEK/ERK signaling pathway without reducing elevated cAMP. These data suggest that reduction of elevated cAMP is not an absolute requirement for amelioration of cystic disease and that Src inhibition may provide therapeutic benefit in both ARPKD and ADPKD.

RESULTS

BPK

Developmental Profile of c-Src (pY418).

A developmental profile of total c-Src in BPK kidneys (Figure 1A) demonstrates that the level of pan Src is essentially un-

![Figure 1. Developmental expression of active c-Src (pY418).](image1)

A Western analysis of the renal expression of immunoprecipitated total/pan Src in BPK kidneys from PN0 (lane 1) to PN14 (lane 4) demonstrates little change with age or disease progression in BPK mice. (B) In contrast, renal expression of active Src (pY418) in BPK mice increases in parallel with disease progression from PN0 (lane 1) to PN14 (lane 4) in the absence of changes in total Src expression (A). (C) Western analysis of the original tissue extracts for β-actin before immunoprecipitation of pan-Src demonstrated that the original tissue extracts were accurately adjusted to equal protein concentrations, and equivalent quantities of protein were subjected to immunoprecipitation. Relative densitometry (minus background) shown below each lane were obtained by NIH Image 1.62, and both Westerns and respective densitometry data shown represent three independent, reproducible experiments.

![Figure 2. A and B) Correlation of renal size (A) with Src activity (B). (A) Lane 3 demonstrates that large cystic kidneys from BPK mice correlate with increased expression of active Src (pY418) (B, lane 3) compared with age-matched Balb/C kidneys (A, lanes 1 and 2) and corresponding Src (pY418) activity (B, lanes 1 and 2). (B) Lane 4 demonstrates that reduction in renal Src activity with SKI-606 treatment (compared with lane 3) results in significant reduction in cystic kidney size as shown in A, lane 4. These data demonstrate that inhibition of c-Src activity (pY418) results in considerable reduction of renal growth in cystic BPK mice. (C) The original tissue extracts were accurately adjusted to equal protein concentrations, and equivalent quantities of protein were subjected to immunoprecipitation. Relative densitometry (minus background) shown below each lane was obtained by NIH Image 1.62, and both Westerns and respective densitometry data shown represent three independent, reproducible experiments.](image2)
changed developmentally. The level of active Src (pY418) (Figure 1B), after immunoprecipitation of total Src, demonstrates that increasing activity of Src correlates with disease progression. Figure 1C demonstrates that the original tissue extracts were accurately adjusted to equal protein concentrations and equivalent quantities of protein were subjected to immunoprecipitation. Relative densitometry (minus background) shown below each lane was obtained by National Institutes of Health (NIH) Image 1.62 and represents three independent, reproducible experiments.

Correlation of Renal Size with Src (pY418) Levels.
Figure 2 demonstrates the effect of SKI-606, a specific inhibitor of c-Src (pY418), on whole kidneys and the correlation of renal size with the levels of active Src after immunoprecipitation of total Src. Figure 2A1 shows a representative vehicle-treated kidney from a Balb/C mouse, and Figure 2B, lane 1, shows the corresponding basal level of renal Src activity. As Figure 2B demonstrates, treatment of Balb/C mice reduced active Src levels (Figure 2B, lane 2) without significant reduction in kidney size (Figure 2A2). The data also demonstrate that enlarged cystic kidneys from BPK mice (Figure 2A3) were associated with high levels of Src activity (Figure 2B, lane 3) compared with age-matched Balb/C kidneys (Figure 2, A1 and B, lane 1). Figure 2B, lane 4, demonstrates that reduction in renal Src activity with SKI-606 (compared with Figure 2B, lane 3) resulted in significant reduction in cystic kidney size as shown in Figure 2A4 compared with an untreated BPK kidney (Figure 2A3).

Histologic Evaluation of SKI-606 Treatment.
SKI-606 treatment of cystic BPK mice resulted in reduction of renal CT cysts (Figure 3B) compared with the cystic CT lesions present in postnatal day 21 (PN21) untreated cystic kidneys (Figure 3A). This reduction in renal CT cysts occurred without morphologic evidence of renal toxicity and directly correlated with the reduction in renal Src activity and kidney size shown in Figures 2, A4 and B, lane 4. Microscopic analysis of hepatic tissue from SKI-606–treated BPK mice (Figure 4C) revealed near-normal biliary ductal development when compared with Balb/C livers (Figure 4A) and amelioration of the significant biliary ductal ectasia (BDE) characteristic of untreated BPK (Figure 4B).

