PI3K/mTOR Dual Inhibitor VS-5584 Preferentially Targets Cancer Stem Cells

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Abstract

Cancer stem cells (CSC) have been implicated in disease recurrence, metastasis, and therapeutic resistance, but effective targeting strategies for these cells are still wanting. VS-5584 is a potent and selective dual inhibitor of mTORC1/2 and class I PI 3-kinases. Here, we report that VS-5584 is up to 30-fold more potent in inhibiting the proliferation and survival of CSC compared with non-CSC in solid tumor cell populations. VS-5584 preferentially diminished CSC levels in multiple mouse xenograft models of human cancer, as evidenced by marked reduction of tumor-initiating capacity in limiting dilution assays. Likewise, VS-5584 treatment ex vivo preferentially reduced CSC in surgically resected breast and ovarian patient tumors. In contrast, chemotherapeutics such as paclitaxel and cisplatin were less effective in targeting CSC than bulk tumor cells. Mechanistic investigations revealed that preferential targeting of CSC required inhibition of multiple components of the PI3K–mTOR pathway: coordinate RNAi-mediated silencing of PI3Kα, PI3Kβ, and mTOR phenocopied the effect of VS-5584, exhibiting the strongest preferential targeting of CSC, while silencing of individual PI3K isoforms or mTOR failed to replicate the effect of VS-5584. Consistent with CSC ablation, VS-5584 delayed tumor regrowth following chemotherapy in xenograft models of small-cell lung cancer. Taken together, the preferential targeting of CSC prompts a new paradigm for clinical testing of VS-5584: clinical trials designed with CSC-directed endpoints may facilitate demonstration of the therapeutic benefit of VS-5584. We suggest that combining VS-5584 with classic chemotherapy that debulks tumors may engender a more effective strategy to achieve durable remissions in patients with cancer. Cancer Res; 75(2); 1–10. ©2014 AACR.

Introduction

Chemoresistance and cancer relapse represent significant challenges in cancer management, and are attributable in part to the presence of subpopulations of cancer cells termed cancer stem cells (CSC) or tumor-initiating cells. CSC were first identified in acute myeloid leukemia (1, 2) and are operationally defined by their self-renewal and tumor initiation capacities. The presence of CSC in solid tumors was first described in breast cancer (3) and has since been identified in a wide variety of solid tumors, and implicated in resistance to anticancer therapies, cancer recurrence, and metastasis. (4–7).

The phosphoinositide 3-kinase–mammalian target of rapamycin (PI3K–mTOR) pathway is one of the most frequently activated signaling pathways in cancer, playing a central role in tumorigenesis by regulating the proliferation, survival, differentiation, and migration of cancer cells as well as tumor angiogenesis (8). AKT and mTOR are the major effector kinases in the PI3K–mTOR pathway. mTOR exists in two distinct protein complexes, mTORC1 and mTORC2, each with different downstream substrates (9). The PI3K–mTOR pathway also plays a role in CSC (8, 10–14). Activation of the PI3K–mTOR pathway in adult blood cells through PTEN deletion led to the generation of leukemia-initiating cells (11). Similarly, activation of PI3K–mTOR signaling achieved by knocking down PTEN, enriched breast CSC (13).

VS-5584 is a potent and selective dual PI3K–mTOR inhibitor, exhibiting approximately equal low nanomolar potency against mTOR kinase and all four class I isoforms of PI3K. VS-5584 was shown to target the mTORC1 and mTORC2 complexes as well as PI3K in cells, as evidenced by inhibition of phosphorylation of cellular targets of these kinases, including AKT (Ser473 and Thr308) and ribosomal protein S6 (Ser240/244; ref. 15). VS-5584 was shown to target the PI3Kα–mTOR pathway: coordinate RNAi-mediated silencing of PI3Kα, PI3Kβ, and mTOR phenocopied the effect of VS-5584, exhibiting the strongest preferential targeting of CSC, while silencing of individual PI3K isoforms or mTOR failed to replicate the effect of VS-5584. Consistent with CSC ablation, VS-5584 delayed tumor regrowth following chemotherapy in xenograft models of small-cell lung cancer. Taken together, the preferential targeting of CSC prompts a new paradigm for clinical testing of VS-5584: clinical trials designed with CSC-directed endpoints may facilitate demonstration of the therapeutic benefit of VS-5584. We suggest that combining VS-5584 with classic chemotherapy that debulks tumors may engender a more effective strategy to achieve durable remissions in patients with cancer. Cancer Res; 75(2); 1–10. ©2014 AACR.
Materials and Methods

