FAK Inhibition Abrogates the Malignant Phenotype in Aggressive Pediatric Renal Tumors

Michael L. Megison, Lauren A. Gillory, Jerry E. Stewart, Hugh C. Nabers, Elizabeth Mrozcek-Musulman, and Elizabeth A. Beierle

Abstract

Despite the tremendous advances in the treatment of childhood kidney tumors, there remain subsets of pediatric renal tumors that continue to pose a therapeutic challenge, mainly malignant rhabdoid tumors and nonosseous renal Ewing sarcoma. Children with advanced, metastatic, or relapsed disease have a poor disease-free survival rate. Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that is important in many facets of tumor development and progression. FAK has been found in other pediatric solid tumors and in adult renal cellular carcinoma, leading to the hypothesis that FAK contributes to pediatric kidney tumors and would affect cellular survival. In the current study, FAK was present and phosphorylated in pediatric kidney tumor specimens. Moreover, the effects of FAK inhibition upon G401 and SK-NEP-1 cell lines were examined using a number of parallel approaches to block FAK, including RNA interference and small-molecule FAK inhibitors. FAK inhibition resulted in decreased cellular survival, invasion and migration, and increased apoptosis. Furthermore, small-molecule inhibition of FAK led to decreased SK-NEP-1 xenograft growth in vivo. These data deepen the knowledge of the tumorigenic process in pediatric renal tumors, and provide desperately needed therapeutic strategies and targets for these rare, but difficult to treat, malignancies.

Implications: This study provides a fundamental understanding of tumorigenesis in difficult to treat renal tumors and provides an impetus for new avenues of research and potential for novel, targeted therapies. Mol Cancer Res; 12(4); 514–26. ©2014 AACR.

Introduction

Significant improvements in the outcome of children with kidney malignancies have been achieved over the past 20 years. Advances in medical and surgical care, and the use of cooperative trials have resulted in 5-year survival rates for Wilms tumor that exceed 90% (1). There remain, however, subsets of pediatric solid renal tumors that have not recognized a significant improvement in survival. These tumors include malignant rhabdoid kidney tumors (MRKT) and renal Ewing sarcomas. MRKTs are aggressive malignancies that comprise 2% of all pediatric renal tumors (2). Children diagnosed with these tumors often have metastatic disease (2, 3) and despite aggressive treatment, their overall survival rate is less than 30% (2). Solid organ sarcomas such as extrarenal Ewing sarcomas are also rare and difficult pediatric kidney tumors (4) with a 5-year disease-free survival rate less than 50% (5).

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that localizes to focal adhesions and controls a number of cellular pathways involved in cell adhesion, migration, invasion, proliferation, and survival (6–9). FAK activation occurs when cell surface integrins bind to β subunits of FAK, resulting in phosphorylation and binding of Src family kinases (6). FAK also has an autophosphorylation site at the tyrosine 397 (Y397) residue (10), which leads to increased cell survival via inhibition of detachment-activated apoptosis, or anoikis (11). FAK is overexpressed in many human adult tumors, including breast and colon cancer (12–14) has been shown to decreased tumor cell survival. FAK inhibition with siRNA (14, 15) has been shown to decrease tumor cell survival. However, it is believed that Y397 autophosphorylation site is the primary site responsible for the role of FAK in tumor cell migration, invasion, and survival. Abrogation of FAK phosphorylation at this site using dominant negative constructs (16) and small-molecule inhibitors (15, 17–19) has been shown to decreased tumor cellular migration, invasion, and survival.

In the current study, we hypothesized that rare pediatric renal tumors would express FAK, and that inhibition of FAK would result in a less aggressive phenotype in these cell lines. We demonstrated that abrogation of FAK in renal tumor cell lines resulted in decreased tumor cell survival in vitro and...
decreased xenograft growth in vivo. From these studies, we concluded that targeting FAK might prove to be a useful therapeutic modality in the treatment of these rare, but aggressive, pediatric renal tumors.

Materials and Methods

Cells and cell culture
The renal tumor cell lines G401 and SK-NEP-1 were used. The MRKT cell line, G401 (CRL-1441; American Type Culture Collection, ATCC; ref. 20) was maintained in McCoy’s medium (30-0207; ATCC) containing 10% non–heat-inactivated FBS (Hyclone), 1 μg/mL penicillin (Gibco), 1 μg/mL streptomycin (Gibco), and 2 mmol/L L-glutamine (ThermoFisher Scientific Inc.) at 37°C and 5% CO2. SK-NEP-1 cells, previously described as a renal Ewing sarcoma (21), were obtained from ATCC (HTB-48) and maintained in McCoy’s medium (ATCC) containing 15% FBS, 1 μg/mL penicillin/streptomycin and 2 mmol/L L-glutamine (ThermoFisher Scientific) at 37°C and 5% CO2. Mouse endothelial fibroblasts with (MEF^{FAK/−/−}) and without (MEF^{FAK/−+}) FAK expression provided a control for FAK detection and were a kind gift from Dr. Elena Kurenova; Roswell Park Cancer Institute, Buffalo, NY (22). They were maintained under standard conditions in Dulbecco’s Modified Eagle Medium with 10% FBS, 2 mmol/L L-glutamine, and 1 μg/mL penicillin/streptomycin.

