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Immunoassays for the quantification of ALK and phosphorylated ALK support the evaluation of on-target ALK inhibitors in neuroblastoma

Elizabeth R. Tucker[1†], Jennifer R. Tall[1,2†], Laura S. Danielson[1], Sharon Gowan[3], Yann Jamin[4], Simon P. Robinson[4], Udai Banerji[2] and Louis Chesler[1]

1 Paediatric Solid Tumour Biology and Therapeutics Team, Division of Clinical Studies and Cancer Therapeutics Division, The Institute of Cancer Research, Sutton, Surrey, UK
2 Clinical Pharmacodynamic Biomarker Team, Cancer Therapeutics Division, The Institute of Cancer Research, Sutton, Surrey, UK
3 Tumour Biology and Metastasis, Cancer Therapeutics Division, The Institute of Cancer Research, Sutton, Surrey, UK
4 Division of Radiotherapy and Imaging, The Institute of Cancer Research, Sutton, Surrey, UK

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Correspondence
L. Chesler, Paediatric Solid Tumour Biology and Therapeutics Team, Division of Clinical Studies and Cancer Therapeutics Division, The Institute of Cancer Research, 15 Cotswold Road, Sutton, Surrey SM2 5NG, UK
Tel: +44 0208 722 4176
E-mail: louis.chesler@icr.ac.uk

†Joint first authors

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Targeted inhibition of anaplastic lymphoma kinase (ALK) is a successful approach for the treatment of many ALK-aberrant malignancies; however, the presence of resistant mutations necessitates both the development of more potent compounds and pharmacodynamic methods with which to determine their efficacy. We describe immunoassays designed to quantitate phosphorylation of ALK, and their use in preclinical models of neuroblastoma, a pediatric malignancy in which gain-of-function ALK mutations predict a poor overall outcome to conventional treatment. Validation of the immunoassays is presented using a panel of neuroblastoma cell lines and evidence of on-target ALK inhibition provided by treatment of a genetically engineered murine model of neuroblastoma with two clinical ALK inhibitors, crizotinib and ceritinib, highlighting the superior efficacy of ceritinib.

1. Introduction

The rapid development of targeted therapeutics against anaplastic lymphoma kinase (ALK) has already resulted in significant changes to the up-front treatment of patients with ALK-rearranged non-small-cell lung cancer (NSCLC) (Camidge *et al.*, 2012), and a current aim is to bring these personalized therapies to the benefit of childhood cancer patients, including those with neuroblastomas that harbor point mutations of ALK (Chen *et al.*, 2008; George *et al.*, 2008; Janoueix-Lerosey *et al.*, 2008; Mosse *et al.*, 2008). The broad range of ALK mutations found either as a secondary resistance mechanism to ALK inhibition in NSCLC or as a primary resistance mechanism in neuroblastoma necessitates thorough interrogation of the ability of individual compounds to inhibit ALK phosphorylation and subsequent survival signaling downstream of ALK, enabling the rapid translation of the most promising compounds from the laboratory to clinical trials (Bresler *et al.*, 2011, 2014). To achieve this, highly relevant preclinical models should be coupled with accurate methods to report pharmacodynamic responses.

Abbreviations
ALK, anaplastic lymphoma kinase; MSD®, Meso Scale Discovery.

We have therefore developed sensitive immunoassays to detect and quantitate the expression of total ALK protein and phosphorylated forms of ALK using the mesoscale technology (MSD®) platform. This format allows for the development of assays that are appropriate for both *in vitro* and *in vivo* studies, and MSD® assays are already routinely incorporated into clinical studies in order to measure pharmacodynamic end points (Basu *et al.*, 2015). We sought to validate our ALK immunoassays using both neuroblastoma cell lines and tumor tissue from the Th-ALK^{F1174L}/*MYCN* transgenic model, which has previously demonstrated the inadequacy of the first-generation ALK inhibitor, crizotinib, to elicit therapeutic responses in ALK F1174L-driven neuroblastomas (Berry *et al.*, 2012).

Our results demonstrate the successful application of MSD® immunoassays to measure ALK and phosphorylated ALK as pharmacodynamic biomarkers following treatment with small-molecule ALK inhibitors in both *in vitro* and *ex vivo* tissues. We show that in addition to autophosphorylation of ALK at Y1278 and Y1604, following the fate of phosphorylated ALK at Y1586 also provides a marker of active ALK levels.