Cell lines, primary human tumor tissues, compounds, and reagents—All cancer cell lines used were obtained from the ATCC with the exception of SUM159, which was acquired from Asterand. Cell lines were authenticated by STR (short tandem repeat) analysis at either ATCC or IDEXX Radil. V12H-Ras–transformed human mammary epithelial cells (HMLE) were obtained from the Broad Institute and maintained as described previously (17). Primary human breast and ovarian tumor specimens were obtained from Tissue Solutions Ltd. after patients’ consent and institutional IRB approval. VS-5584 was synthesized by Selleckchem. Other compounds were purchased from Selleckchem. Reverse-Phase Protein Microarray (RPMA) was conducted at Theranostics Health Inc.

Aldefluor assays—An imaging-based Aldefluor assay was conducted using the Aldefluor Assay Kit (STEMCELL Technologies) with the following modifications: cells were plated in collagen-treated plates. Following compound treatment, Aldefluor reagents supplemented with 10 ng/mL Hoechst 33342 dye were added. Plates were incubated at 37°C for 20 minutes. After washing, Aldefluor+ and total cells (in buffer containing Hoechst 33342) were quantified using Celigo (Nexcelom Bioscience). ALDH inhibitor diethylamino benzaldehyde (DEAB)–treated cells were used as a negative control. FACS-based Aldefluor assay was conducted following the manufacturer’s instructions (STEMCELL Technologies).

Side Population assays—Hoechst 33342 exclusion (Side Population) assay was carried out as previously described (18).

HMLER assay—HMLER cells were treated with compounds for 4 days. Following a 4-day compound wash-off and cell recovery period, cells were stained with PE-labeled anti-CD24, APC-labeled anti-CD44 antibody (BD Biosciences), and 7-AAD+/− live cells were subject to FACS analysis. APC-labeled IgG 2,​κ and PE-labeled IgG2,​κ (BD Biosciences) were used as isotype controls for CD44 and CD24, respectively.

Tumorsphere assay—To determine tumorsphere forming efficiency, cells from tissue culture or dissociated tumors were plated in tumorsphere forming medium as previously described (17). Spheres were enumerated using Celigo.

Apoptosis assays and siRNA transfection—Standard methods were used for Annexin V and caspase-3/7 assays and siRNA transfection with details in Supplementary Materials and Methods.

Results—Preferential targeting of CSC by VS-5584 in vitro—In a previous screening of more than 300,000 compounds using an HMLE CSC assay (17), a number of PI3K–mTOR pathway inhibitors were found to preferentially target CSC. This historic observation prompted our initial interest in exploring the PI3K–mTOR pathway to target CSC. We therefore determined the effect of VS-5584, a highly potent and selective dual inhibitor of PI3K–mTOR (15), on CSC in multiple orthogonal CSC assays that have been validated by limiting dilution implantation in mice.

