Published in final edited form as:

J Neurooncol. 2011 January ; 101(2): 215–226. doi:10.1007/s11060-010-0259-9.

Sunitinib induces PTEN expression and inhibits PDGFR signaling and migration of medulloblastoma cells

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Abstract

We previously showed that inhibition of the platelet-derived growth factor receptor (PDGFR) blocks the survival and migration of medulloblastoma cells. Identification of in vitro PDGFR-targeting pharmacologic agents that are suitable for preclinical testing in medulloblastoma models in vivo will be critical for efficiently translating these agents to clinical investigation in children with medulloblastoma. In this study, we investigated whether the multi-tyrosine kinase inhibitor sunitinib, effectively inhibits PDGFR signaling required for medulloblastoma cell migration. Daoy and D556 human medulloblastoma cells pre-treated for 1 h with 0.2 μ M sunitinib demonstrated induction of PTEN expression and significant inhibition of PDGFR signaling activity and transactivation of EGFR, in a RAS-independent manner, in response to PDGF-BB stimulation. Sunitinib pre-treatment markedly reduced medulloblastoma cell migration in response to both PDGF-BB and 10% serum at 4 and 24 h after treatment. Pre-treatment with sunitinib for 1 h also resulted in detachment and decreased viability of D556, but not Daoy, cells and only after 48 h following treatment. However, sunitinib did not induce apoptosis in either cell line at any time point, indicating that the anti-migratory effects of sunitinib were not due to impeding cell survival. Sunitinib similarly inhibited PDGFR signaling and migration of primary murine Smo/Smo medulloblastoma cells, suggesting that the Smo/Smo mouse is an appropriate model for preclinical testing of sunitinib. These results indicate that sunitinib may be an important pharmacologic agent for the treatment of invasive medulloblastoma, particularly given evidence of its ability to cross the blood–brain barrier to target tumor cells, and thus warrants further in vivo testing for confirmation of efficacy.

Keywords

Medulloblastoma; Sunitinib; Migration; PDGFR; PTEN

Introduction

In treating medulloblastoma, the most common malignant brain tumor in children, prophylactic irradiation of the entire brain and spine is commonly used to protect against CNS metastasis [1,2]. As a result of this therapy, the survival rate of children with this disease has improved considerably; however, treatment-related neurological impairment of survivors remains unacceptably high, especially in infants and toddlers, while the outcome for those who relapse with metastasis, despite having received this treatment, remains dismal [2,3]. Thus, innovative strategies to more safely and effectively prevent medulloblastoma growth and spread are needed.

We recently showed that inhibition of the platelet-derived growth factor receptor (PDGFR) effectively blocks the survival and migration of medulloblastoma cells in vitro [4]. Identification of PDGFR-targeting pharmacologic agents that are suitable for preclinical testing in medulloblastoma models in vivo, will be critical for assessing relative efficacy and translating these agents to clinical investigation in children with medulloblastoma. The oral multi-tyrosine kinase inhibitor, sunitinib, which targets PDGFR, vascular endothelial growth factor receptor-2 (VEGFR-2), and the proto-oncogenes RET, KIT, FLT3, and CSF-1, is FDA-approved in first-line treatment for adult patients with advanced renal cell carcinoma or progressive gastrointestinal stromal tumors (GIST) resistant to imatinib, and has been reported to significantly shrink renal cell carcinoma CNS metastases [5–7]. This suggests that sunitinib crosses the blood–brain-barrier (BBB) to target tumor foci within the CNS and therefore is a particularly attractive drug candidate for the development of pre-clinical testing in primary brain tumor cells that are dependent on these signaling molecules for their survival. In this study, we sought to determine whether sunitinib is indeed a biologically active inhibitor of medulloblastoma cell signaling, survival, proliferation and migration at the concentrations that are considered the clinically safe and effective dosages.

