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Irina M. Shapiro *et al.*

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Editor's Summary

Therapeutic Magic Without Merlin

Mesothelioma is an aggressive cancer of the pleura (lung lining), which is very difficult to treat and often recurs after therapy. Now, Shapiro and coauthors have discovered that mesotheliomas and other tumors that are low in a tumor suppressor called Merlin are particularly sensitive to treatment with a new drug called VS-4718, which blocks the activity of the enzyme focal adhesion kinase. Moreover, in preclinical testing, VS-4718 was particularly effective at killing cancer stem cells, which are the hardest to eradicate with conventional chemotherapy and can give rise to recurrent tumors. These results suggest that VS-4718 or a similar drug may make a valuable addition to the standard treatment regimen for mesothelioma and may reduce the risk of relapse in this cancer.

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CANCER

Merlin Deficiency Predicts FAK Inhibitor Sensitivity: A Synthetic Lethal Relationship

Irina M. Shapiro,[1] Vihren N. Kolev,[1] Christian M. Vidal,[1] Yuwaraj Kadariya,[2] Jennifer E. Ring,[1] Quentin Wright,[1] David T. Weaver,[1] Craig Menges,[2] Mahesh Padval,[1] Andrea I. McClatchey,[3] Qunli Xu,[1] Joseph R. Testa,[2] Jonathan A. Pachter[1]*

The goal of targeted therapy is to match a selective drug with a genetic lesion that predicts for drug sensitivity. In a diverse panel of cancer cell lines, we found that the cells most sensitive to focal adhesion kinase (FAK) inhibition lack expression of the neurofibromatosis type 2 (*NF2*) tumor suppressor gene product, Merlin. Merlin expression is often lost in malignant pleural mesothelioma (MPM), an asbestos-induced aggressive cancer with limited treatment options. Our data demonstrate that low Merlin expression predicts for increased sensitivity of MPM cells to a FAK inhibitor, VS-4718, in vitro and in tumor xenograft models. Disruption of MPM cell-cell or cell–extracellular matrix (ECM) contacts with blocking antibodies suggests that weak cell-cell adhesions in Merlin-negative MPM cells underlie their greater dependence on cell-ECM–induced FAK signaling. This provides one explanation of why Merlin-negative cells are vulnerable to FAK inhibitor treatment. Furthermore, we validated aldehyde dehydrogenase as a marker of cancer stem cells (CSCs) in MPM, a cell population thought to mediate tumor relapse after chemotherapy. Whereas pemetrexed and cisplatin, standard-of-care agents for MPM, enrich for CSCs, FAK inhibitor treatment preferentially eliminates these cells. These preclinical results provide the rationale for a clinical trial in MPM patients using a FAK inhibitor as a single agent after first-line chemotherapy. With this design, the FAK inhibitor could potentially induce a more durable clinical response through reduction of CSCs along with a strong antitumor effect. Furthermore, our data suggest that patients with Merlin-negative tumors may especially benefit from FAK inhibitor treatment.

INTRODUCTION

Focal adhesion kinase (FAK) is an important cancer target because *FAK* gene amplification and protein overexpression have been demonstrated in a wide range of malignancies (*1*). FAK is a nonreceptor protein tyrosine kinase that integrates signals from integrins and growth factor receptors to regulate cell proliferation, survival, migration, invasion, and cancer stem cell (CSC) renewal (*1*–*3*). FAK inhibitors have been shown to decrease tumor growth and metastasis in preclinical models, and have shown initial clinical activity in cancer patients (*4*–*6*).

Although elevated FAK expression is often observed in human tumors, no specific mutations or translocations have been identified to predict which patient population is most likely to respond to a FAK inhibitor. Successful targeted therapies that pair small-molecule inhibitors with specific activated oncogenes include agents targeting *BCR-ABL* and *EML4-ALK* translocations, *HER2* gene amplification, and activating mutations in EGFR (epidermal growth factor receptor) and B-RAF (*7*). Alternatively, identification of a synthetic lethal relationship between a drug target and loss of a tumor suppressor is exemplified by the efficacy of poly(adenosine diphosphate–ribose) polymerase (PARP) inhibitors in breast cancer bearing *BRCA1* or *BRCA2* mutations (*7*). An analogous therapeutic strategy could greatly facilitate the clinical development of a FAK inhibitor.

The neurofibromatosis type 2 (*NF2*) tumor suppressor gene encodes the Merlin protein, which has sequence homology with the ezrin/radixin/moesin (ERM) protein family (*8*). Merlin mediates tumor suppression and cell contact inhibition through reduction of Rac-PAK, mTORC1, Hippo, EGFR, and FAK-Src signaling (*9*). Merlin localizes to cell-cell boundaries, where it plays a role in the maturation of adherens junctions (*10*, *11*). Germline mutations in *NF2* contribute to development of type 2 neurofibromatosis, which is characterized by growth of meningiomas, ependymomas, and schwannomas (*12*). In addition, *NF2* is frequently inactivated in human malignant pleural mesothelioma (MPM), where biallelic inactivation of *NF2* occurs in 40 to 50% of tumors (*12*, *13*). MPM is an aggressive tumor of the pleural lining of the lung and is often associated with previous exposure to asbestos (*13*). It has been estimated that as many as 43,000 people worldwide die from MPM each year (*14*). Median overall survival following frontline chemotherapy with pemetrexed and cisplatin is about 12 months (*15*). New therapies are urgently needed to improve the prognosis of patients with MPM.

CSCs comprise a subpopulation of tumor cells that have self-renewal capacity, exhibit elevated resistance to chemotherapeutic agents, and are often responsible for tumor recurrence (*16*). CSCs have been identified in many cancer types, including colorectal, breast, ovarian, pancreatic, prostate, and head and neck cancers (*17*). Several studies found cells with CSC properties in MPM (*18*, *19*). Moreover, an elevated CSC population has been demonstrated in a mouse model of aggressive NF2-deficient asbestos-induced mesothelioma (*20*). FAK plays a role in self-renewal, tumorigenicity, and maintenance of mammary CSCs (*2*). Therefore, therapeutic targeting of FAK may diminish CSCs in a variety of malignancies including MPM.

Here, we aimed to identify cancers most sensitive to FAK inhibition and discover biomarkers to identify patients most likely to benefit from a FAK inhibitor treatment. VS-4718, previously known as PND-1186 (*21*), is a potent and selective FAK inhibitor (fig. S1). We found

[1]Verastem Inc., Cambridge, MA 02142, USA. [2]Fox Chase Cancer Center, Philadelphia, PA 19111, USA. [3]Massachusetts General Hospital Center for Cancer Research and Department of Pathology, Harvard Medical School, Charlestown, MA 02129, USA.
*Corresponding author. E-mail: jpachter@verastem.com

that VS-4718 is especially effective against Merlin-negative cell lines of certain cancer types including MPM in vitro and in vivo, and have uncovered a mechanism governing sensitivity to the FAK inhibitor. The preferential inhibitory effect of VS-4718 on CSCs, in addition to eradication of non-CSCs, provides a rationale for clinical use of a FAK inhibitor as a single agent after first-line chemotherapy. Together, these studies establish the effectiveness of a FAK inhibitor for treatment of Merlin-negative tumors and identify MPM as a promising setting for a Merlin-stratified clinical trial.

RESULTS

Effects of FAK inhibitor on Merlin-positive and Merlin-negative cancer cell lines

Depletion of FAK by short hairpin RNA or pharmacological inhibition of FAK protein kinase activity has been demonstrated to reduce cell proliferation in three-dimensional (3D) culture more effectively than in 2D culture (*22*). To assess the effects of VS-4718 treatment on cell growth, we modeled the tumor microenvironment using the "Matrigel on top" (MoT) method [adapted from (*22*), see Materials and Methods]. A panel of 47 human cancer cell lines representing diverse tumors, including renal, thyroid, ovarian and breast carcinomas, non–small cell lung cancer, melanoma, and mesothelioma, was tested for sensitivity to VS-4718 in MoT culture. Seven of the 10 most sensitive cell lines [average EC_{50} (half-maximal effective concentration) = 0.24 μM] lack Merlin protein expression (Fig. 1A and fig. S2). Among the 37 less sensitive cell lines (average EC_{50} = 1.85 μM), most (92%) expressed high Merlin levels, whereas only a minority (8%) exhibited loss of Merlin protein expression (Fig. 1A and fig. S2). Hence, loss of Merlin correlated with increased sensitivity to the VS-4718 FAK inhibitor in vitro.

