Combined Antitumor Therapy with Metronomic Topotecan and Hypoxia-Activated Prodrug, Evofosfamide, in Neuroblastoma and Rhabdomyosarcoma Preclinical Models

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Abstract

Purpose: Tumor cells residing in tumor hypoxic zones are a major cause of drug resistance and tumor relapse. In this study, we investigated the efficacy of evofosfamide, a hypoxia-activated prodrug, and its combination with topotecan in neuroblastoma and rhabdomyosarcoma preclinical models.

Experimental Design: Neuroblastoma and rhabdomyosarcoma cells were tested in vitro to assess the effect of evofosfamide on cell proliferation, both as a single agent and in combination with topotecan. In vivo antitumor activity was evaluated in different xenograft models. Animal survival was studied with the neuroblastoma metastatic tumor model.

Results: All tested cell lines showed response to evofosfamide under normoxic conditions, but when exposed to hypoxia overnight, a 2- to 65-fold decrease of IC50 was observed. Adding topotecan to the evofosfamide treatment significantly increased cytotoxicity in vitro. In neuroblastoma xenograft models, single-agent evofosfamide treatment delayed tumor growth. Complete tumor regression was observed in the combined topotecan/evofosfamide treatment group after 2-week treatment. Combined treatment also improved survival in a neuroblastoma metastatic model, compared to single-agent treatments. In rhabdomyosarcoma xenograft models, combined treatment was more effective than single agents. We also showed that evofosfamide mostly targeted tumor cells within hypoxic regions while topotecan was more effective to tumor cells in normoxic regions. Combined treatment induced tumor cell apoptosis in both normoxic and hypoxic regions.


Introduction

Neuroblastoma is the most common extracranial childhood malignancy, responsible for 15% of all childhood cancer-related deaths (1). Despite intensive treatment protocols including megatherapy with hematopoietic stem cell transplantation and immunotherapy, 3-year disease-free survival is only about 50% for metastatic disease compared with 95% for localized tumors (2). Rhabdomyosarcoma is the most common pediatric soft tissue sarcoma and the third most common extracranial solid tumor in children, with an annual incidence of 4 to 7 cases per million children under the age of 16 years (3). Prognosis of metastatic rhabdomyosarcoma remains poor despite aggressive therapies.

There is strong evidence that tumor cells residing in the tumor hypoxic regions cause drug resistance and tumor relapse (4). Tumor hypoxic zones, which occur in tumors due to nonuniform and inefficient vasculature, hinder the accessibility of chemotherapeutics to tumor cells. Hypoxia can be a direct cause of therapeutic resistance due to limited blood supply and drug distribution (5). Hypoxia also inhibits tumor cell proliferation and induces cell-cycle arrest, further enhancing chemoresistance through regulating drug transporters, antiapoptotic proteins, and proangiogenic factors (6). Our previous studies have shown that tumor hypoxic microenvironment may serve as a niche for the highly tumorigenic fraction of tumor cells which exhibit the cancer stem cell features in a diverse group of solid tumor cell lines, including neuroblastoma, rhabdomyosarcoma, and small-cell lung carcinoma (7). We also found that hypoxia induces drug resistance to etoposide, melphalan, doxorubicin, and cyclophosphamide in neuroblastoma cells, which is mediated through an expanded autocrine loop between VEGF/Flt1 and HIF-1α expression (8). There is evidence that some cells in hypoxic regions remain alive in a dormant state for prolonged duration and...
Translational Relevance

Tumor hypoxia is known to correlate with increased malignancy, metastatic potential, drug resistance, and poor patient prognosis in a number of tumor types. Targeting tumor hypoxia with hypoxia-activated prodrugs (HAP), such as evofosfamide, may improve cancer therapy. So far, the effect of evofosfamide has been studied in combination with cytotoxic agents in adult cancers, whereas the impact of evofosfamide on pediatric cancers has not been investigated. This study focuses on the preclinical evaluation of efficacy of evofosfamide and its combination with topotecan, a topoisomerase I inhibitor widely used in recurrent neuroblastoma and rhabdomyosarcoma. We observed that targeting tumor hypoxia with HAPs improves antitumor effects of topotecan in our tumor models, which provides a new therapeutic approach for the treatment of pediatric solid tumors. All data gathered from this study will help us build the rationale for future phase I clinical trials in treating patients with relapsed/refractory neuroblastoma and rhabdomyosarcoma.

become drug resistant as anticancer drugs preferentially target rapidly proliferating cells (6). Hypoxia-inducible factors also induce stem cell phenotype and suppress differentiation of neuroblastoma cells (9). In rhabdomyosarcoma, HIF-1α, induced by hypoxia, upregulates antiapoptotic proteins and glycolytic enzymes (10). HIF-1α inactivation by the inhibition of PI3K/Akt pathway is reported to sensitize tumor cells to apoptosis in rhabdomyosarcoma (11). Moreover, HIF-1α stabilization as the result of hypoxia increases VEGF expression, increases glycolysis and resistance to chemotherapy (12).

