Dual ALK and CDK4/6 Inhibition Demonstrates Synergy against Neuroblastoma

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Abstract

Purpose: Anaplastic lymphoma kinase (ALK) is the most frequently mutated oncogene in the pediatric cancer neuroblastoma. We performed an in vitro screen for synergistic drug combinations that target neuroblastomas with mutations in ALK to determine whether drug combinations could enhance antitumor efficacy.

Experimental Design: We screened combinations of eight molecularly targeted agents against 17 comprehensively characterized human neuroblastoma-derived cell lines. We investigated the combination of ceritinib and ribociclib on in vitro proliferation, cell cycle, viability, caspase activation, and the cyclin D/CDK/CDK6/IB and pALK signaling networks in cell lines with representative ALK status. We performed in vivo trials in CB17 SCID mice bearing conventional and patient-derived xenograft models comparing ceritinib alone, ribociclib alone, and the combination, with plasma pharmacokinetics to evaluate for drug–drug interactions.

Results: The combination of ribociclib, a dual inhibitor of cyclin-dependent kinase (CDK) 4 and 6, and the ALK inhibitor ceritinib demonstrated higher cytotoxicity ($P = 0.008$) and synergy scores ($P = 0.006$) in cell lines with ALK mutations as compared with cell lines lacking mutations or alterations in ALK. Compared with either drug alone, combination therapy enhanced growth inhibition, cell-cycle arrest, and caspase-independent cell death. Combination therapy achieved complete regressions in neuroblastoma xenografts with ALK-F1174L and F1245C de novo resistance mutations and prevented the emergence of resistance. Murine ribociclib and ceritinib plasma concentrations were unaltered by combination therapy.

Conclusions: This preclinical combination drug screen with in vitro validation has provided the rationale for a first-in-children trial of combination ceritinib and ribociclib in a molecularly selected pediatric population.

Introduction

Neuroblastoma is a pediatric malignancy of the sympathetic nervous system. Despite recent intensification of therapy, the long-term survival for patients with high-risk disease continues to lag significantly behind that of other common childhood cancers (1). The most frequently mutated oncogene tractable for targeted therapy is anaplastic lymphoma kinase (ALK), occurring as germ-line mutations in hereditary neuroblastomas (2, 3), and as somatically acquired mutations in sporadic disease (2–5). The most common mechanism of oncogenic ALK activation across all cancers is chromosomal translocation of the ALK tyrosine kinase domain to a homodimerization partner, including EML4-ALK in non–small cell lung cancer (6) and NPM-ALK in lymphoma (7). However, in neuroblastoma, the most common ALK aberrations are activating point mutations in the tyrosine kinase domain of full-length ALK occurring in 8% of tumors across all stages of disease (8). Within the high-risk subset of patients, the overall frequency is 14% (10% mutation, 4% amplification) at diagnosis, and the presence of an activating ALK alteration is independently prognostic of inferior outcome (8). Moreover, ALK mutations are enriched at relapse, further heightening the attractiveness of ALK as a tractable therapeutic target (9, 10).

Small-molecule ALK inhibitors are clinically active against malignancies with ALK translocations, (11–13), and there is known dependence of ALK as a driver in neuroblastoma (2–5). Yet in the pediatric phase I study of crizotinib, the objective response rate was comparatively modest in patients with relapsed neuroblastoma and activating ALK mutations (14). Ongoing studies examining the biochemistry of ALK mutations in neuroblastoma have demonstrated that crizotinib and ceritinib, as well as future generation inhibitors, face significant challenges in overcoming ALK mutations with innate relative resistance to ATP-competitive inhibitors, most notably at the F1174 codon (8, 12, 15). Secondary mutations at codon F1174 in translocated ALK were also associated with both acquired crizotinib resistance during preclinical in vitro screens (16) and progression of ALK-rearranged inflammatory myofibroblastic tumor (17). Although
Translational Relevance

Activating mutations in full-length anaplastic lymphoma kinase (ALK) provide a drug target in the pediatric cancer neuroblastoma, but in a phase I study, an ALK inhibitor did not achieve objective responses in the majority of patients. To improve antitumor activity and durability of responses, we used high-throughput screening to identify synergistic drug combinations. The combination of a CDK4/6 and ALK inhibitor showed selective synergy and efficacy against ALK-mutated neuroblastomas, with dual inhibition of constitutively activated ALK and the downstream cyclin D–CDK4/6 cell cycle switch. The prevalent ALK F1174 hotspot mutations cause preclinical and clinical resistance to ATP-competitive ALK inhibitors; however, combined therapy achieved complete sustained regressions against F1174L xenografts. This work provides the preclinical rationale for a first-in-human trial of combination ALK and CDK4/6 inhibition in ALK-mutated neuroblastomas.

there is considerable enthusiasm about the preclinical data observed with lorlatinib (18), this drug has not yet been tested in children, and the toxicity profile has not been defined; in addition, single-agent kinase inhibition has often proven to be of only transient benefit.