Results

Sunitinib inhibits PDGFR signal transduction in a RAS-independent manner in medulloblastoma cells

One hour sunitinib pre-treatment of serum-deprived Daoy and D556 human medulloblastoma cells resulted in a marked dose-dependent inhibition of PDGFRB and downstream PI3K, AKT and ERK phosphorylation in response to PDGF-BB (Fig. 1a, b). PDGFRB phosphorylation was completely abolished between 0.2 and 0.4 μ M of sunitinib. In a time-course study, we observed in Daoy and D556 cells that following a single treatment dose of sunitinib (0.2 μ M) the phosphorylation of PDGFRB was inhibited for 96 h and the phosphorylation of PI3K, AKT and ERK was inhibited for 48 h (data not shown). Daoy and D556 cells pre-treated with 0.2 μ M sunitinib for 1 h showed no significant difference in the level of total RAS protein or active RAS-GTP expression compared to untreated PDGF-stimulated control cells, indicating that the observed inhibitory effects of sunitinib on PDGFR-mediated PI3K, AKT and ERK activation are independent of RAS (Fig. 1c). In response to 10% serum, 0.2 μ M sunitinib pre-treatment also ablated PDGFRB phosphorylation and significantly inhibited the activation of AKT and ERK in Daoy cells; however, downstream activation of AKT and ERK could not be completely abolished under these conditions, suggesting that in addition to PDGFR and possibly other targets of sunitinib, AKT and ERK signaling is partly regulated by non-sunitinib targeted receptors of serum growth factors (Fig. 1d).

Sunitinib increases PTEN expression in PDGF-treated medulloblastoma cells

PTEN inhibits AKT activation and we have previously observed that within 15–30 min following PDGF-BB treatment of medulloblastoma cells there is a significant decrease in the level of PTEN protein expression, presumably through activation of proteosomal degradation. To confirm that the induced PTEN loss is the direct result of PDGFR activation, we tested the effect of 1 h sunitinib pre-treatment (0.2 μ M) on the level of PTEN expression in serum-starved cells stimulated with PDGF-BB. We found that sunitinib pre-treatment not only abrogates the PDGF-mediated effect but also significantly induces PTEN expression in response to PDGF and increases the relative basal level PTEN expression, indicating that PDGFR activity regulates PTEN expression in medulloblastoma cells and that sunitinib can effectively block this regulation (Fig. 2). There appears to be a transient compensatory increase in p-PI3K (Fig. 1b) as a result of maintained PTEN expression by sunitinib and hence a decreased ability of PI3K to phosphorylate AKT; however, over time and increasing drug concentrations, p-PI3K levels also decrease.

Sunitinib inhibits PDGF-mediated EGFR transactivation in medulloblastoma cells

We previously showed that in medulloblastoma cells, PDGF stimulation results in transactivation of the epidermal growth factor receptor (EGFR) [4]. Here we demonstrate that 1 h 0.2 μ M sunitinib pre-treatment of Daoy and D556 cells abolishes PDGF-BB induced EGFR transactivation compared to untreated PDGF-stimulated control cells (Fig. 3). To verify that sunitinib was not directly impacting EGFR activation, cells were treated for 1 h with sunitinib (0.2 μ M) and then stimulated with EGF. We show that sunitinib treatment does not inhibit EGF-induced EGFR phosphorylation, confirming the specificity of sunitinib to block PDGFR activation, and as a result, prevents PDGF-BB mediated transactivation of EGFR (Fig. 3).

Sunitinib inhibits medulloblastoma cell migration

To determine whether sunitinib inhibits PDGF-mediated medulloblastoma cell migration, we used a haptotaxis cell migration assay to measure Daoy and D556 cell migration across a fibronectin coated membrane in response to PDGF-BB (20 ng/ml), with and without sunitinib (0.2 μ M) treatment. Cell migration after 4 h was significantly decreased ($P < 0.05$) in sunitinib pre-treated Daoy and D556 cells compared to untreated control cells (Fig. 4a). We further assessed migration using an in vitro 'wound healing assay' to determine the effect of sunitinib pre-treatment on the migration of Daoy and D556 cells over a longer time interval (24 h) in the presence of 20 ng/ml PDGF-BB or 10% serum. Sunitinib pre-treatment resulted in a significant decrease in the migration ($P < 0.005$) of both Daoy and D556 cells, as assessed by their movement into the 'wound' 24 h after wound induction, in response to either PDGF-BB (Fig. 4b, c) or 10% serum (Fig. 4c) compared to untreated controls. F-actin immunostaining revealed that sunitinib blocked actin cytoskeletal rearrangements associated with a pro-migratory cellular phenotype (data not shown).