To test whether enhanced FAK inhibitor sensitivity of Merlin-negative cells occurs in vivo, we assessed the antitumor efficacy of VS-4718 in Merlin-negative MDA-MB-231 and Merlin-positive MDA-MB-468 human triple-negative breast cancer xenograft models. In vitro, MDA-MB-231 cells were >60-fold more sensitive to VS-4718 than MDA-MB-468 cells (Fig. 1, B and C). In vivo,

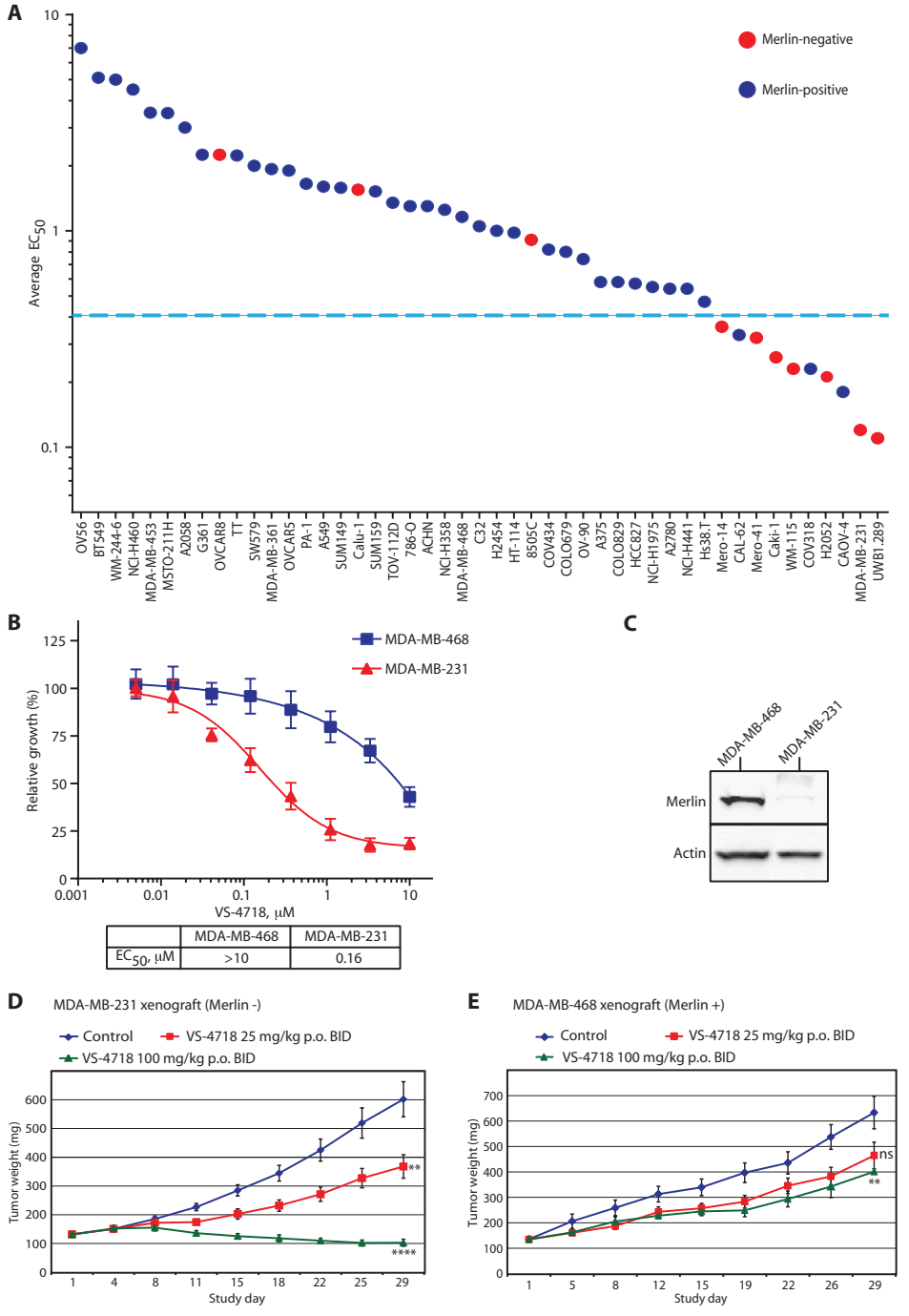


Fig. 1. Cancer cell lines especially sensitive to VS-4718 in vitro and in vivo have low Merlin expression. (**A**) Average EC_{50} values for VS-4718 sensitivity in a panel of cancer cell lines. Blue dotted line marks EC_{50} = 0.4 μM. n = 3 biological replicates. (**B**) Relative growth of MDA-MB-468 and MDA-MB-231 breast cancer cells in response to VS-4718 shown as percent of control. Data are representative of three experiments and are means ± SEM (n = 6). (**C**) Cell lysates from MDA-MB-468 and MDA-MB-231 cell lines, as indicated, analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) and blotted with anti-Merlin antibody. Actin was used as a loading control. (**D**) TGI of MDA-MB-231 subcutaneous xenograft tumors in response to VS-4718 treatment [orally (p.o.), twice daily (BID)] at 25 or 100 mg/kg, as indicated. Data are means ± SEM (n = 10). ****P < 0.0001, **P = 0.006 (unpaired t test with Welch's correction). (**E**) TGI of MDA-MB-468 subcutaneous xenograft tumors in response to VS-4718 treatment (orally, twice daily) at 25 or 100 mg/kg, as indicated. Data are means ± SEM (n = 10). **P = 0.0092; P = 0.056 [nonsignificant (ns); unpaired t test with Welch's correction].

in the MDA-MB-231 xenograft model, mice dosed orally twice daily with VS-4718 at 25 mg/kg had significantly smaller tumors ($P = 0.006$) than those receiving vehicle control after 29 days of treatment. Moreover, tumor regression was observed in mice treated with VS-4718 (100 mg/kg) ($P < 0.0001$; Fig. 1D), with corresponding significant reduction of tumor FAK (pY397) autophosphorylation ($P = 0.03$; fig. S3). In contrast, the Merlin-positive MDA-MB-468 human breast cancer xenograft model showed substantially less antitumor efficacy after 29 days of treatment with VS-4718 at 25 or 100 mg/kg (Fig. 1E). Final average tumor weights in VS-4718–treated groups were reduced only by ~30% relative to the vehicle control group despite significant inhibition of tumor pFAK-Y397 ($P = 0.0002$; Fig. 1E and fig. S3). Hence, tumor growth was suppressed in a dose-dependent manner in the Merlin-negative MDA-MB-231 xenograft model, with less tumor growth inhibition (TGI) in the Merlin-positive MDA-MB-468 model. Thus, we conclude that Merlin-negative cancer cells are especially sensitive to the FAK inhibitor VS-4718 in vitro and in vivo.

FAK inhibitor sensitivity of Merlin-negative mesotheliomas in vitro

NF2 mutations are common in MPM, with about 40 to 50% of tumors exhibiting biallelic inactivation of *NF2*, leading to absent or low Merlin expression (*12*, *13*). Accordingly, we examined a panel of mesothelioma cell lines for their FAK inhibitor sensitivity and assessed Merlin expression by Western blotting. Mero-25, MSTO-211H, H28, and H2452 MPM cell lines had high levels of Merlin, whereas Merlin expression was essentially undetectable in Mero-41, Mero-48a, Mero-83, MM87, and MM129 mesothelioma cell lines (Fig. 2A). Mero-14 expressed a short (~56 kD) isoform 7 variant of Merlin.

We assessed the effect of VS-4718 on the proliferation of these MPM cell lines in 3D MoT culture. Merlin-positive MPM cell lines exhibited an average EC_{50} of ~3.5 μM, whereas the average EC_{50} for Merlin-negative MPM cell lines was 0.3 μM (Fig. 2B and fig. S4). In addition to greater potency of VS-4718, all Merlin-negative cell lines demonstrated a greater magnitude of response, exhibiting cell viability curves that approached zero, suggesting a cytotoxic effect of VS-4718 (Fig. 2B). Together, these data demonstrated that Merlin-negative mesothelioma cells are more sensitive to VS-4718 than Merlin-positive cells in vitro in 3D culture.