Cancer drug development is increasingly focused on combinatorial therapies (19, 20) in recognition that single-agent tyrosine kinase inhibition frequently leads to the development of resistance and relapse (21–23). Inhibition of multiple signaling pathways is also in keeping with the current understanding of cancer cell signaling networks characterized by activation of multiple pathways, cross-talk, feedback, and redundancy (24). In metastatic melanoma with BRAF V600 mutations, combined BRAF and MEK pathway inhibition significantly improved survival compared with BRAF inhibition alone in treatment-naïve (25, 26) and BRAF monotherapy–resistant disease (26, 27). The use of tumor genomics to select patients suitable for combinatorial kinase inhibition is also consistent with preclinical models demonstrating that synergistic combinations are context specific (28). We postulate that targeting of key kinases and parallel pathways is necessary for cooperative and durable inhibition of ALK-mutant neuroblastoma. Therefore, we hypothesized that simultaneous inhibition of ALK and aberrant pathways may lead to superior antitumor activity in neuroblastomas with ALK mutations, while potentially minimizing the emergence of resistance and relapse. In this study, we sought to identify drug combinations with synergistic inhibition of ALK-mutated neuroblastoma cell lines by performing a pairwise combination screen of molecularly targeted agents. Here, we provide the rationale for a clinical trial of combination ceritinib (ALKi) and ribociclib (CDK4/6i) in ALK-driven neuroblastomas.

Materials and Methods

Cell lines and reagents

Cell lines were obtained from the Children’s Hospital of Philadelphia (Philadelphia, PA) cell line bank or ATCC (Manassas, VA) and were maintained according to recommendations.

Cell identity was authenticated using AmpFLSTR Identifiler (Applied Biosystems). Cells were grown to 70% to 80% confluency before experiments and plated at their predetermined cell density. Ceritinib and ribociclib were provided by Novartis, dissolved in DMSO at 10 mmol/L stock concentrations for in vitro experiments, and further diluted in culture medium before use.

Synergy screen

To measure the effects of chemical combinations on cell viability, cells were seeded into 1,536-well assay plates at a density of 300 cells in a 7 μL per well final volume and incubated at 37°C for approximately 18 hours before compound addition. Test compounds were prepared in a 384-well acoustic transfer compatible source plate (Labcyte) with a top concentration of 5 mmol/L, followed by an additional five 3-fold serial dilutions. Compound combinations were generated by transferring 7.5 nL of each compound/dilution from the prediluted source plates into the appropriate assay plate well using an acoustic dispenser (Labcyte ECHO555). Two replicate compound treatments were prepared for each cell line. Single-agent dose–response curves were prepared using 7.5 nL of test compound combined with 7.5 nL of DMSO to keep a consistent 0.2% DMSO concentration for all wells. Following compound addition, plates were returned to the incubator for 120 hours. Next, cellular viability was assessed by the measurement of cellular ATP levels with the addition of 3 μL per well Cell Titer-Glo (Promega), and the luminescent signal was quantitated using a ViewLux (PerkinElmer). The raw data were normalized using the signal obtained from the DMSO-treated cell control wells within each plate. Synergy score and excess synergy score calculations were performed using the Loewe additivity model based on the methods of Lehar and colleagues (28). Efficacy was calculated using the lowest normalized Cell Titer-Glo signal (maximum cytotoxicity) for a given test compound cross, regardless of compound concentration. Combina- tion pairs that had (i) a Loewe synergy score greater than 1.75; (ii) an average Loewe excess greater than 0 across all doses tested; and (iii) a maximal cytotoxicity greater than 80% (defined as the cellular viability compared with vehicle-treated controls) were scored as being positive (Fig. 1A).

Combination index analysis assay

In vitro proliferation was measured using Real-Time Cell Sensing Impedance xCELLigence system (RT-CES, ACEA) using fixed dose molar ratios based on the D50 (absolute IC50). The RT-CES is a microelectronic cell sensor system. The core of the system uses microelectronic cell sensor arrays that are integrated into the bottom of the microtiter plates. For cell-based assays, cells are grown in the individual, sensor-containing wells of the microtiter plates. The electronic sensors provide continuous, quantitative information concerning the biological status of the cells present in the well. Changes to the biological status of the cells are measured automatically and in real time by the system. Cell index values, derived from the measured impedances, are continuously displayed and plotted by the RT-CES software. Results were analyzed at 72 hours posttreatment with CalcuSyn software (Biosoft) using the combination index (CI) method developed by Chou and Talalay (29): CI <1 = synergism, 1 = additivity, and >1 = antagonism. Fraction affected is the fraction of cells inhibited by combination therapy at a given dose compared with vehicle control, where higher values indicate greater effectiveness.
RNAi-mediated knockdown of ALK and CDK4

NB-1691 cells were transfected with siRNA targeting ALK and CDK4 (Origene) alone or in combination, with scrambled siRNA used as a scramble. All samples were cotransfected with two siRNAs (e.g., siRNA CDK4 and siRNA CTRL, siRNA ALK and siRNA control) to ensure equal toxicity independent of their function in regulation of gene expression. Forty-eight hours after transfection, NB-1691 cells were harvested and counted using the Trypan blue exclusion method. The number of total and dead cells was calculated and results represented as fold change of dead cells normalized to the total number of cells. Subsequently, cells were evaluated for expression of phosphorylated Rb (pRB; S780) and ALK using immunoblotting analysis. β-Actin was used as a loading control.

