Downregulation of PDGFRß Signaling Overcomes Crizotinib Resistance in a TYRO3 and ALK Mutated Neuroendocrine-Like Tumor

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A B S T R A C T

Patient-derived xenografts provide significant advantages over long-term passage cell lines when investigating efficacy of treatments for solid tumors. Our laboratory encountered a high-grade, metastatic, neuroendocrine-like tumor from a pediatric patient that presented with a unique genetic profile. In particular, mutations in TYRO3 and ALK were identified. We established a human patient-derived xenoline (PDX) of this tumor for use in the current study. We investigated the effect of crizotinib, a chemotherapeutic known to effectively target both TYRO3 and ALK mutations. Crizotinib effectively decreased viability, proliferation, growth, and the metastatic properties of the PDX tumor through downregulation of STAT3 signaling, but expression of PDGFRß was increased. Sunitinib is a small molecule inhibitor of PDGFRß and was studied in this PDX independently and in combination with crizotinib. Sunitinib alone decreased viability, proliferation, and growth in vitro and decreased tumor growth in vivo. In combination, sunitinib was able to overcome potential crizotinib-induced resistance through downregulation of ERK 1/2 activity and PDGFRß receptor expression; consequently, tumor growth was significantly decreased both in vitro and in vivo. Through the use of the PDX, it was possible to identify crizotinib as a less effective therapeutic for this tumor and suggest that targeting PDGFRß would be more effective. These findings may translate to other solid tumors that present with the same genetic mutations.

Introduction

Cancer is the leading cause of death by disease for children and a limited range of treatment options exists for this vulnerable patient population [1,2]. In the case of pediatric neuroendocrine cancers with unknown primaries, the survival rate is 10% [3]. Surgical excision is often the first step for treatment of pediatric solid tumors, followed by a regimen of chemotherapeutics and/or radiation therapy which are all associated with significant toxicities. Investigation of tumor mutations and signaling cascades is necessary to better target these malignancies and improve outcomes. It is also necessary to develop novel models to study these tumors to better understand the interaction between therapeutic agents and the tumor profile. The use of patient-derived xenolines (PDXs) makes this goal achievable. Tumor PDXs offer the unique opportunity to study both general cancer biology and the recapitulation of the tumor phenotype; conditions that provide an optimal opportunity to investigate and understand the potential effects of unexplored therapies prior to testing in a clinical setting [4].

Genomic sequencing of the PDX in this study identified mutations in anaplastic lymphoma kinase (ALK) and tyrosine kinase 3 (TYRO3) genes. In malignancies with ALK and/or TYRO3 mutations, crizotinib, an ALK/c-Met-inhibitor, has been effective at decreasing tumor cell vi-

Footnote

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ability, growth, and the metastatic properties in pre-clinical investigations [5-7]. Unfortunately, in the clinical setting, crizotinib has been less promising and efficacious. Multiple mechanisms of resistance have been proposed including upregulation of PDGFRα and Axl [8-11]. We hypothesized that similar mechanisms were in play in the PDX under current investigation, and that downregulation of PDGFRα utilizing sunitinib might overcome this drug resistance.

The complexity of molecular pathways in tumor cells may impede the ability to effectively treat patients, so understanding these pathways is critical for the successful development of new therapeutics. It is also crucial to appreciate the individual nature of each tumor and the differences amongst tumors, especially between adult and pediatric populations. The work presented addresses the ability to investigate a rare tumor on the molecular level and supports a mechanism of resistance while proposing a chemotherapeutic solution.

Methods

Establishing patient-derived xenolines

A high-grade neuroendocrine-like tumor from a pediatric patient who presented with multiple metastases was identified for potential use as a patient-derived xenograft, referred to as COA109 (Supplementary Figure 1 A). Following written informed consent and under an institutional review board approved protocol [University of Alabama at Birmingham (UAB) IRB 130627006], a fresh piece of the excised tumor was obtained from the surgical resection specimen and placed in iced Roswell Park Memorial Institute 1640 (RPMI) medium. The specimen was partitioned and placed in (i) formalin for paraffin embedding; (ii) liquid nitrogen for storage at −80 °C; and (iii) sterile media for implantation. Under a UAB institution animal care and use committee protocol, (IACUC 009186), implantation was accomplished using female athymic nude mice (Envigo, Pratville, AL). Briefly, animals were anesthetized with 3% inhalational isoflurane and under sterile conditions, 16 mm² tumor pieces were placed into the subcutaneous space of the animal’s flank. Animals were housed in a pathogen-free environment and monitored for overall health and tumor growth. Tumor volumes were measured weekly and harvested when IACUC parameters were met. At the time of tumor harvest, the tumors were partitioned as described above, with an addition of the portion of the tumor being utilized for experimentation. For in vitro experiments, tumors were dissociated using a Tumor Dissociation Kit (Miltenyi Biotec, San Diego, CA) per manufacturer’s protocol. Cells were resuspended in neurobasal (NB) medium (Life Technologies, Carlsbad, CA) supplemented with B-27 without Vitamin A (Life Technologies), N2 (Life Technologies), amphotericin B (250 μg/mL), gentamicin (50 μg/mL), l-glutamine (2 mM), epidermal growth factor (10 ng/mL, Miltenyi Biotec), and fibroblast growth factor (10 ng/mL, Miltenyi Biotec). Next-Generation Sequencing of COA109 xenograft tumor tissue and COA109 cells was completed (Genomics Core, UAB, Birmingham, AL) and results compared with FoundationOne® testing that had been completed on the patient’s tumor to validate a similar tumor profile. Identified mutations were utilized for further testing. To ensure integrity of the PDXs through multiple passages, we performed short tandem repeat analysis (UAB Genomics Core, Birmingham, AL), real-time PCR (qPCR) to detect potential murine contamination of the PDX (TREND DNA/RNA Isolation and TaqMan QPCR/Genotyping Core Facility, UAB, Birmingham, AL), and histology and immunohistochemistry to confirm recapitulation of the patient’s tumor.