Evofosfamide (previously known as TH-302), a hypoxia-activated prodrug (HAP) has been designed to penetrate to hypoxic regions of tumors. Evofosfamide is reduced at the nitroimadazole site of the prodrug by intracellular reductases and when exposed to hypoxic conditions, leads to the release of the alkylating agent bromoisophosphoramide mustard (Br-IPM). Br-IPM can then act as a DNA crosslinking agent at the tumor hypoxic region and may diffuse to adjacent normoxic regions via a bystander effect (13). Consequently, evofosfamide can target the malignant cells residing in hypoxic zones, while having little or no cytotoxic effect on the normal cells (14, 15).

The antitumor efficacy of evofosfamide correlated with hypoxic fractions in tumor xenografts of lung cancer, melanoma, pancreatic cancer, renal cell carcinoma, and hepatocellular carcinoma. In adult soft tissue sarcoma phase II trial, evofosfamide in combination with doxorubicin demonstrated 2% complete response (CR) and 34% partial response (PR; ref. 16). As a single maintenance therapy, it had limited hematologic toxicity and cardiotoxicity, whereas with doxorubicin, it caused manageable hematologic toxicity without aggravating cardiotoxicity. A phase III study in advanced and metastatic adult soft tissue sarcoma is ongoing (NC101440088). To date, no studies investigating the efficacy of evofosfamide in pediatric cancers have been conducted.

Topotecan, a topoisomerase-I inhibitor has shown activity against both neuroblastoma and rhabdomyosarcoma as a single agent (17). The combination of cyclophosphamide plus topotecan has been active in rhabdomyosarcoma, neuroblas-
toma, and Ewing sarcoma patients with recurrent or refractory disease who have not received topotecan previously (18–20). The combination of topotecan, cyclophosphamide, and etoposide is tolerable and effective in relapsed and newly diagnosed neuroblastoma in phase II clinical trials and upfront phase III clinical trials (18, 21). Single-agent topotecan has also been found to be antiangiogenic in preclinical models of pediatric cancers (22, 23). Despite initial response, tumors eventually acquire resistance to topotecan. Mechanisms of resistance include mutations in topoisomerase-I and involvement of transporters like BCRP, P-glycoprotein, and multidrug resistance associated protein-type IV (24–26).

In this study, we selected the metronomic dose of topotecan for in vivo studies. Low metronomic dose (LDM) chemotherapy usually refers to administration of low dose of cytotoxic agent at close intervals without drug free breaks. LDM chemotherapy has lower acute toxicity due to lower exposure of the cytotoxic agents. It has been shown active in diverse tumor types, including metastatic disease, especially when combined with antiangiogenic drugs (27–30). The availability of the oral topotecan, along with our previous studies combining metronomic topotecan with pazopanib in neuroblastoma preclinical models (23), suggest that oral metronomic topotecan may be an ideal candidate for pediatric solid tumors. Considering the limited effects on hypoxic tumors with conventional chemotherapy, hypoxia-targeting cytotoxic agents along with topotecan therapy may achieve greater antitumor activities. Therefore, in this study, we investigated the effect of combined therapy with topotecan and evofosfamide in neuroblastoma and rhabdomyosarcoma preclinical models.
5 ng/ml selenous acid (ITS Culture Supplement; Collaborative Biomedical Products) and 20% FBS (complete medium). SK-N-BE(2) and SH-SY5Y neuroblastoma cells were cultured in AMEM with 10% FBS.

Test animals
NOD/SCID mice (Jackson Laboratory) were used for xenograft mouse models at 4 weeks of age. The mice were housed in an isolated sterile containment facility. All animal studies were approved by Animal Care Committee at the Hospital for Sick Children (Animal Use Protocol#1000019356). During the study, the mice were observed daily for possible adverse effects due to treatments. Morbidity signs of ill health, such as ruffled/thinning fur, abnormal behaviors, or local erosion from the tumor were noted and experiments terminated as indicated.

Cell viability assay
Cell viability was assessed by Alamar Blue assay as previously described (37). Alamar Blue cell proliferation assay is based on a reducing environment that indicates metabolically active cells. Briefly, exponentially growing tumor cells was seeded into 48-well plates at $1 \times 10^5$ cells per well and grown overnight prior to initiating treatment. On the day of the test, cells were exposed to increasing concentrations of evofosfamide and topotecan either as single agents or combined treatment for 72 hours. Alamar Blue was added to a final concentration of 5%, incubated for 4 hours at 37°C and quantitated on a plate reader at 530/590 nm. IC$_{50}$ was determined by GraphPad Prism software.