Antibodies and Western blot analysis

Cells were treated 24 hours after plating, incubated with drug for 24 hours, versenized, centrifuged, washed once with cold PBS, and stored in −80°C. Each treatment experiment was repeated 3 times. Cells were lysed on ice with hypotonic lysis buffer with protease/phosphatase inhibitor cocktail (Cell Signaling Technology) for 15 minutes and then centrifuged for 10 minutes at 4°C. The amount of protein sample was calculated using the Bradford method, and 30 to 50 μg of proteins was separated on 4%-12% Bis-Tris gradient gels, transferred to PVDF membranes (Millipore) overnight at 4°C, blocked in 5% BSA (for phosphoproteins) or 5% milk, and immunoblotted against pALK Y1604 (Cell Signaling Technology, catalog numbers 3341S), pALK Y1278 (6941S), total ALK (3333S), pRb S795 (9301S), pRb S780 (9307S), total Rb (9309S), cyclin D1 (2922S), cleaved caspase-3 (9664L), cleaved PARP (9541S), GAPDH (2118S), and β-actin (4967S) overnight at 4°C. All primary antibodies were diluted 1:1,000, except GAPDH and β-actin, which were diluted 1:5,000. Secondary antibodies (Santa Cruz Biotechnology) were diluted 1:10,000 and incubated, shaking, at room temperature for 1 hour. Enhanced chemiluminescence substrates were used for detection (Thermo Fisher Scientific).

Cell death, viability assays, and caspase inhibition

Cells were treated with 100 or 500 nmol/L of Actinomycin-D, or 0.5 or 1 × of the D₅₀ values of ceritinib, ribociclib, or ceritinib + ribociclib for 24 hours, plus or minus a 1-hour pretreatment with Q-VD-OPh, a caspase-3, -7, and -9 inhibitor (Sigma-Aldrich). Following 24 hours of drug incubation, cell viability was assayed with CellTiter-Glo (Promega), and paired caspase-3/7 activation was assessed using Caspase-Glo 3/7 Assay (Promega). Luminescence and fluorescence were measured on the GloMax-Multi Microplate Multimode Reader (Promega). Time course effects of caspase inhibition were determined following incubation with...
vehicle, ceritinib, ribociclib, ceritinib + ribociclib, or ceritinib + ribociclib for 24, 48, or 72 hours plus or minus a 1-hour pre-treatment with Q-VD-OPh (Sigma-Aldrich). Cells were stained with Trypan blue and counted using an automatic cell counter that gave the proportion of viable cells.

Cell-cycle analysis
Cell lines in 12-well plates were treated with DMSO control, equipotent serial dilutions of ceritinib or ribociclib, or equipotent combination of both drugs for 72 hours. Floating and adherent cells were collected, washed with PBS, and fixed with Fix and Perm Cell Permeabilization Kit Fixing Medium A (Life Technologies). Cell-cycle analysis was performed with FxCycle Violet Stain Kit (Life Technologies). The fixed cells were washed, and the sample concentration was adjusted to $1 \times 10^6$ cells/mL. Flow cytometry samples were prepared by adding 1 mL cell suspension and 1 μL of FxCycle Violet stain to each sample, incubated for 30 minutes, and analyzed without washing on Attune Acoustic Focusing Cytometer using 405 nm excitation and emission collected in a 450/50 bandpass or equivalent. Analysis was carried out using VenturiOne software (Applied Cytometry).

In vivo pharmacokinetic studies
CB17 SCID female mice bearing NB-EBC1 xenografts were treated daily by oral gavage for 10 consecutive days with 50 mg/kg ceritinib, 150 mg/kg ribociclib, or both agents in combination by administering the compounds in 100 μL/10 g body-weight. Terminal plasma samples were collected by retro-orbital bleeding from 3 mice per each time point after the final dose at 0, 4, 8, and 24 hours. Plasma concentrations of ribociclib and ceritinib were quantified using an LC/MS-MS assay.