Antibodies and reagents

The following antibodies were utilized per manufacturers’ recommendations: rabbit monoclonal anti-phospho-Stat3 (Tyr705, D3A7, 914S), anti-Stat3 (79D7, 4904), anti-PARP (46D11), anti-Caspase-3 (9662), anti-cleaved Caspase 3 (Asp175, SA1E, 9664), anti-Tyro3 (D38C6, 5585), anti-phospho-Akt (Ser473, D9E, 4060), anti-PDGFRβ (28E1, 3169), anti-Akt (11E7, 4685), anti-cyclin D1 (92G2, 2978), and anti-Vinculin (E19Y9, 13,901) from Cell Signaling (Danvers, MA); rabbit monoclonal anti-PDGFR Receptor beta (Y92, ab32570) from Abcam (Cambridge, MA); mouse monoclonal β-actin (A1978), and anti-phospho-Erk 1/2 (AW39R) from Sigma Aldrich (Millipore Sigma, St. Louis, MO). Crizotinib was purchased from Sigma Aldrich and LC Laboratories (Woburn, MA) and sunitinib was purchased from Selleckchem (Houston, TX).

Immunohistochemistry

Paraffin-embedded COA109 tissue was cut into 4 μm sections onto positive slides and processed according to a previously published protocol [12]. Briefly, the primary antibody for Chromogranin A (ab15160, Abcam) was added at a 1:100 dilution and incubated overnight in a humidity chamber at 4 °C. The slides were then washed with PBS and secondary antibody for rabbit (R.T.U. biotinylated universal antibody, Vector Laboratories, Burlingame, CA) was added for 30 min at 22 °C. Staining reaction was applied for 30 min at room temperature with VECTORSTAIN Elite ABC reagent (PK-7100, Vector Laboratories) and Metal Enhanced DAB Substrate (Thermo Fisher Scientific) for 2 min. Slides were counterstained with hematoxylin. Each antibody had a negative control (rabbit IgG, 1 μg/mL, EMD Millipore).

Immunoblotting

COA109 cells (1 × 10⁶) were plated in non-adherent conditions in NB media and various drug treatments were applied for 24 h. Treated cell pellets were flash frozen in liquid nitrogen for later use. Whole cell lysates or homogenized tumor specimens lysates were made using either radioimmunoprecipitation (RIPA) buffer [10 mmol/L Tris base pH 7.2, 150 mmol/L NaCl, 1% Na-deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS)] supplemented with phenyl-methane-sulfonyl-fluoride (PMSF, Sigma), protease inhibitor (Sigma), and phosphatase inhibitor (Sigma), or mammalian target of rapamycin (mTOR) buffer [40 mmol/L HEPES pH 7.5, 120 mmol/L NaCl, 1 mmol/L EDTA, 10 mmol/L pyrophosphate (Sigma, P8010), 10 mmol/L glicerophosphate (Sigma, G9891), 1.5 mmol/L Na₂VO₄, 0.3% CHAPS, and EDTA-free protease inhibitors (Roche)] supplemented with protease inhibitor, 50 mmol/L NaF, and PMSF according to protocols previously described [12]. Protein concentrations were determined with a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Proteins were separated on SDS polyacrylamide (SDS-PAGE) gels via electrophoresis. Precision Plus Protein Kaleidoscope molecular weight marker (Bio-Rad, Hercules, CA) was loaded adjacent to samples for reference. Gels were transferred to a blotting membrane using Trans-Blot Turbo Transfer System (Bio-Rad) per manufacturer’s protocol. Antibodies were used per manufacturers’ instructions. Blots were developed with either Immobilon Classic or Crescendo Western HRP Substrate (EMD Millipore, Millipore Sigma, Burlington, MA). Immunoblots were stripped with stripping buffer (Bio-Rad) at 65 °C for 20 min prior to re-probing. β-actin or vinculin were used to confirm equal protein loading.

Cell survival and proliferation

COA109 cells were suspended in Accutase (Fischer Scientific), incubated at 37 °C for 10 min, re-suspended in NB media, and counted. Cells (1.5 × 10⁵) were plated in 96-well plates and treated with increasing doses of crizotinib (0, 0.5, 1, 2.5, 5, 10, 20 μm), sunitinib (0, 2.5, 5, 10 μm), or an equivalent concentration of dimethyl sulfoxide (DMSO) for a vehicle control. After 24, 48, and 72 h of treatment, an alamarBlue® colorimetric assay (Thermo Fisher Scientific) was used to assess viability. alamarBlue® dye (10 μL, Thermo Fisher Scientific) was added and absorbance was read at 570 nm and 600 nm on a microplate reader (Epoch Microplate Spectrophotometer, BioTek Instruments, Winooski,
VT). To assess proliferation, cells (5 × 10^5) were plated in 96-well plates and treated with control, crizotinib, or sunitinib in increasing concentrations (as above) for 24, 48, and 72 h and the colorimetric CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (10 μL, Promega, Madison, WI) was utilized. Absorbance was read at 490 nm with a microplate reader. Cell viability and proliferation from at least three biologic replicates were reported as fold change ± standard error of the mean (SEM).

**Cell growth**

To assess in vitro cell growth over time, COA109 cells (5 × 10^5) were plated in non-adherent conditions and treated with control (DMSO) or crizotinib (1 μM) for single treatment studies. For combination studies, COA109 cells (2.5 × 10^5) were plated in non-adherent conditions and treated with control (0 μM), crizotinib (0.5 μM), sunitinib (0.5 μM), or a combination of crizotinib and sunitinib (0.5 μM each). Each time point (24, 48, 72, 96 h) had three wells of cells and all wells were treated at 0 h. Cells were brought to a single cell suspension, stained with trypan blue, and counted with a hemocytometer. Total cell population was compared across time points and presented with at least three biologic replicates.

**Cell cycle**

COA109 cells (5 × 10^5) were plated in 6-well plates with increasing doses of crizotinib (0, 2.5 μM). Following a 24-hour incubation period, cells were washed twice with PBS, fixed in ethanol for 24 h at 4 °C, stained with propidium iodide (1 mg/mL, Invitrogen), 0.1% TritonX (Active Motif, Carlsbad, CA), and RNase A (0.1 mg/mL, Qiagen, Valencia, CA), and analyzed by Attune Next™ Flow Cytometer (Thermo Fisher Scientific). Collected data were analyzed using FlowJo software (Ashland, OR) and presented with at least three biologic replicates.