Sunitinib induces cell detachment, but not apoptosis, of D556 medulloblastoma cells

To determine whether the observed inhibitory effect of sunitinib treatment on medulloblastoma cell migration at 4 and 24 h was due in part to an effect on cell survival, serum-deprived Daoy and D556 cells were pre-treated with sunitinib (0.2 μ M) for 1 h and analyzed for apoptosis by annexin-V immunostaining at 24, 48, and 72 h after treatment. Sunitinib treatment did not induce a significant increase in apoptosis at any time-point in neither Daoy nor D556 cells compared to untreated control cells (data not shown). Likewise, increased caspase-3 activity in sunitinib-treated Daoy and D556 cells was not detected at 24 or 48 h after drug treatment (data not shown).

However, interestingly we observed a dose-dependent increase in cell detachment, as determined visually by the presence of rounded up and floating cells, in D556 but not Daoy cells, and only after 48 h following sunitinib pre-treatment (Fig. 5a). Together, these findings indicate that sunitinib's inhibition of PDGF- and serum-mediated medulloblastoma cell migration at 4 and 24 h is not due to induction of cell death; however, sunitinib-targeted effectors appear to be critical for maintaining longer-term attachment and cell morphology. Since Daoy and D556 cells do not express KIT or FLT3, and western blot of whole cell lysates revealed very little protein expression of VEGFR2 (data not shown), the effect of sunitinib on cell morphology and attachment is likely mediated through PDGFR or one of the other targets such as RET or CSF-1 (expression not analyzed).

Sunitinib inhibits viability of D556 medulloblastoma cells

To assess whether the effect of sunitinib treatment on medulloblastoma cell migration assessed at 4 and 24 h after treatment was in part due to inhibition of cell viability, cell counts of Daoy and D556 cells following 1 h pre-treatment with 0.2 μ M sunitinib were compared to untreated control cells at 24, 48, and 72 h after sunitinib treatment. Daoy and D556 sunitinib-treated cells showed similar viability as the untreated control cells at 24 h, however, by 48 and 72 h after sunitinib treatment, D556, but not Daoy cells, exhibited significant ($P < 0.05$) reduction in cell counts compared to untreated controls (Fig. 5b).

Sunitinib inhibits migration, PDGFR signaling and viability of PS125 primary murine medulloblastoma cells

As further confirmation of the results and to establish a potential model for future studies of sunitinib in vivo, we tested migration using the in vitro 'wound healing assay' to determine the effect of sunitinib pre-treatment on the migration of PS125 primary murine medulloblastoma cells over a long time interval (24 h) in the presence of 20 ng/ml PDGF-BB or 10% serum. Sunitinib pre-treatment resulted in a significant decrease in the migration ($P < 0.005$) of PS125 cells, as assessed by their movement into the 'wound' 24 h after wound induction, in response to either PDGF-BB or 10% serum (Fig. 6a) compared to untreated controls.

To confirm that the inhibition of the PS125 medulloblastoma cell migration was due to inhibition of PDGFR, we tested the effect of sunitinib on PDGFR signaling in PS125 cells. Similar to that observed in human medulloblastoma cells, 1 h sunitinib pre-treatment of serum-deprived PS125 cells resulted in a marked inhibition of PDGFRB and downstream SHP2, AKT and ERK phosphorylation in response to PDGF-BB (Fig. 6b). PDGFRB, SHP2 and AKT phosphorylation was completely abolished with 0.2 μ M of sunitinib treatment. PS125 cells pre-treated with 0.2 μ M sunitinib for 1 h showed no significant difference in the level of total RAS protein expression compared to untreated PDGF-stimulated control cells, indicating that the observed inhibitory effects of sunitinib on PDGFR-mediated SHP2, AKT and ERK activation are independent of RAS (Fig. 6b). Likewise, as seen in the human medulloblastoma cell lines, 1 h sunitinib pre-treatment (0.2 μ M) increased the level of PTEN expression in serum-starved cells stimulated with PDGF-BB (Fig. 6b).