SKI-606 Treatment of BPK: Morphometrics and Renal Function.
Balb/C normal and BPK cystic mice received SKI-606, by intraperitoneal (i.p.) injections, at 30 mg/kg per d starting at PN7. This dosage was determined in preliminary studies to maximize efficacy with minimal toxicity. Kidney and liver tissues harvested at PN21 were assessed for the parameters listed. SKI-606 treatment of BPK mice resulted in significant improvement of all assessed parameters compared with untreated or vehicle-treated BPK (Table 1). SKI-606 treatment of Balb/C

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Histologic evaluation of SKI-606 treatment and immunohistochemical analysis of cyst localization. Cyst localization was studied by segment-specific lectin binding using Dolichos biflorus agglutinin (red label) as a marker for CT and Lotus tetragonolobus agglutinin (brown label) as a marker for proximal tubules. These data demonstrate that SKI-606 treatment of cystic BPK animals results in reduced size and number of renal CT cysts (B) compared with the cystic CT lesions present in PN21 untreated cystic kidneys (A). This reduction in renal CT cysts (B) occurs without morphologic evidence of renal toxicity and directly correlates with the reduction in renal Src activity and kidney size shown in Figure 2, B, lane 4, and A, lane 4, respectively. Magnification, ×40.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Microscopic analysis of hepatic tissue from SKI-606 treated animals (C) reveals amelioration of BDE characteristic of untreated BPK (B) and closely resembles biliary ducts from control (A) liver. These data along with renal morphology shown in Figure 3 demonstrate that Src inhibition is effective in ameliorating PKD-associated proliferation in both renal and hepatic organs. These data also demonstrate that SKI-606 treatment at 30 mg/kg per d results in no morphologic evidence of renal (Figure 3B) or hepatic (Figure 4C) toxicity. Magnification, ×40.
Table 1. Src inhibition in BPK: Morphometrics and functional parametersa

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated (n = 42)</th>
<th>SKI-606 (n = 42)</th>
<th>Untreated (n = 41)</th>
<th>SKI-606 (n = 30)</th>
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<td>Body weight (g)</td>
<td>10.60 ± 1.00</td>
<td>10.50 ± 1.30b</td>
<td>10.90 ± 0.80</td>
<td>9.80 ± 1.00d</td>
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<td>Kidney weight (g)</td>
<td>0.15 ± 0.02</td>
<td>0.14 ± 0.02b</td>
<td>2.25 ± 0.30b</td>
<td>0.84 ± 0.30d</td>
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<td>Kidney weight/body</td>
<td>1.44 ± 0.10</td>
<td>1.50 ± 0.20b</td>
<td>21.00 ± 2.00b</td>
<td>8.57 ± 2.00d</td>
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<tr>
<td>Cystic index (CI)</td>
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<td>0.00 ± 0.00</td>
<td>4.70 ± 0.60b</td>
<td>1.35 ± 0.60d</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>19.00 ± 1.40</td>
<td>19.00 ± 2.20b</td>
<td>108.00 ± 33.00c</td>
<td>23.10 ± 3.00d</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.21 ± 0.10</td>
<td>0.21 ± 0.10b</td>
<td>0.54 ± 0.10b</td>
<td>0.30 ± 0.10d</td>
</tr>
<tr>
<td>Max urinary</td>
<td>1066.00 ± 57.00</td>
<td>1069.00 ± 74.00b</td>
<td>439.00 ± 63.00c</td>
<td>863.00 ± 194.00d</td>
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<td>concentrating h</td>
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Data are means ± SD. Results demonstrate that cystic animals treated with SKI-606 (n = 30) showed a 60% (P < 0.001) reduction in kidney weight/body weight ratio, reduction in CI of 71% (P < 0.001), and a 44% decrease in BDE (P < 0.001) with untreated cystic animals (n = 41). Renal functional parameters also demonstrated significant improvement with SKI-606 treatment compared with untreated BPK animals. BUN levels decreased from 108 to 23 (P < 0.001), creatinine levels decreased by 45% (P < 0.001), and maximum urinary concentrating ability increased by 52% (P < 0.001). These data correlate with the decreased levels of active c-Src shown in Figure 2B, lane 4, and the reduced kidney size shown in Figure 2A, lane 4. In wild-type Balb/C kidneys, there was no statistical difference in any parameter assessed with SKI-606 treatment.

**Comparison of Targeted Therapies on Active EGFR (ErbB1) in BPK.**

Previous published therapies directly targeting the activity of the EGFR axis demonstrated promising effectiveness in inhibiting the progression of both renal and biliary abnormalities in the BPK model.13,24 Figure 5 compares the effectiveness of Src inhibition (SKI-606) on EGFR activity with published EGFR targeted therapies. Figure 5A, lanes 2 through 5, demonstrates the increased levels of total EGFR in BPK kidneys compared with Balb/C kidneys (Figure 5A, lane 1). Figure 5B demonstrates the renal levels of active EGFR (pY1068) at PN21 with and without therapy. Figure 5B, lane 5, demonstrates that Src inhibition is as effective as the EGFR targeted therapies (Figure 5B, lanes 3 and 4) at reducing EGFR activity.