**Migration and invasion**

Corning® Transwell® plates (Corning Inc., Corning, NY) with 8 μm pore inserts were utilized. For both assays, the bottom of the inserts was coated with laminin (10 μg/mL, Millipore) overnight at 37 °C. Following treatment with increasing doses of crizotinib (0, 1, 2.5 μM) for 24 h, COA109 cells (2.5 × 10^5) were seeded into the inserts and allowed to migrate for 120 h. For invasion assays a similar method was used, but the top of the inserts was coated with Matrigel™ (1 mg/mL, 50 μL, BD Biosciences, Franklin Lakes, NJ) overnight at 37 °C. Following treatment with increasing doses of crizotinib (0, 1, 2.5 μM) for 24 h, COA109 cells (2.5 × 10^5) were seeded into the inserts and allowed to invade for 120 h. For both assays, inserts were fixed with 4% paraformaldehyde and stained with crystal violet. Image J (https://imagej.nih.gov/ij) was used for analysis and results were reported as fold change in number of cells migrating or invading ± SEM. Each assay had at least three biologic replicates.

**In vivo tumor studies**

PDX tumors were dissociated and cultured in NB media for 24 h. COA109 cells (5 × 10^4 in 25% Matrigel™, BD Biosciences) were implanted subcutaneously in the right flank of 6-week-old female athymic nude mice (Envigo). The mice were maintained in a pathogen-free environment with access to both chow and water ad libitum and under 12-hour light/dark cycles. Animals were monitored daily for overall body condition and weighed three times per week. Tumor volumes were measured three times a week with a caliper. Tumor volume was calculated with the formula (width^2 x length)/2 with width being the smaller value.

Animals were randomized into two treatment groups for the single drug study: control (4% DMSO, n = 7) or crizotinib (75 mg/kg of body weight/day, n = 7, LC Laboratories). For drug combination studies, animals were randomized into four treatment groups: control (4% DMSO, n = 5), crizotinib (75 mg/kg of body weight/day, n = 5, LC Laboratories) [13], sunitinib (40 mg/kg of body weight/day, n = 5, Sellickchem) [14], and crizotinib + sunitinib (same doses as single treatments, n = 7). Treatment was initiated one week after tumor cell implantation and was administered in a metronomic fashion: three consecutive days of each treatment with one day off per week. Drugs were delivered via oral gavage in 100 μL of corn oil and all animals received 100 μL of sterile saline subcutaneous injection daily for rehydration. The mice were treated for 17 days or until the tumors met IACUC parameters for animal euthanasia. Mice were humanely euthanized with CO₂ and cervical dislocation in their home cages one hour after their last treatment. Harvested tumors were saved for histologic sectioning and flash frozen for further experimentation.

**Data analysis**

Each in vitro experiment was repeated with at least three biologic replicates (PDXs from three different mice) and data was displayed as mean ± SEM. To determine statistical significance, a student’s t-test or analysis of variance (ANOVA) was used as appropriate, with p ≤ 0.05 determined to be statistically significant (SPSS V26, IBM®).

**Results**

**Successful establishment of a high-grade pediatric neuroendocrine-like PDX**

A high-grade pediatric neuroendocrine-like tumor, herein referred to as COA109, was successfully propagated as a PDX in immunodeficient mice. The PDX, COA109, was compared to the original patient tumor via histology. Hematoxylin and eosin staining demonstrated that the histology of the PDX (Fig. 1A, top right panel) recapitulated that of the parent tumor (Fig. 1A, top left panel). Both sections had round cells in a ribboning pattern and densely stained nucleoli (Fig. 1A, top panels, black boxes). Chromogranin-positive staining of the patient (Fig. 1A, bottom left panel) and PDX (Fig. 1A, bottom right panel) tumors suggested neuroendocrine features. IgG staining was included as a negative control (Fig. 1A, insert, bottom right panel). FoundationOne® sequencing was initially completed on the patient’s tumor to identify mutations that might affect oncogenesis and to identify potential chemotherapeutic targets. FoundationOne® sequencing revealed mutations in 13 genes including TYRO3 (Supplementary Figure 1B). To confirm replication of the genotype of the parent tumor in the PDX, whole genome sequencing was performed on both COA109 dissociated cells that had been maintained in culture 24 h and directly from ex vivo tumor tissue. Next generation sequencing confirmed the alterations in TYRO3 in the COA109 PDX tumors and cells from dissociated tumors, and detected mutations of unknown significance in ALK. There was over a 75% homology between in vitro and ex vivo samples, verifying the use of this PDX for experimentation (Supplementary Figure 1 C). Analysis showed all thirteen mutations identified by FoundationOne® were present in both the COA109 flank tumor and dissociated cells. These investigations validated a similar genotype between the PDX and the parent tumor. These findings supported the successful recapitulation of a rare tumor to be used for in vitro and in vivo studies as well as identified areas of investigation for therapeutics.

**Crizotinib decreased viability, proliferation, growth, and cell invasiveness in vitro**

ALK inhibitors have been studied in other tumors with TYRO3 or ALK mutations, including non-small lung cancer [8, 15, 16], acute large cell lymphoma [17], leiomyosarcoma [7, 18], and neuroblastoma [5, 6]. Crizotinib is a rigorously researched ALK/c-Met-inhibitor. Based on its
Fig. 1. Crizotinib decreased viability, proliferation, and growth in patient-derived xenograft cells of a highly malignant neuroendocrine tumor. A. Hematoxylin and eosin staining was performed on both the original patient tumor and the established PDX. Staining confirmed that the PDX morphology (top right panel) recapitulated that of the patient tumor (top left panel). Morphology of the tumor cells demonstrated densely stained nuclei and a gyrfowl-like pattern (black boxes). Chromogranin A immunohistochemistry confirmed the neuroendocrine features in the PDX (bottom right panel) and patient (bottom left panel) tumors. IgG staining was included as a negative control (inset, bottom right panel). B. COA109 cells were treated with increasing doses of crizotinib (0 - 20 μM) for 24 h. Viability was assessed using an alamarBlue® assay. Viability was significantly decreased with increasing doses of crizotinib. Crizotinib had a measured LD50 of 4.87 μM. C. COA109 cells were treated with increasing doses of crizotinib (0 - 20 μM) for 24 h. Proliferation was evaluated using a CellTiter 96® assay. Proliferation was significantly decreased with increasing doses of crizotinib. Crizotinib had a measured IC50 of 2.24 μM. D. COA109 cells were treated with 1 μM of crizotinib and their growth was compared to a control group of COA109 cells over a 96-hour period. At 72 h, there was significantly less growth seen in COA109 cells treated with crizotinib. E. Immunoblotting showed an increase in both cleaved Caspase 3 and cleaved PARP, while there was a decrease in total PARP, indicating an upregulation of the apoptotic cascade. F. There was also a decrease in phosphorylation of AKT, consistent with decreased cell proliferation.