Antibodies and reagents
Monoclonal mouse anti–FAK (4.47, 05-0537) and anti–phospho–Src (Tyr 416, 05-677) antibodies were obtained from Millipore (EMD Millipore) and rabbit polyclonal anti–phospho–FAK (Y397, 71-7900) and anti–phospho–Erk 1/2 (05-797R) antibodies were from Invitrogen (Invitrogen Corp.). Rabbit polyclonal anti–PARP (9542S), anti–Akt (9272), anti–phospho–Akt (Ser 473, 9271), anti–Erk 1/2 (9102), and anti–cleaved caspase-3 (9662) antibodies were obtained from Cell Signaling Technology. Monoclonal mouse anti–Jnk (F-3, sc-1648), anti–phospho–Jnk (G-7, sc-6254), and rabbit polyclonal anti–c–Src (sc-18) were from Santa Cruz Biotechnology. Monoclonal mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Millipore (MAB374) and anti–β-actin was from Sigma (A1978; Sigma-Aldrich Corp.). The small molecule PF-573,228 (PF; C_{32} H_{29}F_{3}N_{5}O_{5}S) was obtained from Pfizer, and the small molecule 1,2,4,5-benzenetetramine tetrahydrochloride (Y15; C_{6}H_{10}N_{4}·4ClH) was obtained from Sigma.

Human tissue specimens
Formalin-fixed, paraffin-embedded pediatric renal tumor specimens were obtained under waiver of informed consent from the tumor repository at our institution and from the Children’s Oncology Group after institutional review board approval (X111123007).

Immunohistochemistry
Formalin-fixed, paraffin-embedded human tumor or xenograft tumor specimens were sectioned into 6 μm sections and baked at 70°C for 1 hour on positive slides. Slides were deparaffinized, steamed, sections quenched with 3% hydrogen peroxide and blocked with BPS blocking buffer (bovine serum albumin, powdered milk, Triton X-100, PBS) for 30 minutes at 4°C. The primary antibodies anti–FAK (4.47), 1:100 (05-537; Millipore) and anti–phospho–FAK (Y397), 1:100 (04-974; Millipore) were added and incubated overnight at 4°C. After washing with PBS, the secondary antibodies for mouse (1μmPress MP-77402; Vector Laboratories) and rabbit (Super Picture HRP and 87-9263 from Zymed Laboratories) were added 1:250 dilution for 1 hour at 22°C. The staining reaction was developed with the VECTASTAIN Elite ABC Kit (PK-6100; Vector Laboratories), TSA (biontin tyramide reagent, 1:400; PerkinElmer Inc.) and DAB (Metal Enhanced DAB Substrate; ThermoFisher Scientific). Slides were counterstained with hematoxylin. Negative controls [mouse immunoglobulin G, IgG (1 μg/mL; Invitrogen) or rabbit IgG (1 μg/mL; EMD Millipore)] were included with each run.

Immunohistochemical scoring
Stained slides of human tumors were reviewed and scored by a pediatric pathologist (E. Mrozek-Musulman) blinded to the patients. The staining was evaluated using a weighted stain score, that graded staining intensity (0, none; 1, weak; 2, moderate; 3, strong) and multiplied it by the percentage of tumor cells within each category. For example, if the specimen showed moderate staining (2) in 40% of the cells, the stain score would be 80 (2 × 40 = 80).

Immunoblotting
Western blots were performed as previously described (19). Briefly, whole cell lysates or homogenized xenograft specimens were isolated using radioimmunoprecipitation assay buffer (10 mmol/L Tris base pH 7.2, 150 mmol/L NaCl, 1% Na-deoxycholate, 1% Triton X-100, 0.1% SDS) or mTOR lysis buffers supplemented with protease inhibitors (Sigma), phosphatase inhibitors (Sigma), and phenylmethylsulfonylfluoride. Lysates were cleared by centrifugation at 14,000 rpm for 30 minutes at 4°C. Protein concentrations were determined using BCA Protein Assay Reagent (Pierce) and separated by electrophoresis on SDS-PAGE. Antibodies were used according to manufacturer’s recommended conditions. Molecular weight markers (Precision Plus Protein Kaleidoscope Standards; Bio-Rad) confirmed the expected size of the target proteins. Immunoblots were developed with Luminata Classico or Crescendo ECL (EMD Millipore). Blots were stripped with stripping buffer (bovine serum albumin, powdered milk, Triton X-100, PBS) for 30 minutes at 4°C for 1 hour and then reprobed with selected antibodies. Equal protein loading was confirmed with immunoblotting with antibody to GAPDH or β-actin.

siRNA transfection
siRNAs were obtained from Qiagen for the following FAK target sequence: 5’-CCGGTGCAAATGATAAGGTGTA-3’. Cells were plated (3 × 10^5 cells per well) and allowed

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to attach overnight. Cells were treated with HiPerFect (Qiagen) alone, HiPerFect plus 20 nmol/L negative control siRNA (1027310; Qiagen), or HiPerFect plus FAK siRNA [Hs_PTK2_10 FlexiTube siRNA (NM_005607; Qiagen)] according to the manufacturer’s protocol, incubated for 24 to 48 hours following transfection and then used for experiments. FAK inhibition by siRNA was confirmed using immunoblotting.