**Toxicology.**

Toxicology studies demonstrated no compound-related deaths and no microscopic changes in heart, spleen, stomach, pancreas, or thymus with dosing at 30 mg/kg per d (data not shown). The lack of renal toxicity is supported by the fact that mice did not significantly alter any functional parameters assessed or demonstrate any morphologic evidence of toxicity.

Results summarized in Table 1 demonstrate that treated cystic mice (n = 30) showed a 60% (P < 0.001) reduction in kidney weight/body weight ratio, reduction in CI of 71% (P < 0.001), and a 44% decrease in BDE (P < 0.001) compared with untreated cystic mice (n = 41). SKI-606 treatment of BPK also produced significant improvement in renal function. Blood urea nitrogen (BUN) levels decreased from 108 to 23 (P < 0.001), creatinine levels decreased by 45% (P < 0.001), and maximum urinary concentrating ability increased by 52% (P < 0.001). Treatment of Balb/C controls demonstrated no significant differences in assessed parameters compared with untreated controls.

**Figure 5.** Comparison of EGFR targeted therapies and Src inhibition on active EGFR (pY1068). (A) Western analysis of immunoprecipitated total EGFR (ErbB1) in Balb/C (lane 1) and BPK (lanes 2 through 5) PN21 kidneys. (B) PN21 renal levels of active EGFR (pY1068) after immunoprecipitation of total EGFR with and without therapy as indicated. As seen in lane 5, Src inhibition is as effective as previously published EGFR targeted therapies (lanes 3 and 4) at reducing the activity of the receptor in the BPK model. (EKB is an EGFR tyrosine kinase inhibitor, and WTACE2 inhibits the processing of preproEGFR ligands, thereby reducing the availability of ligand. EKB was tested alone or in combination with WTACE2). (C) Western blot analysis of the original tissue extracts for β-actin before immunoprecipitation of total EGFR demonstrates that equivalent quantities of protein were subjected to immunoprecipitation. Relative densitometry (minus background) shown below each lane were obtained by NIH Image 1.62, and both Westerns and respective densitometry data shown represent three independent, reproducible experiments.
the functional data of PN21 Balb/C treated mice was not significantly different from untreated controls.

**PCK**

*Renal Expression of ErbB2 in PCK.*

We previously reported the lack of EGFR overexpression in the PCK rat; however, the reported hyperactivity of PCK biliary epithelial cells to EGFR ligands in the absence of increased EGFR (ErbB1) expression suggested that a second member of the EGFR family of receptors plays a pivotal role in disease progression. The developmental increase in active ErbB2 (pY1221/1222) that correlates with PCK disease progression. Immunohistochemical staining of PN90 PCK kidney (Figure 6B) illustrates the unusually high expression levels of p-ErbB2 on basolateral and apical cell surfaces of cystic epithelia.

**Developmental Profile of Active Src and Active p44/p42 MAPK (p-ERK1/2).**

A developmental profile of active Src (pY418) after immunoprecipitation of total Src, in both Sprague-Dawley (PCK background) and PCK kidneys, is shown in Figure 7A. Figure 7A, lanes 1 through 4, demonstrates a marginal developmental increase in active Src in Sprague-Dawley kidneys, whereas Figure 7B, lanes 1 through 4, demonstrates a modest developmental decrease in renal p-ERK1/2. In contrast, Figure 7A, lanes 5 through 8 (PCK), demonstrates a sizeable developmental increase in Src (pY418) that correlated with a substantial increased p-ERK1/2 in PCK kidneys (Figure 7B, lanes 5 through 8).

**Correlation of Renal Size with Src (pY418) Levels after SKI-606 Therapy.**

Figure 8 demonstrates the correlation of renal size (Figure 8A) with the levels of phosphorylated (active) Src, ErbB2, B-Raf, and ERK1/2 in Figure 8, B through E, respectively, after immunoprecipitation of the total protein of interest at PN90. Figure 8B demonstrates that SKI-606 treatment of Sprague-Dawley rats results in a slight decline in renal Src (pY418) levels (Figure 8B, lane 2) and reduced p-ErbB2 (Figure 8C, lane 2) without significant reduction in kidney size (Figure 8A, lane 2). Src inhibition of Sprague-Dawley rats did not significantly alter the renal basal levels of either p-B-Raf (Figure 8D, lanes 1 and 2) or p-ERK1/2 (Figure 8E, lanes 1 and 2).