To confirm that sunitinib treated PS125 mouse medulloblastoma cells maintain viability during the timeframe that the migration assays are conducted, and to further assess whether sunitinib effects viability over time, an XTT cell viability assay was performed at 0, 24, 48, 72, 96, and 192 h post increasing concentrations of sunitinib treatment compared to untreated controls. Cells were treated in six replicates with increasing concentration of sunitinib ranging from 0.1 to 0.8 μ M. There was no significant reduction in cell viability as determined by the XTT colorimetric absorbance in all the concentrations of sunitinib used at 24 or 48 h post treatment. However, there was a significant reduction ($P < 0.05$) in viability

in all the treatment groups compared to the untreated controls at 72, 96, and 192 h post sunitinib treatment (Fig. 7). This finding suggests that while longer term sunitinib treatment does inhibit the viability, as observed with human D556 cells at 48 h, this effect is not observed until 72 h post treatment in PS125 cells. Thus, the immediate (24 h) inhibition of cell migration in sunitinib treated cells compared to untreated controls is not a result of reduced cell viability.

Discussion

Identifying small molecule inhibitors that at clinically relevant concentrations can target critical medulloblastoma signaling pathways, cross the BBB, and have minimal off-target CNS toxicity is important for designing pre-clinical in vivo drug testing platforms with an aim toward translation to novel clinical investigational treatments for medulloblastoma. Previously, we demonstrated that PDGFR signaling is critical for medulloblastoma migration [4]. Herein, we show that the multi-tyrosine kinase inhibitor sunitinib effectively blocks PDGFRB activation and RAS-independent downstream signaling through PI3K, AKT and ERK, presumably by preventing PDGF-mediated decrease of PTEN, and significantly inhibits the migration of medulloblastoma cells in response to PDGF and serum at clinically useful drug concentrations (0.2 μ M). Interestingly, although similar blockade of PDGFR pathway signaling in medulloblastoma cells was observed in our previous studies investigating the effect of imatinib, an inhibitor of PDGFR, KIT and BCR-ABL, in contrast to those studies, sunitinib did not induce toxic cell death. The reasons for this are unclear; however, it is possible that imatinib may have additional off-target effects not yet described since sunitinib similarly targets PDGFR and these cells do not express KIT or the mutant fusion kinase BCR-ABL. However, in addition to blocking migration, sunitinib treatment appeared to cause cell detachment without inducing apoptosis, although the precise mechanism for this is unclear, and exhibited a cytotoxic effect on D556 and PS125 cells, a response that was not observed in prior studies with imatinib treatment. Again, the reason for these discrepant results among the agents and among the three cell types is not known; however, it is possible that sunitinib's inhibition of other targets such as RET may be responsible for the delayed anti-proliferative effect observed in D556 and PS125 cells. Activation of an auto-crine loop whereby D556 and PS125 have more endogenous PDGFR activity relative to Daoy could explain these differences; however, this does not appear to be the case since basal levels of PDGFR and downstream effector phosphorylation are not appreciably different among the cells.

Whether or not those differences are attributable to RET inhibition by sunitinib remains to be determined as, to our knowledge, the presence and activity of RET in the cells has not been investigated. Interestingly, the RET protooncogene is a receptor tyrosine kinase that transduces signals for cell growth and differentiation and functional interactions between RET and VEGFR and EGFR has been recently described. EGFR and RET have been found to coimmunoprecipitate and ligand-mediated EGFR activation was shown to result in the phosphorylation of a kinase-dead RET, while kinase inhibitors of EGFR inhibited cell growth induced by constitutively active mutants of RET in thyroid cancer and NIH/3T3 cells [8]. We did not detect appreciable levels of VEGFR in the cells tested in our study, but we did demonstrate that sunitinib effectively ablated PDGF-mediated transactivation of EGFR and thus similar proliferative regulating interactions between RET and EGFR may exist in D556 cells. Previous studies have reported a similar interaction between PDGFRB and EGFR, where the two receptors were reported to be extracted from the same caveoli and a report by Saito et al. suggested the importance of heterodimerization between the two receptors as a requirement for transactivation of EGFR [9–13].