Cell viability assays

Cell viability was measured with alamarBlue assays and trypan blue exclusion. Briefly, 1.5 × 10³ cells per well were plated on 96-well culture plates, allowed to attach, and treated with RNA interference inhibition, PF-573,228 (Pfizer), Y15 (Sigma), or both PF-573,228 and Y15. Following treatment, either 10 μL of alamarBlue dye (Invitrogen) was added and after 4 to 6 hours, the absorbance at 595 nm was measured using a kinetic microplate reader (BioTek Gen5; BioTek Instruments), or cell viability was determined using trypan blue exclusion and cell counting with a hemacytometer. Viability was reported as fold change.

Migration assay

Twelve-well culture plates (TransWell; Corning Inc.) with 8-μm micropore inserts were used. The bottom side of the insert was coated with Matrigel (BD Biosciences) (1 mg/mL, 50 μL for 4 hours at 37°C). G401 cells were treated with PF-573,228 or Y15 and were placed into the upper well at a concentration of 5 × 10³ cells per well. Cells were allowed to migrate through the micropore insert for 24 hours. The inserts were then fixed with 3% paraformaldehyde, stained with crystal violet, and migrated cells counted with a light microscope. Migration was reported as fold change. This assay was only performed with the G401 cells because the SK-NEP-1 cells propagate in a nonadherent fashion.

Cellular invasion assay

Similar to migration, 12-well culture plates (TransWell; Corning) with 8-μm micropore inserts were used. The top side of the insert was coated with Matrigel (BD Biosciences) (1 mg/mL, 50 μL for 4 hours at 37°C). G401 cells were treated with PF-573,228 or Y15 and plated into the upper well at a concentration of 5 × 10³ cells per well. Cells were allowed to invade into the Matrigel (BD Biosciences) layer for 48 hours. The inserts were then fixed with 3% paraformaldehyde, stained with crystal violet, and cells counted with a light microscope and invasion reported as fold change.

Attachment-independent growth assay

Attachment-independent growth was determined by soft agar assay. A base layer of complete culture media in 1% noble agar was established in 60-mm culture dishes. SK-NEP-1 cells were plated at 1 × 10³ cells per dish in the top layer composed of the same culture media and agar mixture. Dishes were treated with graduated concentrations of Y15, and retreated every 4 days. After incubation for 6 weeks, colonies were imaged and quantified using the Gel Dock Imager (Bio-Rad) and Quantity One Software (Bio-Rad), and colony counts reported as fold change.

In vivo tumor growth

Six-week-old, female, athymic nude mice weighing approximately 20 grams, were used (Harlan Laboratories Inc.). The mice were maintained in the specific pathogen-free animal facility with standard 12-hour light/dark cycles and allowed chow and water ad libitum. All experiments were performed after obtaining protocol approval by the Institutional Animal Care and Use Committee (120209355) and in compliance with the institutional, national, and NIH animal use guidelines. Human renal Ewing sarcoma cells, SK-NEP-1 (1.5 × 10⁶ cells/50 μL sterile PBS) were injected into the subcapsular space of the left kidney. After 2 weeks, the animals were randomized and began twice daily treatments with intraperitoneal injection of either control vehicle (saline, 100 μL, n = 7) or Y15 (15 mg/kg, 100 μL, n = 8). Previous experiments with various doses and dosing schedules of the compound proved this dosage to be well tolerated (19, 23). After 2 weeks of treatment, the animals were euthanized with CO₂ and bilateral thoracotomy and the kidney tumors and lungs were harvested.

Determination of metastases

Three levels from each lung of the animals from the SK-NEP-1 xenografts (above) were formalin-fixed (10% buffered formalin), paraffin-embedded and stained by routine hematoxylin and eosin (H&E). Three levels from each lung were examined by a board-certified pediatric pathologist (E. Mrozcek-Musulman) to determine the presence or absence of metastases.

Data analysis

Experiments were repeated at least in triplicate, and data reported as mean ± standard error of the mean. Densitometry of Western blots was performed using the image histogram analysis feature of Adobe Photoshop software (Adobe Systems Inc.). The Student t test, Fisher exact test, or ANOVA was used as appropriate to compare data between groups. Statistical significance was determined at the P < 0.05 level.

Results

FAK was present in pediatric renal tumor specimens and cell lines

Immunohistochemistry was performed on 55 human pediatric renal tumor specimens, including 12 human MRKT tissue specimens. Other tumor types included Wilms tumor (19), clear cell sarcoma (12), and mesoblastic nephroma (12). FAK staining was detected in 75% of MRKTs, compared with 33% of clear cell sarcomas, 32% of Wilms tumors, and 8% of mesoblastic nephromas (Table 1). The number of MRKT specimens staining positive for FAK was significantly greater than those of Wilms tumor or mesoblastic nephroma (Table 1). The median FAK stain score for all tumor types, except the MRKT, was 0 with...
scores ranging from 0 to 300 for the Wilms, 0 to 160 for clear cell sarcoma, and 0 to 60 for the mesoblastic nephroma tumor specimens. For the MRKT specimens, the median FAK stain score was 15 (range, 0–300; Fig. 1B). FAK was phosphorylated in 50% of MRKTs, compared with 21% of Wilms tumors and 8% of clear cell sarcomas (Table 1). FAK was not phosphorylated in any of the mesoblastic nephroma specimens (Table 1). Representative photomicrographs are presented in Fig. 1A. There was weakly positive FAK staining in the normal kidney specimens (no tumor) localized to the renal tubules (Fig. 1A, closed arrows, top left). Staining for phosphorylated FAK was absent in the normal

