Oral Metronomic Topotecan Sensitizes Crizotinib Antitumor Activity in ALK^{F1174L} Drug-Resistant Neuroblastoma Preclinical Models[1]

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Abstract

BACKGROUND: Anaplastic lymphoma kinase (ALK) inhibitor crizotinib has proven to be effective in the treatment of ALK-mutated neuroblastoma, but crizotinib resistance was commonly observed in patients. We aimed to overcome crizotinib resistance by combining with the MEK inhibitor trametinib or low-dose metronomic (LDM) topotecan in preclinical neuroblastoma models. *METHODS:* We selected a panel of neuroblastoma cell lines carrying various ALK genetic aberrations to assess the therapeutic efficacy on cell proliferation *in vitro*. Downstream signals of ALK activation, including phosphorylation of ERK1/2, Akt as well as HIF-1α expression were evaluated under normoxic and hypoxic conditions. Tumor growth inhibition was further assessed in NOD/SCID xenograft mouse models. *RESULTS*: All NBL cell lines responded to crizotinib treatment but at variable ED50 levels, ranging from 0.25 to 5.58 μM. ALK-mutated cell lines SH-SY5Y, KELLY, LAN-5, and CHLA-20 are more sensitive than ALK wild-type cell lines. In addition, we demonstrated that under hypoxic conditions, all NBL cell lines showed marked decrease of ED50s when compared to normoxia except for KELLY cells. Taking into consideration the hypoxia sensitivity to crizotinib, combined treatment with crizotinib and LDM topotecan demonstrated a synergistic effect in ALK^{F1174L}-mutated SH-SY5Y cells. *In vivo*, single-agent crizotinib showed limited antitumor activity in ALK^{F1174L}-mutated SH-SY5Y and KELLY xenograft models; however, when combined with topotecan, significantly delayed tumor development was achieved in both SH-SY5Y and KELLY tumor models. *CONCLUSIONS:* Oral metronomic topotecan reversed crizotinib drug resistance in the ALK^{F1174L}-mutated neuroblastoma preclinical model.

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Introduction

Neuroblastoma (NBL) is the third most common cancer in childhood after leukemia and brain tumors, accounting for 7% to 11% of all pediatric cancers [1]. The development of multimodal chemotherapy/radiotherapies/immunotherapy during the past several decades has demonstrated an improvement of the 5-year survival rates for high-risk neuroblastoma, reaching about 70% for children aged 1 to 14 years [2]. However, some patients will continue to relapse because of *de novo* or acquired drug resistance [3,4], especially for patients carrying anaplastic lymphoma kinase (ALK) mutations.

ALK mutations occur in different cancer types, including lung cancer, renal cell carcinoma, rhabdomyosarcoma, colorectal cancer, etc. [5–8]. ALK has been identified as a major familial predisposition gene in neuroblastoma. Activating mutations within the ALK tyrosine

kinase domain have also been detected in approximately 10% of cases of neuroblastoma, including the most commonly described F1174L, R1275Q, and F1245L mutations which account for 85% of ALK mutations [9–11]. ALK mutations also occur in the absence of

familial predisposition in a context of relapsed neuroblastoma [12]. ALK mutations allow constitutive phosphorylation and activation of downstream signaling, which has become a validated tyrosine kinase target in cancer cells.

ALK inhibitors have proven to be effective in the treatment of ALK-mutated neuroblastoma [13]. Crizotinib, an oral small-molecule tyrosine kinase inhibitor, was originally developed as a c-MET inhibitor, and it was later found to inhibit ALK phosphorylation [14]. From NBL preclinical and phase I studies, R1275Q-mutated ALK and ALK-amplified NBL were sensitive to crizotinib treatment. By contrast, NBL cells harboring F1174L-mutated ALK were relatively resistant to crizotinib [9,15–17]. Inhibition of ALK^{F1174L} mutation remains a therapeutic challenge in neuroblastoma. In this study, we evaluated the therapeutic effects of the ALK inhibitor crizotinib and its combined treatment with MEK inhibitor (trametinib) or low-dose metronomic (LDM) topotecan in preclinical neuroblastoma tumor models.

