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Targeting ALK in pediatric RMS does not induce antitumor activity in vivo

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Abstract

Purpose—The anaplastic lymphoma kinase (ALK) has been demonstrated to be a valid clinical target in diseases such as anaplastic large cell lymphoma and non-small cell lung cancer. Recent studies have indicated that *ALK* is overexpressed in pediatric rhabdomyosarcoma (RMS) and hence we hypothesized that this kinase may be a suitable candidate for therapeutic intervention in this tumor.

Methods—We evaluated the expression of *ALK* in a panel of pediatric RMS cell lines and patient-derived xenografts (PDX), and sensitivity to ALK inhibitors was assessed both in vitro and in vivo.

Results—Essentially all RMS lines were sensitive to crizotinib, NVP-TAE684 or LDK-378 in vitro, and molecular analyses demonstrated inhibition of RMS cell proliferation following siRNA-mediated reduction of *ALK* expression. However, in vivo PDX studies using ALK kinase inhibitors demonstrated no antitumor activity when used as single agents or when combined with standard of care therapy (vincristine, actinomycin D and cyclophosphamide). More alarmingly however, crizotinib actually accelerated the growth of these tumors in vivo.

Conclusions—While ALK appears to be a relevant target in RMS in vitro, targeting this kinase in vivo yields no therapeutic efficacy, warranting extreme caution when considering the use of these agents in pediatric RMS patients.

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Compliance with Ethical Standards

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Keywords

Rhabdomyosarcoma; ALK; patient-derived xenografts; ALK inhibitors; crizotinib

INTRODUCTION

Rhabdomyosarcoma is the most common extracranial solid tumor seen in children and usually presents as a mass in either in the head and neck, the genitourinary system, or the limbs. At least two subtypes exist, including embryonal tumors (ERMS) that tend to be seen in younger patients and are characterized by reduced cellular density and myogenin staining [1]. The second histotype, alveolar RMS (ARMS), demonstrates increased cellularity and 90% contain specific chromosomal translocations that juxtapose either the *PAX3* or *PAX7*, and the *FOXO1* genes [2–7]. The chimeric proteins that result from these rearrangements are transcription factors that demonstrate unique transactivation properties. Response to therapy for all RMS is generally poor and over the last 35 years, the 5 year survival for patients diagnosed with this disease has essentially not changed (~45–65% depending upon age [8]). This dramatically contrasts the improvements seen in leukemia (61% to 89%) and non-Hodgkin's lymphoma (45% to 88%) over the same time frame [8]. This is despite the use of new anticancer agents, dose intensification and repurposing strategies, as well as the application of novel drug combinations and scheduling. Clearly therefore, new cellular targets that are amenable to drug discovery are required for the effective treatment of this disease.

Recent reports indicate that ARMS cells expressing the *PAX/FOXO1* fusion protein may have increased expression of *ALK* (anaplastic lymphoma kinase) [9–13]. Since crizotinib has recently been approved for the treatment of *EML4-ALK* positive non-small cell lung cancer [14], we postulated that ARMS may demonstrate upregulation of this kinase potentially allowing for therapeutic intervention. However, crizotinib was originally developed to target MET and this drug also targets the ROS1 kinase [15,16]. As part of the childhood solid tumor network (CSTN; <https://hospital.stjude.org/dbstp/>; [17]), St. Jude has developed a program to sequence and molecularly characterize a series of pediatric RMS tumors [18]. This includes detailed genomic and microarray analyses, as well as the parallel development of a panel of patient-derived xenografts (PDX; see Table 1), where tumors are intramuscularly implanted into the quadriceps of donor mice directly following surgery. Using established RMS cell lines and these PDX, we sought to evaluate the expression profiles of *ALK*, *MET* and *ROS1* in these samples. Therefore in the following studies, we describe the detailed analysis of kinase expression in a panel of RMS samples and assess their sensitivity to drugs that target ALK and MET. Our results indicate that while ALK is overexpressed in RMS, resulting in in vitro sensitivity of cells to inhibitors of this protein, the growth of PDX expressing this kinase are not impacted by drug treatment in vivo. Our preclinical data therefore, suggests that ALK is not a valid target for the treatment of pediatric RMS, and that the identification of potentially novel tumor targets, using molecular approaches, requires complete detailed validation in representative, in vivo, preclinical models.

MATERIALS AND METHODS

Cell lines and patient-derived xenografts

Detailed information concerning the cell lines and PDX used in this study are presented in Table 1. The presence of *PAX3/FOXO1* or *PAX7/FOXO1* transcripts was confirmed by RT/PCR [19]. All tumor lines were grown in DMEM containing 10% fetal calf serum under an atmosphere of 10% CO₂ at 37C. The immortalized human myoblast line LHCN-M2 [20] was grown in 80% DMEM/20% Medium 199 containing 15% fetal calf serum, supplemented with 0.03mg/L ZnSO₄, 1.4mg/L vitamin B12, 55µg/L dexamethasone and 2.5µg/L human growth hormone. These cells were grown in an atmosphere of 10% CO₂ at 37C.

RMS PDX derived from pediatric patients were grown in the quadriceps of CD1 scid mice by direct intramuscular injection of tumor cells [18]. For biochemical studies, tumors were excised and cells were isolated by combining mechanical dissociation with enzymatic degradation of the extracellular matrix. Resulting cells were then used for biochemical studies. Routinely, FACS analysis indicated that greater than 95% of the cell population was of human origin.