The HPLC system, consisting of Agilent 1200 series LC binary pump (Agilent Technologies Inc.), Agilent 1200 series LC vacuum degasser (Agilent Technologies Inc.), CTCPAL_HTS autosampler (Leap Technologies), and VICI two position rotary valve (Valco Int.) was interfaced to a AB SCIEX API-4000 triple quadruple mass spectrometer (AB SCIEX). Mass spectral analyses were carried out using atmospheric pressure chemical ionization in the positive ion mode. Ribociclib (435.24 > 322.20) and ceritinib (556.30 > 433.07) were monitored using multiple reaction monitoring. Data collection and peak integration were performed using Analyst 1.4 software (SCIEX). Standard curves were generated in the concentration range of 1 ng/mL to 5,000 ng/mL. The lower limit quantitation in plasma was 1 ng/mL. The area under the plasma concentration–time curve (AUC0–96h) was calculated by noncompartamental regression analysis using WinNonlin 4.0 software (Certara Inc.).

Statistical analysis for in vivo studies
A linear mixed-effects model was used to test the difference in the rate of tumor volume change over time between different groups. The model included group, day, and group-by-day interaction as fixed effects and included a random intercept and a random slope for each mouse. A significant group-by-day interaction would suggest that the tumor volume changes at different rates for the comparison groups. The model used vehicle group as the reference group and created separate group indicators and interaction terms for other groups. If a mouse was sacrificed or removed for use of biology studies were considered censored at the time of removal.

Results
High-throughput screen identifies synergistic drug combinations in neuroblastoma
We evaluated 10 molecularly targeted small-molecule therapeutics, in pairwise combinations against a panel of 17 well-characterized human neuroblastoma–derived cell lines, 9 of which harbored either an activating ALK mutation or amplification (Table 1). Because of the large number of drug combinations and cell lines screened, we required stringent criteria to identify synergy hits and diminish experimental noise. Synergy score determination, using an algorithm based upon the Loewe additivity model (30), identified combinations of compounds that gave greater efficacy than predicted from the single-agent efficacy at the same concentrations, indicating that the compounds were interacting in a synergistic manner. A synergy score threshold of 1.75 was set to differentiate between experimentally defined additivity, that is, the activity observed when
Table 1. Drug combination screen identifies synergistic drug targets in human neuroblastoma-derived cell lines

<table>
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<tr>
<th>Cell line</th>
<th>ALK</th>
<th>CDK4 CDK6</th>
<th>MYCN amplified</th>
<th>TP53</th>
<th>Synergy score</th>
<th>Maximum efficacy</th>
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<tr>
<td>LANS3*</td>
<td>R1275Q</td>
<td>WT</td>
<td>Yes</td>
<td>WT</td>
<td>3.09</td>
<td>0.87</td>
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<td>NB-1643*</td>
<td>R1275Q</td>
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<td>Yes</td>
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<td>NB-SD</td>
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<tr>
<td>COG-N-415*</td>
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<tr>
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<td>Yes</td>
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<tr>
<td>SH-SYSY</td>
<td>FI174L</td>
<td>WT</td>
<td>No</td>
<td>WT</td>
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<tr>
<td>KELLY</td>
<td>FI174L</td>
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<td>Yes</td>
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<td>SK-N-BE(2)</td>
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<td>WT</td>
<td>Yes</td>
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<tr>
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<td>0.27</td>
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NOTE: Cell lines used in this study with their ALK, CDK4/6, MYCN, and p53 status. Synergy and efficacy scores obtained with ceritinib and ribociclib are noted.

*Defined by FoundationOne next-generation sequencing solid tumor panel.

**Defined by FISH.

**Synergy screen hit as defined by synergy score over 1.75 and maximum efficacy over 0.80.

Validation of the synergistic interaction between ALK and CDK4/6 inhibitors

To validate and extend the results of the synergy screen, we performed independent experiments in an orthogonal fashion using real-time cell impedance and calculated synergy using the Chou–Talalay method (Supplementary Table S1; ref. 29). We confirmed synergy in the three cell lines with a greater effect observed in NB-1643 with an ALK R1275Q mutation compared with SH-SYSY with the comparatively ceritinib-resistant ALK F1174L mutation (8, 12, 15, 16). NB-1691 with CDK4 amplification and wild-type ALK showed synergy on repeated independent experiments, but pALK was expectedly undetectable by
ALK and CDK4/6 Inhibition in Neuroblastoma

immunoblot, and the mechanism of interaction is unknown. To determine the direct effect of targeting ALK and CDK4, we employed siRNA to knockdown expression of ALK and CDK4 in these cells (Supplementary Fig. S3A). The knockdown of ALK increased the number of dead cells (Supplementary Fig. S3B); in contrast and as expected, the knockdown of CDK4 did not disturb cell viability (Supplementary Fig. S3B) but reduced the total number of cells as a consequence of cell-cycle arrest (Supplementary Fig. S3C). We also examined the effects of dual knockdown of ALK and CDK4 in these cells and observed a decrease in both cell viability (Supplementary Fig. S3B) and cell number (Supplementary Fig. S3C).

We then included two additional cell lines in the discovery screen that were not part of the original synergy screen (Supplementary Table S1). NB-EBC1, an ALK wild-type neuroblastoma cell line with robust pALK expression and ALK addiction (2, 15), showed synergy and efficacy at low doses. The SKNBE2C cell line with wild-type ALK, CDK4, CDK6, and intact Rb did not demonstrate synergy. Taken together, synergy was achieved in cell lines with pathogenic activation of the drug targets, consistent with an on-target mechanism.