Enlarged PN90 PCK kidneys (Figure 8A, lane 3) demonstrated significantly increased levels of Src (pY418) (Figure 8B, lane 3) in Figure 8, B through E, respectively, after immunoprecipitation of the total protein of interest at PN90. Figure 8B demonstrates that SKI-606 treatment of Sprague-Dawley rats results in a slight decline in renal Src (pY418) levels (Figure 8B, lane 2) and reduced p-ErbB2 (Figure 8C, lane 2) without significant reduction in kidney size (Figure 8A, lane 2). Src inhibition of Sprague-Dawley rats did not significantly alter the renal basal levels of either p-B-Raf (Figure 8D, lanes 1 and 2) or p-ERK1/2 (Figure 8E, lanes 1 and 2).

Enlarged PN90 PCK kidneys (Figure 8A, lane 3) demonstrated significantly increased levels of Src (pY418) (Figure 8B, lane 3)
compared with age-matched Sprague-Dawley kidneys (Figure 8B, lane 1). SKI-606 treatment of PCK rats resulted in a reduction of whole-kidney size (Figure 8A, lane 4) that correlated with reduced levels of Src (pY418) (Figure 8B, lane 4), as well as reduced p-ErbB2 (Figure 8C, lane 4), p-B-Raf (Figure 8D, lane 4), and p-ERK1/2 (Figure 8E, lane 4) compared with levels of untreated PN90 PCK kidneys (Figure 8, A through E, lane 3).

**Histologic Evaluation of SKI-606 Treatment.**
SKI-606 treatment of PCK rats resulted in reduction of the size of renal CT cysts (Figure 9C) compared with the cystic CT lesions present in PN90 untreated cystic kidneys (Figure 9A). This reduction in renal cysts directly correlated with the reduction in renal Src, ErbB2, B-Raf, and ERK1/2 activity and kidney size shown in Figure 8, A through E, lane 4, after SKI-606 treatment. Microscopic analysis of Masson’s Trichrome–stained hepatic tissue from SKI-606–treated rats (Figure 9D) revealed a significant reduction in both biliary ductal cyst development and fibrosis when compared with livers of untreated PCK (Figure 9B).

**SKI-606 Treatment of PCK: Morphometrics and Renal Function.**
Results summarized in Table 2 demonstrate that PCK rats treated with SKI-606 (n = 15) showed a 27% (P < 0.02) reduction in kidney weight/body weight ratio, reduction in renal cyst volume of 52% (P < 0.01), and a 17% decrease in liver weight/body weight ratios (P < 0.02) compared with untreated PCK (n = 15). A particularly striking result was the lack of change in renal cAMP levels in the PCK after Src inhibition. This suggests that the effect of Src inhibition in the aberrant signal transduction pathway of ARPKD occurs below the level of cAMP and that amelioration of renal cystic changes can...
Table 2. Src inhibition in PCK: Morphometrics and functional parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD Untreated (n = 10)</th>
<th>SKI-606 (n = 10)</th>
<th>PCK Untreated (n = 15)</th>
<th>SKI-606 (n = 15)</th>
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<tr>
<td>Body weight (g)</td>
<td>335.00 ± 22.00</td>
<td>330.00 ± 18.00</td>
<td>387.60 ± 40.00</td>
<td>375.00 ± 18.00</td>
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<td>Kidney weight (g)</td>
<td>4.40 ± 0.30</td>
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<td>4.10 ± 0.60</td>
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<tr>
<td>Kidney weight/body weight (%)</td>
<td>0.70 ± 0.10</td>
<td>0.70 ± 0.10</td>
<td>1.50 ± 0.10</td>
<td>1.10 ± 0.30</td>
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<tr>
<td>PN90 renal cystic volume</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>30.90 ± 11.00</td>
<td>14.80 ± 7.00</td>
</tr>
<tr>
<td>PN90 liver weight/body weight (%)</td>
<td>3.50 ± 0.40</td>
<td>3.40 ± 0.40</td>
<td>4.80 ± 0.70</td>
<td>4.00 ± 0.60</td>
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<td>BUN (mg/dl)</td>
<td>16.00 ± 1.50</td>
<td>17.00 ± 1.20</td>
<td>36.00 ± 2.80</td>
<td>23.00 ± 1.40</td>
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<td>Creatinine (mg/dl)</td>
<td>0.22 ± 0.10</td>
<td>0.22 ± 0.10</td>
<td>0.59 ± 0.40</td>
<td>0.36 ± 0.30</td>
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<td>Renal cAMP pmol/mg protein</td>
<td>7.72 ± 0.30</td>
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<td>13.63 ± 1.00</td>
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<td>Max urinary concentrating 12 h</td>
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<td>1299.00 ± 55.00</td>
<td>949.00 ± 108.00</td>
<td>1087.00 ± 153.00</td>
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</table>