To test for a causal contribution of Merlin expression in regulating the effect of VS-4718 on cell viability, we created isogenic MPM and breast cancer cell lines by ectopically expressing full-length *NF2* fused to green fluorescent protein (GFP). Merlin expression was confirmed by Western blot analysis (Fig. 2C). Cell growth inhibition analysis

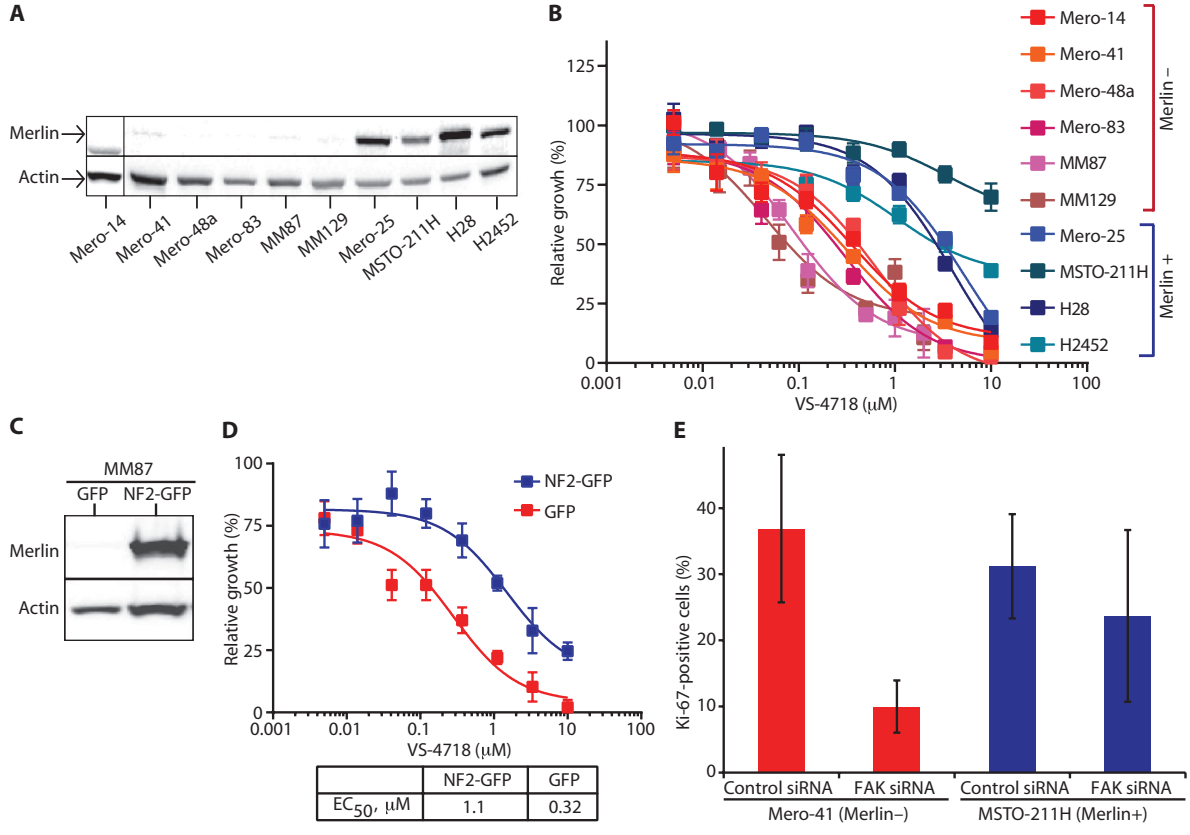


Fig. 2. The FAK inhibitor is most efficacious in Merlin-negative mesotheliomas in vitro. (**A**) Cell lysates from MPM cell lines analyzed by SDS-PAGE, as indicated, and blotted with anti-Merlin antibody. Actin was used as a loading control. (**B**) Relative growth of MPM cells in response to VS-4718 shown as percent of control. Data are representative of three experiments and are means ± SEM ($n = 6$). (**C**) Cell lysates from MM87/GFP or MM87/NF2-GFP cell lines, as indicated, analyzed by SDS-PAGE and blotted with anti-Merlin antibody. Actin was used as a loading control. (**D**) Relative growth of MM87/GFP or MM87/NF2-GFP cells in response to VS-4718 shown as percent of control. Data are representative of two experiments and are means ± SEM ($n = 6$). (**E**) Bar graph depicting percent of Ki-67–positive cells in Mero-41 and MSTO-211H cell lines treated with siRNA, as indicated. Data are means ± SD ($n = 6$, 150 cells each).

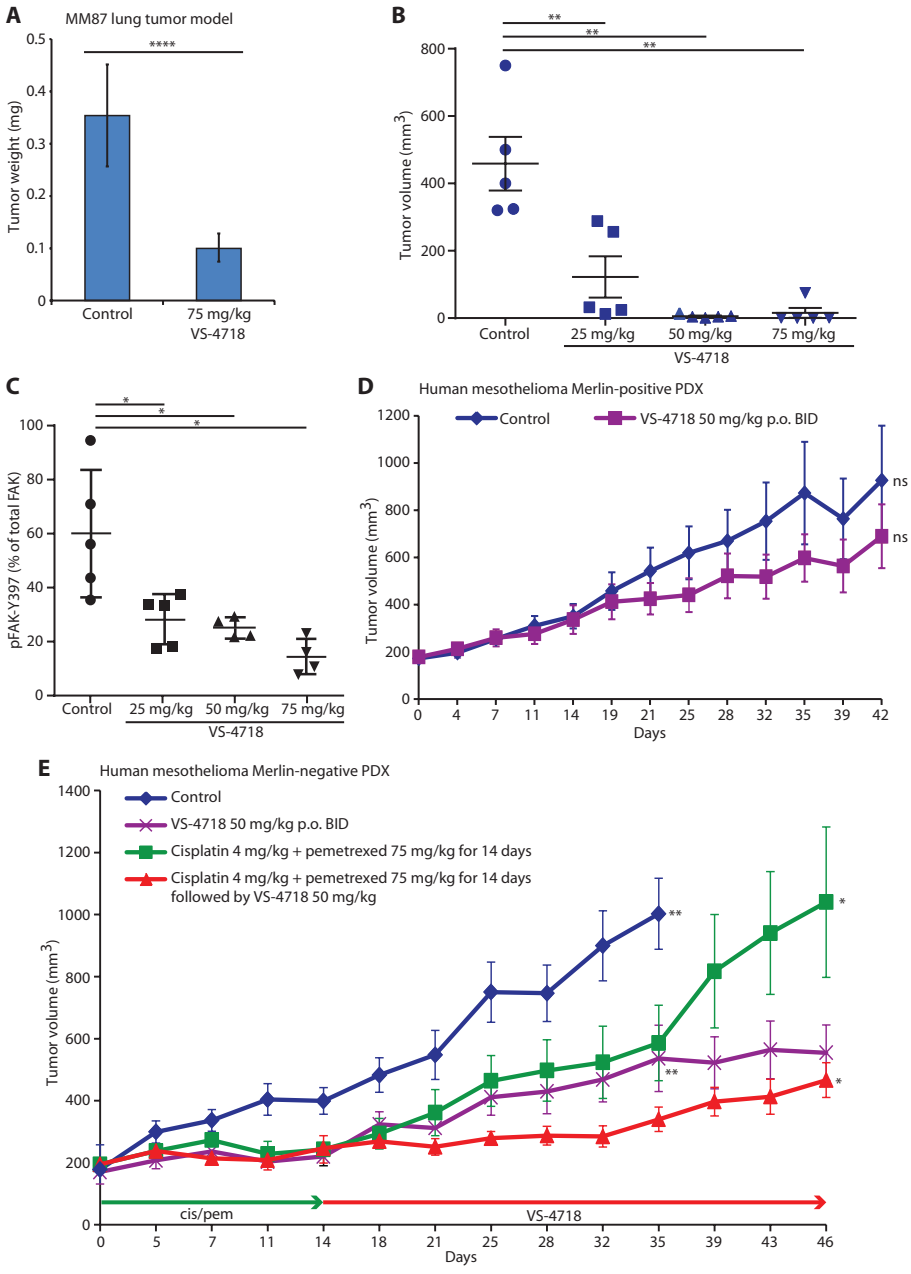


Fig. 3. Merlin-negative mesothelioma cells are especially sensitive to VS-4718 in vivo. (**A**) Bar graph depicting average tumor weights from an MM87 orthotopic lung tumor model treated with VS-4718, as indicated. Data are means ± SD (n = 10). ****P < 0.0001 (Mann-Whitney test). (**B**) Dot plot of tumor volumes in MM87 intraperitoneal xenograft model treated with VS-4718, as indicated. Black lines represent the mean; whiskers depict 25 and 75% of the distribution; n = 5. **P = 0.0079 (Mann-Whitney test). (**C**) Relative pFAK-Y397 levels measured by enzyme-linked immunosorbent assay (ELISA) in tumor samples from (B). Black lines represent the mean; whiskers depict 25 and 75% of the distribution; n = 5. *P = 0.0159 (Mann-Whitney test). (**D**) Tumor volumes from Merlin-positive human MPM PDX model treated with VS-4718 at 50 mg/kg (orally, twice daily). Data are means ± SEM (n = 10). Tumor weights on day 42 are not significantly different (ns). P = 0.19 (unpaired t test with Welch's correction). (**E**) Tumor volumes from a Merlin-negative human MPM PDX model treated with control (blue), VS-4718 at 50 mg/kg (orally, twice daily) (purple), cisplatin at 4 mg/kg (intraperitoneally, once every 7 days) and pemetrexed at 75 mg/kg (intraperitoneally, once daily, 5 days on, 2 days off) for 14 days (green), and cisplatin at 4 mg/kg (intraperitoneally, once every 7 days) and pemetrexed at 75 mg/kg (intraperitoneally, once daily, 5 days on, 2 days off) for 14 days followed by VS-4718 at 50 mg/kg (orally, twice daily) starting on day 16 (red). Data are means ± SEM (n = 10). **P = 0.0089 (Mann-Whitney test); *P = 0.035 (unpaired t test with Welch's correction).

demonstrated decreased sensitivity of MM87 MPM cells and MDA-MB-231 breast cancer cells expressing NF2-GFP to VS-4718, in contrast to cells infected with GFP vector control (Fig. 2D and fig. S5). The VS-4718 EC_{50} in control MM87/GFP cells was ~300 nM, whereas in MM87/NF2-GFP cells the EC_{50} was increased by about threefold (Fig. 2D). This demonstrated that ectopic expression of full-length Merlin reduced sensitivity to the FAK inhibitor VS-4718, consistent with our hypothesis that FAK inhibitor sensitivity in Merlin-negative mesothelioma and breast cancer cells is enhanced by loss of Merlin.