<table>
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<th>Tumor type</th>
<th>n</th>
<th>FAK 4.47 (%)</th>
<th>pFAK Y397 (%)</th>
<th>P (vs. MRKT)</th>
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<td>12</td>
<td>9 (75)</td>
<td>6 (50)</td>
<td>—</td>
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<tr>
<td>Clear cell sarcoma</td>
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<td>4 (33)</td>
<td>1 (8)</td>
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<td>6 (32)</td>
<td>4 (21)</td>
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<tr>
<td>Mesoblastic nephroma</td>
<td>12</td>
<td>1 (8)</td>
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NOTE: P < 0.05 considered significant (bolded).

Abbreviations: FAK 4.47, total FAK protein; NS, not statistically significant; pFAK Y397, FAK phosphorylated at tyrosine 397 residue.

Figure 1. FAK in pediatric renal tumor specimens and cell lines. A, immunohistochemistry staining with antibodies specific for FAK and phospho-FAK was performed on 55 formalin-fixed, paraffin-embedded human pediatric renal tumor specimens (12 MRKT, 12 clear cell sarcoma, 19 Wilms tumor, and 12 mesoblastic nephroma). Representative photomicrographs presented show weak FAK staining in the epithelium of the renal tubules (top left, closed arrows) but no FAK phosphorylation (top right) in normal human kidney. Staining for FAK and phospho-FAK was not present in the majority of Wilms tumor specimens (middle). Strong staining for FAK and phospho-FAK was noted in most of the MRKT specimens (bottom). Negative controls were included with each run (insets, bottom). B, stain scores were calculated for the immunohistochemistry specimens listed above and reported as the range and median (bar). All tumor types, except for the MRKT, had a median stain score of 0. The median stain score for the MRKT specimens was 15. C, immunoblotting for Y397 FAK and total FAK was performed on SK-NEP-1 and G401 renal tumor cell lysates. FAK was detected in both of the renal tumor cell lines and was phosphorylated. Lysates from mouse endothelial fibroblasts with (MEF^+/+) and without (MEF^−/−) FAK served as controls.
kidney specimens (Fig. 1A, top right). FAK and phosphorylated FAK staining was also not detected in many Wilms tumor specimens (Fig. 1A, middle). There was strong staining, both for total and phosphorylated FAK, in most of the MRKT specimens (Fig. 1A, brown stain, bottom). Negative controls responded appropriately (Fig. 1A, small box insets, bottom).

Immunoblotting was used to evaluate FAK expression in the SK-NEP-1 and G401 renal tumor cell lines. SK-NEP-1 and G401 whole cell lysates were compared with those of mouse endothelial fibroblasts with (MEF^FAK+/+) and without (MEF^FAK−/−) FAK. FAK was detected in both of the renal tumor cell lines and was phosphorylated (Fig. 1C). The FAK expression and phosphorylation in the positive (MEF^FAK+/+) and negative (MEF^FAK−/−) cell lysates were appropriate (Fig. 1C).

**FAK inhibition with siRNA decreased cell survival, migration, and invasion**

We initially studied the effects of FAK silencing with siRNA on G401 and SK-NEP-1 cell viability. G401 and SK-NEP-1 cells were treated with HiPerFect alone (control), HiPerFect plus Negative Control siRNA, or HiPerFect plus siRNA specific for FAK (siFAK) for 24 hours. A, immunoblotting for FAK showed decreased FAK expression in the G401 cells with 20 nmol/L siFAK and in the SK-NEP-1 cells with 40 nmol/L siFAK. Treatment with negative control siRNA did not affect FAK expression. B, G401 and SK-NEP-1 cells were treated with siFAK for 24 hours (20 and 40 nmol/L, respectively), cellular viability was measured using trypan blue exclusion, and reported as fold change in viability. In both cell lines, viability decreased significantly following treatment with siFAK. Cell viability was not affected by the negative control siRNA treatment. Experiments were repeated at least in triplicate and data reported as mean fold change ± SEM. C, G401 and SK-NEP-1 cells were treated with increasing doses of siFAK for 24 hours and lysates examined with immunoblotting for PARP cleavage products. Increasing amounts of siFAK led to PARP cleavage, indicating apoptosis. Negative control siRNA did not result in PARP cleavage in either cell line. *, P < 0.05 siFAK vs. Control or siNeg.