We also evaluated drug efficacy under the condition of hypoxia. To simulate tumor hypoxia in vitro, after drug addition, the plates were incubated for 2 hours at 37°C in an anaerobic chamber. The anaerobic chamber was evacuated and gassed with the hypoxic gas mixture (94% N2/5% CO2/1% O2) to create a hypoxic environment. After 2 hours or overnight exposure to hypoxic condition, cells were removed from the hypoxia chamber and further incubated for 3 days in standard tissue culture incubator. Cell viability was assessed by Alamar Blue assay as described above.

Mouse xenograft models
Two neuroblastoma cell lines [CHLA-20 and SK-N-BE(2)] and two rhabdomyosarcoma cell lines (RH4 and RD) were used to establish murine models (38). Briefly, tumor cells were washed three times with Hank balanced salt solution before injection. Cell suspensions were mixed 1:1 with Matrigel (BD Biosciences). Subcutaneous xenografts were developed by injecting growing cells at $1 \times 10^6$ cells in 100 μl volume. Mice were randomized into four groups: control, evofosfamide, topotecan, and the combination of evofosfamide and topotecan. The doses of evofosfamide and topotecan were 50 mg/kg (every day x 5 days/week, i.p.) and 1 mg/kg (every day x 5 days/week by oral gavage), respectively. The injection or gavage volume was 200 μl. Control mice received the same volume of the vehicle (saline). Tumor growth was measured three times a week in two dimensions, using a digital caliper. Tumor volume was calculated as $\text{width}^2 \times \text{length} \times 0.5$. When tumor volume reached 1.5 cm$^3$, mice were sacrificed, and tumors were dissected and weighed. 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. Potential drug toxicity was assessed in tumor-bearing mice by monitoring animal body weight, appetite, diarrhea, signs of animal distress, or any marked neurologic symptoms.

Animal survival study
SK-N-BE(2) cells were prepared and injected intravenously into the lateral tail vein (26-gauge needle, 1 x $10^6$ cells in 100 μl total volume). Mice were randomized into four groups: control, evofosfamide, topotecan, and the combination of evofosfamide and topotecan, with eight mice in each group. All the treatments were initiated 14 days after tumor cell inoculation at the same schedule and dosage as above. Animals were monitored for body weight and any signs of stress. Tumor-bearing mice were euthanized at the endpoint when there were signs of distress, including 20% of body weight loss, fur ruffling, rapid respiratory rate, hunched posture, reduced activity, and progressive ascites formation.

IHC
Double immunofluorescence staining was performed on 5-μm-thick frozen sections. Tissue sections were fixed for 10 minutes in acetone and air dried for 5 minutes. Endogenous biotin, biotin receptors, and avidin sites were blocked with the Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories). The tissue sections were incubated with rabbit anti-cleaved caspase-3 antibody (1:50; #9661, Cell Signaling Technology) or rabbit anti-Ki67 antibody (1:200; ab16667, Abcam) for 1 hour at room temperature. Detection of the rabbit antibodies was performed by incubation with Texas Red goat anti-rabbit IgG antibody (1:200; TI-1000, Vector Laboratories) for 30 minutes. After the washing of the tissue sections, the mouse IgG1 anti-pimonidazole mAb clone 4.3.11.3 (1:50; Hypoxprobe, Inc.) was added overnight at 4°C. This antibody was detected with Fluorescein horse anti-mouse IgG Antibody (1:200; #FI-2000, Vector Laboratories) for 30 minutes. The tissue sections were washed and then mounted with Vectashield mounting medium with DAPI (H-1200, Vector Laboratories).

Statistical analysis
Data from different experiments were presented as mean ± SD. For statistical analysis, Student t test for independent means was used. A $P$ value of $<0.05$ was considered significant. To compare the effects of different treatments on tumor growth in vivo, one-way ANOVA with Dunnett multiple comparison test was used. Survival curve comparisons were performed using GraphPad Prism software for Kaplan–Meier survival analysis. Statistical differences between the various groups were compared pair-wise using log-rank (Mantel–Cox) analysis.