2. Materials and methods

2.1. Cell lines

Neuroblastoma cell lines and HeLa cells were obtained from the American Type Culture Collection, CLB-GA was a gift from V. Combaret (Lyon), and these were shown to be mycoplasma-free using a PCR-based assay (Minerva Biolabs, Berlin, Germany). The Ba/F3 ALK F1174L cells were a gift from R. George (Boston, USA) and were transduced as described previously (George *et al.*, 2008). Cells were cultured in RPMI 1640 media supplemented with 2 mM glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 1 × MEM nonessential amino acids, and 10% FBS (Gibco) and grown at 37 °C with 5% CO_2 in a humidified incubator.

2.2. Drug treatments

Cell lines were treated at 50–70% confluency at the indicated drug concentrations or with DMSO at a concentration that matched the greatest DMSO for the drug-treated cells. Crizotinib and ceritinib were purchased from Shanghai Haoyuan Chemexpress Company.

2.3. GI_{50} determination

In order to calculate the half maximal growth inhibitory concentration (GI_{50}) of individual compounds, neuroblastoma tumor cells were seeded into 96-well plates in a total volume of 100 μL and allowed to attach overnight. Compound (dissolved in DMSO) was added to wells in six replicates of 100 μL, across a concentration gradient including a DMSO-only control, the next day. The cells were exposed to drug for 72 h. Thereafter, the cell number in treated versus control wells was estimated after cell fixation with 10% trichloroacetic acid and staining with sulforhodamine B in 1% acetic acid. The GI_{50} was calculated as the drug concentration that inhibits cell growth by 50% compared with control growth, according to nonlinear regression analysis, using GRAPHPAD PRISM (La Jolla, CA, USA).

2.4. Preparation of protein lysates

Cell lines were harvesting by scraping, spun at 500 *g* for 5 min, and washed once in phosphate-buffered saline, and the cell pellets were resuspended in CHAPS lysis buffer [50 mM Tris/HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% CHAPS, 0.2 mM PMSF, 1 : 50 Phosphatase Inhibitor Cocktail 2 and 3 (Sigma-Aldrich, St. Louis, MO, USA), 1 : 100 Protease Inhibitor Cocktail (Sigma-Aldrich)]. Frozen tissue samples were homogenized in CHAPS lysis buffer prepared as for cell lysates. After incubation for 30 min on ice, lysates were spun at 16 000 ***g*** for 15 min and the supernatant was collected. Protein concentrations were determined using BCA protein assay (Thermo Fisher Scientific) by comparison with bovine serine albumin standard.

2.5. ALK Meso Scale Discovery® immunoassays

Multiarray 96-well plates (Meso Scale Discovery) were coated overnight at 4 °C with 0.5 $\mu g \cdot mL^{-1}$ mouse total ALK antibody (Clone 31F12; Cell Signaling Technology Inc., Danvers, MA, USA) diluted in 50 mM carbonate buffer. Plates were washed 5 × in wash buffer (0.1% Tween 20 in Tris-buffered saline) and incubated for 1 h with blocking buffer (5% BSA in wash buffer). After washing, samples were added with cell lysates being diluted to 20 μg per well (*in vitro*) or 30 μg per well (*in vivo*) in 1 × Tris Lysis Buffer (Meso Scale Discovery) or recombinant ALK protein (Thermo Fisher Scientific) diluted in phosphate-buffered saline and incubated overnight at 4 °C. After washing, the plates were then incubated for 1 h with the appropriate antibody (0.4 $\mu g \cdot mL^{-1}$ total ALK D5F3, 0.2 $\mu g \cdot mL^{-1}$ pY1278 ALK D59G10, 0.4 $\mu g \cdot mL^{-1}$ pY1586 ALK 3B4, 0.2 $\mu g \cdot mL^{-1}$ pY1604 ALK D96H9, or 0.2 $\mu g \cdot mL^{-1}$ phospho-tyrosine P-Tyr-1000, all purchased from Cell Signaling

Technology Inc.), washed, and incubated for a further 1 h with 0.5 μg·mL^{-1} anti-rabbit SULFO-tag antibody. The plates were washed, 2 × Read Buffer T (Meso Scale Discovery) was added to wells, and electrochemiluminescence counts were made using a MSD SECTOR Imager 6000.