Figure 2. FAK inhibition with siRNA in G401 and SK-NEP-1 human renal tumor cell lines. G401 and SK-NEP-1 cells were treated with HiPerFect alone (control), negative control siRNA (siNeg), or siRNA specific for FAK (siFAK, 20, 40, 60 nmol/L) for 24 hours. A, immunoblotting for FAK showed decreased FAK expression in the G401 cells with 20 nmol/L siFAK and in the SK-NEP-1 cells with 40 nmol/L siFAK. Treatment with negative control siRNA did not affect FAK expression. B, G401 and SK-NEP-1 cells were treated with siFAK for 24 hours (20 and 40 nmol/L, respectively), cellular viability was measured using trypan blue exclusion, and reported as fold change in viability. In both cell lines, viability decreased significantly following treatment with siFAK. Cell viability was not affected by the negative control siRNA treatment. Experiments were repeated at least in triplicate and data reported as mean fold change ± SEM. C, G401 and SK-NEP-1 cells were treated with increasing doses of siFAK for 24 hours and lysates examined with immunoblotting for PARP cleavage products. Increasing amounts of siFAK led to PARP cleavage, indicating apoptosis. Negative control siRNA did not result in PARP cleavage in either cell line. *, P < 0.05 siFAK vs. Control or siNeg.
PF-573,228 inhibited FAK and led to decreased cell survival and motility in G401 and SK-NEP-1 cell lines

To further illustrate the effects of FAK knockdown and prepare for in vivo studies, we studied FAK inhibition with a small molecule in both the G401 and SK-NEP-1 cell lines. PF-573,228 is a small molecule that targets the ATP-binding pocket of FAK and has been shown in multiple cell lines to block FAK phosphorylation at the tyrosine 397 (Y397) site (24). Cells were treated with PF-573,228 at increasing concentrations. Immunoblotting was used to confirm FAK abrogation. After 24 hours of treatment, PF-573,228 decreased FAK phosphorylation in both cell lines (Fig. 3A). AlamarBlue assays were used to assess the effects of PF-573,228–induced FAK inhibition on cell survival. Both G401 and SK-NEP-1 cell lines showed significantly decreased cell survival following treatment with PF-573,228 (Fig. 3B). The calculated LD_{50} for PF-573,228 in the G401 cell line was 4.7 μmol/L and in the SK-NEP-1 cell line was 11.4 μmol/L. There was an increase in cleaved PARP expression in both cell lines after treatment with PF-573,228 (Fig. 3C), indicating that decreased cell viability was due to apoptosis. Caspase-3 cleavage further confirmed apoptosis in the SK-NEP-1 cell line following PF-573,228 treatment (Supplementary Fig. S1A and S1B).

FAK also affects cellular migration and invasion (15, 25); therefore, we wished to determine if these effects were also present in our cell lines. Figure 3, A–E, shows the effects of PF-573,228 on cell migration and invasion. Immunoblotting showed increased cleaved PARP following treatment with PF-573,228 in both cell lines, indicating apoptosis. D, G401 cells were treated with increasing concentrations of PF-573,228 and allowed to migrate through a micropore insert. Migration was reported as fold change in the number of cells migrating through the membrane. Cellular migration was significantly diminished following treatment with PF-573,228. These effects were seen at a concentration of 1 μmol/L PF-573,228. E, G401 cells were treated with increasing concentrations of PF-573,228 and allowed to invade through a Matrigel-coated micropore insert. Cells were counted and invasion reported as fold change. Invasion, similar to migration, was significantly decreased after exposure to 1 μmol/L PF-573,228. All experiments were repeated at least in triplicate and data reported as mean fold change ± SEM.

Figure 3. PF-573,228 (PF) inhibition of FAK in human renal tumor cell lines. A, G401 and SK-NEP-1 cell lines were treated for 24 hours with increasing concentrations of PF-573,228. Cell lysates were harvested and evaluated with immunoblotting for total FAK and FAK Y397. Densitometry was performed, and FAK phosphorylation was reported as a ratio between the densities of the Y397 band to the total FAK band. Increasing concentrations of PF-573,228 led to decreased FAK phosphorylation (Y397) in both cell lines. B, AlamarBlue assays were used to assess cell survival. Both G401 and SK-NEP-1 cell lines showed significantly decreased cell survival following treatment with PF-573,228 (Fig. 3B). The calculated LD_{50} for PF-573,228 in the G401 cell line was 4.7 μmol/L and in the SK-NEP-1 cell line was 11.4 μmol/L. There was an increase in cleaved PARP expression in both cell lines after treatment with PF-573,228 (Fig. 3C), indicating that decreased cell viability was due to apoptosis. Caspase-3 cleavage further confirmed apoptosis in the SK-NEP-1 cell line following PF-573,228 treatment (Supplementary Fig. S1A and S1B).

FAK inhibition decreases kidney tumor growth.
entities would be affected by abrogation of FAK. Due to the nonadherent nature of the cell line, the SK-NEP-1 cells were not amenable to the Transwell method of studying migration or invasion. G401 cells were treated with increasing concentrations of PF-573,228 and allowed to migrate through the micropore membrane. There was a significant decrease in cellular migration following PF-573,228 treatment (Fig. 3D), and this decrease occurred at concentrations of PF-573,228 below the calculated LD₅₀.

For invasion, G401 cells were treated with increasing concentrations of PF-573,228 and allowed to invade through a Matrigel layer. Similar to the migration findings, there was a significant decrease in cellular invasion following PF-573,228 treatment in the G401 cells (Fig. 3E), that again, occurred at concentrations below the calculated LD₅₀.