Drugs, antibodies and siRNA

Crizotinib, NVP-TAE684 and LDK-378 for use in tissue culture studies were purchased from Selleck Chemicals (Houston, TX), dissolved in DMSO and stored at –20C. For in vivo use, crizotinib was obtained from Aok Bio (Shanghai, China), and NVP-TAE684 and LDK-378 were sourced from MedChemExpress (Monmouth Junction, NJ). The purity of all drugs were greater than 95%, as assessed by LC-MS analysis. ALK inhibitors were suspended in capryol 90 (Gattefosse Corp., Paramus, NJ) for oral gavage of animals. Vincristine (V), actinomycin D (A) and cyclophosphamide (C) were all obtained from the St. Jude pharmacy and were dissolved in sterile saline. They were administered by i.p. injection using a schedule deemed efficacious in pediatric patients (V given weekly at 0.38mg/kg; A given once at 0.5mg/kg; C given once at 125mg/kg).

Antibodies for ALK (31F12 mouse monoclonal, cat# 3791) and G3PDH (D16H11 rabbit HRP-conjugated monoclonal, cat# 8884) were purchased from Cell Signaling (Danvers, MA). siRNA targeting *ALK* and GFP-tagged RNA used as a control for transfection efficiency were obtained from OriGene (Rockville, MD). The former consists of 3 unique 27mer siRNA duplexes targeting different regions of the *ALK* mRNA and scrambled siRNA were used as negative controls.

Western analyses

Extracts were prepared by vortexing samples in lysis buffer containing phosphatase and protease inhibitors and following sonication, were centrifuged (14,000g, 10min) to remove debris. Samples were separated on 4–12% gradient NuPAGE Bis-Tris SDS-PAGE gels (Invitrogen/Thermo Fisher, Waltham, MA) and proteins were transferred to Immobilon-P PVDF membrane (EMD Millipore, Billerica, MA). Immuno-detection was accomplished using specific primary antibodies (1:1000) and assessed by chemiluminescence with anti-

mouse HRP-conjugated secondary antibodies (1:5000). For the latter, SuperSignal West Dura Extended Duration Substrate (Thermo Fisher) was used and cross-reactivity visualized using BIOMAX MR Film X-ray film (Carestream Health, Inc., Rochester, NY). Multiple exposure times were employed to ensure that very weak and very strong signals could be observed in all samples.

qPCR

qPCR was undertaken using validated gene specific primer sets, and RT²SYBR green master mix containing Hot Start Taq polymerase (Qiagen, Valencia, CA). Analyses were run at least twice with 4 replicates for each sample. Delta C_t values were determined as previously described [21]. Both *TBP* and *ACTB* were used as controls and results for LHCN-M2 were arbitrarily set to 1. All data for RMS cell lines and xenografts were expressed relative to this value.

Growth inhibition assays

Growth inhibition assays were performed essentially as previously described [22]. Routinely, 1×10^5 (1×10^6 for SJRHB012_Y) cells were aliquoted into individual wells of a 6-well plate, allowed to attach overnight and following aspiration of the media, drug was added. At least 7 concentrations of the latter were used for each analysis and samples were repeated in triplicate. After 3 days, cells were harvested and counted using a Vi-Cell XR counter (Beckman Coulter, Fullerton, CA).

IC₅₀ values (concentrations of drug that resulted in 50% growth inhibition) were calculated by determining the relative cell survival as compared to untreated cells and plotting these data as a function of drug concentration. Numerical values were obtained from sigmoidal curve fits using Prism software (GraphPad, San Diego, CA).

Statistical analyses

Statistical correlations were evaluated using GraphPad Prism software with default program settings being employed for each analysis. Analysis of animal survival curves was performed using the log rank test.

siRNA transfection

Cells were transfected with siRNA (75pmoles) using Lipofectamine RNAiMAX (ThermoFisher Scientific, Grand Island, NY). Typically, 0.5×10^6 cells were plated into 6-well plates and *ALK* siRNA and GFP-tagged RNA were added in serum-containing media. The following day, the medium was changed and cells were allowed to grow for up to 9 days. Cell number was determined at 2 day intervals as described above, and transfection efficiency was documented by fluorescence microscopy after 24 hours.

Xenograft studies

Luciferase-labeled tumor cells (1×10^6), obtained from freshly dissociated RMS xenografts, were injected into the quadriceps of CD1 nude mice. Animals were given food and water ad libitum and mice were subjected to imaging on a weekly basis. After 7 days, tumors were visible by bioluminescence imaging using an IVIS 200 instrument (Perkin Elmer, Waltham,

MA). ALK inhibitors were then given by oral gavage daily at their MTD for 20 days and tumor growth was monitored by imaging up to twice weekly. When combined with VAC, the latter was given i.p. using the schedule described above. All studies used 10 mice per group and were conducted in compliance with the St. Jude Children's Research Hospital Institutional Animal Care and Use Committee, consistent with the practices and procedures prescribed by OLAW (Office of Laboratory Animal Welfare, Public Health Services, US).