aData are means ± SD. Results demonstrate that cystic animals treated with SKI-606 (n = 15) showed a 27% (P < 0.02) decrease in kidney weight/body weight ratio, reduction in renal cyst volume of 52% (P < 0.001), and a 17% decrease in LW/body weight ratios (P < 0.02) compared with untreated cystic animals (n = 15). Renal functional parameters also demonstrated improvement with SKI-606 treatment. BUN levels decreased 36% (P < 0.001), creatinine levels decreased by 45% (P < 0.001), and maximum urinary concentrating ability increased by 12% (P < 0.05). A particularly striking result was the lack of change in renal cAMP levels in the PCK after Src inhibition. This suggests that the effect of Src inhibition in the aberrant signal transduction pathway of ARPKD occurs below the level of cAMP. This also suggests that amelioration of renal cystic changes can occur without reduction of renal cAMP. Treatment of Sprague-Dawley controls demonstrated no significant changes in assessed parameters compared with untreated controls.

bNS, Sprague-Dawley untreated versus Sprague-Dawley treated (SKI-606).

cP < 0.01, dP < 0.02, eP < 0.05, Sprague-Dawley untreated versus PCK untreated.

 src PCK treated (SKI-606).

The use of Src inhibition as a therapeutic intervention in PKD is based on an extensive body of experimental evidence regarding the pathophysiology of both ARPKD and ADPKD. This includes the involvement of Src activation in EGFR family–mediated signaling and proliferation, as well as the role of Src in the cAMP-mediated proliferation of cystic renal epithelia. In vitro, normal renal epithelial cells are growth-inhibited by cAMP. In contrast, renal cystic epithelia are growth-stimulated by cAMP in both ARPKD and ADPKD. This “PKD phenotype” has been shown to be directly dependent on activation of B-Raf, resulting in activation of the MEK/ERK pathway,14,15,17,28,29 and in M1 cells, c-Src was required for cAMP-dependent ERK activation.17

DISCUSSION

The results of this study demonstrate that increasing Src activity (pY418) correlates with disease progression in both the BPK and PCK models. Inhibition of Src activity with SKI-606 resulted in substantial amelioration of renal cystic disease, improvement in renal function, and reduction of biliary abnormalities.

Despite that the BPK is not an orthologous model of human ARPKD, good rationale exists for its use in screening therapeutic interventions. Notwithstanding the development of numerous murine models with targeted mutations in orthologous PKD genes, the BPK model remains the most consistent phenocopy of human ARPKD. The BPK displays the classic phenotypic abnormalities including the dual biliary plate and renal CT pathology, abnormal apical expression of EGFR, and the rapid development of renal cystic lesions typically seen in human ARPKD. The phenotypic similarities, in addition to the accelerated development of ESRD in the BPK, provide an excellent model for rapidly screening interventional therapies in a well-characterized phenocopy of ARPKD.

The toxicology included the involvement of Src activation at the ERK family–mediated signaling and proliferation, as well as the role of Src in the cAMP-mediated proliferation of cystic renal epithelia. In vitro, normal renal epithelial cells are growth-inhibited by cAMP. In contrast, renal cystic epithelia are growth-stimulated by cAMP in both ARPKD and ADPKD. This “PKD phenotype” has been shown to be directly dependent on activation of B-Raf, resulting in activation of the MEK/ERK pathway,14,15,17,28,29 and in M1 cells, c-Src was required for cAMP-dependent ERK activation.17

Previous reports from our laboratory demonstrated a lack of increased expression of EGFR (ErbB1) in the PCK kidneys.25 Published reports on the hyperactivity of PCK biliary epithelial cells to EGFR ligands in the absence of increased EGFR expression suggested that a second member of the EGFR family of receptors plays a pivotal role in disease progression.26 The developmental increase in active ErbB2 (pY1221/1222) (Figure 6A) and its correlation with disease progression along with the abnormal localization shown in Figure 6B suggests that ErbB2 plays a pivotal role in disease progression in the PCK.