Materials and Methods

NBL Cells and Cell Culture

LAN-5, SK-N-BE (2), and SH-SY5Y neuroblastoma cells were kindly provided by Dr. Herman Yeger (The Hospital for Sick Children, Toronto). KELLY and SK-N-AS neuroblastoma cell lines were a kind gift of Dr. Meredith Irwin (The Hospital for Sick Children, Toronto). CHLA-20 was obtained from the Children's Oncology Group Cell Culture and Xenograft Repository under a signed and approved Material Transfer Agreement. Cell line authentication was performed using short tandem repeats (STR) DNA profiling (Promega's GenePrint 10 System) [18] conducted by the Genetic Analysis Facility at the Centre for Applied Genomics of The Hospital for Sick Children (Toronto, Canada). The DNA (STR) profile for all cell lines matched the profile listed in the Children's Oncology Group STR Database (http://strdb.cogcell.org). CHLA-20 neuroblastoma cells were cultured in Iscove's modified Dulbecco's medium supplemented with 3 mM L-glutamine, 5 μg/ml of insulin, 5 μg/ml of transferrin, 5 ng/ml of selenous acid (ITS Culture Supplement; Collaborative Biomedical Products, Bedford, MA), and 20% fetal bovine serum (FBS) (Thermo Fisher Scientific). SK-N-BE (2), SH-SY5Y, and LAN-5 neuroblastoma cells were cultured in α-MEM supplemented with 10% FBS. KELLY cells were cultured in RPMI supplemented with 10% FBS and 2 mM L-glutamine (Thermo Fisher Scientific). SK-N-AS cells were cultured in DMEM supplemented with 10% FBS, 1% nonessential amino acids (Thermo Fisher Scientific), and 1% sodium pyruvate (Thermo Fisher Scientific).

Cell Viability Assay

Cells were seeded into 24-well tissue culture plates at a density of 200,000 cells/well in culture medium and incubated for 24 hours at 37°C before starting drug treatment. Cells were exposed to increasing concentrations of drugs for 72 hours. The viability of proliferating cells in the control and treated media was measured with the Alamar Blue assay according to manufacturer's protocol (Trek Diagnostics Systems Inc.). Briefly, Alamar Blue was diluted 1 to 10 in the cell culture media, and the fluorescent color change was monitored after 3 hours. Colorimetrical evaluation of cell proliferation was performed using a SPECTRAmax Gemini spectrophotometer with 540 nm as

excitation wavelength and 590 nm as emission wavelength, and values were expressed as relative fluorescence units. Cell viability was measured in triplicate and calculated relative to control nontreated cells. Hypoxia was achieved by placing tissue culture plates in a hypoxia chamber filled with 5% CO_2, 1% O_2, and 94% N_2 at 37°C.

Western Blot

The protein lysates were analyzed by Western blot for p-Akt, p-ERK1/2, HIF-1α, and β-actin. Briefly, cells were lysed in lysis buffer and denatured. Samples were separated using 10% Bis-Tris precast gels (Invitrogen), followed by transferring to PDVF membranes. After blocking, all membranes were incubated overnight at 4°C in TBST buffer containing the primary antibodies. Primary antibody complexes were then detected using HRP-conjugated secondary antibodies. Protein bands were revealed with SuperSignal West Pico Chemoluminescent Substrate. HIF-1α protein expression before and after crizotinib treatment was quantified densitometrically using ImageJ software (NIH, USA) and normalized in respect to the corresponding expression of β-actin.

Test Animals

Female NOD/SCID mice 4 to 6 weeks old were obtained from Charles River. Animals were housed in the animal facility of the Hospital for Sick Children, Toronto, Ontario, Canada. These studies were approved by the Hospital for Sick Children Animal Care Committee.

Xenograft Development

The antitumor activity of crizotinib and/or topotecan was investigated *in vivo* against subcutaneous neuroblastoma (SH-SY5Y and KELLY) xenografts using NOD/SCID mice. Briefly, tumor cells were washed three times with Hanks' balanced salt solution before injection. Mice were given a subcutaneous injection of 1×10^6 tumor cells. Tumor growth was monitored and measured in two dimensions using a digital caliper, and tumor volume was calculated as width^2 × length × 0.5. Once the tumor diameter reached about 1 cm, mice were randomized into treatment groups with five animals in each group. Crizotinib was administered daily by gavaging at the dose of 50 mg/kg. Topotecan was administered daily by gavaging at 1 mg/kg. Control mice received the same volume of saline.

Tumor volume, mouse body weight, and signs of animal distress were evaluated twice or three times a week for any potential drug toxicity. Animals were sacrificed once the tumor size reached 1.5 cm^3. Tumor growth curves were plotted with the relative tumor volumes at different time points. The relative tumor volume of each tumor is defined as the tumor volume divided by its initial volume.

Statistical Analysis

Data from different experiments were presented as mean ± S.E. To compare the effects of different treatments on tumor growth *in vivo*, statistical significance was determined by Student's *t* test or one-way ANOVA with Dunnett multiple comparison test with the Graphad Prism (version 5) software. All statistical tests were two-sided; $P < .05$ was considered to be statistically different, and $P < .01$ was a significant difference.

In vitro synergism was defined as a greater-than-expected additive effect, whereas antagonism was defined as less-than-an-expected additive effect. Thus, combination index (CI) = 1 indicated an additive effect, CI < 1 indicated a synergistic effect, and CI > 1

indicated antagonism. The CI values were calculated using CalcuSyn software (Biosoft, Cambridge, UK) at each effective dose (ED) of ED10, ED50, and ED90, respectively.