In all studies, mice were evaluated daily by veterinary staff with no knowledge of the experimental protocol, and animals demonstrating undue tumor burden were humanely sacrificed by CO₂ asphyxia. Death was not used as an endpoint in these experiments.

Determination of crizotinib concentrations in plasma muscle and tumor tissue

To determine levels of crizotinib in plasma, muscle and tumor tissue, mice bearing xenografts were dosed with 150mg/kg of drug for three consecutive days. Four hours following the final dose, the animals were sacrificed, the plasma was collected and the quadriceps containing the tumor was removed. The tumor was dissected away from normal tissue and a fragment of muscle distant from the xenograft was saved. Tumor and muscle was frozen at -196C prior to processing. To determine drug concentrations, the solid tissues were ground under liquid nitrogen and the powders (~10mg) were transferred to preweighed vials. After the addition of 500µl of 50mM Hepes pH7.4, the samples were sonicated on ice (4 × 5 sec) using a probe ultrasonic homogenizer (Cole Palmer model #4710, Chicago, IL). Following dilution with an equal volume of acetonitrile, vortexing and centrifugation (32,000g × 15min), the supernatants were analyzed using a Waters Acquity UPLC system with a SQ2 MS detector. Plasma samples were treated in an identical fashion, with the obvious exclusion of the homogenization step. Separation and quantitation of crizotinib was achieved using an acetonitrile:water:formic acid gradient using an Acquity UPLC BEH 2.1mm × 50mm C18 1.7µm column. Drug recovery rates for this assay using plasma spiked with known amounts of crizotinib were > 99%, with a detection limit of 1nM using a standard calibration curve ($r^2=0.99$) over a concentration range of 1nM to 500nM. Densities of tissues were assumed to be 1 such that all results could be presented in molar units.

RESULTS

Expression of *ALK*, *MET* and *ROS1* in primary tumor samples

To assess the expression of these kinases in primary human RMS tumor samples, the fragments per kbp of exon per million mapped fragments (FPKM) values for each identified sequence was plotted for both ERMS and ARMS. As indicated in Figure 1A–C (panels on the left hand side), a significant difference was observed in the expression of *ALK* in these specimens ($P<0.001$) with ARMS demonstrating markedly increased expression (mean ± SEM, 12.500 ± 2.412 for ARMS, vs 0.621 ± 0.267 for ERMS). In contrast, no statistically significant difference was seen for the expression of either *MET* or *ROS1* (kinases that are targeted by *ALK* inhibitors) between the two different histotypes. However there was a considerable difference in the overall levels of expression of these latter two genes (mean ± SD, 11.453 ± 11.296 for *MET* and 0.452 ± 1.37 for *ROS1*).

Expression of ALK in cell lines and xenografts

To determine whether the increased levels of *ALK* observed in the primary tumor specimens occurred in RMS cell lines and PDX, we undertook qPCR and western analyses. For the qPCR analyses, we used two different control primer sets (*TBP* and *ACTB*). While the latter is frequently employed as a reference gene for these types of studies, since the actin pathway (and therefore the expression of this gene) could be disrupted in tumors that arise in the muscle lineage, we also used the *TBPRNA* as a control. Results using both control primer sets are displayed in Figures 1A–C.

As can be seen, essentially all RMS samples demonstrated higher expression of *ALK* as compared to either the normal myoblast control (LHCN-M2), or the fibroblast line (BJ). In addition, generally expression in ARMS lines (samples on the right hand side of the graph in Figures 1A–C) was higher than that seen in ERMS. It was also observed that there was little difference in the relative levels of expression when using either *TBP* or *ACTB* as controls, with all of the latter values being higher except for RNA obtained from BJ cells. However, due to the possibility that *ACTB* expression might be mis-regulated in one or more samples, all subsequent qPCR analyses used *TBP* as a control.

To confirm the qPCR studies, protein extracts isolated from these samples were subjected to western analyses using an antibody specific for ALK. As indicated in Figures 1D and 1E, expression of ALK is readily detectable in approximately half of the RMS cell lines and the majority of the PDX. It should be noted that the samples with low ALK expression were ERMS, consistent with the results obtained from the RNAseq analyses. However, it was also observed that xenograft samples SJRHB012_Z, SJRHB011_X and SJRHB010927_X1 demonstrated very low levels of this protein, as exemplified by the much longer exposure time required for the membrane (2hr vs 10 sec; Figure 1E).

MET and *ROS1* expression in RMS cell lines and PDX

Since drugs targeting ALK in current clinical trials also inhibit the *MET* and *ROS1* kinases, we evaluated the expression of these genes in the RMS specimens. As indicated in Figure 1B and 1C, for the vast majority of the samples, the RNA levels for *MET* and *ROS1* were lower than that seen in the control cell lines LHCN-M2 and BJ. Two ARMS did demonstrate increased levels of *MET* expression (Rh30 and SJRHB010463_X16), however the relative qPCR signal values for these samples were only 1.33- and 1.97-fold higher than the control myoblast line, respectively. As depicted in Figure 1C, none of the samples demonstrated any appreciable expression of *ROS1*.