CSC have high aldehyde dehydrogenase (ALDH) activity, which can be measured by the Aldefluor enzymatic assay (20). Aldefluor+ cells from several breast cancer cell lines have been shown to display CSC characteristics (21). We also show that Aldefluor+ SUM159 triple-negative breast cancer cells display enhanced self-renewal and tumor-initiating capability relative to Aldefluor− cells in immunodeficient mice (Supplementary Fig. S1). To quantify CSC that harbor high ALDH activity, we developed an imaging-based Aldefluor assay. As shown in Fig. 1A, although VS-5584 dose-dependently reduced both Aldefluor+ and Aldefluor− cell populations, it was far more potent against the Aldefluor+ CSC within the SUM159 cell line. A similar preferential effect of VS-5584 on Aldefluor+ CSC was observed using the estrogen receptor–positive (ER+) MCF7 and carcinosarcoma HS578T cell lines (Fig. 1A), indicating that VS-5584 exerts an approximately 10- to 30-fold more potent inhibitory effect on CSC than bulk tumor cells across these cell lines. In contrast, exposure to the cytotoxic agents paclitaxel or cisplatin increased the percentage of Aldefluor+ cells (Fig. 1B), consistent with CSC being resistant to conventional chemotherapy. Importantly, VS-5584 attenuated paclitaxel-induced enrichment of Aldefluor+ cells (Fig. 1C).

A tumorsphere assay can be used to measure the self-renewal of CSC (22). SUM159 and MCF-7 tumorspheres were treated with VS-5584, paclitaxel, or combination of both agents, and the effect of these treatments on CSC was determined in a secondary tumorsphere assay. Tumorsphere forming efficiency was decreased by VS-5584 but increased by paclitaxel. Strikingly,
Figure 1.
VS-5584 preferentially targets CSC in vitro. A, VS-5584 markedly reduced the viability of Aldefluor\(^+\) cells but had weaker effect against Aldefluor\(^-\) cells. SUM159, MCF7, and Hs578T breast cancer cells were treated with VS-5584 for 2 days and an imaging-based Aldefluor assay was carried out. B, paclitaxel and cisplatin enriched for Aldefluor\(^+\) CSC. SUM159 cells were treated with paclitaxel or cisplatin for 2 days followed by an Aldefluor assay. C, VS-5584 attenuated paclitaxel-induced Aldefluor\(^+\) cells. (Continued on the following page.)
combination of VS-5584 and paclitaxel almost completely abolished tumorsphere formation, indicating a marked reduction of CSC (Fig. 1D).

Enhanced drug efflux is another attribute of CSC. Thus CSC, when assayed by their ability to exclude the Hoechst 33342 dye, are found to reside in a side population (SP) with greater dye exclusion capacity (18). As with the Aldefluor assay, we first validated that SP of SUM159 cells indeed exhibited elevated tumor-initiating capability relative to non-SP (Supplementary Fig. S2). To determine the effect of VS-5584 on SP CSC, SUM159 cells were cultured under hypoxic conditions (1% O2) to enrich for CSC (23). VS-5584 markedly reduced the proportion of SP cells with less than 1% SP remaining following treatment with 100 nmol/L VS-5584 and no detectable SP CSC following 1 mmol/L VS-5584 treatment. Similarly, VS-5584 also reduced the proportion of SP cells in MCF-7 cells (Fig. 1E).

The HMLER cell line model, the CD44hi/CD24lo subpopulation has been demonstrated to possess characteristics of CSC (17). A distinct CD44hi/CD24lo population was most evident in the HMLER cells that survived paclitaxel treatment, and this population was preferentially targeted by VS-5584 (Fig. 2).

(Continued.) SUM159 cells were treated with 100 nmol/L VS-5584 and 10 nmol/L paclitaxel alone or in combination and analyzed as in B, D, VS-5584 alone or in combination with paclitaxel decreased tumorsphere forming efficiency. SUM159 and MCF-7 tumorspheres were treated with 100 nmol/L VS-5584, 10 nmol/L paclitaxel alone or in combination. Results of secondary tumorsphere assay are shown. E, preferential targeting of SP CSC by VS-5584. SUM159 and MCF-7 cells were cultured under hypoxic conditions (1% O2) to enrich for SP CSC (23). VS-5584 markedly reduced the proportion of SP cells with less than 1% SP remaining following treatment with 100 nmol/L VS-5584 and no detectable SP CSC following 1 mmol/L VS-5584 treatment. Similarly, VS-5584 also reduced the proportion of SP cells in MCF-7 cells (Fig. 1E).