To further delineate the possible mechanism responsible for crizotinib’s effect on the survival and proliferation of COA109 cells, we investigated downstream signaling targets of Tyro3. We found a decrease in phosphorylation of STAT3 following treatment with crizotinib, suggesting a decrease in the activity of STAT3 (Fig. 2C). STAT3 regulates genes affecting cancer cell survival and proliferation, and inhibiting the function of STAT3 through de-phosphorylation at the TY705 site prevents the transcription of these oncogenes [21]. We propose that the results seen with crizotinib were due to alteration of the STAT3 signaling [18] as depicted in the cartoon (Fig. 2D).

Crizotinib did not affect in vivo tumor growth

Based on in vitro findings, the next step was to study crizotinib in an animal model. COA109 cells (5 x 10⁶) were implanted into the right flank of female athymic nude mice. One week after injection, mice were randomized to receive either control [4% DMSO, 100 μL, per os (po), n = 7] or crizotinib (75 mg/kg, 100 μL, po, n = 7). The tumors were measured three times per week over a period of 37 days or until the mice reached IACUC parameters for euthanasia. Following 37 days of treatment, neither tumor volume (Fig. 3A, C) nor fold change in tumor volume (Fig. 3B) were significantly different between crizotinib and control animals. Additionally, crizotinib did not improve the survival of mice bearing COA109 tumors (Fig. 3D). These findings demonstrated the inability of crizotinib to decrease COA109 tumor growth and rate of tumor progression in vivo and posed the need to identify an alternative pathway that may be responsible.
Fig. 2. Crizotinib treatment led to decreased Tyro3 and STAT3 activity. A. COA109 cells were treated with crizotinib (0, 5, 10 μM) for 24 h. Immunoblotting revealed a decrease in expression of Tyro3. B. Since Tyro3 affects cell motility, we studied migration and invasion using modified Boyden chambers. Following increasing doses of crizotinib, migration and invasion of COA109 cells was significantly decreased. C. STAT3 phosphorylation was evaluated with immunoblotting. There was a decrease in phosphorylated STAT3, indicating a downregulation of the JAK/STAT pathway. D. There is crosstalk between Tyro3 and ALK signaling cascades. This diagram represents a proposed mechanism.

Fig. 3. Crizotinib did not decrease tumor growth in vivo. A-B. COA109 cells (5 × 10^6 in 25% Matrigel™) were placed subcutaneously in the right flank of athymic nude mice and tumor volume was measured three times a week and calculated as (width^2 × length)/2. One week after implantation, mice were treated with crizotinib (75 mg/kg, 100 μL, per os, n = 7) or control (4% DMSO, 100 μL, per os, n = 7). Mice treated with crizotinib had similar (A) tumor volume and (B) fold change in growth compared to the mice treated with control. C. Representative photos of excised tumors treated with control (left) and crizotinib (right). D. There was no statistically significant difference in survival between the control and crizotinib treated mice (NS, not significant).
Increased PDGFRβ in a crizotinib resistant cell subpopulation

Outcomes from the in vivo study contradicted those seen in the in vitro studies, prompting investigations for mechanisms to explain these findings. Other investigators have shown that malignancies treated with small molecule tyrosine kinase inhibitors have an upregulation of growth factors [22], and PDGFRβ upregulation has been identified in some ALK mutation-driven cancers [10]. Since mutations in ALK were also noted in the COA109 PDXs we used immunoblotting to analyze COA109 cells treated with crizotinib for alterations in cell signaling. We noted that PDGFRβ increased with increasing concentrations of crizotinib (Fig. 4A).

Due to the changes in PDGFRβ, we chose to investigate downstream signaling cascades involved in tumor cell proliferation. There was an increase in phosphorylation of ERK 1/2 in COA109 cells treated with crizotinib (Fig. 4B). Consistent with increased ERK 1/2 activity, there was an increase in downstream cyclin D1 (Fig. 4C). The phosphorylation of ERK suggests that crizotinib causes some cancer cells to undergo proliferation while other cells, as previously shown, undergo apoptosis. We propose that this select subpopulation that was able to avoid cell death and proliferate may represent the cells responsible for the lack of efficacy seen in vivo.

Since we noted an increase in cyclin D1 following treatment with crizotinib, we investigated the cell cycle. Cyclin D1 is important for cell cycle progression from G1 phase to S phase. In cancer cells, halting this progression may prevent proliferation [23]. Using flow cytometry, cell cycle analysis was performed on COA109 cells treated with 2.5 μM of crizotinib. In control cells, 61% of the cell population was in G1 and 36% in S phase, while in the crizotinib-treated cells, 48% of cells were in G1 and 50% in S phase (Fig. 4D). The significant difference between G1 (p<0.05) and S (p<0.001) indicated cell cycle progression with crizotinib treatment. We then investigated the inhibition of upstream targets, such as PDGFRβ, attempting to decrease the expansion of these resistant cells.