Sensitivity of RMS cells to ALK inhibitors

Having established that the RMS lines demonstrated overexpression of *ALK*, relative to the normal myoblast and the fibroblast controls, we assessed the sensitivity of these cells to 3 different ALK inhibitors (crizotinib, NVPTAE-684 and LDK-378). The IC₅₀ values for these drugs with these lines, along with several controls (LHCN-M2, BJ, Sup-M2 and NCI-H2228) are presented in Table 2. As can be seen, all RMS lines were more sensitive to these drugs than the control myoblast LHCN-M2, with the exception of RD and Rh18 with LDK-378. Indeed, IC₅₀ values ranged from 1.3- (Rh36 with NVPTAE-684) to 12.2-fold

(LDK-378 with Rh28) lower when compared to LHCN-M2. A representative series of dose response curves for the Rh lines are depicted in Figure 1F.

As expected, the anaplastic large cell lymphoma cell (ALCL) line Sup-M2, which expresses high levels of *ALK*, demonstrated the lowest IC₅₀ value. This line was included as a *bona fide* positive control for these studies. Additionally, the NSCLC line, NCI-H2228, which contains an *EML4-ALK* translocation (the target of ALK inhibitors in lung cancer patients [23]), also demonstrated sensitivity to crizotinib, NVPTAE-684 and LDK-378. Furthermore, the IC₅₀ values for these agents with this cell line, were comparable with that seen for the RMS lines, indicating that *ALK* would be an effective drug target in these pediatric tumors. Statistical analyses for the correlation between the levels of *ALK* expression in cell lines as determined by qPCR, and the IC₅₀ values for ALK inhibitors, were also undertaken. As indicated in Table 3, rank order analysis confirmed a significant correlation between *ALK* expression and IC₅₀ values for LDK-378 ($p < 0.006$, Spearman's correlation). Additionally, a statistically significant linear relationship was observed between the log-transformed *ALK* expression values and the IC₅₀ results for LDK-378 ($p < 0.007$, $R^2 = 0.56$, Pearson's correlation). These results argue that for this drug and these cells lines, the levels of *ALK* expression are an important determinant for sensitivity.

Sensitivity of RMS cell lines to MET inhibitors

Due to the potential overlap of kinases targeted by the ALK inhibitors (which can also inhibit MET and ROS1), we assessed the ability of three different MET inhibitors (SU11274, foretinib and cabozantinib) to induce growth inhibition in the RMS cell lines. As indicated in Table 2, no obvious differential was observed between the tumor cell lines and the controls. Indeed, for foretinib and cabozantinib, the sensitivity of LHCN-M2 and BJ cells was greater than all of the RMS lines by as much as 75-fold (Rh6 vs LHCN-M2 with cabozantinib). While some increased sensitivity to SU11274 was observed, the maximal differential was only 2-fold. In summary, these results argue that the cytotoxicity of the ALK kinase inhibitors is unlikely to be mediated via inhibition of MET.

siRNA targeting *ALK* inhibits RMS cell proliferation

Having demonstrated that small molecules targeting *ALK* could induce growth inhibition in RMS cell lines, we used a molecular approach to confirm these results. Rh3, Rh28 or LHCN-M2 cells were transfected with pooled siRNAs directed towards *ALK* and cell numbers were determined at various time points. To control for the transfection efficiency, cells were co-transfected with a GFP-tagged control RNA and visualized using a fluorescent microscope. As indicated in Figure 2, the tumor lines Rh3 and Rh28 demonstrated almost complete inhibition of cell proliferation following siRNA-mediated loss of *ALK* expression. The normal myoblast line LHCN-M2 also demonstrated a reduction in cell proliferation, however cell number continued to increase over the course of the experiment (Figure 2A). No obvious effect was observed on cells transfected with a scrambled siRNA. The transfection efficiency was similar amongst the cell lines (Figure 2B) and the siRNA had no obvious effects on cell morphology or viability at 24hr. As expected, the levels of ALK protein in all cell lines, as determined by western analysis, was significantly reduced in samples transfected with siRNA targeting *ALK* (Figure 2C).

To assess whether siRNA targeting ALK could also effect reduction in cell growth in a pediatric RMS PDX, we transfected dissociated cells from SJRHB012_Y tumors and generate cell growth curves. As indicated in Figure 2A, cells isolated from this tumor was considerably less sensitive to loss of this protein. However it should be noted, that downregulation of ALK was not complete in this sample, even though the transfection efficiency was relatively high (Figure 2C).

Growth inhibition analyses for RMS cells obtained from dissociated PDX samples

To determine whether tumors cells obtained from intramuscularly grown PDX demonstrated similar drug sensitivities to established RMS lines, we assessed the IC₅₀ values for SJRHB012_Y in an in vitro assay. Briefly, cells were dissociated from xenograft samples and cell growth inhibition assays were undertaken using crizotinib, NVPTAE-684 or LDK-378. This xenograft was chosen as a donor since it contains moderate levels of ALK expression as validated by qPCR and western analyses (Figure 1A). As can be seen in Table 2, the growth inhibition constants were higher than that seen for the established cell lines, but generally comparable to the latter. While the IC₅₀ value for crizotinib was higher than expected, since it is lower than the plasma concentrations that can be achieved for this drug in mice (at least 9μM [24]) this PDX was chosen for in vivo studies.