**Y15 treatment inhibited FAK and led to decreased cell survival**

PF-573,228 was not formulated for use in vivo (24) and we wished to advance these studies to an animal model. Therefore, we chose to use Y15, one of only a few small-molecule FAK inhibitors that can be used in animals (18, 19). Y15 has been previously described and was designed to inhibit Y397 phosphorylation of FAK (17). Using immunoblotting, we showed that Y15 treatment resulted in decreased FAK phosphorylation in both the G401 and the SK-NEP-1 cell lines (Fig. 4A). Next, we examined how Y15...
treatment affected cell survival using alamarBlue assays. Both G401 and SK-NEP-1 cell lines showed significantly decreased cell survival following treatment with Y15 (Fig. 4B). The calculated LD_{50} for Y15 was 3.3 μmol/L in the G401 and 18.2 μmol/L in the SK-NEP-1 cell line. Additionally, the cell death caused by Y15 in both cell lines was via apoptosis, as demonstrated by decreased total PARP and increased PARP cleavage by immunoblotting (Fig. 4C and D). In the SK-NEP-1 cell line following Y15 treatment there was cleavage of caspase-3 further showing apoptosis (Supplementary Fig. S1B).

To determine whether the two FAK inhibitors would have synergistic effects when used in combination, we performed alamarBlue assays with the G401 and SK-NEP-1 cell lines following treatment with PF-573,228 or Y15 alone and in combination (Supplementary Fig. S1D and S1E). The combination index was calculated using the method described by Chou (26) and was nearly one for both cell lines, indicating that these two inhibitors primarily had an additive effect rather than a synergistic effect on cell viability when used together (Supplementary Fig. S1D and S1E).

FAK inhibition with Y15 decreased cell migration, invasion, and attachment-independent growth

Phenotypic changes in the G401 and SK-NEP-1 tumor cells following Y15 treatment were further evaluated with cellular migration, invasion, and attachment-independent growth assays. G401 cells were treated with increasing concentrations of Y15. After 24 hours, the cells showed a marked decrease in migration at concentrations of Y15 below the LD_{50} concentration (Fig. 5A). Invasion was also significantly decreased in the G401 cells after Y15 treatment (Fig. 5B). Attachment-independent growth using soft agar assays is considered one of the best measures of cellular invasion, and was used to measure invasion following Y15-induced FAK inhibition in the SK-NEP-1 cell line. SK-NEP-1 cells were treated with increasing concentrations of Y15, placed into soft agar and colonies were allowed to grow for 6 weeks. The number of cell colonies detected at the end of the studies was decreased by 37.2% at a concentration (10 μmol/L, Y15) compared with controls (Fig. 5C) at a concentration below the calculated LD_{50} for Y15 (18.2 μmol/L).

Figure 5. 1,2,3,4-benzenetetraamine tetrahydrochloride (Y15) decreased cell migration, invasion, and attachment-independent growth. A, G401 cells were treated with increasing concentrations of Y15 for 24 hours and allowed to migrate through a micropore insert. Migration was reported as fold change in number of cells migrating through the membrane. Cellular migration was significantly decreased with Y15 treatment, beginning at 0.5 μmol/L concentration. B, G401 cells were treated with increasing concentrations of Y15 and allowed to invade through a Matrigel-coated micropore insert. Invasion was reported as fold change. Cellular invasion was significantly decreased with increasing concentrations of Y15. C, attachment-independent growth in soft agar was used to characterize tumor invasiveness in the SK-NEP-1 cells. Cells were treated with increasing concentrations of Y15, grown in soft agar for 6 weeks, and colonies were quantified. Colony count was significantly decreased with Y15 treatment compared with untreated cells. All experiments were repeated at least in triplicate and data reported as mean fold change ± SEM.
Y15 treatment resulted in decreased in vivo tumor growth in a nude mouse model of a pediatric renal tumor

An in vivo model of renal tumor growth following FAK inhibition was used to study tumor growth using female athymic nude mice. SK-NEP-1 renal Ewing sarcoma cells (1.5 × 10⁶) were injected into the subcapsular space of the left kidney of each mouse (n = 15). After 2 weeks, intraperitoneal injections with either control (saline, n = 7) or Y15 (n = 8) at 15 mg/kg bid were initiated. This dose was chosen based upon prior in vivo studies with Y15 (17–19, 27). Y15 treatment continued for 3 weeks, at which time the animals were euthanized and the tumors harvested (Fig. 6A). The incidence of tumor occurrence was not different between the treatment groups and all animals (n = 15) developed tumors. Animals treated with Y15 had significantly smaller tumor volumes compared with controls (6,198 ± 1,500 mm³ vs. 2,706 ± 635 mm³; control vs. Y15; P = 0.02; Fig. 6B), but Y15 treatment did not affect the weight of the animal (24.8 ± 1.0 g vs. 24.2 ± 1.3 g; control vs. Y15; P = 0.7). Y15 treatment did not affect the weight of the contralateral kidney (0.17 ± 0.02 g vs. 0.21 ± 0.01 g; control vs. Y15; P = 0.09). Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tumor samples for FAK Y397. There was less FAK Y397 staining in the tumors from animals treated with Y15 compared with vehicle-treated animals.
Discussion