Combination synergism was analyzed with MacSynergy II software (kindly provided by M.N. Prichard) with 95% confidence limits according to the Bliss independence model (39). The effect attributed to combination is determined by subtracting the experimental values from theoretical additive values defined by the equation: $E_{xy} = E_x + E_y - E_{xy}$, where $E_{xy}$ is the additive effect of drugs x and y as predicted by their individual effects $E_x$ and $E_y$. 
Results

Improved antiproliferation effects of evofosfamide under prolonged hypoxic conditions with neuroblastoma and rhabdomyosarcoma cell lines

We used a panel of neuroblastoma and rhabdomyosarcoma cell lines that represented heterogeneity and different stages of therapy, including five neuroblastoma [CHLA-15, CHLA-20, CHLA-90, SK-N-BE(2), and SH-SY5Y] and three rhabdomyosarcoma cell lines (RH4, RH30, and RD), to assess the effects of evofosfamide on cell proliferation in vitro. SH-SY5Y is MYCN nonamplified. CHLA-15 and CHLA-20 cell lines are MYCN nonamplified cell lines obtained from tumors of treatment-naïve and chemotherapy-treated neuroblastoma patients, respectively. Both CHLA-90 (MYCN nonamplified) and SK-N-BE(2) (MYCN-amplified) cell lines are obtained from bone marrow metastases of patients who relapsed after chemotherapy with mutant p53 (35). RH4 and RH30 cell lines are alveolar rhabdomyosarcoma cell lines, whereas RD is an embryonal rhabdomyosarcoma cell line.

All these cell lines were exposed to increased concentrations of evofosfamide in vitro for 72 hours. Under normoxic condition, all the tested lines responded to evofosfamide treatment in a dose-dependent manner, with IC₅₀ values ranging from 4.6 to 151 μmol/L. When exposing neuroblastoma and rhabdomyosarcoma cells under hypoxic conditions (1% O₂) for 2 hours, no significant IC₅₀ change was documented. However, when tumor cells were exposed to hypoxia (1% O₂) overnight, there was a 2- to 65-fold decrease of evofosfamide IC₅₀ (Fig. 1A and B; Table 1) with the IC₅₀ values ranging from 0.07 to 21.9 μmol/L.

Improved antiproliferation effects of evofosfamide when combined with topotecan in vitro

In our previous studies, we showed that maximal plasma concentration (Cₘₐₓ) of topotecan was 19.8 ng/mL with the metronomic oral dose of 1 mg/kg drug administration in NOD/SCID mouse model (23). In patients, Cₘₐₓ of topotecan...
Table 1. Comparison of IC_{50} concentrations of evofosfamide when tested alone and in combination with 20 nmol/L topotecan in neuroblastoma and rhabdomyosarcoma cell lines

<table>
<thead>
<tr>
<th>IC_{50} (nmol/L)</th>
<th>Neuroblastoma cell lines</th>
<th>Rhabdomyosarcoma cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Evofosfamide</td>
<td>Topotecan</td>
</tr>
<tr>
<td>CHLA-15-normoxia</td>
<td>4.6 ± 5.8</td>
<td>3 ± 3.5</td>
</tr>
<tr>
<td>CHLA-15-1% O_2; 2 hours</td>
<td>9.5 ± 6.3</td>
<td>7.4 ± 4.1</td>
</tr>
<tr>
<td>CHLA-15-1% O_2; O/N</td>
<td>0.07 ± 0.03</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>CHLA-20-normoxia</td>
<td>8.9 ± 2.7</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>CHLA-20-1% O_2; 2 hours</td>
<td>5.6 ± 1.1</td>
<td>0.83 ± 0.12</td>
</tr>
<tr>
<td>CHLA-20-1% O_2; O/N</td>
<td>0.16 ± 0.01</td>
<td>0.3 ± 0.01</td>
</tr>
<tr>
<td>CHLA-90-normoxia</td>
<td>13.9 ± 4.3</td>
<td>4.9 ± 2.5</td>
</tr>
<tr>
<td>CHLA-90-1% O_2; 2 hours</td>
<td>10.7 ± 2.7</td>
<td>3.5 ± 1.3</td>
</tr>
<tr>
<td>CHLA-90-1% O_2; O/N</td>
<td>0.47 ± 0.10</td>
<td>0.24 ± 0.03</td>
</tr>
<tr>
<td>SK-N-BE(2)-normoxia</td>
<td>51.9 ± 9.3</td>
<td>46.2 ± 16.9</td>
</tr>
<tr>
<td>SK-N-BE(2)-1% O_2; 2 hours</td>
<td>54.9 ± 5.8</td>
<td>49 ± 15.6</td>
</tr>
<tr>
<td>SK-N-BE(2)-1% O_2; O/N</td>
<td>2.4 ± 0.59</td>
<td>1.7 ± 0.47</td>
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<tr>
<td>SH-SY5Y-normoxia</td>
<td>66.1 ± 9.2</td>
<td>57.5 ± 13.8</td>
</tr>
<tr>
<td>SH-SY5Y-1% O_2; 2 hours</td>
<td>66.1 ± 5.9</td>
<td>39.8 ± 5.3</td>
</tr>
<tr>
<td>SH-SY5Y-1% O_2; O/N</td>
<td>1.3 ± 0.23</td>
<td>0.5 ± 0.07</td>
</tr>
<tr>
<td>Rhabdomyosarcoma cell lines</td>
<td></td>
<td></td>
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<tr>
<td>RH4-normoxia</td>
<td>20.9 ± 1.4</td>
<td>16.2 ± 1.1</td>
</tr>
<tr>
<td>RH4-1% O_2; 2 hours</td>
<td>24 ± 1.1</td>
<td>13.8 ± 1.7</td>
</tr>
<tr>
<td>RH4-1% O_2; O/N</td>
<td>3.3 ± 0.27</td>
<td>1.7 ± 0.22</td>
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<tr>
<td>RH30-normoxia</td>
<td>51.5 ± 34.7</td>
<td>120 ± 6.5</td>
</tr>
<tr>
<td>RH30-1% O_2; 2 hours</td>
<td>115 ± 3.8</td>
<td>95.5 ± 2.7</td>
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<tr>
<td>RH30-1% O_2; O/N</td>
<td>21.9 ± 8.2</td>
<td>15.1 ± 1.5</td>
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<tr>
<td>RD-normoxia</td>
<td>19.1 ± 4.5</td>
<td>11 ± 2.0</td>
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<tr>
<td>RD-1% O_2; 2 hours</td>
<td>18.2 ± 4.7</td>
<td>11.9 ± 19</td>
</tr>
<tr>
<td>RD-1% O_2; O/N</td>
<td>9.55 ± 0.33</td>
<td>4.07 ± 0.15</td>
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NOTE: P values represent statistically significant differences using the extra sum-of-squares F test.