In vivo PDX response to ALK inhibitors as single agents

Having established that RMS lines were sensitive in vitro to the ALK inhibitors and that siRNA targeting the mRNA encoding this kinase resulted in growth inhibition, we assessed the antitumor activity of crizotinib, NVP-TAE684 and LDK-378 as single agents towards intramuscular implanted, luciferase-expressing SJRHB012_Y RMS PDX. For these studies, drug was given daily at the MTD (150, 10 and 50 mg/kg for crizotinib, NVP-TAE684 and LDK-378, respectively) for 20 days, and tumor response was documented by bioluminescent imaging. As indicated in Figure 3B, no antitumor activity was observed with any of the drugs when given as single agents. Alarming however, while the difference in median survivals was small (Figure 3B), it appeared that crizotinib may have actually accelerated the growth of this xenograft (Figures 3C and 3D). Even in this relatively small group of animals (n=10), we observed a statistically significant difference in the median survivals between the control and the crizotinib-treated mice, suggesting that detailed, and well controlled, studies would be needed to confirm such an effect does not occur in cancer patients.

To confirm that levels of crizotinib were present within animals that should have induced an antitumor effect, animals bearing SJRHB012_Y RMS PDX were dosed orally with the drug (150mg/kg - the MTD) for three consecutive days. Four hours following the final dose, plasma, muscle and tumor tissue were harvested, and levels of crizotinib in the samples were determined by UPLC/MS. As can be seen in Figure 3E, the concentrations of drug in both muscle and tumor were significantly higher than that seen in the plasma, but also much higher than the in vitro IC₅₀ for SJRHB012_Y RMS cells ($6.0 \pm 1.2\mu\text{M}$). Indeed, the mean concentrations of crizotinib for plasma, muscle and tumor were $2.8 \pm 2.0\mu\text{M}$, $24.4 \pm 11.9\mu\text{M}$ and $40.0 \pm 15.1\mu\text{M}$, respectively. In some cases, these exceeded the in vitro cellular IC₅₀ value by as much as 10-fold. More importantly however, all tumor samples contained higher

concentrations of drug as compared to either the plasma or normal muscle for the same animal.

In vivo PDX response to ALK inhibitors when combined with VAC therapy

Since new clinical trials would be unlikely to use the ALK inhibitors as single agents, we combined them with the current standard of care treatment for pediatric RMS (VAC). Mice were implanted with SJRHB012_Y RMS tumors, and therapy was initiated on day 10. In these studies, VAC was given i.p. over a 20 day period with the ALK inhibitors being administered orally on a daily schedule. As depicted in Figures 3F and 3G, animals treated with VAC demonstrated a good antitumor response, resulting in marked growth delay (~40 day increase in median survival) as compared to control mice. However, the addition of any of the kinase inhibitors to this regimen did not significantly increase the effectiveness of the therapy (Figure 3G). These results suggest that ALK does not represent a viable target for chemotherapy of this tumor.

DISCUSSION

Recent whole genome sequencing and gene expression analyses have indicated that *ALK* is expressed in RMS and may represent a valid target for chemotherapy of this disease [18]. Therefore we assessed the expression of this gene in primary tumor specimens, in an extensive collection of pediatric RMS cell lines and in intramuscularly-grown xenografts. The latter were generated by direct injection of primary tumor cells into the quadriceps of immuno-deficient mouse [18]. The description of these xenografts has recently been reported, however since they have never been grown in cell culture and are constantly maintained in an intramuscular site, they represent a unique resource in which to evaluate chemotherapeutic response. Using these reagents and a variety of assays (RNAseq read quantitation, qPCR, immunoblotting; Figure 1), we have demonstrated that *ALK* is highly expressed in RMS and that higher levels exist in ARMS as compared to ERMS. This is compared to both a normal human myoblast line (LHCN-M2) and to immortal fibroblasts (BJ). Based upon these results, we hypothesized that RMS cells should be sensitive to ALK inhibition by small molecules that target this kinase. This was indeed the case as demonstrated using standard growth inhibition assays (Table 2). Indeed for LDK-378, both the Pearson and Spearman statistical analyses demonstrated statistical significance between the levels of *ALK* expression by qPCR with the IC₅₀ value of the drug (Table 3).

More importantly however, the IC₅₀ values obtained from RMS cells were comparable to those seen for the NSCLC line NCI-H2228. As the latter was used as one of the controls for the in vivo development of the ALK inhibitors for lung cancer [23], similar IC₅₀ values would argue that sensitivity in animal models should be observed towards the RMS samples with crizotinib, NVP-TAE684 or LDK-378.

The lack of overt, selective cytotoxicity afforded by the MET inhibitors in the RMS lines (Table 2), indicated that it was unlikely that the toxicity of the ALK targeting agents resulted from the inhibition of the former kinase. Potentially, the cytotoxicity of crizotinib, NVP-TAE684 and LDK-378 might arise by the inhibition of one or combination of other kinases, but since these molecules demonstrate significantly different inhibition constants for ALK,

MET and ROS1, we believe that this possibility is remote. Additionally, selective reduction of ALK protein using siRNA resulted in an inhibition of RMS cell proliferation that was not as pronounced in the normal myoblast line (Figure 2). *In toto*, these results strongly implicate ALK as the drug target in the RMS cell lines. It should be noted that cells dissociated from an RMS PDX xenograft (SJRHB12_Y) were considerably less sensitive than the established RMS lines to loss of protein mediated by either molecular approaches (Figure 2) or by small molecules (Table 2). However, since the IC₅₀ values for growth inhibition for the latter were below levels that could be achieved in plasma from patients treated with these drugs, we reasoned that these tumors would likely be susceptible to these agents *in vivo*.

