Combined inhibition of PI3K isoforms and mTOR kinase is critical for cancer stem cell inhibition by VS-5584

#3906

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ABSTRACT

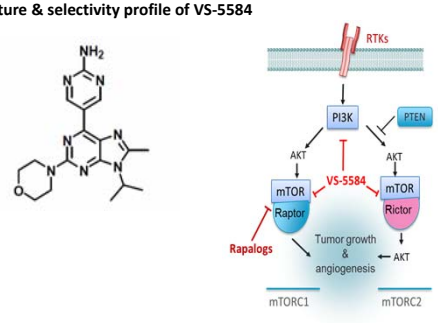
Cancer stem cells (CSCs) represent a subpopulation of cancer cells that have tumor-initiating capability, are particularly resistant to chemotherapy, and can mediate tumor recurrence both locally and at metastatic sites. As such, these cells represent a critical challenge for effective treatment of cancer. High-throughput screening for small molecules that preferentially target CSCs identified inhibitors of the PI3K/mTOR pathway, suggesting the importance of this signaling pathway for CSC biology.

Here we demonstrate that ablation of mTOR or individual PI3K isoforms by isoform-specific siRNA is not sufficient to reduce the proportion of CSCs. In contrast, combined knock down of PI3K isoforms and mTOR effectively reduced the proportion of CSCs in tumor cell lines. VS-5584 is a potent and selective PI3K/mTOR inhibitor with equipotency against all four human Class I PI3K isoforms and the mTOR kinase. We demonstrate that VS-5584 preferentially targets CSCs in multiple orthogonal assays both *in vitro* and in human tumor xenograft models.

Cancer stem cells express high levels of aldehyde dehydrogenase, and an Aldefluor assay that measures activity of this enzyme was used to identify CSCs. VS-5584 decreased the percentage of Aldefluor-positive cells across multiple breast cancer cell lines. We demonstrated that VS-5584 preferentially induced apoptosis in Aldefluor-positive SUM159 cells relative to Aldefluor-negative cells as measured by Annexin V and Caspase 3/7 assays. In contrast, paclitaxel induced more apoptosis in Aldefluor-negative than Aldefluor-positive cells and enriched the percentage of CSCs. Another characteristic of CSCs is their enhanced ability to efflux cytotoxic agents. This CSC population, which is drug resistant and is called side population (SP), can be monitored by exclusion of Hoechst dye. VS-5584 effectively eliminated the SP CSCs across multiple cancer types, while cisplatin and etoposide increased this subpopulation. Furthermore, *ex vivo* treatment of primary breast and ovarian tumor specimens with VS-5584 decreased the proportion of CSCs as measured by the Aldefluor assay and cell surface markers. Significantly, VS-5584 also targets CSCs *in vivo* in MDA-MB-231 triple negative and MCF7 ER+ breast cancer xenograft models as evidenced by decreases in the percentage of Aldefluor-positive cells, tumorsphere-forming efficiency, and tumor-initiating capability in an *in vivo* limiting dilution re-implantation assay. Consistent with the notion that combined inhibition of PI3K isoforms and mTOR is critical for exerting a strong anti-CSC effect, the mTORC1-selective inhibitor everolimus did not reduce CSCs in the MCF7 xenograft model. The potent anti-CSC activities in primary patient cancer tissue and in xenograft models provide strong rationale for the clinical development of VS-5584 in combination with agents targeting the bulk tumor to achieve durable clinical responses for cancer patients.