is 10.6 ± 4.4 ng/mL with the oral dose of 2.3 mg/m^2/day. As the terminal half-life of oral topotecan (3.9 ± 1.0 hours; ref. 40) is significantly longer than evofosfamide (0.6 ± 0.1 hours; ref. 41), a constant concentration of topotecan (20 nmol/L = 8.4 ng/mL) was selected for in vitro combined treatment.

All selected tumor cell lines were treated in vitro with increasing concentrations of evofosfamide with or without the presence of topotecan (20 nmol/L). The dose–response curves were generated by GraphPad software and IC_{50} values were compared between IC_{50} concentrations of evofosfamide when tested alone and in combination with 20 nmol/L topotecan in neuroblastoma and rhabdomyosarcoma cell lines. As shown in Tables 1 and 2, with the presence of topotecan, evofosfamide-induced cytotoxicity was significantly enhanced under overnight hypoxia as indicated by decreased IC_{50} in most tested tumor cell lines.

To understand whether the combination of the drugs was additive or synergistic, a Bliss analysis was performed with MacSynergy II software with 95% confidence limits. The effect of the combination was additive in all cell lines.

Topotecan and evofosfamide delay tumor growth and enhance animal survival in neuroblastoma model

After verifying in vitro activity of evofosfamide and topotecan, the in vivo effectiveness of topotecan/evofosfamide as single agents or combination therapy was evaluated in CHLA-20 and SK-N-BE(2) xenograft models. As both CHLA-20 and SK-N-BE(2) cell lines are derived from samples of patients who relapsed after chemotherapy, we use them to develop aggressive tumor models for rigorous screening of anticancer agents. All treatments commenced when the tumor sizes reached 0.25 cm^3. In both neuroblastoma models, after 2 weeks of treatment, tumor growth delay was observed with evofosfamide or topotecan as monotherapies (Fig. 2A). In SK-N-BE(2) xenograft model, combined regimen induced complete tumor regression, while topotecan induced partial tumor regression. When we compared the tumor sizes between these two arms, the difference is statistically significant (P < 0.05) at different time points (from day 14 to day 33). In CHLA-20 xenograft model, both single-agent topotecan and combined treatment induced complete tumor regression, but all those tumors relapsed after drug treatment was stopped. Therefore, we compared tumor recurrence between single-agent topotecan and combination groups after we stopped drug treatment. As shown in Figure 2B, the combination group had significantly longer recurrent-free interval compared with single-agent topotecan group (P < 0.05). For topotecan-treated mice, median time of recurrence was 30 days, while median time of recurrence for combined therapy was postponed to 57 days. In addition, when we challenged those relapsed tumors with the combined evofosfamide/topotecan therapy, those tumors remained responsive with the original treatment schedule (Fig. 2A).

Treatment was well tolerated, with weight loss >10% observed in single-agent and combined treatment groups within the first 8 days but returned to baseline thereafter. No animal death was observed in any of the groups during the experimental period (Fig. 2C).