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helped to set the gate for FACS analysis across all samples. Whereas paclitaxel treatment significantly increased the percentage of CD44hi/CD24lo cells compared to the control sample, VS-5584 abolished the CD44hi/CD24lo cells with greater than 10-fold reduction (Fig. 1F). Taken together, these data demonstrate that VS-5584 has a strong preferential inhibitory effect on CSC in direct contrast with the cytotoxic agents paclitaxel and cisplatin, suggesting that CSC have an increased dependency on PI3K–mTOR signaling compared with non-CSC.

Preferential induction of apoptosis by VS-5584 in CSC

Because VS-5584 dramatically reduced the proportion of CSC-containing population, we surmised that VS-5584 might induce stronger apoptosis in CSC. To test this hypothesis, SUM159 cells were treated with VS-5584 or DMSO control for 24 hours and the percentage of Annexin V–positive apoptotic cells was determined for both Aldefluor+ and Aldefluor− subpopulations. VS-5584 (500 nmol/L) induced apoptosis in 8% of Aldefluor+ cells compared with 2% of Aldefluor− cells (Fig. 2A), indicating that VS-5584 selectively induced apoptosis in CSC. In addition, SUM159 cells were sorted for Aldefluor+ and Aldefluor−/C0 subpopulations. VS-5584 induced apoptosis in 8% of Aldefluor+ cells compared with 2% of Aldefluor−/C0 cells (Fig. 2A), indicating that VS-5584 preferentially induces apoptosis in CSC.

Preferential targeting of CSC by VS-5584 in the MDA-MB-231 triple-negative breast cancer model in vivo

We subsequently determined whether similar preferential targeting of CSC occurs in vivo. We first used MDA-MB-231 triple-negative human breast cancer cells implanted orthotopically in

Figure 3.
VS-5584 preferentially abolishes CSC in two human breast tumor xenograft models in vivo. A, experimental design. Rx, denotes treatment with either compound or vehicle. B–D, mice bearing MDA-MB-231 tumors were treated with either vehicle or 25 mg/kg VS-5584 once a day for 7 days. Cells were dissociated from tumors and subject to CSC assays shown in A. B, Aldefluor assay data showing that VS-5584 preferentially reduced Aldefluor+ CSC in MDA-MB-231 tumor xenografts. Shown is a scatter plot of percent Aldefluor+ cells. *, P = 0.015, unpaired t test. C, limiting dilution assay showing that VS-5584 reduced tumor-initiating capability of CSC in vivo. Cells dissociated from MDA-MB-231 tumors were re-implanted in limiting dilutions into the mammary fat pad of SHN immunodeficient mice. The number of mice that grew tumors at week 8 out of total of 4 mice per group (in parenthesis) is tabulated. TIF was calculated using the ELDA software. D–G, mice bearing MCF7 tumors were dosed orally with vehicle (control), 20 mg/kg VS-5584, or 5 mg/kg everolimus daily for 10 days. Cells were dissociated from tumors and subject to a panel of CSC assays as shown in A. D, Aldefluor assay results showing preferential reduction of CSC by VS-5584 (*, P = 0.007) but not everolimus. E, secondary tumor sphere assay showing that VS-5584 but not everolimus significantly suppressed self-renewing capability of CSC (**, P = 0.001). F, in vivo limiting dilution assay showing that VS-5584 reduced, whereas everolimus increased TIF. G, results of RPMA showing that both VS-5584 and everolimus reduced pS6 (Ser240/244) levels in MCF-7 tumors.
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Figure 4.