Results

In Vitro Antiproliferation Effects of Crizotinib and Trametinib on NBL Cells

The activities of crizotinib and trametinib were tested *in vitro* using the cell viability assay in different NBL cell lines SK-N-BE (2) (ALK wild type, MYCN amplified), SH-SY5Y (ALK^{F1174L} mutated), KELLY (ALK^{F1174L} mutated, and MYCN amplified), LAN-5 (ALK^{R1275Q} mutated, MYCN amplified), CHLA-20 (ALK^{R1275Q} mutated), and SK-N-AS (ALK wild type, but N-ras mutated) (Table 1 and Figure 1A). It has been reported that the peak plasma concentration (Cmax) of trametinib could reach 22.2 ng/ml (32 nM) with the dose of 2 mg/day. From our *in vitro* cell viability assay (Figure 1), trametinib showed limited effect at the clinically achievable drug concentration, 32 nM. As shown in Figure 1A, all the tested lines responded to crizotinib treatment in a dose-dependent manner, with ED50 values ranging from 0.65 to 5.58 μM. NBL cells with ALK mutations (LAN-5, CHLA-20, SH-SY5Y, and KELLY cells) are more sensitive to crizotinib treatment compared to ALK wild-type cells (SKNBE-2 and SK-N-AS). Cmax of crizotinib could reach 243 ng/ml (540 nM) for a once-daily dose of 400 mg crizotinib and 493 ng/ml (1.10 μM) for a standard schedule of 250 mg twice daily [19].

For combined crizotinib and trametinib treatment, all selected tumor cell lines were treated at increasing concentrations of crizotinib with the presence of trametinib at the mixing concentration ratio of 10:1. Combined treatment showed enhanced cytotoxicity in some NBL cell lines: SK-N-BE (2), CHLA-20, and SK-N-AS. However, synergistic analysis indicated additive affects instead of synergistic effects.

Inhibition of Erk1/2 and Akt Phosphorylation by Crizotinib and Trametinib Treatment

Both Erk1/2 and Akt pathways have been implicated in NBL cell survival and proliferation; we first investigated the phosphorylation levels of Erk1/2 and Akt in all selected NBL cell lines by Western blot. As shown in Figure 1B, different NBL cells expressed viable levels of p-Akt and p-Erk1/2 under *in vitro* cell culture condition. The cellular response to trametinib and crizotinib was assessed by measuring phosphorylation of ERK and Akt (Figure 1C) by Western blot after exposing to crizotinib (1 μM) and/or trametinib (100 nM) overnight. Marked reduction in ERK1/2 phosphorylation was observed in all tested cell lines treated with trametinib, whereas inhibition of p-Akt by crizotinib was observed in all tested cell lines except the ALK wild-type cell line, SK-N-BE (2).

Enhanced Antiproliferation Effects of crizotinib in NBL Cells under Hypoxic Conditions

It has been reported that under hypoxic conditions, ALK directly regulated the hypoxia-inducible factors (HIF) expression [20] which promotes the proliferation and survival of cancer cells. Therefore, we assessed the effects of crizotinib on NBL cell proliferation under hypoxic condition. All tested cell lines were exposed to 1% O$_2$ culture condition with increased concentrations of crizotinib (10 nM to 10 μM) for 72 hours. As mentioned earlier, NBL cell lines have ED50 values ranging from 0.65 to 5.58 μM under normoxic condition. When exposed under hypoxic conditions (1% O$_2$), all NBL cell lines except KELLY cells (ALK^{F1174L} mutated and amplified MYCN) showed marked decrease of ED50s compared to NBL cells under normoxia ($P < .01$) (Figure 2A). There was a 2.6- to 28-fold decrease of crizotinib ED50 with ED50 values ranging from 0.12 to 0.25 μM (Figure 2A; Table 1). We also measured how HIF-1α expression was affected by crizotinib treatment in different NBL cell lines under hypoxic conditions (1% O$_2$). As shown in Figure 2, B and C, we observed a downregulation of HIF-1α in some ALK mutant cell lines, SH-SY5Y, LAN-5, and CHLA-20 cells, after crizotinib treatment. Again, no HIF-1α inhibition was observed in KELLY (ALK^{F1174L} mutated and amplified MYCN) cells and ALK wild-type SK-N-BE [2] cells.