To determine whether biological depletion of FAK would mimic the effect of VS-4718, we depleted FAK by small interfering RNA (siRNA) in MPM cell lines and assessed cell proliferation in 3D MoT culture. Treatment of MPM cell lines with FAK siRNA resulted in reduction of FAK mRNA levels by 89 to 93% (fig. S6). Ki-67 staining was reduced about 3.5-fold in Merlin-negative Mero-41 cells (Fig. 2E), indicating reduced proliferation in response to FAK depletion. In contrast, reduction in Ki-67 positivity in Merlin-positive MSTO-211H cells in response to FAK siRNA treatment was marginal (Fig. 2E). These data demonstrate that depletion of FAK by siRNA mimics VS-4718 treatment and reduces proliferation especially in Merlin-negative cells.

FAK inhibitor efficacy in Merlin-negative mesotheliomas in vivo

To extend our observation to an in vivo mesothelioma model, we first tested the efficacy of VS-4718 in a Merlin-negative MPM lung tumor model. Merlin-negative MM87 murine mesothelioma cells were injected into the tail vein of mice and allowed to seed in the lungs for 7 days, and then animals were treated orally twice daily with VS-4718 at 75 mg/kg for 2 weeks. By initiating treatment 7 days after tail vein injection, this assay was intended to measure tumor growth in the lungs, rather than assessing elements of the initial metastatic process. At the end of the VS-4718 treatment on day 21 after initial tail vein injection, the tumor burden in the lungs of VS-4718–treated animals averaged 3.5-fold less than in the lungs of vehicle control–treated animals (P < 0.0001; Fig. 3A), suggestive of an inhibitory effect of the FAK inhibitor on tumor growth in the lungs.

We also tested the effect of VS-4718 in an MM87 intraperitoneal orthotopic xenograft model. Animals treated with VS-4718 (25 mg/kg) orally twice daily for 2 weeks had small tumors with an average volume of about 100 mm^3, in contrast to control-treated animals that had large tumors with an average volume of about 500 mm^3. A measurable tumor was observed in only 1 of 10 animals treated for 2 weeks with VS-4718 (50 or 75 mg/kg) orally twice daily (Fig. 3B). This indicated that the FAK inhibitor was efficacious in a Merlin-negative mesothelioma model in a dose-dependent manner. pFAK-Y397 was significantly inhibited in the tumors treated with VS-4718 ($P = 0.016$; Fig. 3C), establishing a clear correlation between target inhibition and antitumor efficacy. No significant change in tumor burden was observed in Merlin-positive MSTO-211H xenografts treated with VS-4718 orally twice daily at 75 mg/kg for 11 days (fig. S7), despite ~67% inhibition of tumor FAK-Y397 phosphorylation ($P = 0.0051$; fig. S7).

As a closer approximation of human cancer, we assessed the effect of VS-4718 in mice bearing human MPM patient-derived xenografts (PDXs). Merlin-negative and Merlin-positive PDX models (fig. S8) were chosen for this experiment, and FAK inhibitor treatment was initiated when tumors reached 200 mm^3. FAK autophosphorylation was effectively inhibited in both PDX models treated with VS-4718 (50 mg/kg) (fig. S8). Merlin-positive PDX tumors treated with VS-4718 at 50 mg/kg averaged only 23% smaller than control tumors at the end of the study on day 42 (Fig. 3D). In contrast, in the Merlin-negative PDX study, mice treated orally twice daily with VS-4718 (50 mg/kg) exhibited 56% TGI on day 35 compared to vehicle-treated control animals ($P = 0.0089$; Fig. 3E). Collectively, these observations demonstrate that Merlin-negative mesotheliomas are sensitive to the FAK inhibitor in vivo in mouse xenograft models and in a human PDX model.

Effects of FAK inhibitor treatment on cell proliferation and apoptosis

Reduced cell number in response to VS-4718 could potentially result from reduction of cell proliferation and/or induction of apoptosis. We therefore assessed the effects of VS-4718 on caspase 3/7 activity as a marker of apoptosis, and Ki-67 positivity as an indicator of cell proliferation in mesothelioma cells. We observed two- to threefold induction of caspase 3/7 activity in response to VS-4718 treatment in a panel of human Merlin-negative MPM cell lines—Mero-41, Mero-48a, and Mero-83—with only weak caspase 3/7 activation in Merlin-positive Mero-25 and H28 cells (Fig. 4A). Ki-67 staining was markedly reduced in Merlin-negative Mero-48a and Mero-41 cells treated with VS-4718, whereas little reduction in Ki-67 staining was observed in Merlin-positive MSTO-211H and H28 cells (Fig. 4B). These results indicate that the FAK inhibitor reduced both the proliferation and viability of Merlin-negative cells.

The AKT family of kinases regulates cell survival and proliferation (*23*). To better understand the inhibitory effect of VS-4718 on cell survival, we measured AKT activation in cells grown in 3D MoT culture. After 1 hour of VS-4718 treatment, FAK activation (pFAK-Y397) was inhibited to a similar extent in Merlin-negative Mero-41 and Mero-48a cells as in Merlin-positive H28 and MSTO-211H cells (Fig. 4, C to F, and

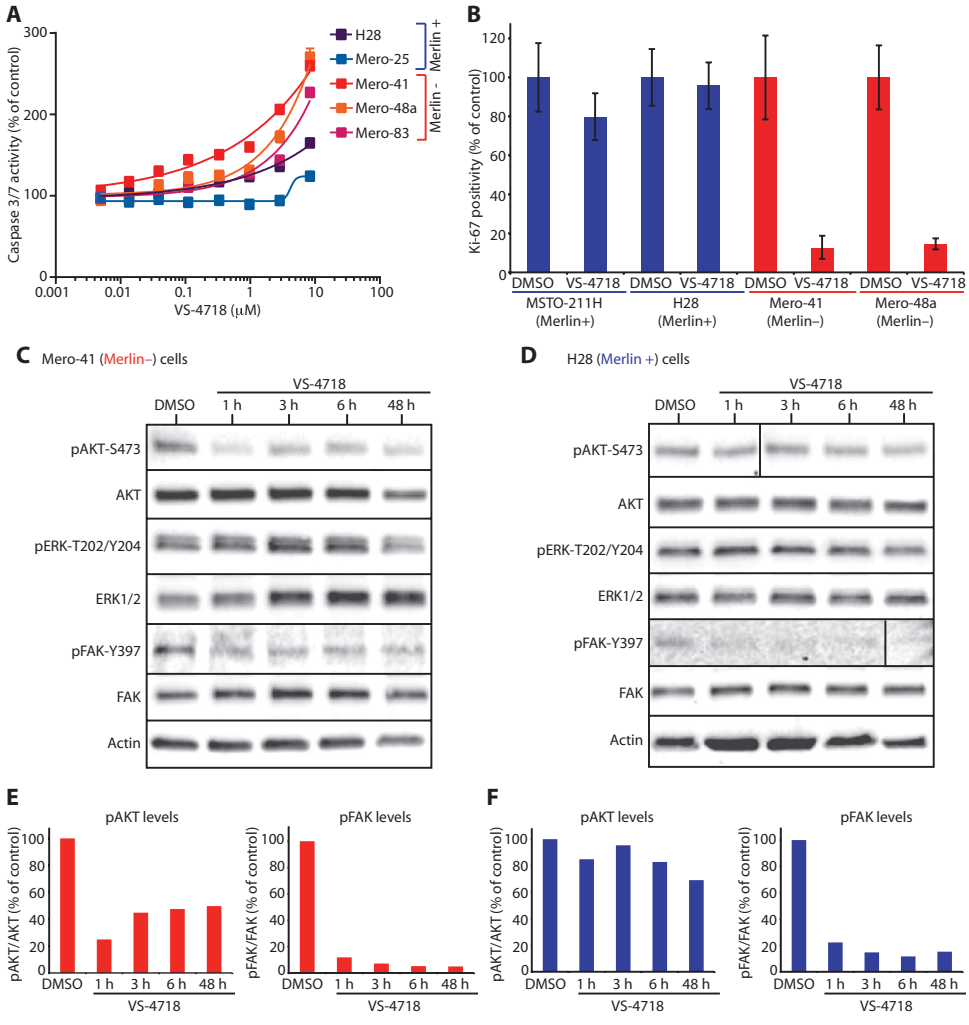


Fig. 4. VS-4718 induces apoptosis and reduces proliferation in Merlin-negative MPM cells. (**A**) Relative caspase 3/7 activation in response to VS-4718 treatment depicted as a percent of control. Data are representative of three experiments and are means ± SEM ($n = 6$). (**B**) Bar graph of an average number of Ki-67–positive cells treated with dimethyl sulfoxide (DMSO) or 1 μM VS-4718 for 4 days, depicted as percent of control. Data are representative of two experiments and are means ± SEM ($n = 6$). (**C** and **D**) Protein lysates from Mero-41 cells (C) or H28 cells (D) treated with 1 μM VS-4718, as indicated, in MoT culture analyzed on SDS-PAGE and blotted with antibodies, as indicated. Actin was used as a loading control. (**E** and **F**) Bar graphs depicting quantification of Western blots in (C) (red) and (D) (blue) represent a ratio of phosphorylated protein band density to the total protein band density normalized to the control in each case, as indicated.