Dual ALK/CDK4/6 inhibition enhances pALK and pRb depletion

To investigate the in vitro effects of combination therapy on proximal drug targets, we compared phosphoprotein signaling with single-agent and combination therapy (Fig. 2). Single-agent ALK inhibition with ceritinib caused robust dose-dependent abrogation of pALK against the most prevalent mutation, ALK R1275Q, in NB-1643 (Fig. 2A). Comparable pALK abrogation required higher doses in SH-SYSY (Fig. 2B) with the de novo resistant ALK F1174L mutation, as reported previously (2, 8). NB-EBC1 (ALK WT, pALK positive) showed modest pALK abrogation with single-agent ceritinib (Fig. 2C), and NB-1691 (ALK WT, no pALK) had no detectable basal pALK expression (Fig. 2D; ref. 2). Single-agent ribociclib did not modulate pALK, even at doses over 3 μmol/L. In NB-1643, the combination of ceritinib and ribociclib enhanced abrogation of pALK compared with single-agent ceritinib, and a similar but less potent effect was evident in SH-SYSY and NB-EBC1 (Fig. 2).

Active cyclin D–CDK4/6 complexes phosphorylate and inactivate retinoblastoma protein (Rb), causing pRb to release bound E2F transcription factors that promote expression of genes that drive progression into late G1 and S phases. Conversely, inhibition of CDK4/6 maintains Rb in an active hypophosphorylated state so E2Fs remain bound, leading to cell-cycle arrest (33, 34). Therefore Rb hypophosphorylation is a surrogate but proximal marker of on-target CDK4/6 inhibition, although the therapeutic significance of disparate pRb status at S780 and S785 is unclear. Single-agent ribociclib caused dose-dependent abrogation of pRb in all cell lines and was most pronounced in NB-1691 with CDK4 amplification (Fig. 2A–D). In NB-1691, single-agent ALK inhibition with ceritinib had no effect on Rb phosphorylation (Fig. 2D), demonstrating that ceritinib had no off-target activity against CDK4/6 even at high doses. In contrast, in NB-1643, single-agent ALK inhibition with ceritinib decreased total Rb and abrogated pRb (Fig. 2A), consistent with pathogenic ALK signaling driving activation of the cyclin–CDK–retinoblastoma pathway. Abrogation of pRb occurred at lower doses when ribociclib was administered with ceritinib, with a less pronounced effect in other cell lines. These data suggest that ceritinib inhibited activated ALK and pathogenic signal transduction to CDK4/6, an effect that was augmented by ribociclib’s well-characterized direct CDK4/6 inhibition.

Synergy with dual ALK–CDK4/6 inhibition is caspase and p53 independent

We next sought to determine whether the efficacy of combination therapy was dependent on intact caspase and p53 pathways, as neuroblastoma at the time of relapse is associated with disruption of caspase and TP53 programmed cell death and cytotoxic chemotherapy resistance (1, 35, 36). Cleavage of caspase-3 and PARP was detected by immunoblotting after treatment with single-agent ceritinib at pharmacologically relevant doses in all three cell lines with activated ALK: NB-1643, SH-SYSY, and NB-EBC1 (Fig. 2A–C). Ribociclib alone did not result in detectable caspase or PARP cleavage by immunoblot, consistent with previous reports (34). Combination therapy enhanced caspase-3 and PARP cleavage at 24 hours in NB-1643 (ALK R1275Q), but not in other cell lines (Fig. 2A–D). To further determine mechanisms of synergy in SH-SYSY (ALK F1174L), we assessed earlier time points but saw no evidence that combination treatment increased caspase cleavage (Supplementary Fig. S1A; ref. 34).

We next tested the effects of Q-VD-OPh, a potent inhibitor of caspase-3, -7, and -9 on combination therapy with ceritinib and ribociclib to further determine whether synergy was caspase dependent. Incubation of NB-1643 and SH-SYSY with the chemotherapeutic agent Actinomycin-D for 24 hours caused potent caspase-3/7 activation and cytotoxicity. The addition of Q-VD-OPh to Actinomycin-D completely suppressed caspase activation and maintained cell viability comparable with untreated controls consistent with caspase-dependent cytotoxicity (Fig. 3A and B). Ceritinib and ribociclib monotherapy and combination therapy activated caspase-3/7 proteolysis as determined by the Caspase-Glo assay, and caspase activation was completely inhibited by the caspase inhibitor Q-VD-OPh. However, Q-VD-OPh did not protect against in vitro cytotoxicity caused by ceritinib alone, or combination ceritinib and ribociclib after 24 hours (Fig. 3A and B) or 72 hours (Fig. 3C and D), consistent with a caspase-independent mechanism.