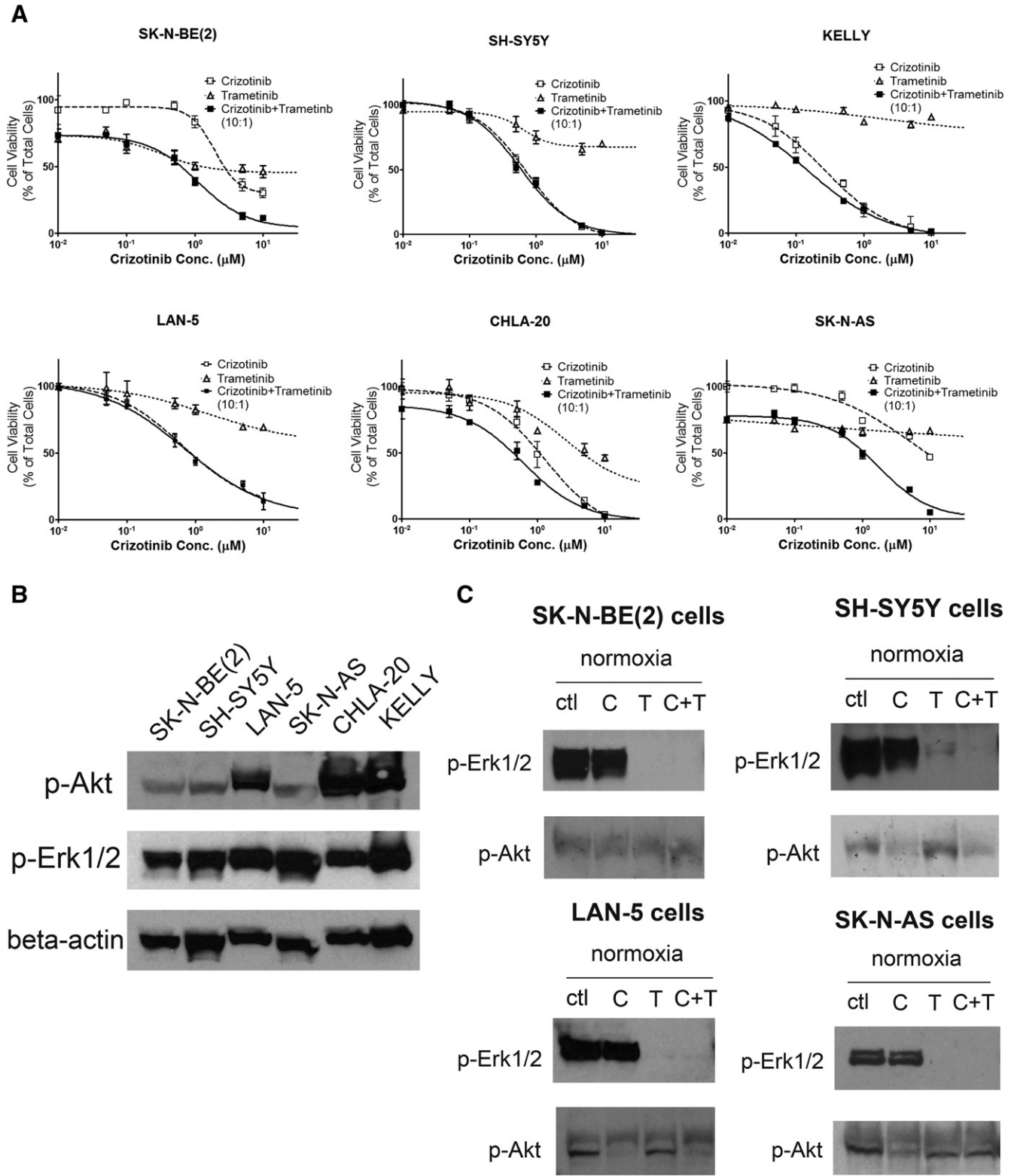### Synergistic Effect When Combining Crizotinib with LDM Topotecan

Because topotecan, especially daily metronomic topotecan, induces oxidative stress and downregulates HIF-1α expression in cancer cells [21–23], we further investigated the efficacy of crizotinib when adding LDM topotecan *in vitro*.

SK-N-BE (2), SH-SY5Y, KELLY, and LAN-5 cells were treated *in vitro* for 6 days with increasing concentrations of crizotinib with or without the presence of topotecan at the concentration ratio of 20:1. As shown in Figure 3 and Table 2, synergistic antiproliferative effects (CI < 1) were observed in SH-SY5Y cells, with CI = 0.12 at ED10, CI = 0.31 at ED50, and CI = 0.83 at ED90. No synergy was observed in SK-N-BE (2), KELLY, and LAN-5 cells. All three cell lines carry amplified MYCN, which supports a potential role of MYCN in the mechanism of drug resistance to ALK inhibitors.

Delayed Tumor Development with Crizotinib and Topotecan Treatment In Vivo

We proceeded to test the antitumor activity of crizotinib and topotecan *in vivo* with two ALK^{F1174L}-mutated cell lines: SH-SY5Y and KELLY.

When SH-SY5Y–xenografted tumors reached about 1 cm in diameter, tumor-bearing NOD/SCID mice were randomized to therapy with crizotinib, topotecan, or combined therapy. We continued daily drug treatment for 9 days until a significant animal body loss (~14%) was observed. With 9-day drug treatment, single-agent topotecan treatment showed potent antitumor activity with a partial tumor regression. Although single-agent crizotinib delayed tumor growth ($P < .05$), the combination of crizotinib and LDM topotecan significantly enhanced the antitumor effect compared to crizotinib or LDM topotecan alone ($P < .01$). With combined treatment, complete tumor regression was observed in all tested animals. Twelve days after we stopped the drug treatment, tumor relapse was observed in combined treatment group. Thereafter, we reinitiated another 9-day treatment with the same schedule and

Table 1. Characterization of Neuroblastoma Cell Lines and ED50 of Crizotinib under Normoxic and Hypoxic Conditions