Inhibition of PI3Kα, PI3Kβ, and mTOR is required for strong preferential targeting of CSC. A, SUM159 cells were transfected with siRNA against PI3Kα, PI3Kβ, and mTOR individually or in combination. Aldefluor+ cells as a percentage of control, averaged from two independent experiments, is shown (Y axis). B, confirmation of siRNA knockdown of PI3K isomers and mTOR. Lysates from cells transfected with various siRNA were subjected to Western blot analysis. β-actin served as a loading control.

the mouse mammary fat pad. After tumors reached a volume of approximately 200 mm³, VS-5584 was administered orally at 25 mg/kg once daily for 9 days. Tumors were harvested and dissociated into single cells and subjected to CSC assays without further compound treatment (Fig. 3A). Results of the Aldefluor assay showed that the percentage of Aldefluor+ cells was relatively low with an average of 0.7% in control tumors, VS-5584 treatment caused significant reduction of the proportion of Aldefluor+ CSC to 0.2% (P = 0.015, Fig. 3B, Supplementary Fig. S5). A more rigorous and functional test for CSC is the limiting dilution assay. Accordingly, cells dissociated from either VS-5584- or vehicle-treated tumors were injected into immunodeficient mice in limiting dilutions. Cells from VS-5584-treated tumors displayed a 7-fold reduction of TIF, confirming reduction of CSC in tumors by VS-5584 treatment (Fig. 3C).

Differential effects of VS-5584 and everolimus on CSC in the ER+ MCF7 breast cancer model in vivo

Similar to our observations using the MDA-MB-231 model, VS-5584 treatment also reduced the proportion of CSC in the MCF7 ER+ breast tumor xenograft model, as evidenced by a significant decrease in the percentage of Aldefluor+ cells, tumorsphere forming efficiency, and TIF in a limiting dilution assay (Fig. 3D–F).

Because VS-5584 inhibits multiple nodes of the PI3K–mTOR signaling pathway, we reasoned that inhibition of CSC by VS-5584 might involve effects on more than one of these pathway components. To test this hypothesis, we investigated effects of the potent mTORC1 inhibitor everolimus on CSC in the same MCF7 xenograft model. In contrast with VS-5584, oral daily dosing of everolimus at 5 mg/kg, shown to inhibit mTORC1 activity in tumors as evidenced by reduction of S6 phosphorylation (Fig. 3G), did not reduce the proportion of CSC, as measured by multiple assays (Fig. 3D–F). Collectively, these data suggest that inhibition of components of the PI3K–mTOR pathway other than mTORC1 might be important for the preferential reduction of CSC observed with VS-5584.

The role of PI3K isoforms and mTOR in the preferential targeting of CSC by VS-5584

To further assess the contribution of individual PI3K isoforms and mTOR in mediating the CSC effect of VS-5584, SUM159 cells were transfected with siRNA specific for PI3Kα, PI3Kβ, or mTOR individually or in combination. Two different siRNA sequences were used per gene. An Aldefluor assay was carried out 3 days after siRNA transfection. PI3Kβ and PI3Kγ were not examined because they are mostly expressed in leukocytes and their role in epithelial cells is not established (24). Single isoform knockdown of PI3Kα, PI3Kβ or mTOR resulted in a moderate increase in the percentage of Aldefluor+ cells compared with scrambled control siRNA. Importantly, double knockdown of PI3Kα and PI3Kβ, PI3Kα and mTOR, or PI3Kβ and mTOR decreased the percentage of Aldefluor+ CSC to some extent, whereas triple knockdown of PI3Kα, PI3Kβ, and mTOR, which mirrors the kinase inhibition profile of VS-5584, showed the most striking 70% reduction of the proportion of Aldefluor+ cells (Fig. 4A). Effective target knockdown by all siRNAs was confirmed by Western blot analysis (Fig. 4B). These data further support the notion that inhibition of multiple components of the PI3K–mTOR pathway by VS-5584 is critical for the observed preferential targeting of CSC.