To determine the antitumor activities of evofosfamide and topotecan on late-stage metastatic disease, we further assess animal survival in the SK-N-BE(2) intravenous metastatic model.
Figure 2.
Antitumor effects of evofosfamide and topotecan in neuroblastoma xenograft models. A, a total of $1 \times 10^6$ cells were implanted subcutaneously into SCID/SCID mice. Once tumors were palpable (about 0.25 cm$^3$), mice were randomized into vehicle control ($n = 10$), or three treatment groups ($n = 10$ for each group). Mice bearing human neuroblastoma [SK-N-BE(2) and CHLA-20] xenografts were treated for 2 weeks with evofosfamide (50 mg/kg; every day x 5 days/week, i.p.) or topotecan (1 mg/kg; every day x 5 days/week, orally) as single agents or in combination. The shaded area indicates the period of drug treatment. Tumor growth curves were plotted with the tumor volumes at different time points. One-way ANOVA was used to compare tumor volumes between experimental groups at different time points during the experimental period (*, $P < 0.05$; **, $P < 0.01$). Data are expressed as mean ± SEM. After 1 cycle of therapy, the combination treatment with evofosfamide and topotecan led to complete tumor regression in both SK-N-BE(2) and CHLA-20 xenograft models. In the CHLA-20 model, tumor recurrence was observed in the combined treatment group. On day 55, we rechallenged the tumor bearing animals with the original treatment dosage and schedule on day 55 (identified by shaded area between day 55 and 69). All the relapsed tumors remained responsive to combined treatment. B, recurrence-free interval curves were estimated in CHLA-20 xenograft model by the Kaplan–Meier method and compared between topotecan and combination of evofosfamide and topotecan using the log-rank (Mantel–Cox) analysis. The tumor-free interval was defined from the end of treatment to the first appearance of a palpable mammary tumor at least 100 mm$^3$ in size. *, $P < 0.05$. C, mouse body weight was measured in CHLA-20 tumor-bearing mice in different treatment groups and control animals. Percentage of body weight loss was calculated and plotted to monitor the potential drug toxicity. D, Kaplan–Meier survival curves of the SK-N-BE(2) metastatic tumor model. NOD/SCID mice ($n = 8$ / group) were injected with $1 \times 10^6$ SK-N-BE(2) cells intravenously. Fourteen days after tumor cell inoculation, mice were treated for 2 weeks with control vehicle, evofosfamide, topotecan, or the combination of evofosfamide and topotecan with the same dose as indicated above. Statistical differences between the various groups were compared pair-wise using log-rank (Mantel–Cox) analysis. *, $P < 0.05$.  

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Fourteen days after tumor cell inoculation, we initiated drug treatment with evofosfamide and topotecan as single agents or combined treatment. As shown in Fig. 2D, evofosfamide or topotecan treatment showed a substantial increase in animal survival compared with the control mice with a median survival of 22.5 days for the control group, 25 days for the evofosfamide group, and 36 days for the topotecan group. However, treatment with a combination of evofosfamide and topotecan had the greatest impact on animal survival ($P < 0.01$) with a median survival of 46 days.

**Topotecan and evofosfamide delays tumor growth in rhabdomyosarcoma models**

To test the antitumor activity of evofosfamide and topotecan in rhabdomyosarcoma, we chose an alveolar rhabdomyosarcoma cell line (RH4) and an embryonal rhabdomyosarcoma cell line (RD) to generate xenograft models. Tumor bearing NOD/SCID mice were randomized to therapy with evofosfamide, topotecan, or combined therapy. In both RH4 and RD xenograft models, both evofosfamide and topotecan monotherapy significantly delayed tumor growth with 2 weeks of treatment (Fig. 3). Compared with neuroblastoma models, single-agent evofosfamide was more effective in rhabdomyosarcoma models with partial tumor regression in all treated xenografts. The combination of topotecan and evofosfamide were superior to the respective drugs given alone at different time points from day 26 to day 30 ($P < 0.01$; Fig. 3). There was no significant drug-related toxicity in any of the treatment arms.

**Colocalization of cell apoptosis and tumor hypoxia with evofosfamide and topotecan treatment**

As combined treatment with evofosfamide and topotecan significantly improved antitumor activity both in vitro and in vivo, we have particular interest to examine its potential mechanism of action in vivo. To this end, tumor cell apoptosis was assessed by cleaved caspase-3 immunoreactivity analysis in our xenograft models. On day 5 of drug treatment, RH4 xenografts from different regimens were harvested for immunohistochemical analysis (Fig. 4A). Hypoxic regions were identified as the area of pimonidazole-positive staining. Apoptotic cells were detected by anti-cleaved caspase-3 antibody. Apoptosis was quantified as the percentage of positive cleaved caspase-3 staining area using ImageJ software (Fig. 4B). As shown in Fig. 4A and B, evofosfamide induced tumor cell apoptosis in hypoxic regions, with a remarkably higher percentage of cleaved caspase-3–positive cells in hypoxic regions compared with normoxic regions (Student $t$ test, $P < 0.01$). On the contrary, topotecan induced more extensive cell apoptosis in normoxic regions than hypoxic regions ($P < 0.05$). With the combination treatment, significant tumor cell apoptosis was observed throughout the tumor, in both normoxic and hypoxic areas.