The renal developmental profile of active Src and p-ERK1/2 in Figure 7, A and B, respectively, demonstrates that increasing Src activity directly correlates with increasing ERK1/2 activity and disease progression in the PCK. Src inhibition of PCK animals leads to amelioration of disease progression as demonstrated by (1) a reduction in whole-kidney size (Figure 8A, lane 4), (2) reduced kidney weight (Table 2), and (3) improved renal (Figure 9C) and hepatic (Figure 9D) morphology of treated animals compared with kidneys (Figure 9A) and liver (Figure 9B) of untreated animals. SKI-606 treatment also results in a substantial reduction in fibrosis surrounding biliary

occur without reduction of renal cAMP. Treatment of Sprague-Dawley controls demonstrated no significant differences in assessed parameters compared with untreated controls.
ducts. Whether this improvement is simply due to decreased biliary epithelial proliferation or involves alterations in complex fibrotic pathways is currently an area of interest within our laboratory.23

Table 2 summarizes and compares the morphometric and functional parameters assessed in SKI-606–treated and untreated PCK. As previously reported,30 PCK cystic kidneys demonstrate increased levels of cAMP compared with Sprague-Dawley controls. This study demonstrated that marked improvement in renal morphology with Src inhibition occurred without a significant reduction in renal cAMP levels. These data provide *in vivo* support of published *in vitro* observations that Src was required for cAMP-dependent ERK activation in M-1 cells.17 In PCK kidneys, reduction of cAMP was not required for inhibition of B-Raf and ERK1/2 or amelioration of renal cyst formation and enlargement.

In comparing the results of Table 1 (BPK) and Table 2 (PCK), the effects of SKI-606 in the PCK model seem to be less striking than that seen in BPK animals; however, it should be noted that these two animal models were treated at different stages of disease. By PN21, the BPK mouse is premorbid and in severe renal failure, emphasizing the value of the rapid pace of disease in this model for preclinical testing. Conversely, by PN90, the PCK rat is still at an early stage of disease with mild renal insufficiency and despite progressive cystic disease has a lifespan that extends for another 9 to 12 mo. Additional studies extending SKI-606 treatment in both the BPK and PCK models beyond the length of this study are under way. These studies will also be of value in assessing any long-term toxicity of SKI-606.

As previously stated, a large body of experimental evidence indicates that abnormalities in the EGFR axis are a common cellular phenotype downstream from a number of primary cystic gene mutations. Despite interesting speculations, the cellular phenotype downstream from a number of primary abnormalities in the EGFR axis are a common cellular phenotype downstream from a number of primary cystic gene mutations. Despite interesting speculations, the cellular phenotype downstream from a number of primary aberrations in the EGFR axis is currently an area of interest within our laboratory.23

Table 2 summarizes and compares the morphometric and functional parameters assessed in SKI-606–treated and untreated PCK. As previously reported,30 PCK cystic kidneys demonstrate increased levels of cAMP compared with Sprague-Dawley controls. This study demonstrated that marked improvement in renal morphology with Src inhibition occurred without a significant reduction in renal cAMP levels. These data provide *in vivo* support of published *in vitro* observations that Src was required for cAMP-dependent ERK activation in M-1 cells.17 In PCK kidneys, reduction of cAMP was not required for inhibition of B-Raf and ERK1/2 or amelioration of renal cyst formation and enlargement.

In comparing the results of Table 1 (BPK) and Table 2 (PCK), the effects of SKI-606 in the PCK model seem to be less striking than that seen in BPK animals; however, it should be noted that these two animal models were treated at different stages of disease. By PN21, the BPK mouse is premorbid and in severe renal failure, emphasizing the value of the rapid pace of disease in this model for preclinical testing. Conversely, by PN90, the PCK rat is still at an early stage of disease with mild renal insufficiency and despite progressive cystic disease has a lifespan that extends for another 9 to 12 mo. Additional studies extending SKI-606 treatment in both the BPK and PCK models beyond the length of this study are under way. These studies will also be of value in assessing any long-term toxicity of SKI-606.

As previously stated, a large body of experimental evidence indicates that abnormalities in the EGFR axis are a common cellular phenotype downstream from a number of primary cystic gene mutations. Despite interesting speculations, the precise mechanisms of this striking relationship remain unresolved.16,31–34

A growing body of data indicates that the Src family of protein tyrosine kinases functionally interacts with a variety of nonreceptor and receptor protein tyrosine kinases, particularly the EGFR family.35,36 From these studies, it is generally agreed that the association of Src (through Src homology 2 domain) with ErbB1 and/or ErbB2 results in the mutual stimulation of Src catalytic activity and enhanced phosphorylation of the receptors’ downstream targets to promote mitogenic and, at times, tumorigenic growth.

Traditionally, the EGFR axis is considered to act primarily through activation of the MAPK (RAS/RAF/MEK/ERK) pathway. It has become increasingly clear that the EGFR axis is much more complex, and recent data suggest that there is considerable cross-talk between the MAPK pathway and the cAMP-mediated pathway.36,37

In summary, inhibition of c-Src (pY418) was effective in ameliorating the dual renal and biliary abnormalities of ARPKD in both the BPK and orthologous PCK rodent models without evidence of organ toxicity. This effect was due to inhibition of well-characterized proliferative signaling pathways mediated by increased p-ErbB1/B2 and p-B-Raf. Preliminary studies suggest Src inhibition demonstrates similar antiproliferative potential in human ADPKD.38,39 We speculate that c-Src (pY418) is a potential therapeutic target for both human ARPKD and ADPKD.