In clinical trials sunitinib has been reported to inhibit PDGFR on pericytes leading to tumor regression and inhibition of metastatic progression [7,14]. To determine its efficacy on brain tumors, one study evaluated the antitumor activities of sunitinib on orthotopic models of glioblastoma multiforme (GBM) in vitro and in vivo [15]. Sunitinib treatment at concentrations as low as 10 nM, effectively inhibited angiogenesis induced by implantation of U87MG and GL15 GBM cells into organotypic brain slices. At higher doses (10 μ M), sunitinib directly induced antiproliferative and proapoptotic effects on GL15 cells and decreased invasion of these cells implanted into brain slices by ~50%. In addition, athymic mice bearing intracerebral U87 GBM that were treated with sunitinib showed improved median survival [15]. Thus, sunitinib may be a novel therapeutic agent in the treatment of brain tumors considering it has a greater ability to cross the BBB than similar multi-kinase targeting agents such as imatinib [5,7,15].

In summary, the primary goal of this study was to identify a tumor-specific agent capable of preventing medulloblastoma cell migration at a low, non-toxic dose with minimal off-target effects. Our results show that sunitinib blocks both PDGF- and serum-mediated human D556, Daoy and murine PS125 medulloblastoma cell signaling and migration and can inhibit D556 and PS125 cell viability following 72 h after treatment. While we acknowledge the limitations of the in vitro results, the purpose of this preliminary investigation was to establish the rationale for future confirmatory pre-clinical in vivo studies. Our findings, and the results reported by others demonstrating that sunitinib crosses the BBB and can impact tumor growth, indicate that sunitinib is an ideal drug for pre-clinical in vivo testing in medulloblastoma animal models, especially in models evaluating the efficacy of drugs targeting medulloblastoma migration and metastasis. Thus, our findings provide compelling rationale for validating sunitinib's effect in the Smo/Smo mouse model of medulloblastoma.

Methods

Medulloblastoma cells

Daoy and D556 metastatic human medulloblastoma cells were grown in EMEM media (Biowhitaker) containing 10% fetal bovine serum (FBS) at 37°C with 5% CO₂ according to the specification of the American Type Culture Collection (ATCC) until near confluence before initiation of experiments. D556 expresses relatively higher levels of PDGFRB protein compared to Daoy cells and was used to compare the effect of PDGFRB inhibition on signal transduction and biological function in a relatively high versus low-PDGFRB expressing cell line, respectively. PS125 primary mouse medulloblastoma cells derived from the Smo/Smo transgenic mouse [16] were kindly provided by Dr. Craig Castellino, Emory University.

Treatment with sunitinib

Cells were cultured under normal conditions and allowed to reach ~80% confluence before they were washed twice with serum-free media and serum-starved overnight at 37°C and 5% CO₂. The next day, cells were treated with vehicle or sunitinib (Pfizer Pharmaceutical). For the dose-curve studies, cells were treated with increasing doses of the drug for 1 h, after which they were stimulated with 10 ng/ml of PDGF for 12 min, lysed and processed for western blot analysis. For the time-point and biologic assay studies, cells were treated with 0.2 μ M sunitinib for the length of the study. Cell stimulation with PDGF took place at each indicated time-point for 12 min before cells were lysed and processed for western blot analysis. Note that during drug treatment, cells were kept in serum-free media at 37°C and 5% CO₂, and sunitinib was not replenished at any time-point after the initial 0.2 μ M dose supplied at the beginning of the experiment.

PDGF-BB stimulation

Cells were serum starved (1×10^6) for 24 h in 6 ml serum-free growth media in T-75 cm² flasks. The next day cells were washed (3×) with serum-free media to remove endogenous growth factor contamination, replenished with 6 ml serum-free medium and then treated with PDGF-BB (10 ng/ml for signaling assays or 20 ng/ml for bioassays conducted over longer periods of time) or control PBS stimulation for 12 min at 37°C prior to cell harvesting by either scraping in 1× Cell Lysis Buffer for immediate cellular protein expression using western blot analysis or by trypsinization for intact cell incubation in bioassays as detailed below.