fig. S9), and this effect was sustained for at least 48 hours. In contrast, after 1 hour of VS-4718 treatment, we observed reduced AKT activation (pAKT-S473) in Merlin-negative Mero-41 and Mero-48a cells sustained for 48 hours, whereas no inhibition of AKT activation was detected in Merlin-positive H28 or MSTO-211H cells (Fig. 4, C to F, and fig. S9). VS-4718 had no effect on extracellular signal–regulated kinase 1/2 (ERK1/2) activation in both Merlin-negative and Merlin-positive cells. These observations suggest that in the absence of Merlin, inhibition of AKT activation may mediate the proapoptotic effect of the FAK inhibitor on MPM cells. Together, these data indicate that Merlin-negative MPM cells are dependent on FAK signaling for both cell survival and proliferation in a 3D environment.

Potential mechanism of FAK inhibitor sensitivity in cells lacking Merlin expression

Merlin has been shown to play an important role in the establishment of stable adherens junctions in epithelial and Schwann cells in vitro and in vivo (*10*, *11*). We hypothesized that survival and proliferation of tumor cells may depend on a balance between cell-cell contacts supported by Merlin and cell–extracellular matrix (ECM) adhesion supported by integrin and FAK signaling. To confirm Merlin's role in cell-cell adhesion, we examined whether Merlin expression status would affect the morphology of cell colonies in MoT culture. Indeed, Merlin-positive MSTO-211H cells exhibited round mass colony morphology in 3D, suggestive of robust cell-cell adhesions (*24*), whereas Merlin-negative H2052 cells formed invasive stellate-like colonies (Fig. 5A). Merlin-negative parental MDA-MB-231 cells formed stellate, poorly consolidated colonies in MoT culture, as expected (*24*), which changed into round, smooth-surfaced morphology upon forced expression of *NF2* (Fig. 5A), consistent with the known role of Merlin in stabilizing cell-cell junctions.

The destabilization of adherens junctions in Merlin-negative cells may result in an up-regulation of integrin-FAK signaling and increased levels of pFAK because cells compensate for the loss of trophic signals normally derived from cell-cell contacts. An inhibitory effect of Merlin expression on FAK activation has been previously demonstrated in mesothelioma and glial cells (*25*, *26*). To confirm this observation, we performed pFAK-Y397 immunofluorescence analysis in Merlin-negative Mero-41 and Mero-48a cells ectopically expressing NF2 fused to GFP. pFAK-Y397 signal was reduced

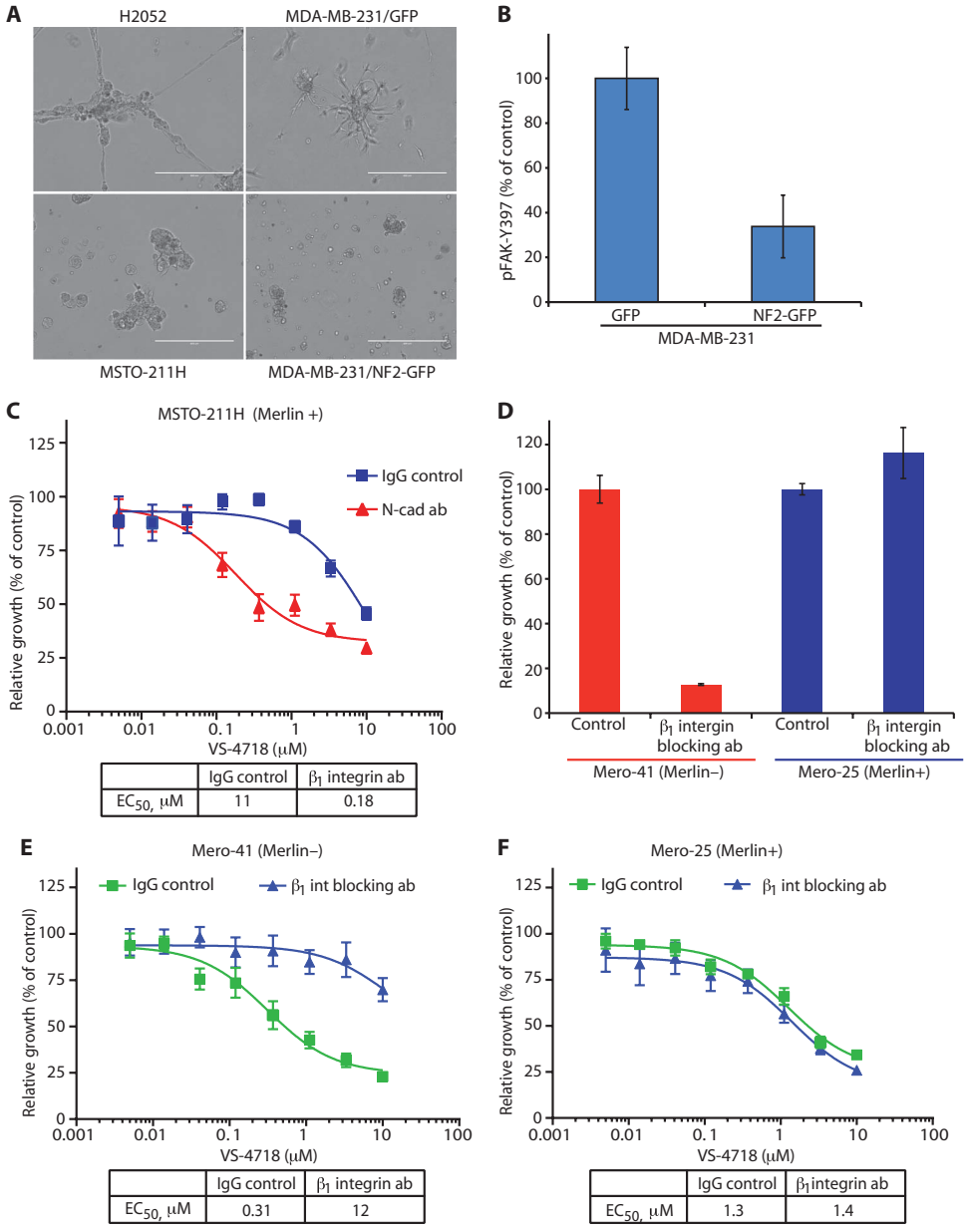


Fig. 5. FAK inhibitor sensitivity of Merlin-negative cells is caused by increased cell-ECM engagement and β_1 integrin activation. (**A**) Representative phase-contrast images of cells grown in 3D MoT culture, as indicated. Scale bars, 400 μm. (**B**) pFAK-Y397 levels measured by ELISA in MDA-MB-231/GFP and MDA-MB-231/NF2-GFP cells and represented as percent of control. Data are representative of two experiments and are means ± SEM (n = 6). (**C**) Relative growth of MSTO-211H cells treated with control immunoglobulin G (IgG) or N-cadherin–blocking antibody in response to VS-4718, depicted as percent of control. Data are representative of two experiments and are means ± SEM (n = 6). (**D**) Relative growth of Mero-41 and Mero-25 cells, as indicated, treated with control IgG or β_1 integrin–blocking antibody, depicted as percent of control. Data are representative of two experiments and are means ± SEM (n = 6). (**E**) Relative growth of Mero-41 cells treated with control IgG or β_1 integrin–blocking antibody in response to VS-4718, depicted as percent of control. Data are representative of three experiments and are means ± SEM (n = 6). (**F**) Relative growth of Mero-25 cells treated with control IgG or β_1 integrin–blocking antibody in response to VS-4718, depicted as percent of control. Data are representative of three experiments and are means ± SEM (n = 6).

at focal adhesions in cells with detectable Merlin expression (fig. S10). To test FAK activation in a 3D environment, we performed an ELISA to detect the activated form of FAK (pFAK-Y397) in Merlin-negative MDA-MB-231 parental cells and in cells engineered to express *NF2*. We found that activated FAK levels were decreased by about 70% upon expression of *NF2* (Fig. 5B), suggesting that ectopic Merlin expression diminished FAK activation.