Preferential targeting of CSC by VS-5584 in primary human tumor tissue samples

Having demonstrated that VS-5584 preferentially reduced CSC in both cell line models and in vivo tumor xenografts, we determined if ex vivo treatment with VS-5584 preferentially targets CSC in surgically removed primary human tumor specimens. Ex vivo treatment of breast tumor tissue fragments with VS-5584 for 5 days reduced the proportion of both CD44hi/CD24lo (Fig. 5A) and Aldefluor+ (Fig. 5B) CSC. The modest effect on the tumor of patient 3 (Fig. 5A and B) may be explained by a sample quality issue, as there were significantly fewer live cells available for FACS analysis compared with tumors of patients 1 and 2 (Fig. 5A). We reasoned that other carcinomas, such as ovarian cancer, might depend on similar CSC signaling pathways. Accordingly, ex vivo treatment with VS-5584 was undertaken using ovarian tumor tissue, surgically removed from 2 patients who had received prior taxane- and platinum-based chemotherapy. Ex vivo treatment with 100 nmol/L VS-5584 for 5 days reduced the proportion of CD44hi/CD117+ cells, previously validated as a CSC marker in ovarian cancer (25), in both ovarian cancer patient tumor specimens (Fig. 5C). To further evaluate the effect of VS-5584 on tumor-initiating cells, 10,000 cells dissociated from the above control- or VS-5584–treated ovarian tumor specimens were implanted subcutaneously into immunodeficient mice. After 17 weeks, mice injected with cells from control-treated ovarian tumor specimens 1 and 2 developed tumors, whereas no tumors formed in mice injected with cells from VS-5584–treated tumor specimens (Fig. 5D). Thus, the results from ex vivo treatment of primary patient tumor specimens are in agreement with our findings with cell lines and xenograft tumors, and indicate that VS-5584 preferentially reduces CSC populations.

Effects of VS-5584 on tumor regrowth after chemotherapy

CSC have been postulated to account for tumor recurrence after chemotherapy (4). We therefore hypothesized that, by
preferentially eliminating CSC, VS-5584 might delay tumor regrowth after initial treatment with chemotherapy. Small-cell lung cancer (SCLC) represents a logical setting to test this hypothesis as most patients with SCLC initially respond to chemotherapy but subsequently experience tumor recurrence (26). The effect of VS-5584 on tumor-initiating CSC of SCLC was, therefore, assessed in the NCI-H841 SCLC xenograft model.

VS-5584 treatment caused significant tumor growth inhibition ($P = 0.003$, Fig. 6A) and reduced the proportion of CSC in NCI-H841 tumors as evidenced by a significant decrease in the percentage of SP cells ($P = 0.006$; Fig. 6B). Furthermore, cells dissociated from NCI-H841 tumors of VS-5584-treated mice showed a striking 67-fold reduction in tumor-initiating frequency when injected in limiting dilutions into immunodeficient mice, indicating a marked depletion of CSC by VS-5584 in xenograft tumors (Fig. 6C).

Subsequently, we sought to determine if VS-5584 can delay tumor regrowth after initial debulking by cisplatin, a standard-of-care first-line chemotherapeutic agent, in human SCLC xenograft models. Before in vivo experiments, we confirmed that cisplatin or etoposide was not effective in depleting CSC of SCLC cell lines; in fact, both agents enriched CSC, whereas VS-5584 depleted CSC in vitro (Supplementary Fig. S3). In the NCI-H69 SCLC xenograft study, weekly i.p. dosing of 5 mg/kg cisplatin for 2 weeks induced initial tumor regression, but these tumors regrew quickly after cessation of cisplatin treatment. Single agent VS-5584 at 15 mg/kg dosed orally once daily caused significant inhibition of NCI-H69 tumor growth ($P < 0.05$). In combination, VS-5584 substantially delayed the regrowth of NCI-H69 tumors following cessation of cisplatin treatment (Fig. 6D). Patient-derived-xenograft (PDX) models, which preserve the histology and heterogeneity of patient tumors, may be more relevant models for CSC research (27, 28). We, therefore, tested VS-5584 in a SCLC PDX model established from a lymph node metastasis of a patient with SCLC. In keeping with our observations with the NCI-H69 model, VS-5584 administered following cessation of cisplatin dosing delayed tumor regrowth (Fig. 6E). These results are consistent with the suppression of cisplatin-resistant CSC by VS-5584, resulting in a longer delay of tumor regrowth after cessation of cisplatin treatment.