Cell Lines	ALK Mutation	MYCN Amplification	ED50 of crizotinib (μM)		P Value
			Normoxia	Hypoxia	
SK-N-BE (2)	WT	Yes	3.80 ± 0.42	0.14 ± 0.03	<.01
SH-SY5Y	F1174L	No	0.71 ± 0.05	0.04 ± 0.002	<.01
KELLY	F1174L	Yes	0.83 ± 0.16	0.93 ± 0.03	>.05
LAN-5	R1275Q	Yes	0.65 ± 0.09	0.25 ± 0.08	<.01
CHLA-20	R1275Q	No	1.16 ± 0.20	0.12 ± 0.01	<.01
SK-N-AS	WT	No	8.65 ± 1.06	0.2 ± 0.04	<.01

Figure 1. (A) Antiproliferation effects of crizotinib and/or trametinib on pediatric tumor cell lines [SK-N-BE (2), SH-SY5Y, KELLY, LAN-5, CHLA-20, and SK-N-AS] *in vitro*. Alamar Blue assays were performed after exposing tumor cells to increasing concentrations of crizotinib and/or trametinib *in vitro* for 72 hours. Cell viability was plotted with GraphPad Prism software. (B) Western blot analysis showing different phosphorylation levels of p-Akt and pErk1/2 in a panel of six NBL cell lines. (C) Phosphorylation of ERK1/2 and Akt (Figure 1) by Western blot after exposing to crizotinib (1 μM) and/or trametinib (100 nM) overnight in SK-N-BE (2), SH-SY5Y, LAN-5, and SK-N-AS cells.

dose in three treatment groups. Single-agent topotecan delayed tumor growth with no tumor regression observed, whereas combined treatment induced complete tumor regression again (Figure 4A).

