5333

# Evofosfamide (TH-302) potentiates the antitumor activity of topotecan in neuroblastoma and rhabdomyosarcoma preclinical models

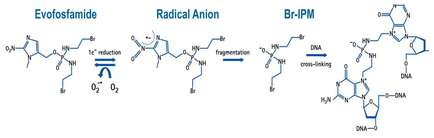

**Libo Zhang [1], Bing Wu [1], Paula Marrano [2], Paul Thorner [2, 4], Sylvain Baruchel [1, 4]**

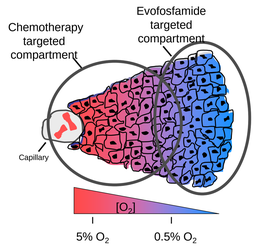
[1]New Agent and Innovative Therapy Program, Division of Hematology and Oncology; [2]Dept of Pathology,

The Hospital for Sick Children; [4]Institute of Medical Sciences, University of Toronto, Toronto, Canada


## Introduction

- There is strong evidence that tumor cells in the hypoxic regions cause drug resistance and tumor relapse. Hypoxia targeting agents may reverse drug resistance and make cytotoxic therapy more effective.
- Evofosfamide (previously known as TH-302), a hypoxia activated prodrug (HAP) has been designed to penetrate hypoxic regions of tumors. When exposed to hypoxic conditions, evofosfamide is reduced at the nitroimadazole site of the prodrug by intracellular reductases leading to the release of the alkylating agent Bromo-IPM. Bromo-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.'Evofosfamide is currently in several clinical trials, including two phase 3 trials (STS and PDAC).




- Topotecan is a potent topoisomerase I inhibitor. It also inhibits HIF-1α expression and tumor angiogenesis. It is primarily used for the treatment of ovarian cancer and small cell lung cancer. It is also used in pediatric cancer chemotherapy such as neuroblastoma (NBL) and rhabdomyosarcoma (RMS).
- In this study, we investigated the efficacy of evofosfamide, both alone and in combination with topotecan, in preclinical models of NBL and RMS

## Methods

***In vitro:***

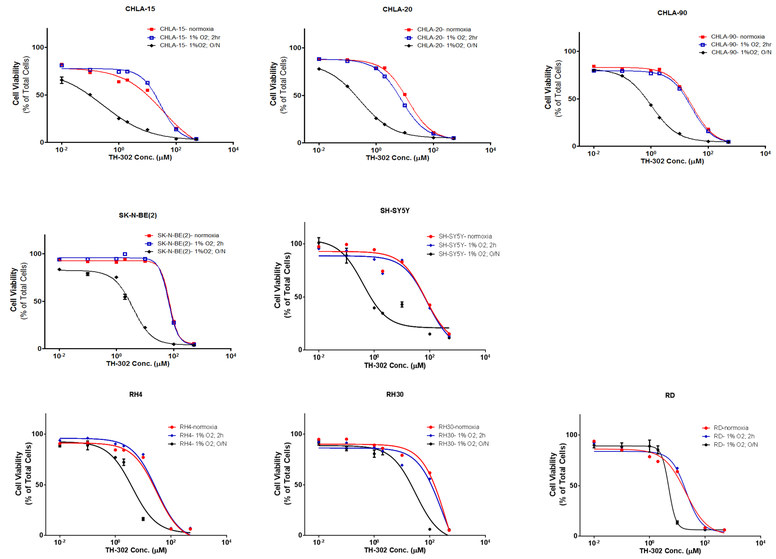
- A panel of five neuroblastoma (CHLA-15, CHLA-20, CHLA-90, SK-N-BE(2) and SH-SY5Y) and three rhabdomyosarcoma (RH4, RH30 and RD) cell lines were selected for in vitro study.
- Cell viability assay (Alamar Blue assay)
- Tumor cells were exposed to increased concentrations of evofosfamide in vitro for 72 hours under nomoxic and two hypoxic conditions (1%$O_2$ 2 hours or 1%$O_2$ overnight). For combined drug treatment, 20nM of topotecan was added.

