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FAK inhibitors VS-6063 (defactinib) and VS-4718 reduce cancer stem cells in models of triple negative breast cancer

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ABSTRACT

Cancer stem cells (CSCs) are an underlying cause of tumor progression and metastasis. In breast cancer, CSCs can be identified by Aldehyde Dehydrogenase 1 (ALDH) or CD44-high/CD24-low expression. Neoadjuvant chemotherapy has been shown to lead to an increase in CSCs in locally advanced breast cancer (Alamgeer et al., 2014, Br. Can. Res. R14). In addition, the presence of CSCs in residual axillary disease is associated with a significantly worse prognosis following neoadjuvant chemotherapy and surgery (Sakakibara et al. 2011, Cancer 3899, 2011). Currently, there are no approved therapies that effectively target and kill CSCs. VS-6063 and VS-4718 are orally bioavailable small molecules that kill cancer stem cells through the inhibition of Focal Adhesion Kinase (FAK). Both VS-6063 and VS-4718 have demonstrated preferential targeting of CSCs in preclinical models and are currently in clinical development.

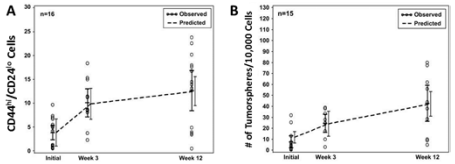
A multiplex assay for multiple CSC markers (ALDH1, CD44 and CD24) was developed and validated in biopsies of primary tumor and matched lymph node taken pre- and post-neoadjuvant chemotherapy. Consistent with previously reported data, here we report that VS-6063 and VS-4718 effectively kill CSCs in multiple models of breast cancer. In an *ex vivo* model, biopsies from human breast tumors were obtained and cultured as primary explants within 24 hours of surgery. The primary explants were incubated with VS-6063, VS-4718 or paclitaxel for 4 days. Treatment with either VS-6063 or VS-4718 decreased the proportion of CSCs in contrast to paclitaxel.

VS-6063 and VS-4718 diminished the self-renewal capacity of primary cultures from established TNBC patient-derived xenografts as measured by tumorsphere assays. In a MDA-MB-231 mouse xenograft model, *in vivo* treatment with VS-6063 decreased CSCs more that 6-fold in limiting dilutions assay. Similarly, using an imaging-based 4T1-luciferase TNBC orthotopic model, both VS-6063 and VS-4718 diminished the size of metastatic nodules within two weeks.

In summary, CSCs are readily detectable in primary breast cancers at surgery, and VS-6063 and VS-4718 diminish the CSC subpopulation *in vitro*, *ex vivo* and in xenograft models using a number of functional and biomarker assays. This critical subpopulation of CSCs is detectable in residual tumor following neoadjuvant therapy. CSC-targeted agents such as VS-6063 or VS-4718 should be clinically tested in the neoadjuvant setting to potentially delay time to relapse and improve patient outcome.

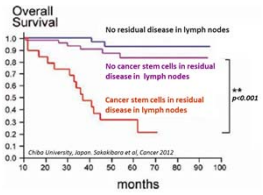
INTRODUCTION

Neoadjuvant chemotherapy in patients with TNBC increases the proportion of CSCs in primary breast tumors



Cancer stem cells in tumors quantified by (A) $CD44^{hi}/CD24^{lo}$ cells or (B) efficiency of tumorsphere formation. *(Li et al., JNCI 100:672, 2008)*

Presence of ALDH1+ CSCs in residual axillary disease after neoadjuvant chemotherapy & surgery correlates with poor overall survival for patients with breast cancer



(Sakakibara et al., Cancer, 2011)

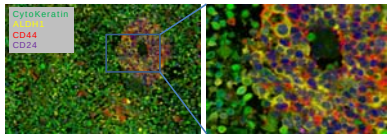
n = 115 patients

Standard neoadjuvant chemotherapy of 4 cycles of anthracycline & cyclophosphamide + 12 weeks of paclitaxel

CSCs identified by ALDH1+

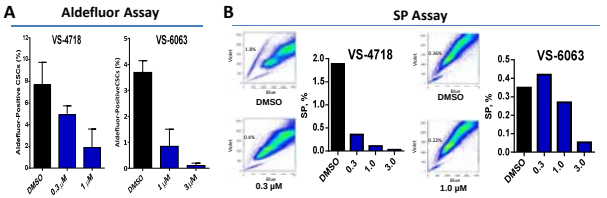
RESULTS

Fig 1: Identification of CSCs in primary TNBC tumors.



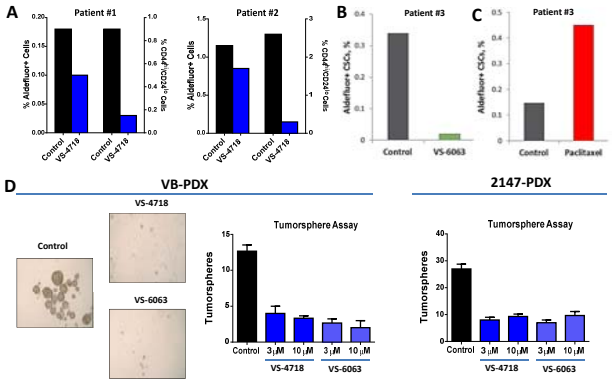
CSCs in breast cancer have been identified as $ALDH1^+$ or $CD44^+/CD24^-$ subpopulations. Sections from primary TNBC tumors were analyzed for expression of CytoKeratin, ALDH1A1, CD44 and CD24 by Opal (Perkin Elmer) multiplex assay.