We hypothesized that Merlin-positive cells forced to lose cell-cell interactions might become more dependent on cell-ECM interactions and thus on FAK signaling. To test this directly, we assessed FAK inhibitor sensitivity in 3D culture of Merlin-positive MSTO-211H and H28 cells, both of which express N-cadherin (fig. S11), in the presence of an N-cadherin–blocking antibody used to disrupt cell-cell adhesions (*27*). The N-cadherin–blocking antibody reduced β-catenin recruitment to cell-cell junctions indicative of defective cell-cell adhesions (fig. S11). Whereas Merlin-positive MSTO-211H cells were only modestly sensitive to VS-4718, exhibiting an EC_{50} of 11 μM, the N-cadherin–blocking antibody increased the potency of VS-4718 by about 60-fold, resulting in an EC_{50} of 180 nM (Fig. 5C). Similarly, the N-cadherin antibody increased the sensitivity of Merlin-positive H28 cells to VS-4718 (fig. S11). These results support the concept that blocking cell-cell contacts in a Merlin-positive context renders cells more dependent on cell-ECM–stimulated FAK signaling.

We further hypothesized that reducing cell-ECM interactions through disruption of β_1 integrin activity in Merlin-negative cells would supplant FAK inhibition, decreasing sensitivity to the FAK inhibitor. Therefore, we assessed whether a β_1 integrin–blocking antibody would inhibit FAK activation and reduce proliferation of MPM cells. In Merlin-negative Mero-41 and Mero-83 cells, β_1 integrin blockade indeed reduced FAK activation in contrast to Merlin-positive H28 and Mero-25 cells (fig. S12). Proliferation of Merlin-negative Mero-41 cells was markedly reduced (90%) by treatment with the β_1 integrin–blocking antibody (Fig. 5D), thus mimicking the effect of the FAK inhibitor. Moreover, blocking of β_1 integrin activity rendered Merlin-negative Mero-41 cells relatively insensitive to further reduction of proliferation by VS-4718, with an EC_{50} of 12 μM compared to an EC_{50} of 0.31 μM in the control IgG–treated cells (Fig. 5E). In contrast, in Merlin-positive Mero-25 cells, the β_1 integrin–blocking antibody had no effect on proliferation (Fig. 5D) or response to VS-4718 (Fig. 5F), suggesting lack of dependence of Merlin-positive cells on β_1 integrin/FAK signaling. Together, we conclude that a balance between cell-cell adhesion and β_1 integrin–mediated cell-ECM interaction regulates sensitivity to FAK inhibitor in MPM cells.

Preferential effect of FAK inhibitor on mesothelioma CSCs in vitro and in vivo

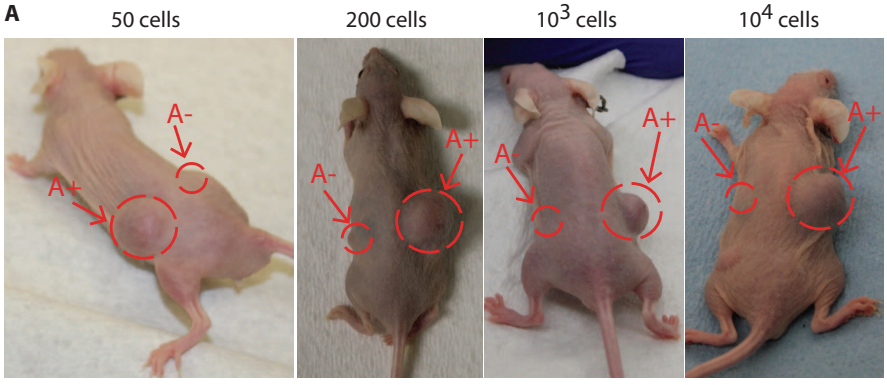
FAK has been shown to play a role in self-renewal and tumor-initiating capability of CSCs in a mouse model of breast cancer (*2*). Therefore, we were interested to assess the role of CSC depletion in the antitumor efficacy of the FAK inhibitor in MPM cells. CSCs are defined as a subpopulation of cancer cells that (i) can self-renew, (ii) has tumor-initiating capability, and (iii) can differentiate into heterogeneous cell types within tumors (*17*). An Aldefluor assay, which measures aldehyde dehydrogenase (ALDH) activity, has been used in various cancer types to define and isolate CSCs (*17*). To validate Aldefluor as a CSC marker in MPM, we assessed self-renewal, tumor-initiating capability, and differentiation within tumors.

The MM87 MPM cell line has, on average, 3.8 ± 1.4% Aldefluor$^+$ cells. The self-renewal potential of Aldefluor$^+$ MM87 MPM cells was assessed in a tumorsphere assay (*17*). Increased tumorsphere-forming efficiency of Aldefluor$^+$ MM87 cells was evident in primary and secondary tumorsphere assays. MM87 cells isolated from primary tumorspheres generated 85.6 ± 6.5 secondary spheres, whereas Aldefluor$^-$ cells only generated 8.6 ± 5.3 spheres (fig. S13), suggesting increased self-renewal capability of Aldefluor$^+$ MPM cells. To assess the tumor-initiating potential of Aldefluor$^+$ MPM cells, we sorted Aldefluor$^+$ or Aldefluor$^-$ MM87 cells and injected them subcutaneously into nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice at various cell numbers per injection from 100,000 down to 50 cells (limiting dilution); tumor burden was evaluated 3 weeks later. Large tumors of ~1000 mm^3 were formed in mice injected with as few as 50 Aldefluor$^+$ cells within 3 weeks, whereas Aldefluor$^-$ cells typically required injection of 1000 cells to initiate tumors (Fig. 6, A and B). Overall, Aldefluor$^+$ cells had ~35-fold higher tumor-initiating potential than Aldefluor$^-$ cells ($P < 0.0001$; Fig. 6B). To assess the ability of Aldefluor$^+$ MPM cells to differentiate within tumors, we analyzed Aldefluor$^+$ and Aldefluor$^-$ cell content in MPM tumors formed from MM87 Aldefluor$^+$ cells. After 3 weeks, tumors from Aldefluor$^+$ cell injection retained only ~1% Aldefluor$^+$ cells, whereas the bulk of the tumor consisted of Aldefluor$^-$ cells (fig. S13), suggesting that Aldefluor$^+$ cells differentiated into Aldefluor$^-$ cells during tumor growth. Together, these data validate Aldefluor as a tumor-initiating cell/CSC marker in MPM.

We next determined whether the FAK inhibitor VS-4718 has an effect on tumor-initiating cells in MPM. Changes in the Aldefluor$^+$ cell subpopulation in Merlin-negative H2052 mesothelioma cells were assessed in response to VS-4718 or treatment with the standard-of-care cytotoxic agents pemetrexed, cisplatin, vinorelbine, or gemcitabine. VS-4718 treatment reduced the percentage of Aldefluor$^+$ cells in direct contrast with the standard cytotoxic agents, which enriched the Aldefluor$^+$ cell subpopulation (Fig. 6C). Similar effects of VS-4718 on Aldefluor$^+$ CSCs were observed in the Mero-83 and Mero-48a MPM cell lines (fig. S14). Moreover, when used in combination, VS-4718 blocked the enrichment of Aldefluor$^+$ cells by pemetrexed (Fig. 6D), suggesting that FAK activity is necessary for survival and/or proliferation of mesothelioma tumor-initiating CSCs in vitro.

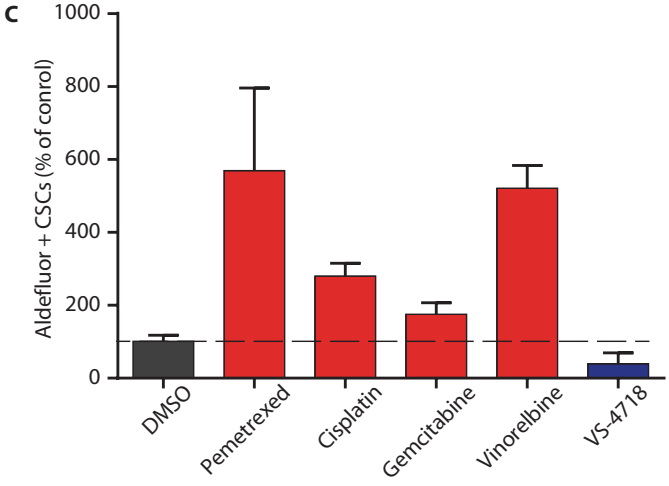
ALDH1 expression, like Aldefluor activity, has been widely used as a CSC marker (*17*). Accordingly, we attempted to extend the above observations to an in vivo model of MPM. ALDH1 immunofluorescence analysis demonstrated that control MPM tumors in an MM87 intraperitoneal xenograft model had dispersed ALDH1-positive cells. In contrast, tumors from animals treated with VS-4718 at 50 mg/kg (orally, twice daily) showed a marked reduction of ALDH1-positive cells (Fig. 6, E and F). Collectively, these data demonstrate that the FAK inhibitor decreased tumor-initiating CSC content in Merlin-negative MPM in vitro and in vivo.

