



Published in final edited form as:

*Pediatr Blood Cancer*. 2020 July ; 67(7): e28284. doi:10.1002/pbc.28284.

## Evaluation of VTP-50469, a Menin-MLL1 Inhibitor, Against Ewing Sarcoma Xenograft Models by the Pediatric Preclinical Testing Consortium

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### Abstract

**Background.**—VTP-50469 is a potent inhibitor of the menin-MLL1 interaction and is implicated in signaling downstream of EWSR1-FLI1.

**Procedure.**—VTP-50469 was evaluated against 7 Ewing sarcoma (EwS) xenograft models and *in vitro* against EwS cell lines.

**Results.**—VTP-50469 showed limited antitumor activity, statistically significantly slowing tumor progression in 4 tumor models but with no evidence of tumor regression. *In vitro*, the IC<sub>50</sub> concentration was 10 nM for the MLL-rearranged leukemia cell line MV4;11, but > 3μM for EwS cell lines.

**Conclusions.**—In contrast to its high level of activity against MLL1-rearranged leukemia xenografts, VTP-50469 shows little activity against EwS models.

### Keywords

Ewing sarcoma; Pediatric Oncology; Xenograft

### Introduction

MLL1, the mammalian homolog of drosophila Trithorax (*trx*), positively regulates *HOX* gene expression during development.<sup>1</sup> The activity of *trx* group proteins is balanced by the repressive activity of Polycomb group (PcG) genes.<sup>2</sup> MLL1 (KMT2A), a lysine methyltransferase, binds promoters of *HOX* genes resulting in H3 Lys 4 methylation and H3 and H4 acetylation.<sup>3</sup> Dysregulation of *HOX* genes occurs in several cancers, suggesting

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Conflict of Interest: Dr. McGeehan is an employee of Syndax Pharmaceuticals. The other authors have no conflicts.

a critical role for developmental programs in transformation.<sup>4–6</sup> While the role of MLL1 translocations is well established for several leukemias<sup>1</sup>, less is known regarding the role of MLL1 in solid tumors. In leukemias, the oncogenic activity of MLL1 fusion proteins is dependent on association with menin, a scaffolding protein that binds MLL1 and MLL4 (KMT2B) in the context of TrxG COMPASS complexes.<sup>7</sup> Consequently, small molecules that inhibit the Menin-MLL1 interaction have potential therapeutic value for treatment of MLL1 rearranged leukemia.<sup>8</sup> Posterior *HOXD* genes are overexpressed in Ewing sarcoma (EwS).<sup>9</sup> Promoter regions for these genes are characterized by MLL-mediated H3K4me3 marks and are devoid of recessive H3K27me3 marks.<sup>9</sup> Recently, evidence has been presented that the tumorigenicity of EwS cells is dependent on the Menin-MLL1 interaction.<sup>10</sup> Here we have evaluated VTP-50469, a potent inhibitor of Menin-MLL1 interactions that shows profound activity against Pediatric Preclinical Testing Consortium (PPTC) MLL-rearranged leukemia models.<sup>11</sup>

## Materials and Methods

### In vivo testing:

C.B.17SC *scid*<sup>-/-</sup> (C.B-*Igh-1<sup>b</sup>*/IcrTac-*Prkdc<sup>scid</sup>*) female mice were maintained under barrier conditions and experiments were conducted using protocols and conditions approved by the institutional animal care and use committee at UTHSCSA as previously described<sup>12</sup> Details of the statistical analytic methods are provided in Appendix 1.

### Drugs and Formulation:

VTP-50469 was provided to the Pediatric Preclinical Testing Consortium (PPTC) by Syndax Pharmaceuticals Inc., through the Cancer Therapy Evaluation Program (NCI). VTP-50469 was suspended in the required amount of vehicle (0.5% Natrosol + 1% Polysorbate-80). The resulting formulation was sonicated in a water bath set to 37°C until completely dissolved or visually uniform suspensions were achieved. Formulated drug was stored at 4°C for up to one month. VTP-50469 was administered by oral gavage (PO) at 120 mg/kg, twice daily (BID) for a planned 28 consecutive days.

### In vitro testing:

The potency of VTP-50469 was evaluated against four EwS cell lines (ES-1, ES-4, ES-6, EW-8). Each line has the EWSR1-FLI1 type 1 translocation. VTP-50469 was also tested against the MV4;11 cell line that has the MLL-AF4 (KMT2A-AFF1) fusion. Cells were exposed to VTP-50469 for 96h and viability assessed by Alamar Blue staining, as described previously.<sup>13</sup>

## Results

For CHLA-258 and EW-8 cell lines the VTP-50469 dose was reduced to 100 mg/kg BID because of excessive toxicity observed in models treated at 120 mg/kg BID. For the five Ewing sarcoma models treated at 120 mg/kg BID, excessive mortality (18 of 50) was observed in the absence of antecedent weight loss. For the remaining two models (CHLA-258 and EW-8) treated at 100 mg/kg BID, 1 of 20 mice in the treatment group died

(5%). VTP-50469 caused a statistically significant growth delay in 4 EwS models, Table 1. Among models with significant slowing of tumor growth, the ratio of median time to event for the treated versus control groups (EFS T/C) ranged from 1.24 to 1.74. There were no tumor regressions, and the mean minimum relative tumor volumes (RTV) for treated groups ranged from 1.2 to 3.5 (Table 1). The overall objective response classification for all models was Progressive Disease 1 (PD1). Kaplan-Meier EFS for each tumor line is shown in Figure 1A. The expression (mRNA) and mutation status of KMT2A and Menin (MEN1) in the PPTC leukemia and solid tumor models is shown in Supplemental Figures 1 and 2.