Histological analyses from other investigators have also indicated that ALK may represent a valid target for chemotherapy in pediatric tumors. This includes both neuroblastoma [25–29] and RMS [30,12,13]. Therefore, results from both pathological and molecular studies would argue that ALK may represent a clinically viable target for therapeutic intervention in these tumors. Based upon this information and our *in vitro* studies, we undertook preclinical studies using a RMS xenograft (SJRHB012_Y; [18]). This xenograft was selected for several reasons. Firstly, it expresses moderate levels of ALK as determined by immunoreactivity (Figure 1E). Secondly, these xenografts have never been grown *in vitro* and have been maintained by serial passage in immunocompromised mice. This would eliminate any artifactual change in gene expression and phenotype due to the stresses of cell culture. Finally, these tumors grow readily in an intramuscular site within the mouse (the quadriceps), reproducibly engraft, and readily allow for the assessment of antitumor activity of different agents.

However, as indicated in Figure 3, we were unable to demonstrate any significant antitumor efficacy of the ALK kinase inhibitors in this model. While VAC was relatively effective, addition of crizotinib, NVP-TAE684 or LDK-378 did not increase median survival of animals bearing bilateral tumors. Indeed, when these agents were used alone, our data suggests that crizotinib may actually accelerate tumor growth. While the cohort sizes were small, the data was statistically significant (Table 3), and indicates that caution will be necessary when applying these drugs in clinical studies when used as single agents.

The reason we did not observe antitumor activity by the ALK inhibitors indicates one or possibly more of the following scenarios. Firstly, that the established RMS cell lines do not represent valid models for assessing antitumor activity. While this is clearly a possibility, the fact that SJRHB012_Y expresses significant levels of ALK protein would argue that it should be sensitive to these drugs. Secondly, while ALK may represent a valid target *in vitro*, *in vivo* this protein may not be a driver and/or required for continued cell proliferation. Thirdly, the three dimensional and tumor stromal interactions that occur *in vivo*, may significantly outweigh the importance of *ALK* expression. As the former would be very difficult to accurately model *in vitro*, it will be hard to ascertain whether this represents a valid concern. Finally, although probably unlikely, the pharmacokinetics of crizotinib, NVP-TAE684 or LDK-378 in this tumor/normal tissue compartment (i.e., the quadriceps muscle) may not provide a sufficient drug concentration to elicit effective antitumor activity. Crizotinib concentrations up to 9μM have been reported in mice treated with 50mg/kg drug

[24] and since the dose we used was 150mg/kg, it would seem unlikely that the circulating concentration in these studies would be as low as 6 μ M (the in vitro determined IC₅₀ value for SJRHB012_Y cells). While detailed tissue-specific PK studies could be confirmed to assess drug concentrations both in plasma and in tumor tissue, since we observed little activity in vivo, it is not apparent if this would be worthwhile.

In summary, our results indicate that while RMS tumor cell lines express *ALK*, and the encoded protein can be targeted with clinically used small molecules to induce cytotoxicity, the drugs are not efficacious in a pediatric patient-derived RMS xenograft model. This is despite the fact that the latter expresses relatively high levels of the ALK kinase. Indeed, as single agents these inhibitors may actually accelerate tumor cell growth. Since the identification of novel targets for cancer chemotherapy is now being based upon large scale genomic sequencing and expression analysis of individual tumors, we caution that extensive preclinical testing must be undertaken to ensure that derived proteins are validated as *bona fide* candidates.

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Abbreviations

A	actinomycin D
ALCL	anaplastic large cell lymphoma
ARMS	alveolar rhabdomyosarcoma
ATCC	American Type Culture Collection
C	cyclophosphamide
CSTN	childhood solid tumor network
ERMS	embryonal rhabdomyosarcoma
FPKM	fragments per kilobase of exon per million reads mapped
GFP	green fluorescent protein
IC₅₀	concentrations of drug that result in 50% growth inhibition)
LDK-378	5-chloro-2-N-(5-methyl-4-piperidin-4-yl-2-propan-2-yloxyphenyl)-4-N-(2-propan-2-ylsulfonylphenyl)pyrimidine-2,4-diamine
MTD	maximal tolerated dose
NSCLC	non-small cell lung cancer

NVP-TAE684	5-chloro-2-N-[2-methoxy-4-[4-(4-methylpiperazin-1-yl)piperidin-1-yl]phenyl]-4-N-(2-propan-2-ylsulfonylphenyl)pyrimidine-2,4-diamine
PDX	patient-derived xenograft
RMS	rhabdomyosarcoma
SJCRH	St. Jude Children's Research Hospital
V	vincristine
VAC	vincristine, actinomycin D and cyclophosphamide therapy with V given weekly at 0.38mg/kg; A given once at 0.5mg/kg; and C given once at 125mg/kg

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Fig 1. Expression of *ALK*, *MET* and *ROS1* in primary pediatric patient tumor samples, RMS cell lines and PDX as determined by RNAseq, qPCR and western analyses

A, B and C. The box plots on the left hand side represent results obtained from RNAseq datasets using 26 ERMS and 14 ARMS patient tumor specimens. The bar charts on the right hand side represent qPCR analyses of RNA isolated from RMS cell lines and PDX. Values represent relative delta Ct numbers using either *TBP* or *ACTB* as a control, with the LHCN-M2 result arbitrarily set to 1.