**Discussion**

Intensive efforts have been devoted to targeting the PI3K–mTOR pathway due to its critical importance in the proliferation and survival of cancer cells. Here, we report that, in addition to exhibiting broad and robust antitumor activity in animal models (15), VS-5584, a potent dual PI3K–mTOR kinase inhibitor, also exerts a preferential effect on CSC present in cell culture, in human tumor xenografts, and in surgically excised human tumor specimens. In stark contrast, the cytotoxic chemotherapeutic agent paclitaxel, cisplatin, and etoposide increased the proportion of CSC. Our observation that VS-5584 inhibited tumor regrowth after effective treatment with cisplatin in SCLC models, including a patient-derived primary tumor model, is consistent with the notion that VS-5584 may delay tumor recurrence at least in part through the suppression of CSC. To our knowledge, this is the first
comprehensive evaluation of the effect of a highly selective PI3K–mTOR inhibitor on CSC.

The PI3K–mTOR pathway has been shown to be important for the proliferation of CSC in both solid tumors and leukemias (10–14). In prostate cancer, a CD133+/CD44+ subpopulation with progenitor/stem cell characteristics was shown to exhibit higher PI3K–mTOR pathway activity, and treatment with the PI3K–mTOR inhibitor BEZ-235 suppressed the proliferation of such prostate CSC (10). PF-04691502, another dual PI3K–mTOR inhibitor, was recently shown to inhibit the proliferation of CSC in vitro and to inhibit tumor growth in a colon CSC-derived xenograft model (29). However, in these various studies, a critical unanswered question remained whether PI3K–mTOR inhibitors exert a general antiproliferative effect against both CSC and non-CSC, or whether the CSC are more dependent on the PI3K–mTOR pathway than are non-CSC. Interestingly, Korkaya and colleagues (13) reported that the AKT inhibitor perifosine reduced the proportion of ALDH+ CSC as well as tumor-initiating cells in breast tumor models, suggesting that perifosine has preferential effects on breast CSC. In light of the fact that perifosine was subsequently reported to also inhibit EGFR and c-Met phosphorylation (30), the CSC effect of perifosine could be due to inhibition of these other molecular targets in addition to AKT.

VS-5584 is highly selective for class I PI3K isoforms and mTOR kinase with no significant activity against more than 400 other protein and lipid kinases profiled (15). We provide clear evidence that inhibition of mTORC1, mTORC2, and PI3K isoforms by VS-5584 confers a strong preferential inhibitory effect on CSC across different carcinoma types, including breast, ovarian, and SCLC. Whereas VS-5584 preferentially abolished CSC in the MCF7 breast tumor model, an mTORC1-selective inhibitor everolimus did not reduce the proportion of CSC. Rapamycin, a closely related mTORC1 inhibitor, also did not affect the self-renewal of mammary stem cells (13). These data, therefore, suggest that

![Figure 6.](image-url)

VS-5584 targets SCLC CSC and delays tumor regrowth after chemotherapy in SCLC models. A–C, mice bearing NCI-H841 SCLC tumors were treated with either vehicle or 20 mg/kg VS-5584 three times weekly for 3 weeks. Cells were dissociated from tumors and subject to CSC assays. A, tumor volume plot showing that VS-5584 caused significant tumor growth inhibition (P = 0.003). B, SP analysis showing that VS-5584 significantly reduced the proportion of SP cells in tumors (P = 0.006). C, in vivo limiting dilution assay showing that VS-5584 caused a 67-fold reduction in TIF (P = 5 × 10^-6). D and E, tumor weight plots showing that VS-5584 delayed tumor regrowth following weekly dosing of 5 mg/kg cisplatin for 2 weeks. VS-5584 was dosed orally at either 15 mg/kg on a once a day schedule for 8 weeks starting on day 1 as a single-agent group (red) and in combination with cisplatin (green; D) or at 25 mg/kg on a three times weekly schedule for 4 weeks starting on day 9 (E).
inhibiting PI3K isoforms, mTORC1, and mTORC2 simultaneously may be important to exert a strong preferential effect on CSC. This hypothesis was further substantiated by our result that triple knockdown of PI3Kα, PI3Kβ, and mTOR by siRNA showed the strongest preferential reduction of CSC, whereas knockdown of PI3Kα, PI3Kβ, or mTOR individually did not exert a preferential effect on CSC (Fig. 4).