2.6. Immunoblotting

Western blotting of 20 μg of denatured lysates was carried out using precast 4–12% Bis/Tris gels in 1 × MOPS running buffer [Thermo Fisher Scientific (Invitrogen)]. A prestained molecular weight marker was loaded alongside the experimental samples (Invitrogen). The gels were transferred to PVDF membranes at 30 V for 3 h, or at 0.05 mA overnight. The membranes were blocked for 1 h in 5% (w/v) nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBST), and then incubated with primary antibody overnight at 4 °C in 2.5% milk TBST (ALK, pY1278 ALK, pY1604 ALK, pY1586 ALK (all equivalent to the respective MSD® immunoassay), ERK1/2, pERK1/2, Akt, pAkt, GAPDH, all from Cell Signaling Technology Inc.). Membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibody and proteins were visualized using electrochemical luminescence (ECL plus) [GE Healthcare Life Sciences (Amersham Biosciences), Buckinghamshire, UK] detection system on a LAS-3000 Imaging System [GE Healthcare Life Sciences (Fujifilm), Buckinghamshire, UK]. Quantification of blots was performed by densitometry using IMAGEJ software analysis (Schneider *et al.*, 2012).

2.7. Immunoprecipitation

Cell lysates were subjected to preclearing before direct incubation with the antibody of interest (ALK; IgG) for an hour at 4 °C. Protein G beads were added to the lysate and incubated overnight at 4 °C. The next day, the samples were spun down for 30 s at 4 °C and the supernatant was discarded. The bead pellet was washed five times with lysis buffer, before denaturing by heating to 95 °C for 5 min in SDS sample buffer.

2.8. Mouse models

All experiments, including the breeding of transgenic animals, were performed in accordance with the local ethical review panel, the UK Home Office Animals (Scientific procedures) Act 1986, the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines (Kilkenny *et al.*, 2010) and the UK NCRI guideline (Workman *et al.*, 2010). Th-*ALK*^{F1174L}/*MYCN* tumor-bearing animals were enrolled into therapeutic trials when their abdominal tumors reached 5 mm in diameter according to palpation. Volumetric MRI was performed as previously described (Jamin *et al.*, 2013), with each animal undergoing imaging on day 0 and day 7. The tumor volume at each time point was then calculated. For *in vivo* oral dosing on days 1–7, crizotinib was dissolved in sterile water with 10% Tween 20. Ceritinib was dissolved in 0.5% methylcellulose, 0.5% Tween 80 with sterile water. Two hours following the final dose of either compound, tumor tissue was excised and snap-frozen prior to analysis.

3. Results

3.1. Detection of recombinant ALK (rALK) protein with immunoassays

Using the MSD® platform, we optimized immunoassays to detect phosphorylated or total ALK protein. Confirmation of the ability of the assays to detect ALK or phosphorylated ALK species was sought through the use of a kinase active recombinant ALK (rALK) protein (Fig. 1). Using a titrating amount of rALK, we found that pan-pY ALK, pY1278 ALK, pY1586 ALK, pY1604 ALK, and total ALK were detected in a linear fashion in each assay (Fig. 1A). The reproducibility of the assays to detect rALK was assessed both within a single experiment (Fig. 1B) and across at least three independent experiments (Fig. 1C). In all cases, the intra-assay percentage coefficient of variation for pY1278, pY1604, and total ALK was less than 2.4%, 6.2%, and 6.2%, respectively. In four of five samples for the pY assay and the pY1586 assay, the coefficient of variation was less than 2.5% and 5.4%, respectively. The interassay variability was less than 30% in all assays, indicating high reproducibility of the assay signals.

3.2. Immunoassays quantitate ALK and phosphorylated ALK forms in neuroblastoma cell lines

Gain-of-function mutations of *ALK*, which lead to constitutive ALK phosphorylation, are known to contribute to the aggressive nature of pediatric neuroblastoma tumors, and therefore, the detection of ALK activity in available neuroblastoma cell lines is of increasing importance. We employed a panel of these cell lines harboring either one of the two most frequent ALK mutations (F1174L or R1275Q), or wild-type ALK, and assessed the total and phosphorylated