Pediatric renal tumors, other than Wilms tumor, continue to pose a therapeutic challenge, highlighting the need for novel therapies. In this study, we explored the role of FAK in the tumorigenesis of two types of aggressive pediatric renal tumors, MRKts, and renal Ewing sarcoma. The rationale for the study of FAK in these tumors was 3-fold. First, FAK has been shown to play a key role in two other aggressive pediatric solid tumors, neuroblastoma (13, 15) and hepatoblastoma (23). In neuroblastoma, FAK was important not only for cell survival (13, 28), but also for development of metastases (15). In hepatoblastoma, FAK inhibition resulted in decreased cell survival in vitro and decreased xenograft tumor growth in vivo (23). Second, FAK has been found to be upregulated in the adult renal tumor, renal cell carcinoma (RCC), an aggressive tumor with survival rates around 50%. Jenq and colleagues compared FAK mRNA abundance in a metastatic Caki-1 RCC cell line with that from normal human renal cortex epithelial cells and found that there was a twice as much FAK mRNA in the Caki-1 cell line (29). Another group examined FAK phosphorylation at Y861 site by immunohistochemistry in 57 human RCC specimens and noted staining in over 30% of the samples, and on multivariate analysis, there was a correlation between FAK phosphorylation and cancer-specific survival (30). In another study, decreased FAK activity resulted in decreased RCC cell proliferation and migration (31). In addition, inhibition of FAK phosphorylation at Y397 in Caki and 786-O RCC cell lines with the small-molecule quinazoline (DZ-50) resulted in decreased viability, reduced adhesion, arrested cell cycle, and reduced metastatic potential in vivo (32). Finally, multiple other kinases that act either upstream or downstream from FAK have been identified as having a significant impact upon the tumorigenicity of other renal tumors (Fig. 6C). Representative photomicrographs are presented in Fig. 6C (40×). Negative controls for both total FAK (mouse IgG) and phospho-FAK (rabbit IgG) reacted appropriately (Fig. 6C, small insets, top). To further confirm target knockdown in the tumor specimens, immunoblotting for FAK was performed on tumor lysates. Tumors treated with Y15 showed a decrease in FAK phosphorylation (Y397) compared with those treated with saline (vehicle). A representative immunoblot is shown in Fig. 6D. Densitometry was used to compare immunoblots from a number of xenograft specimens. Phosphorylated FAK was expressed as a ratio to total FAK for each blot and normalized to β-actin for that blot, allowing for a comparison to be made between the saline-treated and the Y15-treated tumors. There was a significant decrease in the FAK phosphorylation (Y397) in the tumors from the animals treated with Y15 (Fig. 6E). Because these tumors are known to metastasize to the lungs, we also examined the lung tissue with H&E staining for the presence of metastases. There was no difference in the number of pulmonary metastases in the control versus the treated animals (3.3 ± 0.5 vs. 3.7 ± 0.4 metastases per slide, control vs. Y15, not significant).

In the current investigations, we reported that FAK was expressed in human pediatric renal tumor specimens, and was more prominent in tumor types that tended to be more aggressive. Our sample numbers were too small to use the FAK stain score as a disease prognosticator, and that was not the purpose of the current study. It was interesting to note, however, that the MRKT had a median stain score of 15 compared with a median score of 0 in the other types of pediatric renal tumors, including Wilms tumor. As previously mentioned, MRKts are highly aggressive tumors with an extremely poor prognosis, compared with Wilms tumors that often have a survival rate greater than 90%. We obtained two different pediatric renal tumor cell lines, G401 (MRKT) and SK-NEP-1 (renal Ewing sarcoma) and confirmed that FAK was present and was phosphorylated at the Y397 site in these cell lines. A review of the literature revealed that the characterization of FAK in pediatric renal tumors and cell lines has not been previously described.

The number of cell lines available for study of these rare, but deadly, pediatric renal tumors is limited. Both the G401 and SK-NEP-1 cell lines were originally thought to be Wilms tumor. The G401 cell line was characterized as a malignant rhabdoid tumor of the kidney, an entity completely distinct from Wilms tumor (21). Subsequently, this cell line has been used by numerous investigators to study rhabdoid tumor types. Another cell line that we chose was the SK-NEP-1 cell line. Again, originally classified as a Wilms tumor, this cell line has recently been further characterized and found to express EWS-FL1 gene fusion transcripts, changing the classification of SK-NEP-1 cell line from a Wilms tumor to a nonosseous renal Ewing sarcoma (20). The SK-NEP-1 cell line was chosen for the current study because nonosseous renal Ewing sarcomas, like MRKT, are rare and clinically difficult to treat tumors.

In the current investigations, it was noted that the SK-NEP-1 cell line had more FAK expression detected by immunoblotting at baseline than the G401 cell line, although the FAK phosphorylation was greater in the G401 cells. These findings may potentially explain the finding that the SK-NEP-1 cell line required a higher concentration of siRNA to abrogate total FAK expression and increase apoptosis than the G401 cell line. The SK-NEP-1 cells simply had more FAK to knockdown. In...
addition, the cellular responses to inhibition of FAK phosphorylation with PF-573,228 and Y15 may be explained in a similar fashion. It seemed as if the G401 cell line was more dependent upon FAK phosphorylation for function than the SK-NEP-1 cell line, which may simply be due to the degree of FAK phosphorylation. These findings could also be explained by the idea of oncogene addiction. Investigators have postulated that certain tumor cell lines are physiologically more dependent upon specific survival factors, and inhibition of these specific cellular factors will have a more profound effect upon those cells than others even of the same tumor type (38).