**Decreased cell proliferation with evofosfamide and topotecan combined treatment in vivo**

We further evaluated tumor cell proliferation in different treatment groups by immunostaining of the proliferation marker, Ki67. As shown in Fig. 4C and D, the Ki67$^+$ proliferating tumor cell population was mainly located in normoxic regions. After the 4-day in vivo treatment, single-agent topotecan showed a limited effect on cell proliferation as measured by Ki67 positivity. Surprisingly, decreased cell proliferation was observed in evofosfamide-treated tumors in normoxic regions with significantly lower percentage of Ki67$^+$ positive cells than control tumors ($P < 0.05$), suggesting a hypoxia-independent bystander effect. Combined evofosfamide and topotecan treatment led to a further decrease in the Ki67$^+$–proliferating tumor cell population compared with single-agent evofosfamide group (combined group vs. single-agent topotecan, $P < 0.01$; combined group vs. single-agent evofosfamide, $P < 0.05$).

**Discussion**

Topotecan has demonstrated efficacy in preclinical and clinical studies in neuroblastoma and rhabdomyosarcoma. However, total eradication of highly tumorigenic populations of tumor...
cells is required to achieve relapse-free survival in patients with cancer. Incomplete distribution of chemotherapeutics to poorly perfused areas of primary tumors and metastatic sites is a major hindrance in complete eradication of these tumor cells. These sparsely vascularized areas are characterized by hypoxia which confers drug and radiation resistance, and a proangiogenic and invasive phenotype to tumor cells. Despite the advent of novel chemotherapies, complete eradication of tumor cells cannot be accomplished until hypoxic tumor cells are targeted. Therefore, targeting hypoxic zone of solid tumor may reverse drug resistance and make cytotoxic agent more effective. In this study, we are proposing new strategies to improve the efficacy of chemotherapy by incorporating a novel agent targeting tumor hypoxic regions.

HAPs, which deliver the cytotoxic payload in hypoxic zones, have been developed to solve this issue. PR-104, an earlier HAP, was found to have a steep dose–response relationship in a panel of pediatric tumor xenograft models, which raises safety concerns in pediatric patients (42). Evofosfamide, however, has demonstrated survival benefit in various adult cancers without causing prohibitively additive toxicity with conventional chemotherapy in clinical trials (16). Evofosfamide shares the similar active metabolites as ifofosfamide, but it is preferentially activated in hypoxic tissues and does not have the same toxic metabolites as ifosfamide. It is anticipated that evofosfamide should have less hematologic, renal, and CNS toxicity than ifosfamide. There is no direct clinical studies comparing evofosfamide with ifosfamide, but evofosfamide shows
superior efficacy and less toxicity than ifosfamide in preclinical lung carcinoma models (43). In our xenograft models, we observed tumor growth inhibition with single-agent evofosfamide, but no significant antitumor effect was achieved in the CHLA-20 model with ifosfamide (50 mg/kg; every day × 5 days/week, i.p.; Supplementary Fig. S1). In clinical trials (NCT02047500), evofosfamide is administered at a dose ranging from 170 to 340 mg/m². This could be converted to 32–64 mg/kg in the tumor bearing mice, calculated by the pharmacokinetic data from the clinical studies and preclinical mouse studies (44). In this study, we tested evofosfamide at the dose of 50 mg/kg, as single agent and in combination with topotecan in two of the most common pediatric extracranial solid tumors, neuroblastoma and rhabdomyosarcoma. To our knowledge, this is the first study evaluating the effectiveness of evofosfamide in pediatric tumors.

Our observation that single-agent topotecan is more effective in delaying tumor growth in neuroblastoma than in rhabdomyosarcoma is in agreement with our previous finding (23). From our observations in vitro, evofosfamide showed more activity in neuroblastoma cell lines than in rhabdomyosarcoma cells. However, in vivo, rhabdomyosarcoma xenografts were more sensitive to evofosfamide than neuroblastoma xenografts. In this study, we cannot simply translate in vitro observation to in vivo activity. In vitro, we simulated tumor hypoxia by incubating tumor cells inside the hypoxia chamber. Therefore, 100% of cell population was under hypoxia and exposed to activated evofosfamide. In vivo, the percentage of hypoxic tumor cells varies among different tumor types (45, 46). In vivo tumor microenvironment is also more complex in which HAP activation would be affected by many other factors, including the duration and the intensity of oxidative stress, tumor angiogenesis, drug penetration in different
solid tumor types, and acquired drug resistance under prolonged hypoxic conditions. Although it was beyond the scope of this study to examine the tumor microenvironment among different tumor types, it remains an important area for our future investigation.