TP53 activation may contribute to synergy when ALK inhibitors are combined with cytotoxic chemotherapy (37), and TP53 inactivation has been associated with neuroblastoma relapse and multidrug resistance (36). Therefore, we sought to determine whether combination ceritinib and ribociclib acted in a p53-independent manner. The combination did not result in p53 stabilization or phosphorylation, nor did it activate the downstream signaling molecule p21 compared with monotherapy or Actinomycin-D–positive controls (Supplementary Fig. S1B and S1C), excluding p53-mediated apoptosis as a mechanism of synergy. These data suggest combination ribociclib and ceritinib synergy is independent of the caspase and p53 pathways.

Dual ALK–CDK4/6 inhibition promotes cell-cycle arrest

Single-agent ribociclib performed as a cytostatic agent against human neuroblastoma–derived cell lines, leading to accumulation in the G1 phase (34). Therefore, in cell lines where combination therapy was synergistic, we measured the proportion of cells in each phase of the cell cycle following simultaneous treatment with ribociclib and ceritinib (Supplementary Fig. S2A–S2C). Ceritinib monotherapy caused modest G1 arrest in NB-1643 (ALK R1275Q) and SH-SYSY (ALK F1174L) consistent...
Combination ALK and CDK4/6 inhibition enhances pALK and pRb depletion. Western blots of NB-1643 (A), SH-SY5Y (B), NB-EBc1 (C), and NB-1691 (D) cells treated with vehicle, ceritinib, ribociclib, or ceritinib + ribociclib at fractions of the cell lines' predetermined \( \text{IC}_{50} \) values for each inhibitor. CTRL, control. Cells were collected for immunoblotting at 24 hours posttreatment and probed with the indicated antibodies. Untreated NB-1643 was used as a positive control for the two phospho-ALK sites on the top of D as NB-1691 expresses no phosphorylated ALK.

![Figure 2](image-url)
Figure 3.
Synergy between ceritinib and ribociclib in neuroblastoma cell lines does not rely on a caspase-dependent mechanism. Caspase inhibition does not restore cell viability in SH-SY5Y or NB-1643 cells in vitro. **A** and **B**, SH-SY5Y (**A**) and NB-1643 (**B**) cells were treated with 100 or 500 nmol/L Actinomycin-D (as a positive control), ceritinib alone, ribociclib alone, or ceritinib + ribociclib combination at 0.5 or 1× the cell’s D50 value of each inhibitor. Cells were also pretreated for 1 hour with the pan-caspase inhibitor [Q-VD-OPh (Q-VD)] in the same treatment conditions. Cell viability was measured using Cell Titer-Glo (CTG), and caspase-3/7 activation was measured using Caspase-Glo 3/7 Assay Kit (Promega) at 24 hours posttreatment. **C** and **D**, SH-SY5Y (**C**) and NB-1643 (**D**) cells were treated with vehicle, ceritinib, ribociclib, or the combination at 1× the cell’s D50 value of each inhibitor, with Q-VD-OPh pretreatment in a replicate of the combination treatment. Cells were collected at 24, 48, and 72 hours posttreatment to assay percentage of viable cells, measured by Trypan blue exclusion.
with CDK4 and CDK6 being downstream of activated ALK. Single-agent ribociclib caused more pronounced dose-dependent G1 cell-cycle arrest with corresponding decreases in the fraction of cells in S and G2–M phase. Combination therapy caused a greater accumulation in G1 phase compared with either drug alone in all cell lines, indicating that cytostasis may have contributed to in vitro efficacy and synergy.

**Combined ALK–CDK4/6 inhibition is effective for treatment of ALK-driven neuroblastoma xenografts**

The ALK R1275Q mutation has lower ATP affinity compared with mutations at F1245 and F1174 codons, and R1275Q mutations were more susceptible to single-agent ATP-competitive ALK inhibitors in biochemical, preclinical, and clinical settings (6, 14, 15). Therefore, we focused initial in vitro experiments on the resistant ALK F1174L mutation using SH-SY5Y xenografts. The dose of ceritinib 50 mg/kg/daily was selected, as murine drug exposures were comparable with ceritinib exposures at the recommended dose in human adults (38). SH-SY5Y xenografts treated with ceritinib alone had prolonged EFS (Fig. 4A, \( P < 0.0001 \)) compared with vehicle, but not growth delay (\( P = 0.3 \), Supplementary Table S2). SH-SY5Y xenografts treated with ribociclib (CDK4/6i) showed tumor growth delay (\( P < 0.0001 \)) and prolonged median EFS (\( P < 0.0001 \)) versus vehicle controls (Fig. 4A; Supplementary Table S2). All SH-SY5Y xenografts treated with combination ceritinib and ribociclib achieved complete and sustained regressions, and prolonged EFS versus either single agent (\( P < 0.0001 \); Fig. 4A). At predefined cessation of the study at 7 weeks, mice came off treatment and all eventually relapsed.