PDGFRβ inhibition decreased COA109 survival and growth

Sunitinib is a small molecule tyrosine kinase inhibitor that effectively targets PDGFRβ [24] and has been employed in clinical trials to treat pediatric malignancies [25]. Our data showed an upregulation of PDGFRβ following crizotinib treatment and we hypothesized that by using sunitinib, we could counteract the increase in activity of proliferative kinases. To assess the effect of sunitinib on COA109 cells independently, an alamarBlue® assay was performed. Sunitinib significantly decreased viability of COA109 in vitro and had a calculated LD₅₀ of 2.9 μM (Fig. 5A). These results provided substantiation to proceed with further experimentation of sunitinib alone and in combination with crizotinib.

Growth of COA109 cells over a 72-hour period was assessed under the following conditions: sunitinib + crizotinib (0.5 μM each), crizotinib (0.5 μM), sunitinib (0.5 μM), and control. At 48 h, sunitinib had a 24% and sunitinib + Crizotinib (combination) had a 36% decrease in growth compared to control (p ≤ 0.05, p ≤ 0.01, respectively). In comparing the combination group to each treatment alone, for crizotinib and sunitinib there were 32% and 38% and less cell growth at 72 h respectively (p ≤ 0.05, p ≤ 0.01, respectively, Fig. 5B). The findings for crizotinib were comparable with previous in vitro studies. These results suggested that combination treatment may be advantageous due to the cytotoxic effects of both drugs.

We then investigated the downstream mechanisms of crizotinib and sunitinib in combination. As previously shown, there was an increase in PDGFRβ expression and phosphorylation of ERK with increasing doses of crizotinib. At increasing doses of sunitinib alone, there was a decrease in phosphorylated ERK 1/2 (Fig. 5C), coinciding with the decrease in cell proliferation. Together, sunitinib in combination with crizotinib,
decreased the expression of PDGFRβ (Fig. 5C), suggesting sunitinib was reducing the crizotinib-induced PDGFRβ upregulation. These results showed promise for the combined use of sunitinib with crizotinib in vivo.

**PDGFRβ inhibition decreased COA109 in vivo tumor growth**

Because of the in vitro findings, we proceeded with studying sunitinib alone and in combination with crizotinib in a murine model. COA109 cells (5 × 10⁶) were implanted subcutaneously in the right flank of athymic nude mice. At one week, animals were randomized to receive one of the following treatments: control (4% DMSO, 100 μL, po, n = 5), crizotinib (75 mg/kg of body weight/day, 100 μL, po, n = 5), sunitinib (40 mg/kg of body weight/day, 100 μL, po, n = 5), and crizotinib + sunitinib (same doses as in single treatments, 100 μL, po, n = 7). Animals were weighed and tumors measured three times per week for 20 days of treatment. Again, there was no significant difference between the tumor growth rate of the control treated group and those treated with crizotinib (Fig. 6A, B). Sunitinib alone significantly delayed COA109 tumor growth (p ≤ 0.001, Fig. 6A, B). Further, the addition of sunitinib to crizotinib diminished tumor growth compared to the control (p ≤ 0.05) and crizotinib treated groups, but not to sunitinib alone. These findings differed from the in vitro findings of combination treatment but substantiated the lack of efficacy of crizotinib in vivo.

Tumors from the experimental mice were harvested at euthanasia one hour after the final administration of treatment. Proteins were extracted from the tumors and immunoblotting completed. Consistent with our previous findings, tumors from mice treated with crizotinib (Fig. 6C) showed a high expression of PDGFRβ and increased phosphorylation of ERK 1/2, indicating crizotinib had a similar effect on the in vivo tumor as it did in vitro. With the addition of sunitinib to crizotinib, PDGFRβ and phosphorylation of ERK 1/2 were decreased (Fig. 6C). Sunitinib treatment alone decreased PDGFRβ expression (Fig. 6C).

These findings were consistent with the hypothesized mechanism of resistance to crizotinib mediated through PDGFRβ signaling, and provide support for the use of sunitinib in tumors bearing this genotypic profile.

**Discussion**

Genetic mutations have become a driver for the development and use of targeted chemotherapeutics in cancer. We utilized the identified **TYRO3** and **ALK** mutations as a basis to study the use of crizotinib in an established PDX of a rare pediatric neuroendocrine-like tumor (Supplementary Figure 1 A-D). The in vitro data were promising with a decrease in COA109 cell viability, growth, and motility following treatment with crizotinib. When studied in vivo, crizotinib had little effect on the COA109 tumors, contradicting what was expected based on in vitro experiments. PDGFRβ upregulation was noted with crizotinib treatment and suggested a potential resistance mechanism. Sunitinib was then added to override this activity and in vivo analysis suggested a potent combination for COA109. In vivo findings confirmed the lack of efficacy of crizotinib. Addition of sunitinib decreased in vivo tumor growth of the crizotinib resistant cells and on its own.

With the advent of genomic sequencing, it has become possible to pinpoint the specific, altered pro-oncogenic or pro-apoptotic signaling pathways in a cancer and target them for therapy. There are downsfalls to this approach of personalized medicine: (i) the potential toxicity of these molecularly targeted therapies, and (ii) the inability of the targeted therapeutics to fully inhibit a protein’s downstream activity [26]. Additionally, these pathways are subject to adaptability and may vary with the tumor microenvironment, providing an avenue for the development of drug resistance [16,27]. The use of PDX models allows for a more accurate prediction of a clinical outcome in a pre-clinical setting for chemotherapeutics as these models more closely replicate the properties of patient tumors [4]. A limitation to the PDX model is the amount of time required to grow the cancer in a murine model [28]. This barrier
was not encountered with the tumor in this study; COA109 tumors grew predictably within a month’s time and at a similar rate with injection of dissociated cells, eliminating potential intertumoral selection bias that is associated with the implantation of tumor chunks. Other concerns with employing PDXs include genetic and phenotypic deviations of the PDX from the parent tumor after multiple passages; however, these were not encountered in this study. We showed that the COA109 PDX remained consistent with the original tumor through both histology and genotyping. The recapitulation of the parent tumor in a PDX model allowed for an in-depth study of the tumor’s genotype and phenotype in order to better understand and explore potential therapeutic interventions.