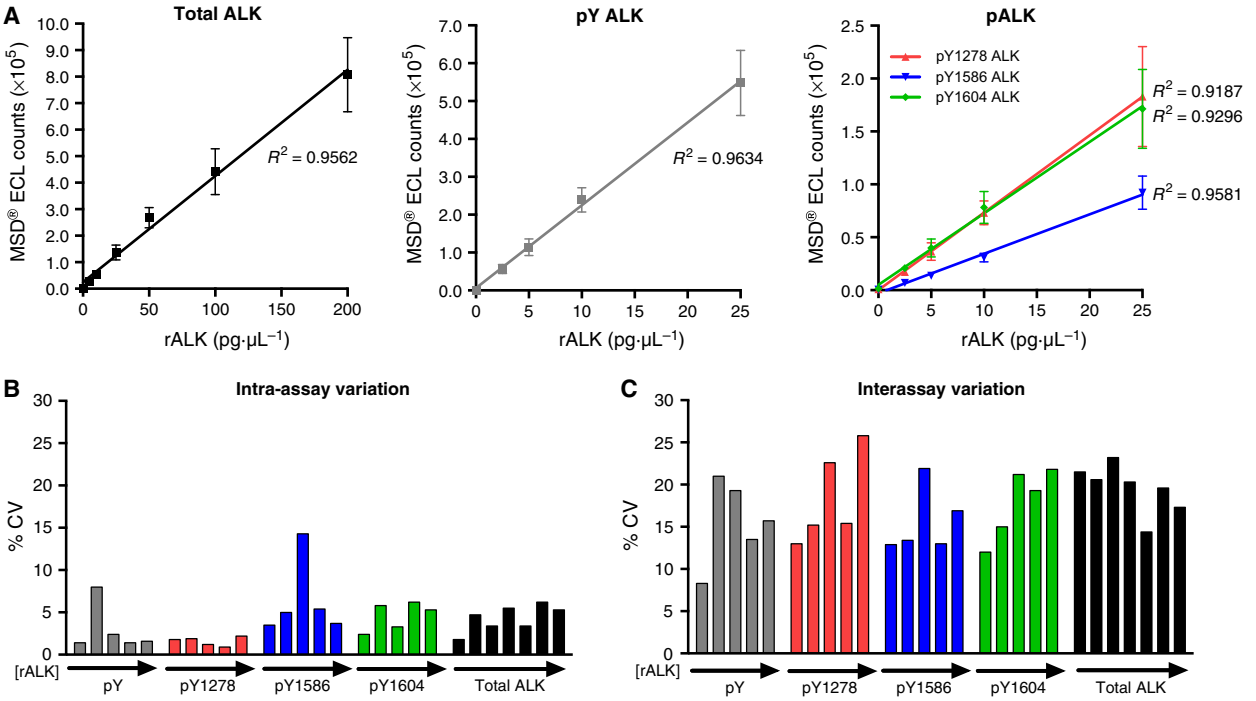


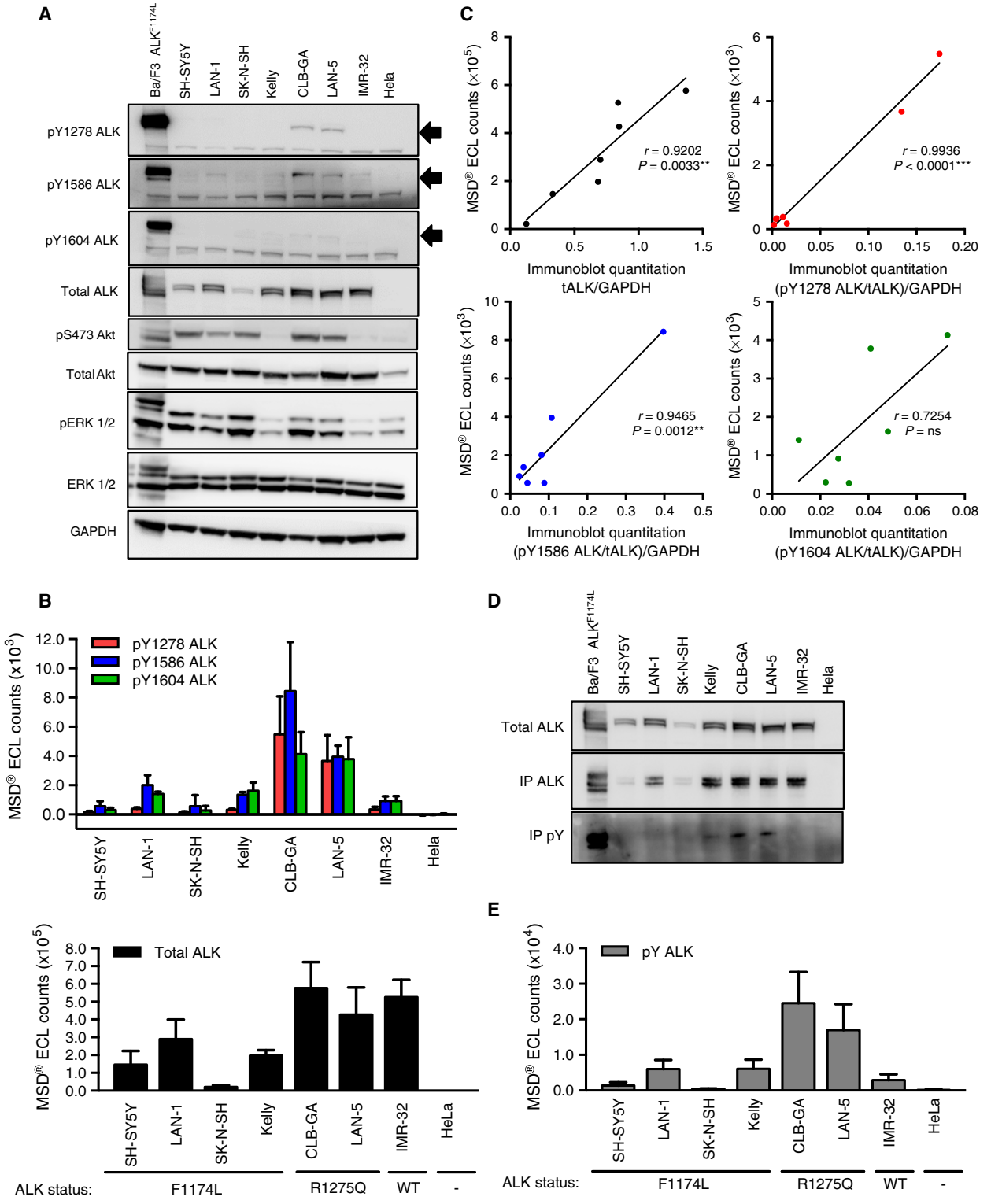
Fig. 1. Immunoassays to quantitate phosphorylated and total ALK protein. (A) Titration of recombinant ALK protein (rALK) in MSD® assays to quantify pan-phospho-tyrosine ALK, phospho-Y1278 ALK, phospho-Y1586 ALK, phospho-Y1604 ALK, and total ALK protein levels. Mean ± SD from ≥ 3 independent repeats. R-squared values for linear regression from each of the assays are indicated. Assay reproducibility was assessed by (B) intra-assay variability calculated across triplicate wells of each ALK MSD® assay with increasing concentrations of recombinant ALK protein (same as A) and (C) interassay variability calculated across from ≥ 3 independent repeats using increasing concentrations of recombinant ALK protein (same as A) and is presented as percentage coefficient of variation (CV).