In NB-1691 (ALK WT, CDK4 amplification), there was no benefit with single-agent ceritinib over vehicle (Fig. 4B), but single-agent ribociclib delayed tumor growth (\( P < 0.0001 \), statistics in Supplementary Table S2) and prolonged survival (\( P < 0.0001 \)) compared with vehicle controls (Fig. 4B). Combination therapy significantly prolonged median survival over ribociclib monotherapy (Fig. 4B; Supplementary Table S3, \( P < 0.0001 \)). The tumor growth linear mixed-effect analysis did not result in a statistically significant \( P \) value at 10 weeks of treatment, but the effectiveness of combination therapy at earlier time (1–6 weeks on treatment) shows a trend toward greater biological efficacy of combination therapy over ribociclib alone. Although NB-1691 xenografts treated with combination therapy showed complete responses, all xenografts recurred on-treatment prior to study cessation at 7 weeks (Fig. 4B).

When ribociclib human phase I pharmacokinetic data became available, the murine ribociclib doses were reduced to achieve murine plasma exposures that were pharmacologically relevant to adult human exposures. To determine whether combination therapy with reduced ribociclib doses achieved complete regressions against ALK F1174L xenografts, we performed a dose-finding combination study with a fixed dose of ceritinib at 50 mg/kg/daily and four different doses of ribociclib (CDK4/6i) at 50, 75, 100, and 125 mg/kg/daily for 21 days in SH-SY5Y xenografts (Fig. 4C). Ribociclib 50 mg/kg/daily in combination with ceritinib resulted in stable disease with significant tumor growth delay (\( P < 0.0001 \); Fig. 4C, statistics in Supplementary Table S3) and prolonged EFS (Supplementary Table S3) compared with vehicle. Combination therapy with ribociclib doses of 75, 100, and 125 mg/kg/daily resulted in complete regressions that were sustained at the a priori study endpoint of 3 weeks (Fig. 4C).

In our synergy screen, the cell line harboring ALK F1245C mutation ranked 14th of 17 cell lines with regard to synergy scores, and combination efficacy grouped with ALK wild-type cell lines (Table 1). Therefore, we used Felix PDXs harboring an ALK F1245C mutation, directly xenotransplanted from human tumor to mice without an in vitro intermediary step, to test the in vivo efficacy of combination therapy with ceritinib and ribociclib (Fig. 4D). Compared with vehicle, monotherapy with ribociclib for 3 weeks showed marginal but statistically significant tumor growth delay at both the 75 mg/kg/daily (\( P = 0.01 \), statistics in Supplementary Table S3) and 125 mg/kg/daily (\( P = 0.001 \), and superior EFS (Supplementary Table S4). Ceritinib monotherapy significantly delayed tumor progression compared with vehicle (\( P < 0.0001 \)). The combination of ceritinib with ribociclib at either 75 mg/kg/daily or 125 mg/kg/daily doses significantly delayed tumor growth compared with ceritinib alone (\( P = 0.04 \)) or ribociclib alone (\( P < 0.0001 \)), and in contrast to ceritinib alone, combination therapy led to complete and sustained regressions of PDX harboring ALK F1245C mutations (Fig. 4D). To explore whether F1245C xenograft responses were durable, daily treatment was continued past the 3-week a priori endpoint until week 8. The majority of xenografts treated with ceritinib alone progressed, indicating the emergence of resistance, whereas xenografts treated with combination therapy using the ribociclib 125 mg/kg/daily dose showed sustained complete regressions.

**Combination therapy did not cause drug accumulation in plasma**

To evaluate whether in vivo efficacy was driven by plasma drug accumulation due to a drug–drug interaction, we treated CB17 SCID mice with NB-E8c1 tumor-bearing xenografts for 10 days with ceritinib 50 mg/kg/daily and ribociclib 150 mg/kg/daily either as single agents or in combination. Terminal plasma samples were obtained with 3 mice per time point after the final dose on day 10 at 0, 4, 8, and 24 hours. The steady-state plasma concentrations of ribociclib varied between 15 and 8 \( \mu \)mol/L, whereas those of ceritinib fluctuated between 3 and 1.9 \( \mu \)mol/L in the 24-hour dosing interval (Fig. 5A). There was no significant difference in plasma levels between single-agent and combination therapy with regard to maximum concentration or AUC (Fig. 5B).