From our study, evofosfamide showed enhanced cytotoxicity under overnight and 3-day (Supplementary Fig. S2) prolonged hypoxia compared with short-term (2-hour) hypoxia exposure. When we test the effect of evofosfamide and its combination with topotecan on five neuroblastoma and three rhabdomyosarcoma cell lines under normoxia, short-term hypoxia and prolonged hypoxia, it is also under overnight hypoxia (1% O2) that adding topotecan significantly lowered IC50 of evofosfamide in most tumor cell line, except SK-N-BE(2). However, in the SK-N-BE(2) xenograft model, combined therapy achieved significant antitumor effects in vivo. Combined treatment induced complete tumor regression in all treated animals, which was superior to evofosfamide or topotecan single-agent treatment. This is likely because SK-N-BE(2) xenografts have more severe and more chronic hypoxic environment than in vitro cell culture (1% O2, overnight), which could more effectively activate evofosfamide to exert its cytotoxic activity in vivo.

In all the neuroblastoma and rhabdomyosarcoma xenograft models tested, combined treatment showed superior antitumor effects compared with single-agent treatments, and induced tumor regression after a 2-week treatment period. The advantage of the combination regimen was also approved in the metastatic neuroblastoma model. Our experimental metastatic model has been developed to simulate residual disease in neuroblastoma. Residual disease refers to disseminated tumor cells which survive after therapy (47). More than 50% of children with high-risk neuroblastoma develop recurrence due to the presence of minimal residual disease. In our metastatic model derived from a disseminated MYCN-amplified cell line, mice treated with combined topotecan and evofosfamide had extended survival compared with single-agent topotecan treatment. This result is significant in promoting new therapies for neuroblastoma minimal residual disease or refractory neuroblastoma by combining HAPs with existing chemotherapeutic drugs. We believe that incorporating HAPs, such as evofosfamide, could potentially eradicate the tumor cells hiding in hypoxic zones which are largely responsible for eventual relapse and metastasis. Eventually, this new regimen might prolong progression-free survival in high-risk neuroblastoma patients.

In this study, we were able to assess the correlation between tumor hypoxia and tumor cell apoptosis with immunostaining of hypoxic marker pimonidazole and apoptotic marker cleaved caspase-3. As expected, in evofosfamide-treated tumors, apoptotic cells were mostly confined to hypoxic zones while those in topotecan-treated tumors were located in normoxic areas. Homogeneous population of tumor cells undergoing apoptosis was observed in both normoxic and hypoxic regions, which supported the mechanism of synergistic efficacy of the combination therapy in vivo. In a previous study, the accessibility of topotecan, doxorubicin, and mitoxantrone was found to decrease with increasing distance from functional blood vessels in breast cancer xenografts (48). Clearly, poor drug penetration into solid tumors limits drug efficacy. In our study, we achieved tumor cytotoxicity in both normoxic and hypoxic regions by combining evofosfamide with topotecan, which explains the superior efficacy of our combination regimen in all tumor models.

An ideal HAP requires selective activation in hypoxic regions as well as good penetration of multiple cell layers to exert a local cytotoxic bystander effect. Using a multicellular tumor spheroid and multicellular layer (MCL) coculture model systems, Meng and colleagues (49) showed that evofosfamide has penetrability and bystander effect. In our study, with evofosfamide single-agent treatment, we observed decreased cell proliferation even at normoxic regions. This phenomenon confirms that once activated in hypoxic tissues, evofosfamide releases Br-IPM which can diffuse into surrounding oxygenated regions of the tumor and kill cells in those regions via a “bystander effect.”

In summary, topotecan and evofosfamide demonstrated anti-tumor activities in our preclinical models of various subtypes of neuroblastoma and rhabdomyosarcoma. Compared with single agents, combination treatment induces more tumor regression, delays tumor relapse, and enhances animal survival in our preclinical tumor models. In this study, we explored the mechanism of action with combined evofosfamide/topotecan therapy. We observed increased reactive oxygen species (ROS) under hypoxic condition (1% O2, overnight; Supplementary Fig. S3). We also found that adding topotecan enhanced the oxidative stress in rhabdomyosarcoma cells under hypoxic condition (Supplementary Fig. S4). In vivo, evofosfamide showed significant cytotoxicity against hypoxic tumor cells, while topotecan plays complementary roles by targeting tumor cells residing at normoxic regions. The ability of this combination to target cells in both normoxic and hypoxic tumor zones, as demonstrated by immunofluorescence, provides a proof-of-principle for its superior efficacy. These preclinical data support the development of a clinical trial in high-risk neuroblastoma and rhabdomyosarcoma.

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S. Baruchel reports receiving commercial research grants from Merck TH302. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Zhang, P. Marrano, B. Wu, S. Kumar, P. S. Thorner, S. Baruchel
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