***In vivo:***

- Two NB cell lines (CHLA-20 and SK-N-BE(2)) and two RMS cell lines (RH4 and RD) were used to establish murine models. Xenograft tumors were established by the subcutaneous inoculation of tumor cells into NOD/SCID mice.
- Drug treatment was initiated when tumors reached a size of 0.2 $cm^3$. Evofosfamide (50 mg/kg; qd × 5/wk, IP) and topotecan (1 mg/kg; qd × 5/wk, orally) were administered as single agents or in combination.
- Animal survival was studied with the SK-N-BE(2) intravenous metastatic tumor model. Endpoint was defined as significant (>20%) body weight loss or presence of any signs of stress.
- Cleaved caspase-3 and a bioreductive agent (*pimonidazole)* were used as apoptotic and hypoxic *markers in vivo.*

## Results

- **Increased cytotoxicity of evofosfamide under prolonged hypoxic conditions with NBL and RMS cells**




- **Enhanced anti-proliferation effects of evofosfamide when combined with topotecan in vitro**

**IC50 of evofosfamide with or without the presence of topotecan (20nM)**

| | IC50 (μM) | | p Value * |
|---|---|---|---|
| | Evofosfamide | Evofosfamide +Topotecan | |
| CHLA-15-normoxia | 4.6 | 2.99 | 0.387 |
| CHLA-15-1% Hypoxia; 2hr | 9.47 | 7.4 | 0.5735 |
| CHLA-15-1% Hypoxia; O/N | 0.07 | 0.037 | <0.01 |
| | | | |
| CHLA-20-normoxia | 8.925 | 1.203 | <0.01 |
| CHLA-20-1% Hypoxia; 2hr | 5.615 | 0.8334 | <0.01 |
| CHLA-20-1% Hypoxia; O/N | 0.1555 | 0.09536 | <0.01 |
| | | | |
| CHLA-90-normoxia | 13.91 | 4.925 | <0.05 |
| CHLA-90-1% Hypoxia; 2hr | 10.74 | 3.496 | <0.05 |
| CHLA-90-1% Hypoxia; O/N | 0.474 | 0.2352 | <0.01 |
| | | | |
| SK-N-BE(2)-normoxia | 51.92 | 46.23 | 0.55 |
| SK-N-BE(2)-1% Hypoxia; 2hr | 54.87 | 48.97 | 0.47 |
| SK-N-BE(2)-1% Hypoxia; O/N | 2.43 | 1.67 | 0.22 |
| | | | |
| SH-SY5Y-normoxia | 13.18 | 69.18 | <0.01 |
| SH-SY5Y-1% Hypoxia; 2hr | 15.85 | 12.02 | <0.01 |
| SH-SY5Y-1% Hypoxia; O/N | 0.66 | 0.44 | <0.01 |

| | IC50 (μM) | | P Value * |
|---|---|---|---|
| | Evofosfamide | Evofosfamide +Topotecan | |
| RH4-normoxia | 20.89 | 16.22 | <0.01 |
| RH4-1% Hypoxia; 2hr | 23.99 | 13.8 | <0.01 |
| RH4-1% Hypoxia; O/N | 3.31 | 1.74 | <0.01 |
| | | | |
| RH30-normoxia | 151.4 | 120.2 | <0.01 |
| RH30-1% Hypoxia; 2hr | 114.8 | 95.5 | <0.01 |
| RH30-1% Hypoxia; O/N | 21.88 | 15.14 | <0.01 |
| | | | |
| RD-normoxia | 19.05 | 10.96 | <0.01 |
| RD-1% Hypoxia; 2hr | 18.2 | 10.96 | <0.01 |
| RD-1% Hypoxia; O/N | 9.55 | 4.07 | <0.01 |