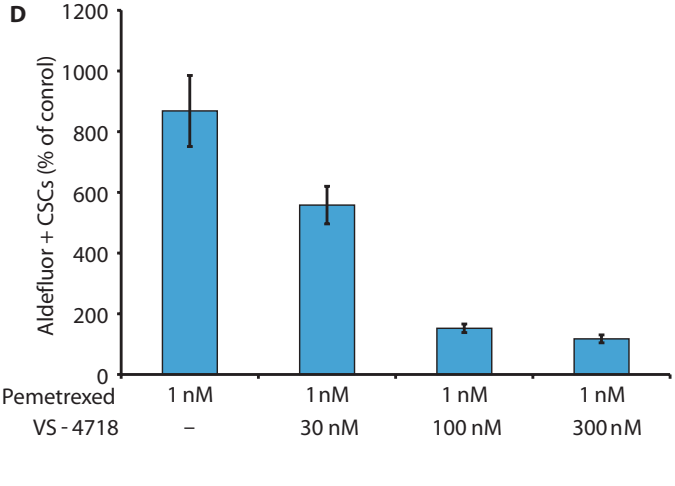
Standard frontline therapy for patients with mesothelioma typically consists of pemetrexed in combination with a platinum agent (*15*). Because pemetrexed and platinum increase the proportion of CSCs in MPM cells (Fig. 6C), we reasoned that the use of a FAK inhibitor as a single agent after frontline chemotherapy to suppress CSCs and tumor recurrence might effectively extend survival. To model this preclinically, we used a Merlin-negative MPM PDX model (Fig. 3E). Mice bearing Merlin-negative PDX tumors were first treated with cisplatin and pemetrexed for 14 days, followed by vehicle control or VS-4718 treatment for an additional 32 days to model maintenance treatment. Combination treatment with cisplatin and pemetrexed resulted in complete TGI on day 14; however, tumors regrew after cessation of treatment, reaching

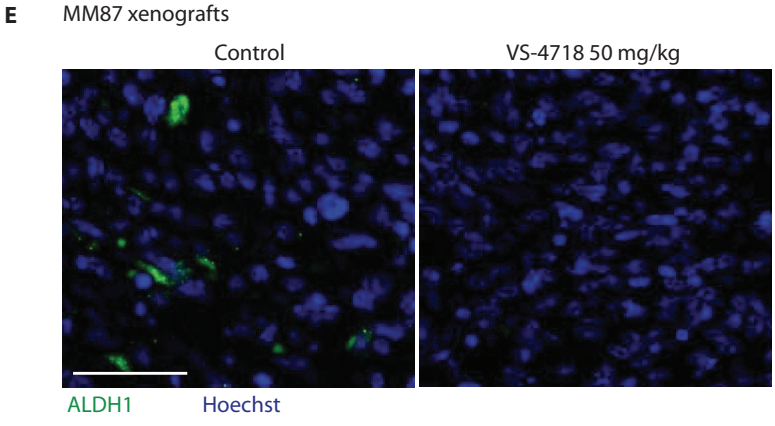


B

Cell #	A+	A–
10^5	3/3	3/3
10^4	3/3	2/4
10^3	4/4	2/3
200	2/4	0/4
50	2/4	1/4
TIF (3 weeks)	1/174	1/6063







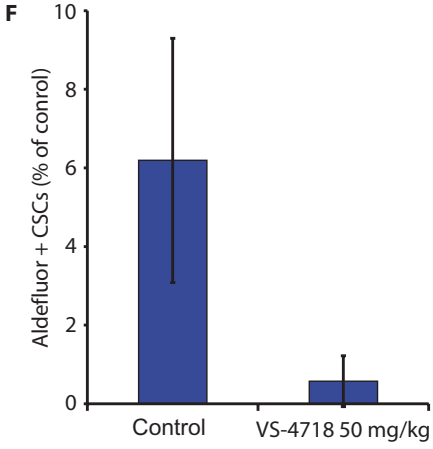


Fig. 6. FAK inhibitor VS-4718 has a preferential effect on CSCs in MPM. (**A**) Representative photographs of mice injected with Aldefluor$^+$ MM87 cells (A+) on one side and Aldefluor$^-$ MM87 cells (A–) on the other, as indicated. (**B**) Table summarizing tumor-initiating frequency (TIF) of MM87 A+ and A– cells ($P < 0.0001$). Total number of injected cells is indicated in the column "Cell #." Total number of tumors/total number of mice is indicated in columns "A+" and "A–." (**C**) Bar graph showing the number of Aldefluor$^+$ H2052 cells in response to drug treatment, as indicated, depicted as a percent of control. Compounds were used at the following concentrations: pemetrexed, 1 nM; cisplatin, 2 nM; gemcitabine, 1 nM; vinorelbine, 1 nM; VS-4718, 300 nM. Data are representative of two experiments and are means ± SEM ($n = 6$). (**D**) Bar graph showing the number of Aldefluor$^+$ H2052 cells, depicted as a percent of control, in response to pemetrexed alone or pemetrexed in combination with VS-4718, as indicated. Data are representative of two experiments and are means ± SEM ($n = 6$). (**E**) Representative immunofluorescence images of MM87 intraperitoneal xenograft tumors treated with VS-4718 and stained with anti-ALDH1 antibody (green) and 4′,6-diamidino-2-phenylindole (DAPI, blue). Scale bar, 20 μm. (**F**) Percent of ALDH1-positive cells in MM87 xenograft tumors from (E) depicted relative to control. Data are means ± SEM ($n = 100$).

1000 mm^3 by day 46 (Fig. 3E). In contrast, treatment with VS-4718 as a single agent starting on day 16, after cessation of the cisplatin/pemetrexed regimen, substantially delayed tumor regrowth, resulting in 77% TGI by day 46 compared to the cisplatin/pemetrexed arm ($P = 0.035$; Fig. 3E). These results demonstrate that a FAK inhibitor can indeed extend the duration of TGI, providing a more durable response when used as a single agent after cisplatin/pemetrexed treatment in a PDX model of mesothelioma.

DISCUSSION

Targeted therapies tailored to cancers with specific genetic lesions have emerged as effective anticancer treatments. In the absence of a specific activating mutation or translocation of the drug target, another promising approach is to establish a "synthetic lethal" relationship wherein pharmacological inhibition of one gene product is especially efficacious in the background of loss of function of a second gene product (*7*). Our studies reveal that a potent and selective FAK inhibitor, VS-4718, is efficacious in several cancer types, and that Merlin deficiency is a potential predictive marker for enhanced response to the FAK inhibitor in MPM. Our work also demonstrates that the FAK inhibitor targets CSCs in MPM, in contrast to cytotoxic agents that enrich the CSC population. Together, our data suggest a synthetic lethal relationship between Merlin loss and FAK inhibitor sensitivity and provide an attractive strategy for clinical testing of FAK inhibitors in patients with MPM.

NF2 expression and function can be lost in tumors by a variety of mechanisms, including mutation, chromosome loss, and focal deletions (*13*). *NF2* is mutated in about 40 to 50% of MPMs, with lower frequency of mutations in several other cancer types (*12*, *13*). Additional promising indications for clinical use of FAK inhibitors may include other tumors with high prevalence of *NF2* loss, such as schwannomas, meningiomas, and ependymomas, resulting from neurofibromatosis type 2 syndrome (89% *NF2* mutations) (*28*) and high-grade malignant meningioma (*29*). Additional studies are needed to unequivocally extend the synthetic lethal relationship between Merlin loss and FAK inhibitor sensitivity to other tumor types beyond the MPM tumors studied here.

A few reports have suggested a potential role of Merlin in regulating FAK activity in MPM and glial cells (*25*, *26*). FAK has been demonstrated to play a role in proliferation, migration, and survival of $NF2^{-/-}$ schwannoma cells through activation of phosphatidylinositol 3-kinase/AKT and ERK signaling pathways (*9*). Ectopic expression of Merlin in $NF2^{-/-}$ MPM cells inhibited invasion, decreased FAK Tyr^{397} phosphorylation, and impaired FAK interaction with its binding partners Src and p85 (*26*). However, an effect of Merlin expression on the antiproliferative and proapoptotic activities of FAK inhibitors has not previously been investigated.