INTRODUCTION

Structure & selectivity profile of VS-5584



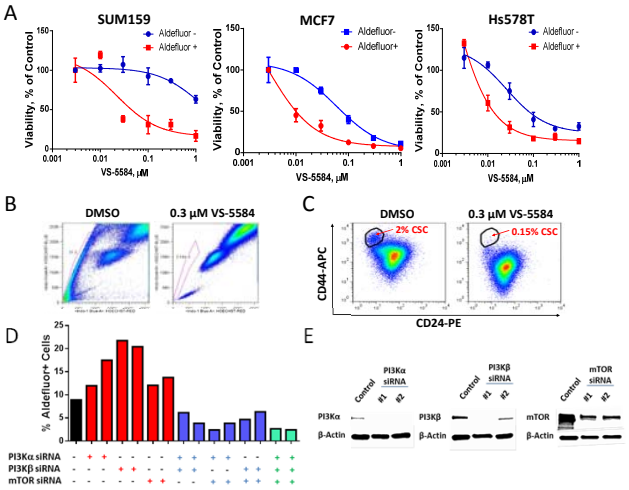
Biochemical Potencies (IC_{50}, nM)*					
mTOR	PI3Kα	PI3Kαmut H1047R	PI3Kβ	PI3Kγ	PI3Kδ
3.4	2.6	3.3	21	2.7	3

* Data on file Verastem

Selective for these kinases among a panel of over 400 kinases (Hart et al., Mol Cancer Ther. 2013 Feb;12(2):151-61.)

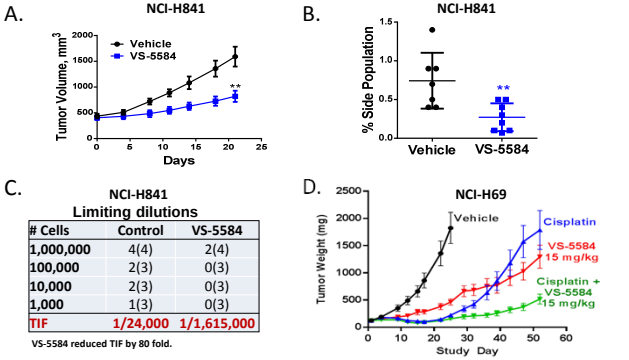
RESULTS

Fig 1: Combined inhibition of PI3K isoforms and mTOR kinase by VS-5584 preferentially targets CSCs *in vitro*.



SUM159, MCF7 and Hs578T cells were treated with VS-5584 for 2 days and Aldefluor+ and Aldefluor– cells counted (A). SUM159 cells were treated with VS-5584 for 2 days under hypoxia before Hoechst dye exclusion assay was carried out (B). Immortalized HMLER breast cancer cells were treated with VS-5584 and the percentage of $CD44^{high}/CD24^{low}$ CSCs was quantified by flow cytometry (C). Sum159 cells were transfected with siRNA against PI3K-α, PI3K-β and mTOR, individually or in combination, 2 different siRNA sequences were used for each gene, and the percentage of Aldefluor+ cells was measured (D). The knockdown was confirmed by western blot.

Fig 2: VS-5584 preferentially targets CSCs *in vivo* and delays tumor regrowth after chemotherapy in small cell lung cancer models.

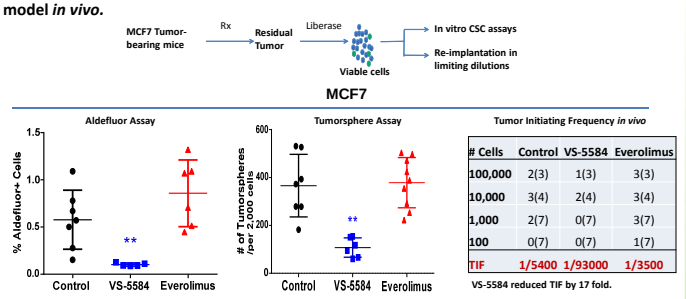


# Cells	Control	VS-5584
1,000,000	4(4)	2(4)
100,000	2(3)	0(3)
10,000	2(3)	0(3)
1,000	1(3)	0(3)
TIF	1/24,000	1/1,615,000

VS-5584 reduced TIF by 80 fold.

Mice bearing NCI-H841 SCLC tumors were treated with either vehicle or 20 mg/kg VS-5584 thrice weekly for 3 weeks. Tumor volume was measured (A). Cells were dissociated from tumors and subject to side population analysis by FACS (B).or implanted in secondary mice in limiting dilution assay (C). Mice bearing NCI-H69 xenografts were treated with vehicle, cisplatin (weekly dosing of 5 mg/kg cisplatin for 2 weeks), VS-5584 (oral gavage at 15 mg/kg on a QDx5 schedule for 8 weeks), alone or in combination (D).