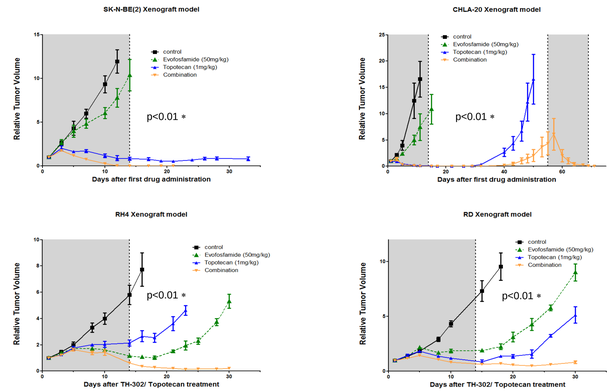
* Statistical comparisons between best-fit $EC_{50}$s for any two curves were performed in Prism using the extra sum-of-squares F test.

**IC50 of topotecan**

| Cell lines | IC50 (nM) |
|---|---|
| RH4 | 89.13 |
| RH30 | 17.54 |
| RD | 17.78 |
| CHLA-15 | 7.59 |
| CHLA-20 | 9.71 |
| SK-N-BE(2) | 100 |
| SH-SY5Y | 12.56 |

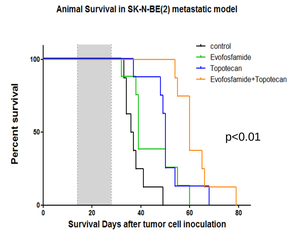
## Results (cont.)

- **In vivo antitumor effects of evofosfamide and topotecan in NBL and RMS xenograft models**



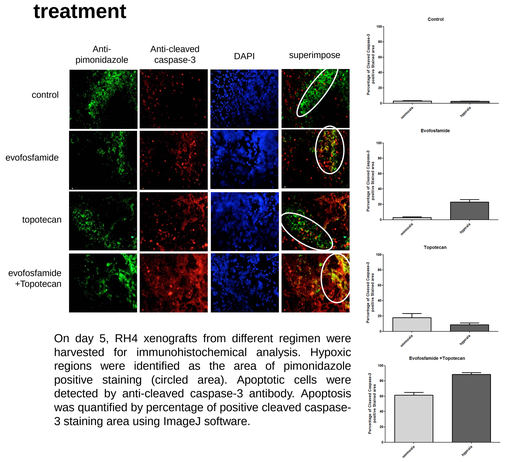

* One-way ANOVA was used to compare tumor volumes between experimental groups at the last time point of control group.

- **Improved animal survival with evofosfamide and topotecan treatment in metastatic NB models**




Statistical differences between the various groups were evaluated by log-rank (Mantel-Cox) analysis. Both evofosfamide and topotecan treatment prolonged survival. Combined treatment significantly improved animal survival compared to single-agent evofosfamide/ topotecan treatment (median survival: Control 36.5 days; evofosfamide, 39 days; topotecan 50 days; and combined treatment, 60 days).

- **Co-localization of tumor cell apoptosis and hypoxia with evofosfamide and topotecan treatment**




On day 5, RH4 xenografts from different regimen were harvested for immunohistochemical analysis. Hypoxic regions were identified as the area of pimonidazole positive staining (circled area). Apoptotic cells were detected by anti-cleaved caspase-3 antibody. Apoptosis was quantified by percentage of positive cleaved caspase-3 staining area using ImageJ software.

## Conclusions

- Evofosfamide shows antitumor effects in neuroblastoma and rhabdomyosarcoma xenografts.
- Evofosfamide increased apoptosis in hypoxic regions, and topotecan increased apoptosis mainly in normoxic region. With the combination treatment, apoptosis is further increased throughout the tumor.
- Compared to single-agent evofosfamide/ topotecan, combination treatment significantly improves tumor response, delays tumor relapse and enhances animal survival in our preclinical tumor models.
- These preclinical data support the development of a clinical trial combining topotecan and evofosfamide in recurrent neuroblastoma and rhabdomyosarcoma.

## Acknowledgement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