**CONCISE METHODS**

**BPK Model**

The BPK model, a murine model of ARPKD, arose as a spontaneous mutation in an inbred colony of BALB/c mice and has been extensively characterized.13,40,41 Affected animals die at PN24 (average) with a range of PN21 to PN29. Extrarenal manifestations include biliary proliferation and BDE. Balb/C controls were used in all experiments. All animal experiments were conducted in accordance with policies of the NIH Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

**BPK Genotyping**

Positional cloning techniques led to the discovery that a 2-bp insertion (GC) in exon 22 of the murine bicaudal-C gene (*Bicc1*) was the causative disease mutation in the *bpk* allele.42 BPK animals were identified using a PCR-based genotyping method that has been described in detail.43

**PCK Model**

PCK rats were originally derived from a spontaneous mutation in a strain of Sprague-Dawley rats maintained at the Education and Research Center of Animal Models for Human Diseases of Fujita Health University.44 The animals used in this study were from a colony established at the Medical College of Wisconsin from PCK founders donated for Dr. V.E. Torres at the Mayo Research Foundation. The PCK are homozygous for the gene and maintained as such for ease of breeding.

Initial characterization of this model demonstrated that the progression and severity of the PKD in this model is more severe in males; however, the polycystic liver disease was not gender dependent.45,46 We therefore use only male PCK and male Sprague-Dawley rats obtained from Charles River (Wilmington, MA). In 2002, Ward et al.2 demonstrated that the mutation in the PCK rat was an exon deletion in the rat homologue of human PKHD1.

**Immunoprecipitation and Western Analysis**

Control and cystic kidneys were first chopped in RIPA buffer supplemented with protease and phosphatase inhibitors (RIPA+), to minimize the contribution of cyst fluid protein to the total cellular protein. Tissue lysates were then extracted in fresh RIPA+ as described previously41 and were adjusted to an equal amount of protein (3.5 to 5 mg determined by BCA assay; Pierce, Rockford, IL) and equal volume.

For studying the active form of the protein of interest, (Src [pY418], EGFR [pY1068], ErbB2 [pY1221/1222], B-Raf [Th598/Ser601], p44/p42MAPK [Thr202/Tyr204], or p-ERK1/2), 700 µg of...
Src Inhibition: In Vivo Studies

BPK.

BPK (cystic) and BALB/c (wild-type control) pups received SKI-606 at 30 mg/kg per d by i.p., starting at PN7. This dosage was based on published pharmacologic and pharmacokinetic data of SKI-606 treatment in mice and preliminary dosage-response studies (data not shown). Animals were treated from PN7 to PN20 (14 doses). Kidney and liver were routinely harvested at P21 and heart, spleen, pancreas, stomach, and thymus were periodically harvested at P21 to evaluate possible toxicity of vehicle or SKI-606. Control animals for these studies included SKI-606–treated and untreated (vehicle only) control (Balg/C) pups and cystic (BPK) animals. A total of 29 cystic and 41 control animals were analyzed at the 30-mg/kg per d dosage. The vehicle used for immunoprecipitation injections was 2% Tween 80 and 0.5% methylcellulose in water.

PCK.

Male PCK (cystic) and male Sprague-Dawley (wild-type control) pups received i.p. SKI-606 at 30 mg/kg per d, starting at PN7. This dosage was based on published pharmacologic and pharmacokinetic data of SKI-606 treatment in rodents. Animals were treated daily from PN7 to PN89 (82 doses). Kidney and liver were routinely harvested at P90, and heart, spleen, pancreas, stomach, and thymus were periodically harvested at P70 to evaluate possible toxicity of vehicle or SKI-606. Control animals for these studies included SKI-606–treated and untreated (vehicle only) Sprague-Dawley and PCK animals. A minimum of 15 cystic and 12 control animals were analyzed at the 30-mg/kg per d dosage. The vehicle used for i.p. injections was 2% Tween 80 and 0.5% methylcellulose in water.

Histology, Immunohistology, and Determination of Segmental Nephron Cyst Localization

All renal and hepatic tissues were fixed in 4.0% paraformaldehyde containing phosphatase inhibitors (04-906-8450-001; PhosSTOP; Roche Applied Science, Indianapolis, IN) in phosphate buffer (pH 7.4) for 90 min at 4°C, dehydrated in graded acetone, and embedded in Immunobed (Polysciences, Warrington, PA). Segmental nephron cyst localization and CT CI were quantified in each experimental group by combining morphometric analysis with light microscopy and immunohistology as described previously.