Synergy of crizotinib and topotecan was also studied in the KELLY xenograft model. Comparing to SH-SY5Y cells, MYCN-amplified KELLY cells were less reactive to either crizotinib or topotecan
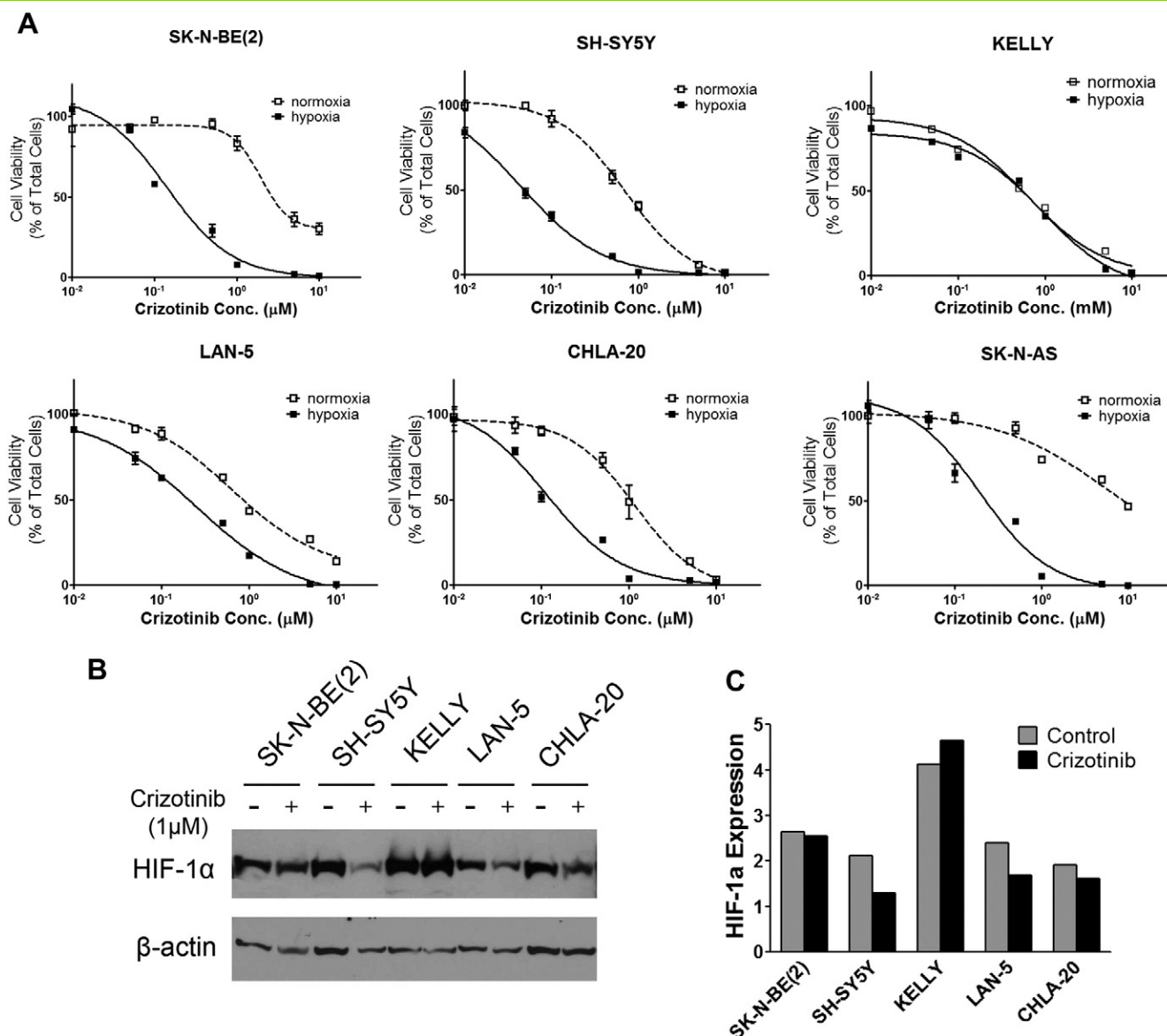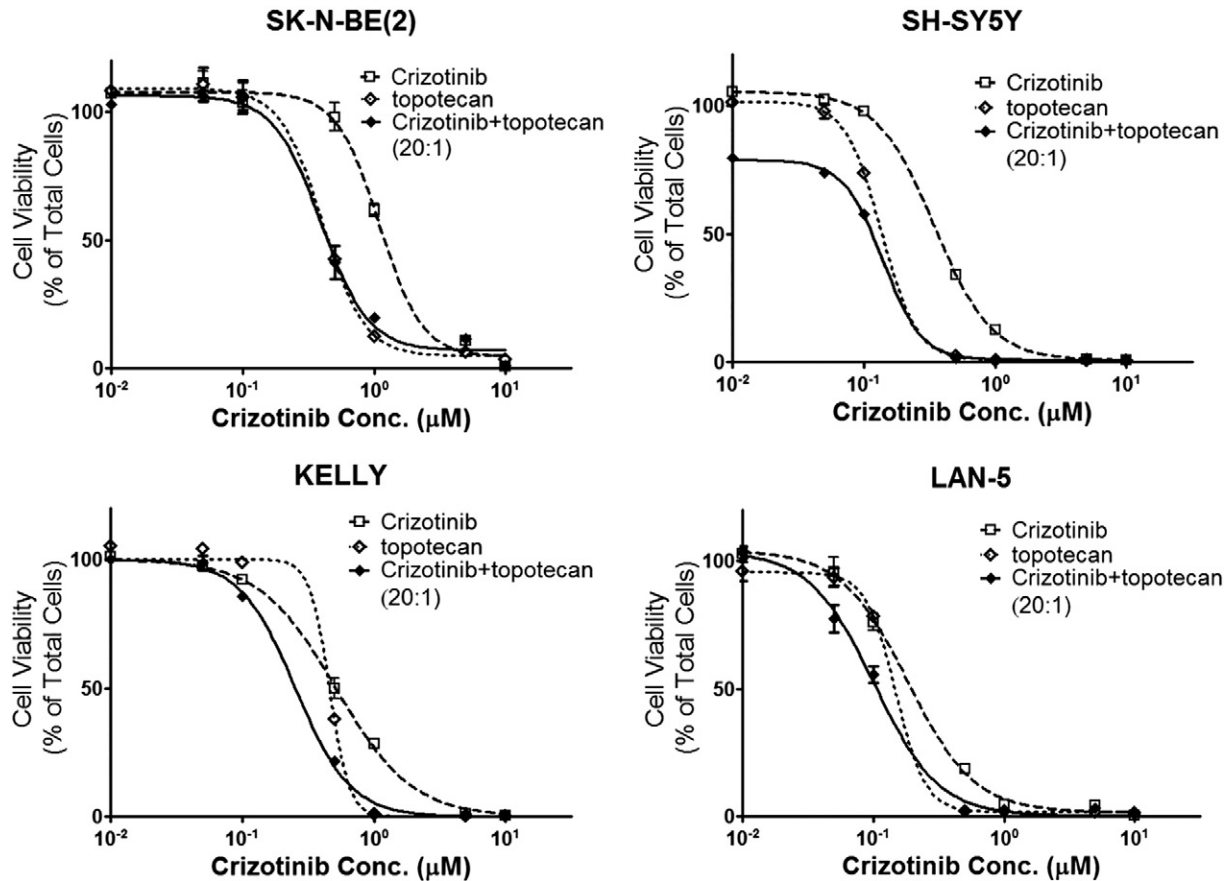
Figure 2. (A) Effects of crizotinib on pediatric tumor cell lines *in vitro* under normoxic and hypoxic conditions. Neuroblastoma cell lines were treated with increasing concentrations of crizotinib *in vitro*. Cell viability was plotted with GraphPad Prism software and compared between normoxic and hypoxic conditions (1% O_2). (B). Western blot analysis for HIF-1α following crizotinib treatment in different NBL cell lines under hypoxic conditions (1% O_2). SK-N-BE (2), SH-SY5Y, KELLY, LAN-5, and CHLA-20 cells were grown in standard growth medium and then treated with 1 μM crizotinib for 72 hours. HIF-1α expression was measured by Western blot analysis. β-Actin was used as internal loading control. (C) Quantitative analysis of HIF-1α expression in different NBL cell lines. HIF-1α protein expression in Western blot images was quantified densitometrically using ImageJ software (NIH, USA) and normalized with respect to the corresponding expression of β-actin. Column bar graph was generated using GraphPad Prism 6.0 Software.

treatment *in vivo*. Although single-agent crizotinib showed no significant antitumor activity, when combined with topotecan, significantly delayed tumor development was achieved in all tested animals ($P < .01$) (Figure 4C).