Fig 2: FAK inhibitors VS-6063 and VS-4718 preferentially reduce CSCs in multiple orthogonal assays *in vitro*



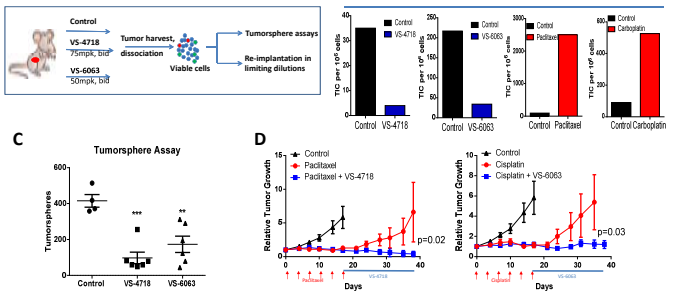
VS-4718 and VS-6063 reduced the proportion of CSCs *in vitro* as assessed by (A) an Aldefluor ALDH enzymatic assay in SUM-159 cell line and (B) Hoechst dye exclusion side population analysis in SUM159 cells.

Fig 3: *Ex vivo* effects of VS-4718, VS-6063 and paclitaxel on proportion of CSCs in primary breast tumor specimens



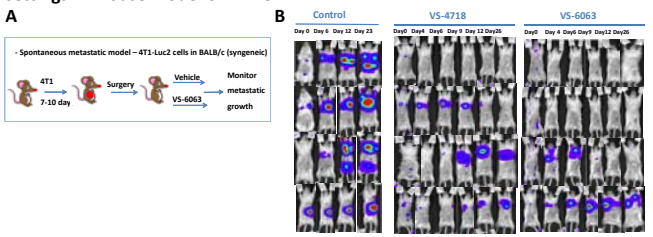
Breast cancer explants were treated with VS-4718 (1 µM) (A), VS-6063 (1 µM) (B), paclitaxel (10 nM) (C) or control *ex vivo* for 4 days. Tumor fragments were dissociated and cells percentage of Aldefluor+ and $CD44^{hi}/CD24^{lo}$ cells was determined by flow cytometry. Both VS-4718 and VS-6063 diminished while paclitaxel enriched for CSCs. (D)Cells from 2 different TNBC PDX models were treated with VS-4718 or VS-6063 in matrigel and live cells were plated in tumorsphere forming assay. FAK inhibitors inhibited formation of tumorspheres in both PDX models.

Fig 4: VS-4718 and VS-6063 target CSCs in TNBC xenograft tumors *in vivo* in contrast to paclitaxel and prevent tumor regrowth.



Mice bearing human MDA-MB-231 TNBC xenograft tumors were treated with 75 mg/kg VS-4718, 50 mg/kg VS-6063, po BID or vehicle control for 25 days and various CSC endpoints were assessed (A). Dissociated tumor cells from VS-4718- and VS-6063-treated animals had decreased tumor-initiating capability following implantation in immunodeficient mice in limiting dilutions (B). In contrast, paclitaxel treatment increased tumor-initiating capability of residual tumor cells under similar conditions. Both FAK inhibitors decreased tumorsphere-forming capability *in vitro* (C). Mice bearing CAL-51 TNBC xenograft tumors were treated with paclitaxel 10 mg/kg or cisplatin 5 mg/kg q3d. Mice were randomized in 2 groups and treatment continued on vehicle or either VS-4718 or VS-6063 50 mg/kg po bid. Both FAK inhibitors prevented tumor re-growth after cessation of chemotherapy (D).

Fig 5: VS-6063 and VS-4718 attenuate development of metastasis in adjuvant settings in mouse model of TNBC.



4T1-Luc2 cells were implanted in mammary fat pads of Balb/c mice. Primary tumor were removed surgically. Mice were randomized and treated with VS-4718 and VS-6063 50 mg/kg po BID (A). Metastasis development was monitored by bioluminescence (B). Representative images are shown. All 8 animals from Control group developed progressive metastasis. Both the VS-4718 and VS-6063 groups included animals that showed regression of metastasis or no metastatic outgrowth.

SUMMARY

- **Neoadjuvant chemotherapy has been shown to enrich CSCs, and presence of CSCs in lymph nodes has been correlated with poor overall survival in patients with breast cancer**
- **VS-4718 and VS-6063 are potent and selective inhibitors of the FAK family kinases FAK & PYK2**
- **VS-4718 and VS-6063 preferentially eliminate CSCs as assessed with multiple orthogonal assays in preclinical TNBC models both *in vitro, ex vivo* and *in vivo***
- **We have developed a multiplex assay to measure $ALDH^+$, $CD44^+/CD24^-$ CSCs in clinical specimens**
- **These preclinical data provide rationale for the clinical development of VS-6063 in combination with chemotherapy in neoadjuvant TNBC**

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