To determine renal cystic volume in PCK kidneys, 4-μM transverse tissue sections, including cortex, medulla, and papilla, were stained with hematoxylin. Image analysis procedures were performed with Meta-Morph software (Molecular Devices, Downingtown, PA). Cystic percentage was measured as a ratio of cystic area to a total section area.

Kidney Weight/Body Weight Ratio and Renal CI

At PN21, control and cystic treated and untreated (vehicle only) animals were weighted, as were both excised kidneys. Total kidney weights to body weight ratios were calculated. The degree of CT cyst formation was quantitatively assessed by segment-specific morphometric analysis as described previously and expressed as the CI. Cyst localization was determined by segment-specific lectin binding using Dolichos biflorus agglutinin as a marker for CT and Lotus tet-

Antibodies

To increase specificity, we used rabbit polyclonal antibodies to pan or total proteins to immunoprecipitate the protein of interest, which was then detected by Western analysis using phospho-specific proteins. Pan c-Src (44-656G) and active c-Src (pY148; 44-660G) were obtained commercially from Biosource/Invitrogen (Camarillo, CA). Antibodies including EGFR (2232; for immunoprecipitation) and phospho-EGFR (Tyr 1068), p44/42 MAPK (9102) and phospho-p44/42-MAPK (Thr202/Tyr204; 9101), and phospho-ErbB2 (Tyr1221/1222) were purchased from Cell Signaling Technology (Beverly, MA). EGFR (sc2003 for Western blot), ErbB2 (sc-284); B-Raf (sc-9002 and phospho-B-Raf (Thr598/Ser601) were obtained commercially from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal to β-actin (ab8227) was purchased from Abcam (Cambridge, MA; or 600-401-886 from Rockland Immunochemicals; Gilbertsville, PA) and used to monitor protein quantities subjected to immunoprecipitation.

Src Inhibition: SKI-606

SKI-606 is 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinase, which was obtained under a standard MTA from Wyeth Research (Pearl River, NJ). SKI-606 inhibits Src activity in an enzyme assay with an IC50 of 1.2 nM, inhibits anchorage-independent growth of Src-transformed fibroblasts with an IC50 of 100 nM, and inhibits Src-dependent protein tyrosine phosphorylation at comparable or lower concentrations.

SKI-606 has an excellent pharmacologic profile after oral administration with a serum half-life of 8.6 h and tissue inhibition of Src (pY-418) >60% at 24 h. SKI-606 inhibits other Src family kinases but does not directly inhibit growth factor receptor tyrosine kinases such as EGFR receptors 1 and 2, PDGF receptor, IGF-I receptor, fibroblast growth factor receptor, and serine-threonine kinases such as AKT and Cdk4.

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Src Inhibition in ARPKD

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ragonolobus as a marker for proximal tubules. For each treatment group, a CT CI was determined on at least 19 affected pups.

Treated and untreated (vehicle only) PKD and Sprague-Dawley animals were weighed at PN90, and kidneys and liver were removed and weighed. Renal cystic volumes were determined by evaluating 10 evenly spaced, 4-µM-thick renal sections (spaced through the tissue block half-kidney) that included cortex, medulla, and papilla, stained with hematoxylin. Image analysis procedures were performed with Meta-Morph software. Cystic percentage was measured as a ratio of cystic area to a total section area.

Hepatic BDE
After routine histologic preparation, eight evenly spaced (at least 32 µM apart), 4-µM-thick, hematoxylin-stained liver sections from the left lobe were graded (0 to 4) for BDE and biliary epithelial proliferation as described previously.

Analysis of Renal Function and Maximal Urinary Concentrating Ability
Animals were deprived of water for 12 h before collection of urine samples for urine osmolarity measurements. Blood samples were obtained by cardiac puncture for serum BUN and creatinine measurements. Serum BUN and creatinine were determined on an automated clinical chemistry analyzer as described previously.

cAMP Content of Whole Kidneys
The kidneys were ground to fine powder under liquid nitrogen in a stainless steel mortar and homogenized in 10 volumes of 0.1 M HCl in a glass-Teflon tissue grinder. After centrifugation at 600 × g for 10 min at room temperature, the supernatants were processed without acetylation using an enzyme immunoassay kit (CA-200; Sigma-Aldrich, St. Louis, MO). The results were expressed in pmol/mg protein.

Statistical Analysis
The significance of differences between experimental groups was determined by a one-way ANOVA with GraphPad Prism 5 (GraphPad, San Diego, CA). Results are expressed as means ± SD.

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