Because VTP-50469 demonstrated limited activity against EwS xenograft models at a dose and schedule that was highly active against MLL-rearranged leukemias, we determined the sensitivity of EwS cell lines in comparison with the MLL-rearranged leukemia cell line MV4;11. Cells were exposed to VTP-50469 at concentrations from 3 nM to 3  $\mu$ M for 96h. The MV4;11 cell line was very sensitive to VTP-50469 ( $IC_{50}$  = 10 nM), whereas for all EwS cell lines the  $IC_{50}$  was >3  $\mu$ M, (Figure 1B).

## Discussion

MLL translocations occur in 5–10% of B-cell ALL with a similar frequency observed for AML. Rearrangements are more frequent in infant leukemias (~70% of infant ALL). Menin inhibitors are highly active in preclinical models of MLL-rearranged ALL,<sup>8,11,14</sup> and clinical trials for this class of agents have been initiated (SNDX-5613 in [NCT04065399](https://clinicaltrials.gov/ct2/show/study/NCT04065399) and KO-539 in [NCT04067336](https://clinicaltrials.gov/ct2/show/study/NCT04067336)).

Recent work has suggested that Menin-MLL1 interactions may be important for tumorigenesis induced by the EWSR1-FLI1 fusion oncogene in EwS and that this effect may be in part mediated through effects on serine biosynthesis.<sup>10,15</sup> MI-503, a small molecule that disrupts the Menin-MLL1 interaction,<sup>8</sup> reduced proliferation and suppressed anchorage independent growth of EwS cell lines.<sup>10</sup> *In vitro*, MI-503 induced loss of both MLL1 and menin, and it also reduced tumorigenicity of EwS cells that were pre-treated with MI-503 prior to inoculation into athymic nude mice.<sup>10</sup> mRNA expression in EwS models for KMT2A and Menin (MEN1) show that expression of KMT2A is lower than in MLL models that have fusions. The only EwS model to have a mutation (missense; E2419K) is SK-NEP-1, a model not used in the current study. Expression of MEN1 was similar in leukemias and EwS models, and no mutations in the EwS models were detected (<https://pedcbiportal.org/study?id=pptc#summary>).<sup>16</sup>

VTP-50469, like MI-503, potentially disrupts the Menin-MLL1 interaction and is highly active over a broad range of doses in infant leukemias that have MLL1 translocations. Against EwS xenografts, VTP-50469 showed little antitumor activity, statistically significantly slowing tumor growth in several models. However, extension of EFS in these models was modest, and tumor regression was not observed. Consistent with the *in vivo* results, EwS cell lines were >300-fold less sensitive to VTP-50469 than the MLL leukemia cell line MV4;11. Pharmacodynamic studies were not undertaken to determine target inhibition, as VTP-50469 administered at these and lower dose levels on the same schedule has robust activity in several infant leukemia models with MLL1 translocations. Our results are consistent with

those of a recent report that found that while MI-503 had *in vitro* activity against a range of leukemia and solid tumor cell lines, the more selective menin inhibitor BAY-155 was primarily active in AML and ALL models.<sup>17</sup>

Our results and an examination of existing literature for menin inhibitors suggest that EwS cells are less dependent on the Menin-MLL1 interaction for survival in comparison to MLL-rearranged leukemias. First, the IC<sub>50</sub> values for EwS cell lines are approximately one log greater than those for MLL-rearranged leukemia cell lines for the menin inhibitor MI-503.<sup>8,10</sup> Similarly, our *in vitro* results show much greater sensitivity for a MLL-rearranged leukemia cell line to VTP-50469 in comparison to that observed for EwS cell lines. Second, the effect of menin inhibitors on the expression of the MLL1 gene fusion target genes (e.g., *Hox9* and *Meis1*) is much greater than the effect of menin inhibition on expression of *HoxD* genes in EwS cell lines.<sup>8,10</sup> Finally, VTP-50469 shows remarkably high *in vivo* activity against MLL-rearranged leukemia xenograft lines, but shows minimal levels of *in vivo* activity against EwS models.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements.

This work was supported by NCI grants: U01 CA199297, U01 CA199222, and P30 CA054174.