Fig 3: VS-5584 reduces CSCs and tumor initiating frequency in an ER+ breast cancer xenograft model *in vivo*.

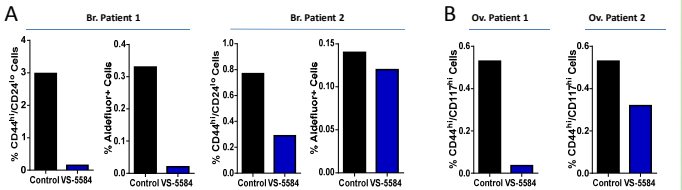


# Cells	Control	VS-5584	Everolimus
100,000	2(3)	1(3)	3(3)
10,000	3(4)	2(4)	3(4)
1,000	2(7)	0(7)	3(7)
100	0(7)	0(7)	1(7)
TIF	1/5400	1/93000	1/3500

VS-5584 reduced TIF by 17 fold.

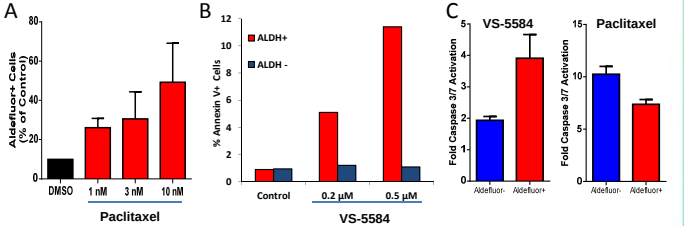
Mice bearing MCF7 tumors were treated with either vehicle, 20 mg/kg VS-5584 or 5 mg/kg everolimus QD for 10 days. Aldefluor assay results showed preferential reduction of CSCs by VS-5584 (p = 0.007) but not everolimus. Secondary tumor sphere assay demonstrated that VS-5584 but not everolimus suppressed self-renewing capability of CSCs. *In vivo* limiting dilution assay showed that VS-5584 caused a 17- fold reduction in tumor-initiating frequency (TIF) while everolimus had no significant effect. Tumor-initiating frequency (TIF) was calculated using the L-CalcTM Software. Similar results were obtained with MDA-MB-231 xenografts (Xu et al. AACR 2013).

Fig 4: VS-5584 reduces CSCs in primary human ovarian and breast tumors in *ex vivo culture*.



Breast tumor tissue from 2 patients was treated *ex vivo* with 100 nM VS-5584 for 5 days. Dissociated cells were subjected to CD44 and CD24 FACS analysis and Aldefluor assay by FACS (A). Ovarian tumor tissue from 2 different patients was treated *ex vivo* with 100 nM VS-5584 for 5 days and subject to CD44 and CD117 FACS analysis (B).

Fig 5: VS-5584 induces apoptosis preferentially in CSCs



SUM159 cells were treated with paclitaxel for 2 days followed by an Aldefluor assay (A). SUM159 cells were treated with VS-5584 or vehicle for 24h and followed by co-staining with Annexin V and Aldefluor reagents (B). SUM159 cells were first FACS-sorted into Aldefluor+ and Aldefluor- cells and treated with VS-5584 for 24h. Apoptosis induction was analyzed using Caspase 3/7 assay (C).

SUMMARY

- VS-5584 is a highly selective dual PI3K/mTOR inhibitor that potently inhibits mTORC1, mTORC2 and all Class I PI3K isoforms.
- VS-5584 preferentially inhibits cancer stem cells as assessed in orthogonal assays *in vitro*, *in vivo* and *ex vivo* using primary human cancer specimens. In contrast, everolimus – a mTORC1 inhibitor does not have an effect on CSCs and the cytotoxic agent paclitaxel enriches for CSCs.
- Consistent with its preferential targeting of CSCs, VS-5584 delays tumor regrowth after treatment with the cytotoxic agent cisplatin in a SCLC xenograft model.
- VS-5584 is currently being evaluated in a Phase 1 clinical trial in patients with solid tumors and lymphoma.

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