D. Western analysis of ALK expression in RMS cell lines

E. Western analysis of ALK expression in selected patient-derived intramuscularly grown RMS xenografts. This data has been presented as supplementary data previously [18], but is included here for comparative purposes. For all gels, size markers are in kDa.

F. Growth inhibition curves for selected cell lines when treated with NVP-TAE684. A representative example is shown to demonstrate the high R^2 values (typically greater than 0.96) for the curve fits for each individual data set. For clarity, error bars are not shown.

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Fig 2. Cell proliferation curves and transfection efficiencies for cell lines following transfection with siRNA targeting *ALK*

A. Cell proliferation curves for LHCN-M2, Rh3, Rh28 and SJRHB012_Y cells following transfection with siRNA directed towards *ALK*. Control samples were transfected with a scrambled siRNA.

B. Phase contrast (upper panel) and fluorescence images (lower panel) of cells either 24hr (LHCN-M2, Rh3, Rh28) or 72hr (SJRHB012_Y) after transfection. A GFP-tagged control RNA was used to monitor transfection efficiency.

C. Western analysis of ALK protein in cells following siRNA transfection. An image for LHCN-M2 is not shown since ALK expression is undetectable in these cells using this approach (see Figure 1D).

Fig 3. Xenograft growth curves and Kaplan-Meier curves for mice bearing SJRHB012_Y tumors when given ALK inhibitors either as single agents or when combined with VAC

A. Individual photon flux curves (as monitored by bioluminescent imaging) for mice bearing SJRHB012_Y xenografts grown in the thigh muscle of CD1 mice. Animals were orally dosed daily at the MTD for crizotinib (red line – CRI), NVP-TAE684 (blue line – TAE) or LDK-378 (green line – LDK) for 20 days. Data points represent the sum of the values for bilaterally implanted tumors following imaging on the dorsal side of the animal.

B. Animal survival curves for mice harboring SJRHB012_Y xenografts following daily dosing with ALK inhibitors. The labeling of the curves is identical to that depicted in panel

A. Median survival (in days) and statistical significance versus the control untreated group are indicated on the legend.

C and D. Representative bioluminescence images of cohorts of mice bearing intramuscular SJRHB012_Y RMS xenografts. Panel C depicts the control group at day 27 and panel D is the crizotinib-treated animals at the same time point.

E. Concentrations of crizotinib in plasma, muscle and tumor for mice bearing SJRHB012_Y RMS xenografts. Crizotinib (150mg/kg) was given orally for three consecutive days and samples harvested 4 hours following the final dose. Samples from the same animals are connected by the lines.

F. Individual photon flux curves for mice bearing SJRHB012_Y RMS xenografts when treated with VAC alone (orange line – VAC), or when combined with oral daily dosing of crizotinib (red line – CRI), NVP-TAE684 (blue line – TAE) or LDK-378 (green line – LDK) for 20 days. Data points represent the sum of the values for bilaterally implanted tumors following imaging on the dorsal side of the animal.

G. Animal survival curves for mice harboring SJRHB012_Y RMS xenografts following VAC therapy and daily dosing with ALK inhibitors. Curves are labeled in an identical manner to that described in panel B. Median survival (in days) and statistical significance vs the VAC alone group are indicated on the legend.

Table 1

Information concerning the cell lines and patient-derived xenografts used in this study

Name	Cell line (C) or xenograft (PDX)	Source	Reference	Translocation
LHCN-M2	C	<i>TERT</i> immortalized normal human myoblast	[20]	None
BJ	C	Human foreskin fibroblasts	American Type Culture Collection (ATCC)	None
RD	C	ERMS primary tumor	ATCC	None
Rh2	C	ERMS - unknown	Dr. P. Houghton, St. Jude Children's Research Hospital (SJCRH)	None
Rh3	C	ARMS lung metastasis	Dr. P. Houghton, SJCRH	<i>PAX3/FOXO1</i>
Rh4	C	ARMS breast metastasis	Dr. P. Houghton, SJCRH	<i>PAX3/FOXO1</i>
Rh6	C	ERMS lymph node metastasis	Dr. P. Houghton, SJCRH	None
Rh18	C	ARMS primary tumor	Dr. P. Houghton, SJCRH	None
Rh28	C	ARMS lymph node metastasis	[31]	<i>PAX3/FOXO1</i>
Rh30	C	ARMS bone marrow metastasis	[2]	<i>PAX3/FOXO1</i>
Rh36	C	ERMS primary tumor	Dr. P. Houghton, SJCRH	None
Rh41	C	ARMS lung metastasis	Dr. P. Houghton, SJCRH	<i>PAX3/FOXO1</i>
Sup-M2	C	ALCL	Dr. S. Morris, SJCRH	<i>NPM/ALK</i>
NCI-H2228	C	NSCLC	ATCC	<i>EML4/ALK</i>
SJRHB011_X	PDX	ERMS from skull mass reoccurrence	[18]	None
SJRHB011_Y	PDX	ERMS from primary neck mass	[18]	None
SJRHB012_X	PDX	ERMS from prostate	[18]	None
SJRHB012_Y	PDX	ERMS from prostate reoccurrence (original tumor SJRHB012_X)	[18]	None
SJRHB012_Z	PDX	ERMS from reoccurrence in the pelvis (original tumor SJRHB012_X)	[18]	None
SJRHB013_X	PDX	ERMS from perineal mass reoccurrence	[18]	None
SJRHB000026_X2	PDX	ERMS from gastric mass reoccurrence	[18]	None
SJRHB010468_X1	PDX	ARMS from right thigh	[18]	<i>PAX7/FOXO1</i>
SJRHB010463_X16	PDX	ARMS from lung biopsy (original tumor maxillary sinus)	[18]	<i>PAX3/FOXO1</i>
SJRHB010927_X1	PDX	ERMS from parapharyngeal mass	[18]	None
SJRHB010928_X1	PDX	ERMS from prostate reoccurrence	[18]	None