The highly malignant features, variety of mutations, and lack of clear diagnostic pathology make this undifferentiated tumor challenging [29]. The focal neuroendocrine features were intriguing as there has been an increasing number of neuroendocrine tumors seen in adults but they remain rare in children [19]. Neuroendocrine tumors, however, are derived from the same embryonic tissue as the pediatric tumor, neuroblastoma, and led us to investigate potential similarities between COA109 and neuroblastoma. Identification of multiple ALK mutations in PDX tissue/cells served as a corroboration for initial studies (Supplementary Figure 2). ALK, an ATP-dependent tyrosine kinase receptor, is sparsely expressed throughout the brain and is believed to play a role in neural development [30]. ALK signaling pathways have been found to be important for oncogenesis in neuroblastoma [31]. Up to 14% of high-risk neuroblastomas harbor ALK mutations and these mutations are known to promote oncogenesis through PI3K/AKT, RAS/MAPK, and STAT3 signaling pathways. F1774L, F1245C, and R175Q make up 85% of the ALK mutations found in neuroblastoma, but these specific mutations were not identified in the COA109 PDX. Additionally, in high-risk neuroblastoma, ALK mutations are often associated with MYCN amplification, the worst prognostic indicator in neuroblastoma, and TP53 mutations [32,33]. The tumor in the current study had multiple TP53 mutations but not MYCN amplification. Thus, the similarities between COA109 PDX and neuroblastoma were limited to sharing a neural ectoderm origin. Ultimately these findings eliminated neuroblastoma as the clinical diagnosis and led to the consideration of other neuroendocrine tumors for diagnosis.

Targeting ALK through tyrosine kinase inhibitors has been successful in ALK mutated malignancies. Crizotinib, a small molecule inhibitor of the ALK receptor, is an FDA approved drug that targets ALK fusion proteins and was initially developed for treatment of non-small cell lung cancer [34]. When studied in a PDX model of neuroblastoma, crizotinib did not significantly decrease in vivo tumor growth [35], similar to the findings presented in the current study. This resistance was attributed to the loss of TP53 function [35]. Studies have since showed a varying sensitivity of different ALK mutations to crizotinib, prompting investigations on the mechanisms of resistance or pathways to overcome resistance [5]. F1174L is the most widely known mutation to have de novo resistance to crizotinib and newer therapies such as lorlatinib have demonstrated improved efficacy over crizotinib in neuroblastoma lines with the F1174L mutation [13,36]. Even with these considerations, promising data with other long-term passage cell lines led to the initiation of a clinical trial. Recent reports of the study show less pronounced effects than anticipated and suggest the use of crizotinib for ALK mutations may not be as beneficial as anticipated (clinicaltrials.gov, NCT00939770). The discrepancy of crizotinib in pre-clinical findings compared to clinical use is similar to the contradiction we found in comparing our in vitro and in vivo results.

Crizotinib has been shown to inhibit other tyrosine kinase receptors such as TAM receptors [7,11]. The TAM receptor family is a group of tyrosine kinase receptors that includes Tyr3, Axl, and Mer [18]. Studies have shown the importance of TAM receptors in promoting cancer cell survival, metastasis, and chemoresistance through STAT3, PI3K/AKT, and RAS/MAPK pathways [11,18,37-40]. The PDX studied here had a TYRO3 mutation (Supplementary Figure 1 C). Investigation of Tyr3 in other neural crest-derived malignancies has been focused on schwannomas and melanoma. Tyro3 has been shown to be overexpressed in schwannomas and activation of the receptor via Gαs6 contributed to...
an increased phosphorylation of ERK 1/2, AKT, and JNK, resulting in schwannoma cell proliferation [41]. In melanoma, Tyro3 activity decreased with focal adhesion kinase inhibition, which resulted in decreased metastases [20]. Finding that crizotinib successfully targeted Tyro3 in other neural crest-derived malignancies [42] prompted the use of crizotinib in these investigations. In TYRO3-mutated leiomyosarcoma, crizotinib was found to decrease tumor cell survival through inducing G2 cell cycle arrest and apoptosis [7]. Those findings were similar to those in the current studies where crizotinib decreased proliferation and increased apoptosis in vitro in the PDX studied but different in that crizotinib did not induce cell cycle arrest.

Coinciding downstream signaling of both ALK and Tyro3, such as PI3K/AKT, MAPK/ERK 1/2, and JAK/STAT, provides a promising area for targeted drug therapy [18,21,32,43,44]. Since the PDX in the current study had mutations in both ALK and TYRO3, it was hypothesized that crizotinib would be an effective agent. However, interpreting one signaling cascade over the other, or the inability to completely inhibit both, may result in the generation of a drug resistant tumor cell subpopulation [26]. This effect has been noted in ALK mutated neuroblastoma and non-small cell lung cancer. When these tumors were treated with ALK inhibitors, there was a subsequent increase in Axl receptor activity [9,45]. Specifically, increased Axl expression resulting from the ELM4-ALK fusion mutation induced resistance to ALK inhibitor in lung cancer. In the absence of ALK mutations and in pediatric solid tumors other than neuroblastoma, crizotinib has been shown to drive growth factor rescue and negate its therapeutic effects through upregulation of ERK 1/2 activity [22]. This finding could explain why PDGFRß expression is increased in ALK-mutated anaplastic large cell lymphoma (ALCL) which is resistant to crizotinib [10]. We showed both an upregulation of PDGFRß and activity of its downstream components including ERK 1/2 and cyclin D1 in COA109 following crizotinib treatment. Furthermore, we showed crizotinib-induced cell cycle progression, which may suggest proliferation of a crizotinib-resistant tumor cell subpopulation. We postulate that this cell subpopulation was responsible for the aberrant tumor growth seen in vivo in the crizotinib treated animals.