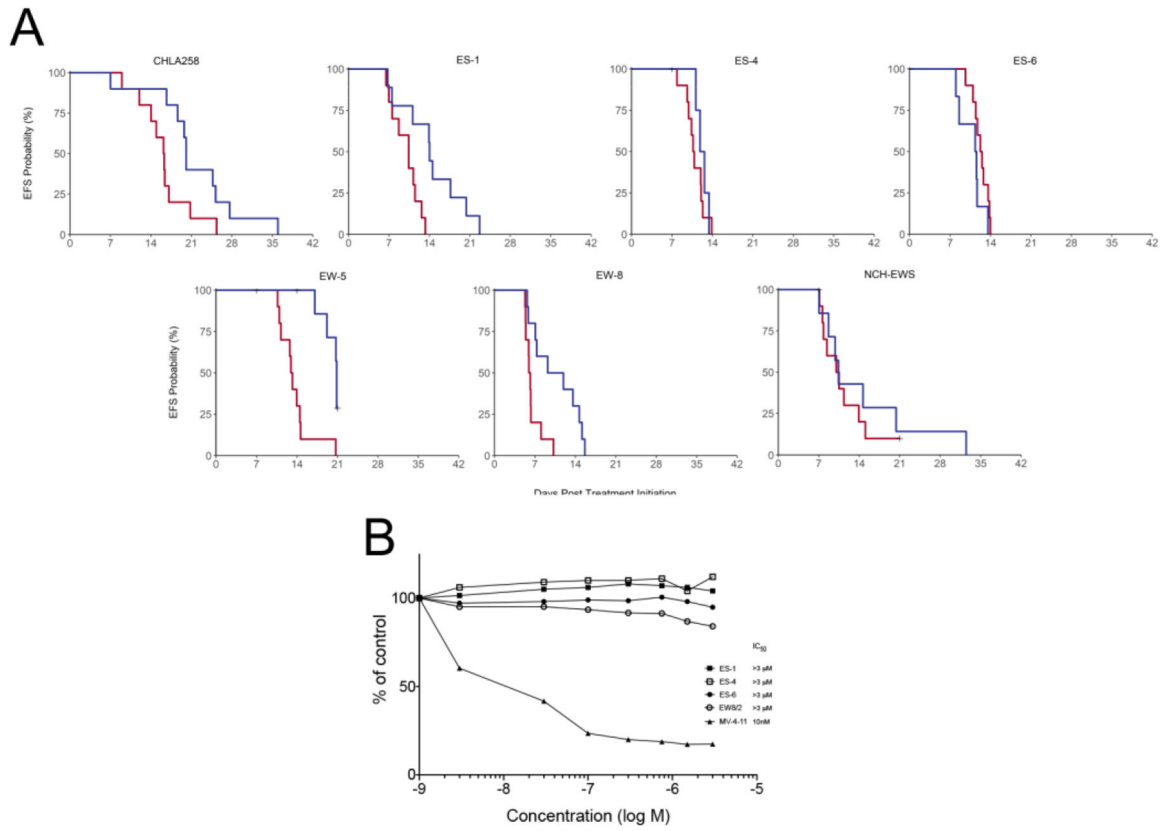
## Abbreviation

<b>EwS</b>	Ewing sarcoma
<b>PPTC</b>	Pediatric Preclinical Testing Consortium

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**Figure 1.** **A**, Kaplan-Meier plots showing probability of being Event-Free with time. Red line: Controls; Blue line VTP-50469 treatment; **B**, Sensitivity of EwS cell lines compared to MV4;11 a cell line that has the t(4;11)(q21;q23) chromosomal translocation leading to fusion of the mixed lineage leukemia (MLL) gene located on 11q23 and AF4 located on 4q21 (MLL/AF4; or KMT2A/AFF1). Cells were exposed to VTP-50469 for 96h and viability assessed by Alamar Blue staining.

**Table 1.**

Summary of Antitumor Activity of VTP-50469 against EwS Xenograft Models

Tumor Line	Treatment	KM Median (days)	EFS T-C (days)	EFS T/C	p-value Gehan-Wilcoxon	Median Response	Minimum Relative Tumor Volume
CHLA258	Control	16.2					1.971±0.771
	VTP-50469	20.1	3.9	1.24	0.036	PD1	1.553±0.430
ES-1	Control	10.4					3.047±0.816
	VTP-50469	14.0	3.6	1.35	0.046	PD1	2.612±0.991
ES-4	Control	10.7					2.494±0.616
	VTP-50469	12.2	1.5	1.14	0.186	PD1	2.092±0.460
ES-6	Control	12.4					2.066±0.280
	VTP-50469	11.5	-0.9	0.93	0.084	PD1	2.333±0.953
EW-5	Control	13.1					1.709±0.370
	VTP-50469	20.8	7.8	1.6	<0.001	PD1	1.241±0.394
EW-8	Control	6.1					5.014±0.931
	VTP-50469	10.6	4.5	1.74	0.015	PD1	3.497±1.177
NCH-EWS-1	Control	10.3					2.741±1.078
	VTP-50469	10.4	0.1	1.01	0.665	PD1	3.112±0.823