The small-molecule FAK inhibitor, PF-573,228, was used in these studies to corroborate the siRNA findings that FAK was an important survival signal and a potential target in these rare and difficult to treat renal tumors. PF-573,228 has been shown to block the catalytic activity of FAK by binding to the ATP pocket of FAK (24). This molecule has been noted to decrease cell survival, migration, and invasion in neuroblastoma cell lines (15) as well as decrease metastatic potential of breast cancer cells (39) and enhance chemotherapy-induced cytotoxicity in pancreatic cancer cell lines (40). Realizing that off-target effects are a risk when using small-molecule inhibitors, PF-573,226 was chosen over a similar FAK inhibitor, PF-562,271, in an effort to minimize off-target effects. PF-562,271, in addition to inhibiting FAK, also affects PyK2 (41), which PF-573,228 does not. In this study, PF-573,226 effectively diminished FAK phosphorylation and increased cell death in both the G401 and SK-NEP-1 cell lines. Additionally, this inhibitor also diminished the migration and invasion of the G401 cells at concentrations below the LD50 for the cells.

Because we wished to advance our studies to an animal model and PF-573,228 was not suitable for use in vivo, another small-molecule FAK inhibitor, Y15 (1,2,4,5, benzenetetraamine tetrahydrochloride), was used. Again, this small-molecule inhibitor was used to corroborate the findings that FAK provided a tumorigenic signal for these tumors. Y15 has been previously described in the literature (17) and has been used for FAK inhibition in vitro (15, 17, 18, 27). Y15 is also one of only a few small-molecule FAK inhibitors available for use in animal studies (17, 19, 27). In a mouse xenograft model using BT474 human breast cancer cells, animals treated with Y15 (30 mg/kg/day) had significantly smaller tumors than vehicle-treated animals (17). The addition of Y15 to gemcitabine treatment was shown to have an additive effect on survival in a nude mouse model of pancreatic cancer (18). In another pediatric solid tumor, neuroblastoma, Y15-induced FAK inhibition resulted in a significant decrease in both flank xenograft tumor growth (19) and in liver metastases (27). These studies provided the rationale for using Y15 as a FAK inhibitor for the current in vivo investigation, in which we noted that Y15 treatment led to decreased tumor volumes of renal subcapsular SK-NEP-1 tumors.

Pulmonary metastases have been reported with the murine subcapsular renal model of SK-NEP-1 tumors. Although the main focus of the current study was on the importance of FAK in primary tumors and not pulmonary metastases, we did note that Y15 treatment did not alter the prevalence of metastases. This finding may be due to the time lag between the injection of tumor cells and the initiation of treatment. Other investigators have noted similar findings with this model. Soffer and colleagues evaluated the efficacy of topotecan in reducing tumor growth with SK-NEP-1 cells injected into the subcapsular space of the kidney. They noted a significant decrease in volume of the primary tumor, but no significant change in the occurrence of pulmonary metastases (42). Lee and colleagues treated mice bearing subcapsular renal SK-NEP-1 xenografts with a selective COX-2 inhibitor, SC-236, and, again, demonstrated a profound decrease in the volumes of the primary renal tumors, but no significant effect upon the presence of pulmonary metastases (43).

The small molecules PF-573,228 and Y15 have been reported to inhibit FAK phosphorylation at the Y397 autophosphorylation site (17, 24) and that is why they were chosen for these investigations. Many consider the FAK autophosphorylation site (Y397) to play the primary role in how FAK affects tumor cell migration, invasion, and survival. In our study, we did see inhibition of FAK Y397 phosphorylation in both cell lines (Figs. 3A and 4A), but at higher concentrations of the inhibitors there was also some inhibition of total FAK expression in both cell lines (Figs. 3A and 4A). Other investigators have reported similar findings in other cell lines. Huanwen and colleagues found that pancreatic cancer cells treated with PF-573,228 had a decrease in total FAK expression following treatment with 10 μmol/L concentration (40). Similar results were noted when colon cancer cells were treated with PF-573,228 (44). Y15 treatment of the human pancreatic cell line, Panc 1, led to a decrease in FAK phosphorylation but also decreased total FAK at higher concentrations (18). Findings were similar in BT474 breast cancer cells (17) and U87 glioma cells (45) treated with Y15, in that, increasing concentrations of the inhibitor and also increasing the time of treatment resulted in decreased total FAK expression (17).

In summary, we showed that FAK abrogation, using siRNA and small molecules, had a significant effect upon the malignant phenotype of G401 and SK-NEP-1 cells. There was a significant decrease in cell survival, increased cellular apoptosis, and decreased migration and invasion. An important observation was that the changes in migration and invasion following small-molecule FAK inhibition occurred at concentrations below the LD50 of the inhibitors, because cells that are not viable will not invade or migrate. Another novel aspect of the current studies was the demonstration that FAK inhibition in a nude mouse model incorporating a subcapsular renal tumor resulted in significantly smaller renal tumors when compared with controls. We believe that the data presented provide justification for further investigations of FAK inhibition as a potential therapeutic strategy for difficult-to-treat pediatric renal tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
FAK Inhibition Decreases Kidney Tumor Growth

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References


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Michael L. Megison, Lauren A. Gillory, Jerry E. Stewart, et al.


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