abundance of ALK by immunoblotting, in addition to pathways downstream of ALK (Fig. 2A). When compared to the Ba/F3-transduced cell lysates, it was apparent that detection and therefore quantification of phosphorylated ALK is challenging in the neuroblastoma cell panel by western blotting as bands were only clearly visible for pY1278 and pY1586 ALK in CLB-GA and LAN-5 lysates. Phosphorylated Akt and phosphorylated ERK 1/2 signals were detected more strongly in CLB-GA and LAN-5 again, as well as in three of the four *ALK F1174L* cell lines, excluding Kelly cells. However, analyzing the same cell lysates in the ALK immunoassays we developed showed detectable levels of pY1278, pY1586, pY1604, and total ALK in all the neuroblastoma cell lines tested, whereas no signals were obtained for HeLa cell lysate in any of the ALK assays consistent with the lack of ALK expression (Fig. 2B). When the levels of phosphorylated ALK in the neuroblastoma cell lines were normalized to total ALK signals from the immunoassays and compared with densitometry of the immunoblots, there was a positive correlation that was statistically significant for the total ALK and pY1278 and pY1586 ALK assays, and although not significant, a positive trend between densitometry and immunoassay for the detection of pY1604 ALK was seen (Fig. 2C). Furthermore, upon immunoprecipitation of ALK and detection of pan-pY from the same lysates (Fig. 2D), there was a stronger pY signal obtained for the two cell lines harboring the R1275Q ALK mutation (CLB-GA and LAN-5). This finding is consistent with CLB-GA and LAN-5 cell lysates giving the highest signals out of the cell line panel in the pY1278, pY1586, and pY1604 ALK assays (Fig. 2B) and the pY ALK MSD® assay (Fig. 2E). Together, these data indicate that the ALK immunoassays can be used to characterize basal levels of ALK activity in neuroblastoma cells in a more quantitative manner and with greater sensitivity than immunoblotting.

3.3. ALK immunoassays quantify the dose- and time-dependent response of neuroblastoma cell lines to therapeutic ALK inhibition

To further assess the ability of the phospho-ALK MSD assays to measure changes in ALK activity, we

made use of two clinical small-molecule ALK inhibitors, crizotinib and ceritinib. Crizotinib was less successful against ALK F1174L neuroblastomas in clinical trial, but showed efficacy in the treatment for ALK R1275Q neuroblastomas (Mosse *et al.*, 2013). Ceritinib is a second-generation ALK inhibitor, currently being evaluated in pediatric clinical studies. We used two neuroblastoma cell lines to compare crizotinib and



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