Inhibition of PDGFRß receptor signaling has also been investigated in ALK-mutated malignancies, as research has demonstrated that ALK mutations can increase transcription of its activator, JunB [8]. In ALCL, the inhibition of PDGFRß by imatinib decreased tumor growth in mice bearing xenografts, and imatinib’s effects correlated with the levels of PDGFRß expression in the tumor. That study also demonstrated PDGFRß inhibition functioned in a synergistic fashion with crizotinib. It was effective at decreasing relapsed lymphoma growth, following an ALK inhibition relapse, when utilized in conjunction with CEP28122 (ALK inhibitor) [10]. Sunitinib is another small molecule inhibitor of PDGFRß and has been shown to both decrease its phosphorylation and expression [46]. Sunitinib also has cytotoxic effects on neuroblastoma as well as off-target effects on Axl [14]. In medulloblastomas, similar to crizotinib, sunitinib decreased STAT3 activity as well as increased apoptosis [47]. Sunitinib also decreased AKT activity whereas crizotinib did not [47]. With this knowledge, we hypothesized that downregulation of PDGFRß may be a potential therapeutic option for this tumor. The in vitro results also suggested that sunitinib in combination with crizotinib functioned in an additive fashion, with in vivo studies demonstrating sunitinib to efficaciously decrease tumor growth independently and in combination with crizotinib.

Despite what our in vitro findings would suggest, combining crizotinib with sunitinib in vivo was not more effective than sunitinib alone. These findings could be explained by the contribution of the tumor microenvironment, which is absent in vitro studies. It is well documented the components of the tumor microenvironment, such as cancer associated fibroblasts and mesenchymal stromal cells, contribute to tumor progression, metastasis, and chemoresistance [48]. For example, in neuroblastoma, cancer associated fibroblasts can increase production of IL-6 [49] and it has been shown that IL-6 signaling leads to chemoresistance of common therapeutics like BCL2 inhibitors [49,50]. Clearly, utilizing models that include the tumor microenvironment, like patient-derived xenografts, are essential in pre-clinical drug testing.

This study only chose to investigate two of the many mutations identified in this tumor. Targeting both ALK and Tyro3 led us to expose mechanisms of resistance and understand the modest outcomes with crizotinib. Future studies will investigate the effects of sunitinib on the COA109 tumor profile. Additionally, there are other mutations with potential for chemotherapeutic targeting that may be explored. Although many of these treatments have targeted the adult population, translation to pediatrics could provide new therapeutic opportunities.

Conclusion

The establishment of a PDX of a rare, pediatric neuroendocrine-like tumor allowed for the exploration of tumor biology and the translation of the results into the investigation of targeted chemotherapeutics. In this study, the results of the in vitro studies using crizotinib were not replicated when tested in vivo, leading to the investigation of potential resistance mechanisms and the identification of PDGFRß signaling. We subsequently targeted PDGFRß to overcome this resistance. The current study has demonstrated some of the potential pitfalls to relying solely on genetic sequencing for treatment and highlighted the need for a more complete understanding of a drug’s effect on a tumor, leading us to contend that caution should be exercised when employing crizotinib in TYRO3/ALK mutated malignancies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Colin H. Quinn: Conceptualization, Methodology, Validation, Investigation, Visualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. Andee M. Beierle: Investigation, Formal analysis, Writing – review & editing. Adele P. Williams: Investigation, Formal analysis, Writing – review & editing. Raoud Marayati: Investigation, Writing – review & editing. Laura V. Bownes: Investigation, Formal analysis, Writing – review & editing. Hooper R. Markert: Investigation, Formal analysis, Writing – review & editing. Jamie M. Aye: Methodology, Resources, Writing – review & editing. Jerry E. Stewart: Investigation, Methodology, Elizabeth Mroczek-Muselman: Investigation, Validation, Visualization. David K. Crossman: Investigation, Formal analysis, Resources, Data curation. Karina J. Yoon: Methodology, Resources. Elizabeth A. Beierle: Supervision, Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition, Resources.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101099.

References

[1] E. Ward, C. DeSantis, A. Robbins, B. Kohler, A. Jemal, Childhood and adolescent cancer statistics, 2014, CA Cancer J. Clin. 64 (2) (2014) 83–103, doi:10.3322/caac.21219.
[2] R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, CA Cancer J. Clin. 68 (1) (2018) 7–30, doi:10.3322/caac.21442.

[3] P. Navalekale, M.S. O’Dorrio, T.M. O’Dorrio, G.K. Zamba, C.F Lynch, Incidence, survival and presence of neuroendocrine tumors versus neuroblastoma in children and young adults: nine standard SEER registries, 1973-2006, Pediatr. Blood Cancer 56 (1) (2011) 50-57, doi:10.1002/blc.22559.

[4] I. Eunmacon, K. Paz, D. Czignadz, I. Sloma, A. Katz, D. Vasquez-Dundtoll, et al., Patient-derived xenografts as preclinical models of high-grade neuroblastoma, Cancer Discov. 4 (8) (2014) 682–694, doi:10.1158/2155-9555 CD-13-0071.

[5] S.C. Bresler, D.A. Weiser, P.J. Huwe, J.H. Park, K. Krytska, H. Ryles, et al., ALK mutations confer on neuroendocrine tumors versus neuroblastoma in children and young adults: nine standard SEER registries, 1973-2006, Pediatr. Blood Cancer 56 (1) (2011) 50-57, doi:10.1002/blc.22559.

[6] C. H. Kim, E. Kang, K. Nakagawa, T. Seto, L. Crino, M.J. Ahn, et al., Zanotinib versus chemotherapy in advanced ALK-positive lung cancer, N. Engl. J. Med. 368 (25) (2013) 2385–2394, doi:10.1056/NEJMoa1209714.

[7] S. Durand, C. Pierre-Eug ne, O. Mirambeau, C. Louis-Bremontet, V. Combaret, L. Birner, et al., ALK inhibitor FF-046992 is effective as a single agent in neuroblastoma driven by expression of ALK and MYCN, Dis. Model. Mech. 9 (6) (2016) 941–952, doi:10.1242/dmm.024428.

[8] L.F. Carpenter, Y.P. Morse, Targeting ALK in neuroblastoma-preclinical and clinical advances, Nat. Rev. Clin. Oncol. 7 (9) (2010) 391–399, doi:10.1038/nrclinonc.2010.72.

[9] C. Dantas-Barbosa, T. Lesley, F.L. Loarer, F. Chibon, I. Treilleux, J.M. Coindre, et al., Expression and role of TYRO3 and AXL as potential therapeutic targets in leukaemogenesis, Br. J. Cancer 117 (12) (2017) 1767–1779, doi:10.1038/bjc.2017.354.