Merlin has been shown to play a role in the establishment of stable adherens junctions in several epithelial cell types (*11*, *30*). Our data support a model in which the well-established cell-cell junctions in Merlin-positive MPM cells provide survival and proliferation signals independent of FAK signaling, explaining their reduced sensitivity to inhibition of FAK kinase activity (Fig. 7). In contrast, Merlin-negative cells with weak cell-cell contacts depend more on cell-ECM–induced FAK activation for proliferation and resistance to apoptosis and are therefore more sensitive to FAK kinase inhibition (Fig. 7). Accordingly, it may be possible to extend this observation to other cancers with weak cell-cell adhesions. For example, others have suggested that another FAK inhibitor, BI 853520, may be most effective in treatment of tumors with low expression of E-cadherin, another essential mediator of adherens junctions (*31*). The enhanced sensitivity of CAL-62, COV318, and CAOV-4 ovarian cancer cell lines to VS-4718 (Fig. 1A) despite wild-type Merlin expression supports the notion that additional molecular markers and mechanisms predictive of FAK inhibitor sensitivity remain to be elucidated.

Our data also demonstrate that a FAK inhibitor preferentially targets tumor-initiating CSCs in MPM. CSCs are thought to play an integral role in metastasis and recurrence after chemotherapy (*17*). Although several putative CSC markers have been proposed in MPM (*18*, *19*), they have not been rigorously validated. ALDH activity has been used to define CSCs in numerous solid tumor types (*17*). We validated ALDH activity (Aldefluor) as a marker of CSCs in MPM through the use of tumor initiation, tumorsphere self-renewal, and in vivo differentiation assays. In murine and human mesothelioma samples, VS-4718 decreased ALDH-positive cells in vitro and in vivo, establishing the FAK inhibitor as an anti-CSC agent in mesothelioma. Recent evidence in the literature implies a potential role for FAK in CSC proliferation, differentiation, motility, and invasion (*32*). Distinct roles for kinase-dependent and kinase-independent activities of FAK have been suggested in regulation of mammary stem cell biology (*33*). Our data demonstrate the importance of FAK kinase activity for the maintenance of CSCs in mesothelioma.

Frontline cytotoxic therapy with pemetrexed and cisplatin is the standard of care for treatment of MPM (*15*). Our data demonstrate that treatment of mesothelioma cells with these agents increases the proportion of CSCs, which may account for the tumor recurrence that invariably follows initial response. Treatment with a FAK inhibitor after completion of first-line therapy might extend survival through MPM TGI coupled with the preferential suppression of CSCs. The striking tumor growth delay observed with FAK inhibitor administration after combination treatment with cisplatin and pemetrexed in an

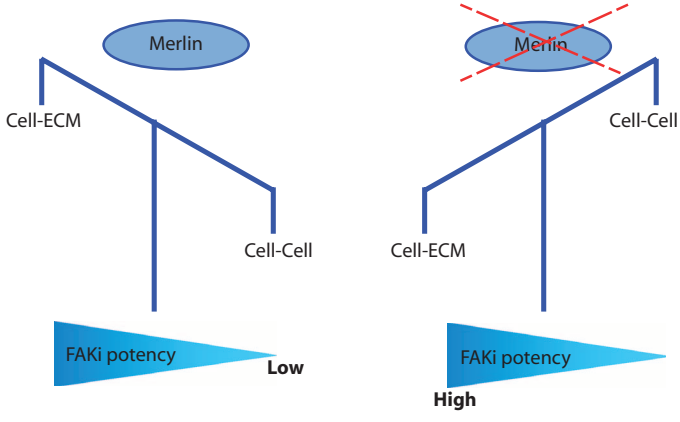


Fig. 7. FAK inhibitor sensitivity depends on the balance between cell-ECM and cell-cell interactions. In Merlin-positive cells, stable cell-cell adhesions decrease dependence on FAK signaling for survival and proliferation, thus causing low sensitivity to FAK inhibition. In Merlin-negative cells, strong dependence on cell-ECM interactions is mediated through β_1 integrin/FAK signaling important for cell survival and proliferation, and leads to greater sensitivity to FAK inhibition. Genetic or pharmacological manipulations of this balance influence FAK inhibitor sensitivity accordingly.

MPM PDX model provides preclinical proof of concept for this strategy. Recent data from a phase 1 clinical trial demonstrated increased progression-free survival of MPM patients treated with a FAK inhibitor (GSK2256098). The disease stabilization was especially pronounced in Merlin-low mesothelioma patients (*34*). Together, the data presented here provide strong rationale for a clinical trial of a FAK inhibitor administered to MPM patients after first-line therapy with stratification based on Merlin protein expression (ClinicalTrials.gov NCT01870609).

MATERIALS AND METHODS

Study design

Mouse models of mesothelioma, human MPM PDXs, MSTO-211H, MDA-MB-468, and MDA-MB-231 subcutaneous xenograft experiments are described in detail in Supplementary Materials and Methods. Briefly, endpoints of each study included assessment of the antitumor activity of compounds based on TGI at the end of the study and quantification of pFAK-Y397 in tumor samples. For subcutaneous models, animals were randomized into treatment or control groups when tumors reached about 125 to 250 mm^3 and dosing was initiated. All of the studies were powered to have an 80% $(1 - \beta)$ chance of detecting a significant difference in tumor burden at the end of the study specifying $\alpha = 0.05$ (two-tailed testing). Control cohorts had tight tumor growth curves, allowing to be adequately powered with experimental recipient cohorts of average size $n = 5$ to 10. Original data for animal studies are provided in table S1. For mouse models of mesothelioma, all animal experiments were performed in accordance with the regulations of Fox Chase Cancer Center's Institutional Animal Care and Use Committee.

Cell culture and compound treatment

Cell lines used in this study and culture conditions are described in Supplementary Materials and Methods. Cells were treated with VS-4718, pemetrexed, cisplatin, vinorelbine, or gemcitabine for 4 days. VS-4718, pemetrexed, vinorelbine, or gemcitabine was added at 15 μl per well in a 96-well plate as 10× DMSO solution ($n = 6$ per concentration point). Cisplatin was diluted from 10× NaCl stock solution.

Plasmids, virus production, and infection of target cells

The pMXs-NF2-IRES-GFP construct was generated by inserting *NF2* cDNA (complementary DNA) (Open Biosystems, MHS1011-202832561) into pMXs-IRES-GFP retroviral vector (Cell Biolabs). Retroviral packaging, infection, and fluorescence-activated cell sorting were performed as previously described (*22*).

FAK siRNA

A mix of four individual PTK2 siRNAs (ON-TARGETplus PTK2 siRNA; Dharmacon, Thermo Scientific) was used for depletion of FAK in mesothelioma cell lines with DharmaFECT reagent as described in the "siRNA Transfection with DharmaFECT Protocol." siGENOME Non-Targeting siRNA #1was used as a negative control.

Statistics

Shapiro-Wilk normality test was used to determine whether the data were normally distributed. Unpaired *t* test with Welch's correction or one-way analysis of variance (ANOVA) was used to determine statistical significance of the results for normally distributed data sets. Mann-Whitney test or Kruskal-Wallis test was used to determine significance of nonnormally distributed data. $\alpha = 0.05$ (two-tailed testing) was used for all statistical measurements. Statistical analysis was performed with Prism 6 software.

Aldefluor assay, limiting dilution assay, immunofluorescence and immunohistochemistry procedures, MoT, Western blotting and antibodies, blocking antibody experiments, and mouse models are described in Supplementary Materials and Methods.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Chemical structure of VS-4718.
Fig. S2. Western blot analysis of lysates from cancer cell lines in Fig. 1A.
Fig. S3. pFAK-Y397 levels in MDA-MB-231 and MDA-MB-468 xenograft tumor samples.
Fig. S4. Average EC_{50} values for Fig. 2B.
Fig. S5. Relative growth of MDA-MB-231/GFP and MDA-MB-231/NF2-GFP cells in response to VS-4718.
Fig. S6. Quantitative polymerase chain reaction analysis of FAK cDNA in MSTO-211H and Mero-41 cells.
Fig. S7. TGI and pFAK-Y397 expression analysis in MSTO-211H xenograft model.
Fig. S8. Merlin and pFAK-Y397 expression in human mesothelioma PDX models.
Fig. S9. pAKT and pFAK activation in Mero-48a and MSTO-211H cell lines.
Fig. S10. pFAK-Y397 immunofluorescence analysis in Mero-41 and Mero-48a cells expressing NF2-GFP.
Fig. S11. N-cadherin–blocking antibody treatment in H28 and MSTO-211H cells.
Fig. S12. Quantification of pFAK-Y397 in H28, Mero-25, Mero-83, and Mero-41 cells.
Fig. S13. Tumorsphere self-renewal and in vivo differentiation analysis of $Aldefluor^+$ MM87 cells.
Fig. S14. Effect of VS-4718 treatment on $Aldefluor^+$ CSCs in Mero-83 and Mero-48a MPM cells.
Table S1. Original data from in vivo studies (provided as an Excel file).

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