Table 2

IC₅₀ values of cells following exposure to ALK or MET inhibitors

Cells	Cell type	IC ₅₀ (± SD), μM					
		ALK inhibitors			MET inhibitors		
		Crizotinib	NVP-TAE684	LDK-378	SU11274	Foretinib	Cabozantinib
LHCN-M2	Myoblast	1.84 ± 0.61	0.45 ± 0.10	1.84 ± 0.51	3.85 ± 0.98	0.12 ± 0.05	0.14 ± 0.06
BJ	Fibroblast	2.11 ± 0.39	0.90 ± 0.17	2.60 ± 0.50	5.19 ± 1.26	0.18 ± 0.07	4.52 ± 2.13
RD	ERMS	0.49 ± 0.10^a	0.25 ± 0.07	2.18 ± 0.51	2.11 ± 0.44	0.22 ± 0.03	1.21 ± 0.40
Rh2	ERMS	0.25 ± 0.05	0.18 ± 0.04	0.77 ± 0.15	1.84 ± 0.31	0.23 ± 0.06	2.48 ± 0.92
Rh3	ARMS	0.45 ± 0.14	0.17 ± 0.05	0.36 ± 0.06	2.72 ± 0.49	0.49 ± 0.20	3.94 ± 1.06
Rh4	ARMS	0.58 ± 0.05	0.09 ± 0.01	0.34 ± 0.08	2.21 ± 0.32	0.22 ± 0.05	3.09 ± 0.42
Rh6	ERMS	0.90 ± 0.15	0.16 ± 0.02	0.72 ± 0.16	1.84 ± 0.70	1.05 ± 0.45	10.37 ± 3.67
Rh18	ARMS	0.55 ± 0.11	0.27 ± 0.06	2.34 ± 0.44	4.07 ± 0.68	0.31 ± 0.11	5.75 ± 0.99
Rh28	ARMS	0.91 ± 0.21	0.08 ± 0.02	0.15 ± 0.04	4.50 ± 1.86	0.48 ± 0.15	4.29 ± 1.13
Rh30	ARMS	0.99 ± 0.20	0.30 ± 0.10	0.64 ± 0.20	4.70 ± 3.04	0.60 ± 0.06	5.90 ± 0.81
Rh36	ERMS	0.82 ± 0.12	0.36 ± 0.06	0.75 ± 0.12	4.32 ± 1.18	0.66 ± 0.09	4.62 ± 0.84
Rh41	ARMS	0.45 ± 0.12	0.07 ± 0.01	0.35 ± 0.06	1.84 ± 0.39	0.59 ± 0.06	2.40 ± 0.96
Sup-M2	ALCL	0.08 ± 0.004	0.02 ± 0.004	0.02 ± 0.003	2.92 ± 0.34	0.52 ± 0.04	7.00 ± 0.76
NCL-H2228	NSCLC	0.48 ± 0.14	0.22 ± 0.07	0.38 ± 0.14	8.32 ± 3.01	0.82 ± 0.11	5.19 ± 0.58
SJRB012_Y	RMS PDX	6.0 ± 1.2	1.3 ± 0.3	1.7 ± 0.9	ND ^b	ND	ND

^a – Values in bold are lower (i.e. more sensitive) than the LHCN-M2 myoblast control for the corresponding drug

^b ND – Not determined.

Table 3

Statistical correlations between IC₅₀ values for cell lines with crizotinib, NVP-TAE684 and LDK-378 with *ALK* expression levels, as determined by qPCR.

Parameters	Drug	Linear regression r ²	Pearson r (P-value)	Spearman r (P-value)
ALK qPCR	Crizotinib	0.017	-0.13 (0.70)	-0.35 (0.29)
	NVP-TAE684	0.11	-0.34 (0.31)	-0.51 (0.11)
	LDK-378	0.22	-0.47 (0.14)	-0.78 (0.006)
Log10 ALK qPCR	Crizotinib	0.26	-0.51 (0.11)	-0.35 (0.29)
	NVP-TAE684	0.36	-0.60 (0.05)	-0.51 (0.11)
	LDK-378	0.56	-0.76 (0.007)	-0.78 (0.006)