[10] A.S. Berghoff, P. Birner, B. Streubel, L. Kenner, M. Preusser, ALK gene aberrations and the JUN-JUNB/PDGFR axis in metastatic NSGCL, APMIS 122 (9) (2014) 867–872, doi:10.1111/apm.12439.

[11] D.N. Debruyne, N. Bhatnagar, B. Sharma, W. Luther, N.F. Moore, N.K. Cheung, et al., ALK inhibitor resistance in ALEF1174-driven neuroblastoma is associated with AXL activation and induction of EM, Oncogene 35 (28) (2016) 3681–3691, doi:10.1038/onc.2016.17.

[12] D. Laimer, H. Dolzg, K. Kollmann, P.W. Vesely, M. Schlederer, O. Merkel, et al., PDGFR blockade is a rational and effective therapy for NPM-ALK-driven lymphomas, Leukemia 18 (11) (2004) 1699–1704, doi:10.1038/sj.leu.2403480.

[13] F. Xu, L. Li, V. Sun et al., Improved the targeted therapy against ALK-mutated neuroblastoma, Biochem. Biophys. Res. Commun. 454 (4) (2014) 556–571, doi:10.1016/j.bbrc.2014.10.120.

[14] L.L. Stafan, A.P. Williams, L.R. marital, I.M. Aye, H.R. Markert, E.F. Garner, et al., Focal adhesion kinase inhibition contributes to tumor cell survival and motility in neuroblastoma patient-derived xenografts, Sci Rep 9 (1) (2019) 1–12, doi:10.1038/s41598-019-49853-z.

[15] B.R. Infanteiro, J.H. Park, K. Krytska, H. Ryles, R. Sano, K.M. Szegety, et al., The ALK/ROS1 inhibitor FF-046992 overcomes primary resistance to crizotinib in ALK-driven neuroblastoma, Cancer Discov. 6 (1) (2016) 96–107, doi:10.1158/2155-9555 CD-15-1056.

[16] A.M. Tew, J.M. Smith, K.J. Chung, D. Stempak, H. Yeger, P. Marrano, et al., in vivo antitumor and antimetastatic activity of Sunitinib in preclinical Neuroblastoma mouse model, Neoplasia 11 (5) (2009) 425–435, doi:10.1016/j.neo.2009.166.

[17] R. Katsayama, A.T. Shaw, T.M. Khan, M. Minas-Kenudson, B.J. Solomon, B. Halmos, et al., Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers, Sci. Transl. Med. 4 (120) (2012) 120ra17, doi:10.1126/scitranslmed.3003316.

[18] T. Yamada, S. Takeuchi, J. Nakade, K. Kita, T. Nakagawa, S. Nanjo, et al., Paracrine receptor activation by microenvironment triggers bypass survival signals and ALK inhibitor resistance in ALK-rearranged lung cancer cells, Clin. Cancer Res. 18 (13) (2012) 3592–3602, doi:10.1158/1078-0432.CCR-11-2972.

[19] P.B. Stabler, P. Vesely, N. Haq, R.G. Ott, K. Funato, I. Bambach, et al., The oncoprotein NPM-ALK of anaplastic large-cell lymphoma induces JUNB transcription via ERK1/2 and Akt mediated MEK/MAPK and PI3K signaling, Bloodlood 110 (9) (2007) 3374–3383, doi:10.1182/blood-2007-02-17125.

[20] S.K. Smart, E. Vasiileiadis, X. Wang, D. Deleyckere, D.K. Graham, The emerging role of TYRO3 as a therapeutic target in cancer, Cancers (Basel) 10 (12) (2018) 1–27, doi:10.3390/cancers10120874.

[21] A.J. Degnan, S. Tocchio, W. Kurtom, S.S. Tadros, Pediatric neuroendocrine carcinoid tumors: management, pathology, and imaging findings in a pediatric referral center, Pediatr. Blood Cancer 64 (9) (2017), doi:10.1002/pbc.26477.

[22] H. Hao, A. Wang, D. Laufenstein, A. Wells, Tyr3-mediated phosphorylation of ACTN4 at tyrosine is FAK-dependent and decreases susceptibility to cleavage by m-Cathep, Int. J. Biochem. Cell Biol. 95 (December 2017) 73–84, doi:10.1016/j.biocel.2017.12.014.

[23] J.S. Rawlings, K.M. Rosler, D.A. Harrison, The JAK/STAT signalling pathway, J. Cell. Sci. 117 (8) (2004) 1281–1283, doi:10.1242/jcs.00963.

[24] M. Sie, W.F.A. Den Dunnen, H.J. Lourens, T.G. Meeuwen-De Boer, F.J.G. Scherber, P.W. Witzeman, et al., Growth factor-driven rescue to Receiver Tyrosine Kinase ii (Rtk) inhibitors through Akt and Erk phosphorylation in pediatric low grade astrocytoma and ependymoma, PLoS ONE 10 (3) (2015) 1–6, doi:10.1371/journal.pone.0122555.

[25] G. Grooin, S.M. Min, J.H. Phadke, M. Chung, J. Moser, I. Miller, et al., A map of protein dynamics during cell-cycle progression and cell-cycle exit, PloS Biol. 15 (9) (2017) e2002368, doi:10.1371/journal.pbio.2002368.

[26] A.I. Vinik, E. Raymond, Pancreatic neuroendocrine tumors: approach to treatment, Am. J. Clin. Endocrinol. Metab. Therap. Adv. Gastroenterol. 6 (5) (2013) 296–311, doi:10.1177/2168383X13493878.

[27] S.G. Dubois, S. Shuterman, A.M. Ingle, C.H. Ahern, J.M. Reid, B. Wu, et al., Phase I pharmacokinetic study of sunitinib in pediatric patients with refractory solid tumors, a children’s oncology group study, Cancer. Cancer Res. 73 (15) (2013) 5113–5122, doi:10.1158/0008-5472.CAN-12-0237.

[28] L.F. Tannock, A.J. Hickman, Limits to personalized cancer medicine, N. Engl. J. Med. 375 (13) (2016) 1289–1294, doi:10.1056/NEJMp1607705.