**Discussion**

Therapy for patients with high-risk neuroblastoma continues to be optimized (39), but even in the immunotherapy era, substantial improvement is required to increase efficacy and decrease toxicity (40, 41). The discovery of activating mutations in the ALK oncogene in a subset of patients with high-risk neuroblastoma (8) has provided an unprecedented opportunity to impact outcome through the development of ALK inhibition strategies. More precise studies of clonal evolution in sequential samples from patients with neuroblastoma have revealed an emergence of ALK mutations at the time of relapse, a finding of utmost clinical importance given the development of ALK inhibition approaches (9, 10). Crizotinib inhibits ALK by competing with ATP for binding to its kinase active site, and we have showed that the reduced susceptibility of F1174L-mutated ALK to crizotinib inhibition results from its increased ATP-binding affinity (15). Our preclinical work and phase 1...
Figure 4.
Dual ALK and CDK4/6 inhibition displays marked antitumor activity in several in vivo models of neuroblastoma. A–C, Mice harboring SH-SY5Y (A), NB-1691 (B), and SH-SY5Y xenografts (C) were treated with vehicle or ceritinib (50 mg/kg) + four different doses of ribociclib (50, 75, 100, or 125 mg/kg) for 3 weeks. D, Felix-PDX tumors were treated daily orally with vehicle, ceritinib (50 mg/kg), ribociclib (75-187.5 mg/kg), or ceritinib + ribociclib for 8 weeks.
Figure 5.
Plasma was collected from CB17 SCID mice bearing NB-EBc1 xenografts and treated for 10 consecutive days to test plasma drug concentrations of monotherapy versus combination therapy. A, Time course of mean plasma concentrations (μmol/L) of ribociclib (LEE011) and ceritinib (LDK378) in mice (n = 3) on day 10 of dosing. B, Mean maximum plasma concentrations (C_{max}) and area under the plasma concentration time (AUC_{0-24h}) of ribociclib (LEE011) and ceritinib (LDK378) in mice (n = 3) on day 10 of dosing.
clinical trial of crizotinib argue that not all clinically observed ALK mutations are functionally relevant as ALK activators, and further that some ALK-activating mutations also cause primary resistance to direct ALK kinase inhibition with crizotinib (8, 15). Consistent with this, while marked antitumor activity was observed in patients with diseases harboring ALK translocations, far fewer objective responses were seen in patients with ALK-mutant neuroblastoma in the Children’s Oncology Group phase I trial of crizotinib (14). We have therefore sought alternative ALK inhibitors, such as lorlatinib, that overcome this intrinsic resistance by simply binding more tightly to ALK (18) or by utilizing a different binding mode that is also enhanced by the mutation, or by novel–novel combination strategies.

The complexities of signaling networks that modify therapeutic vulnerability in cancer cells mandate the study of drug combinations to prevent or reverse tumor drug resistance. In melanoma, dual BRAF and MEK inhibition was superior to single-agent inhibition, with clear biological and clinical rationale to combine these agents (42, 43). These observations motivated us to identify therapeutic combinations to optimize ALK inhibition strategies in neuroblastoma. Here, we performed a cell line–based screen and focused on combinations showing selective efficacy and synergy in ALK-activated cell lines to improve the therapeutic index and durability of response. Our finding of synergy when ceritinib was combined with a PI3K inhibitor was consistent with previous reports of synergy when an ALK inhibitor was combined with a dual PI3K and mTOR inhibitor (44). We identified a novel synergistic interaction between ALK inhibitor ceritinib, and CDK4/6 inhibitor ribociclib. In both active combinations identified by our synergy screen, the cell lines specifically harboring ALK R1275Q mutations, notably the most frequently mutated ALK residue in neuroblastoma, were highly significant, suggesting that the sensitivity of this mutation to ceritinib alone may drive combination efficacy. Although the weak activity that ceritinib has against IGF1R may contribute to the activity observed in these studies, we saw no evidence of IGF1R activation in these models. Mechanistically, we observed that the synergy from dual ALK and CDK4/6 inhibition was independent of caspase and p53 activation, and inhibition of these mechanisms has been associated with resistance to cytotoxic chemotherapeutics. We demonstrate that simultaneous ALK and CDK4/6 inhibition leads to enhanced target modulation and cell-cycle inhibition; notably, ALK inhibition only diminished phospho-Rb levels in cell lines harboring ALK activation. This is consistent with constitutive ALK signaling driving activation of CDK4 and CDK6 and suggests that ceritinib in combination with ribociclib provides upstream and downstream inhibition of the receptor tyrosine kinase and cell-cycle oncopgenic network.

Although there is currently no suitable biomarker to identify patients with wild-type ALK who may benefit from combinatorial ALK inhibition strategies, the potential oncogenic activity of wild-type ALK receptor is under further investigation. Likewise, the synergy observed in cell line EBC1 with evidence for ALK activation in the absence of a mutation or copy number alteration suggests that there may be ligand-dependent activation, a hypothesis that is being addressed.

The actionable synergistic interactions were validated using PDX models harboring ALK F1174L and ALK F1245C mutations with de novo ceritinib resistance and demonstrated complete and sustained tumor regressions. Murine plasma exposures associated with these responses, as best can be extrapolated to available human pharmacokinetics data, appear to be comparable with single-agent therapy in human adults. This preclinical screen in a molecularly defined subset of cell lines provided a responder hypothesis that was mechanistically validated in vitro and in vivo PDX models at pharmacologically relevant doses. These data provide the preclinical rationale for a first-in-children biomarker-driven trial of dual ALK and CDK4/6 inhibition in a pediatric population with shared molecular etiology driven by the presence of an underlying activating ALK lesion.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**


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