Discussion

Several ALK inhibitors, including ceritinib, AP26113, alectinib, PF06463922, X-396, RXDX-101, and TSR-011, have been developed in clinical use for adult patients [24,25]. Among those ALK inhibitors, only crizotinib, ceritinib, and PF06463922 were used in clinical trials for pediatric patients. In NBL clinical trials, crizotinib demonstrated to be responsive in patients harboring ALK

aberrations, although ALK[F1174L] mutant tumors exhibited relative resistance to this inhibitor [16,26]. Our study showed a similar finding that greater concentrations of crizotinib were required to inhibit ALK wild-type cells compared to ALK mutant cells. ALK[F1174L] mutant SH-SY5Y and KELLY cells had higher EC50 than ALK[R1275Q] mutant LAN-5 cells. To overcome the resistance of ALK[F1174L] tumors to crizotinib, we tried to combined crizotinib treatment with different antitumor agents, including MEK inhibitor. Crizotinib and trametinib showed complementary antitumor effects in different NBL cell lines by inhibiting phosphorylation of different downstream kinase targets, Akt and Erk1/2, respectively. However, at the clinically achievable drug concentrations, trametinib showed

Figure 3. Effects of crizotinib and topotecan on pediatric tumor cell lines *in vitro*. Alamar Blue assays were performed after exposing tumor cells to increasing concentrations of crizotinib and topotecan for 7 days. Cell viability was plotted with GraphPad Prism software with four NBL cell lines: SK-N-BE (2), SH-SY5Y, KELLY, and LAN-5.

limited effect; its combined treatment with crizotinib only showed additive antitumor effects. More potent MEK inhibitors may be required to achieve a synergistic effect.

In this study, we observed enhanced cytotoxicity of crizotinib under hypoxic condition. As we know, hypoxia regulates tumor cell proliferation, migration, and invasiveness through the expression of a group of transcription factors called HIFs [27,28]. It has been demonstrated that ALK specifically regulates HIF-1α expression under hypoxia conditions in both anaplastic large cell lymphoma and non–small cell lung cancer [20]. Crizotinib, as a dual inhibitor of the c-MET and ALK receptor tyrosine kinases, may also suppress NBL cell proliferation through c-MET inhibition. Hypoxia has been reported to activate c-MET transcription *in vitro* and *in vivo* [29], possibly through

the upregulation of HIF-1α [30]. In cultured cells, the Met protein and mRNA levels increase substantially after exposure to low oxygen tension. In experimental tumors, Met protein levels dramatically increased in coincidence with HIF-1α–positive, hypoxic areas [29]. From our study, we observed the downregulation of HIF-1α in SH-SY5Y cells after crizotinib treatment, which may explain the enhanced cytotoxicity of crizotinib under hypoxia in NBL cells.

MYCN amplification is the major genetic aberration associated with aggressive tumor phenotype and poor outcome in neuroblastoma [31,32]. *In vitro* studies have shown that constitutively activated ALK upregulates the transcription of MYCN [33]. Compared to ALK[F1174L] and MYCN alone, coexpression of these two oncogenes led to the development of neuroblastomas with earlier onset, higher penetrance, and enhanced lethality [34–36]. In this study, KELLY cells which harbor both ALK[F1174L] mutation and MYCN amplification showed less responsiveness to crizotinib and topotecan single-agent treatment even under hypoxic condition. However, a significant tumor growth inhibition was still achieved with combined treatment of metronomic topotecan and crizotinib.

Topotecan, especially daily metronomic topotecan, has been known to induce oxidative stress and downregulate HIF-1α expression in cancer cells [21–23]. In this study, we first tested the combined treatment of crizotinib with LDM topotecan in ALK-[F1174L]-mutant NBL. We chose LDM chemotherapy because it has lower acute toxicity due to lower exposure of the cytotoxic agents. It also has been shown to be active in diverse tumor types, including

Table 2. CI of SK-N-BE (2), SH-SY5Y, KELLY, and LAN-5 Cells Treated with Crizotinib in Combination with Topotecan *In Vitro*

Cell Lines	CI Values		
	ED10	ED50	ED90
SK-N-BE (2)	1.63	1.56	1.51
SH-SY5Y	0.12	0.31	0.83
KELLY	1.39	1.56	1.76
LAN-5	2.27	1.84	1.54