Multiple, distinct subpopulations of leukemic stem cells have previously been identified (4). In solid tumors, CSC have been identified using a variety of markers and functional attributes even within the same type of cancer, e.g., breast or ovarian cancer. While these markers may identify overlapping states of CSC, a more plausible explanation is that CSC are also heterogeneous in solid tumors. An ideal CSC-targeting agent should optimally reduce all CSC pools. The PI3K/mTOR dual inhibitor VS-5584 appears to be such an agent, as we have demonstrated that VS-5584 reduced CSC based on multiple independent measurements, including Aldefluor, Hoechst-dye exclusion, and CSC surface markers (CD44hi/CD24lo for breast cancer and CD44hi/CD117lo for ovarian cancer). Furthermore, VS-5584 also preferentially reduced the proportion of cells with self-renewal potential as measured by tumorsphere assays and by tumor-initiating capability following limiting dilution re-implantation of cells into secondary immunodeficient mice.

Our findings have implications for the clinical development of VS-5584. The observation that CSC exhibited 10- to 30-fold greater sensitivity to VS-5584 than non-CSC (Fig. 1) suggests that clinical trials designed with CSC-directed endpoints may facilitate demonstration of efficacy at sub-MTD doses. One such clinical trial concept is to test VS-5584 in a maintenance setting following cessation of front-line chemotherapy with survival endpoints, rather than conventional tumor shrinkage endpoints, which primarily assess targeting of the bulk tumor burden. Current first-line chemotherapy generally consists of cytotoxic agents, such as taxanes (e.g., paclitaxel) and platinum agents (e.g., cisplatin). While these agents may effectively debulk tumors and control disease initially, tumors invariably recur due to ineffective control of CSC. A CSC-targeting agent is expected to block CSC-mediated tumor recurrence. Our results generated using two SCLC xenograft models (Fig. 6) provide proof-of-concept that inhibition of PI3K and mTOR kinase activities by VS-5584 after debulking tumors with a cytotoxic agent may substantially extend antitumor response and delay tumor regrowth. Our findings thus provide strong rationale for the clinical development of VS-5584, currently in a phase 1 clinical trial (NCT01991938), for the treatment of cancer with the goal of achieving more durable responses through the preferential targeting of CSC.

Disclosure of Potential Conflicts of Interest
C.M. Vidal, J.E. Ring, J.M. Shapiro, D.T. Weaver, M.V. Padval, J.A. Pachter, and Q. Xu have ownership interest (including patents) in Verastem, Inc. No potential conflicts of interest were disclosed by the other authors.

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Acknowledgments
The authors thank Dr. Robert Weinberg for critical reading of the article and Verastem team for valuable advice throughout this work. The authors also thank David Dombkowski at MGH for assistance with FACS analysis of SP and CD44hi/CD24lo cells.

The NCI-H69 xenograft study was conducted by TGen (Scottsdale, AZ). Animal husbandry and in vivo procedures for Figs 3, 5D, 6, and Supplementary Figs. S1 and S2 were conducted with outstanding support of Sara Little at Vivisource Laboratories (Waltham, MA).

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Received April 23, 2014; revised October 23, 2014; accepted November 10, 2014; published OnlineFirst November 28, 2014.

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Cancer Res  Published OnlineFirst November 28, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-1223

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