CI values were calculated as described in the "Material and Methods." CI = 1 indicated an additive effect, CI < 1 indicated a synergistic effect, and CI > 1 indicated antagonism. ED10, ED50, and ED90 = effective dose 10, 50, and 90, respectively.
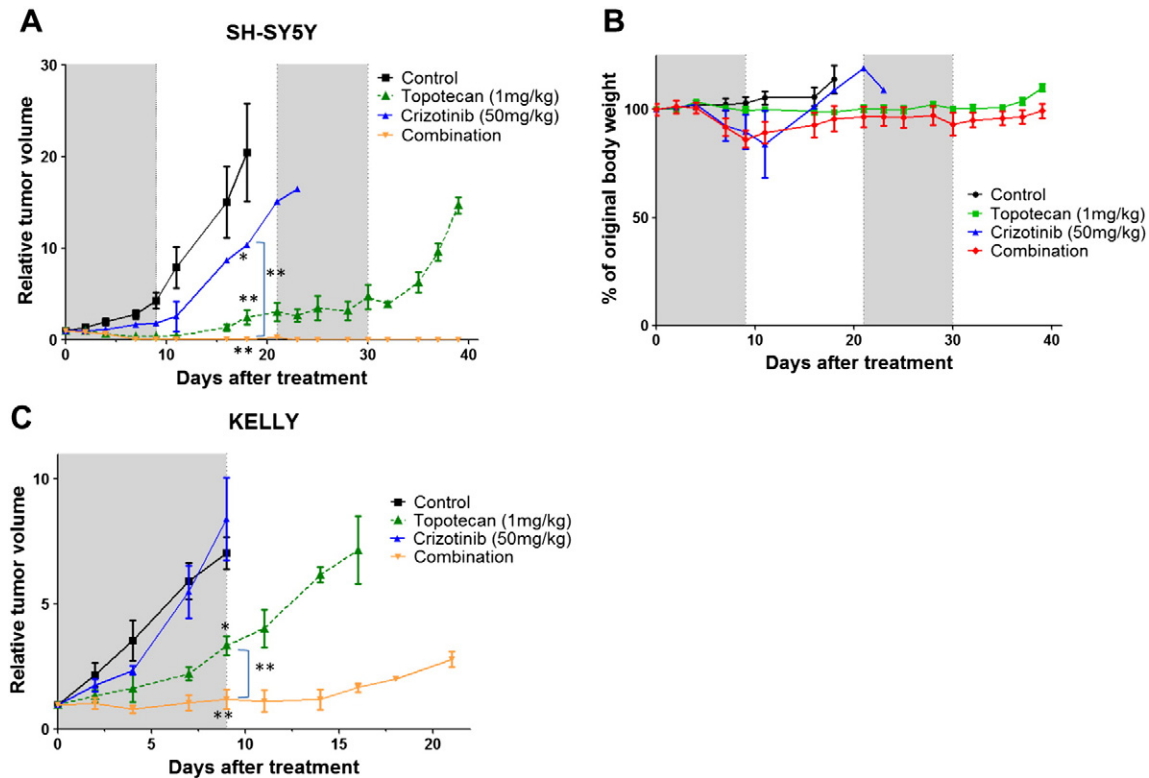
Figure 4. Effects of crizotinib and LDM topotecan on the growth of NBL xenografts. Shaded areas indicate the treatment period. (A) In the SH-SY5Y xenografts, when xenograft tumors reach about 1 cm in diameter, mice were randomized into four groups (control, crizotinib treatment, LDM topotecan treatment, and combined treatment) with five animals in each group. Crizotinib and topotecan were administered daily for 9 days at the dose of 50 mg/kg and 1 mg/kg, respectively, by oral gavaging. Tumor volume was measured and calculated as $width^2 \times length \times 0.5$. Twelve days after we stopped the drug treatment, tumor relapse was observed in combined treatment group. Thereafter, we reinitiated another 9-day treatment with the same schedule and dose in three treatment groups. (B) Animal body weight was measured in the SH-SY5Y xenograft model to monitor the potential drug toxicity. (C) Tumor growth was assessed in the KELLY xenograft model with crizotinib and topotecan treatment. Crizotinib and topotecan were administered at the same dose and schedule as mentioned above. Shaded areas indicate the treatment period. * $P < .05$; ** $P < .01$.

metastatic disease, especially when combined with antiangiogenic drugs [37–40]. Our previous studies with LDM topotecan have demonstrated synergistic effect when combined with pazopanib in NBL preclinical models. Beppu et al. [22] reported that topotecan blocks HIF-1α and vascular endothelial growth factor expression induced by insulin-like growth factor-I in neuroblastoma cell. In this study, we observed the synergistic antitumor effect with combined therapy with crizotinib and topotecan in ALK^{F1174L}-mutant NBL preclinical tumor models. Our data suggest that crizotinib treatment could target tumor cells population residing in hypoxia regions. Adding metronomic topotecan may reverse crizotinib drug resistance in ALK^{F1174L}-mutant NBL tumors. This regimen may also serve as a novel therapy for adult patients with multidrug-resistant ALK mutations.

Acknowledgements

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