Western blot analysis

Whole cell lysates were prepared from cells lysed in 500 µl of 1× Cell Lysis Buffer (Cell Signaling Technology). The protein concentration was determined using the Bradford dye-binding assay (Bio-Rad Laboratories, Hercules, CA). An aliquot of the lysate was mixed with an equal volume of 2× Laemli Sample buffer and heated at 97°C for 10 min. Between 40 and 80 µg of total protein concentration was electrophoresed on a 7.5% SDS-PAGE (depending on what protein was being detected) and transferred to PVDF membrane (Perkin Elmer, Waltham, MA). Target proteins were detected using primary antibodies for total or phosphorylated forms of the PDGFRB (1:500; 1:250), PI3K (1:500; 1:250), PTEN (1:500; 1:500), AKT (1:500; 1:250) and ERK (1:500; 1:250) (Cell Signaling Technology) using the above dilutions. The blots were incubated with the primary antibody overnight at 4°C, washed (3×) 5 min each and incubated with secondary antibody 1 h at room temperature using the anti-rabbit IgG HRP-conjugated (Cell Signaling Technology) at a dilution of 1:2000. The blots were then washed (4×) 15 min each with 1× TBS-Tween (0.1%) and incubated in Lumiglo reagent for ~2 min and then exposed. To control for protein loading variations, a primary antibody for Actin (Santa Cruz Biotechnologies) or GAPDH (Cell Signaling) was used, and the total protein for PDGFRB, PI3K, PTENT, AKT and ERK was used to measure the ratio of total: phosphorylated protein, as quantitated by densitometric analysis using software from Scion Corporated.

RAS activation assay

RAS-GTP was precipitated from total cell lysates using a RAS activation assay kit (Millipore Corporation, Charlottesville, VA) as described in the manufacturer's manual. Briefly, 500 µl of the total cell lysate was incubated with 20 µl of the RAS assay reagent (Raf-1 RBD, agarose) for 45 min at 4°C with gentle agitation. The agarose beads were pelleted by brief centrifugation and supernatant discarded. Beads were washed (3×) with 500 µl wash buffer, taking care to minimize loss of beads during removal of the wash buffer. Agarose beads were resuspended in 40 µl of 2× laemmli reducing sample buffer, boiled for 5 min and pelleted by brief centrifugation. The supernatant (20 µl) was loaded on a SDS-polyacrilamide gel, electrophoresed and transferred to a PVDF membrane. The membrane was blocked with 5% milk and 3% BSA in 0.1% TBS-T (1×), and incubated overnight with anti-RAS (clone RAS10) at 4°C. The next day, the membrane was washed (3×) 5 min each with 0.1% TBS-T, and incubated 1 h in anti-mouse secondary antibody (1:1000) (Santa Cruz Biotechnologies, CA). The membrane was washed (3×) 15 min each with 0.1% TBS-T and protein was detected using SuperSignal Dura chemiluminescent substrate (Pierce, Rockford, IL).

Cell migration assays

Two migration assays were employed, a haptotactic Boyden chamber cell migration assay for 4 h short term evaluation and a 24 h 'wound-healing' scratch assay for longer term evaluation. For short term assays, serum-deprived cells were treated with 0.2 µM sunitinib

or vehicle for 1 h. Cells were then harvested, counted and resuspended to a density of 1×10^6 . Sunitinib treated and un-treated Daoy, D556 and PS125 cells were plated into the upper chamber of a fibronectin-coated haptotaxis cell migration chamber. The lower well of the chamber was either filled with serum-free media or serum-free media supplemented with 20 ng/ml PDGF-BB. After 4 h incubation, non-migrating cells were removed with a cotton swab and the migrated cells stuck to the lower side of the membrane were stained using a cell stain solution (Chemicon, part no. 20294) for 10 min. The stain was extracted using stain extraction buffer (Chemicon, part no. 20295) and 100 μ l of the dye was loaded into wells of a micro-plate and quantitated using a colorimetric plate reader at an absorbance of 570 OD.

For longer term migration assays, Daoy, D556 and PS125 cells plated in a Petri dish at a density of ~70–80% confluence were serum-deprived overnight and the next day treated with either vehicle (H_2O) or 0.2 μ M sunitinib for 1 h. After 1 h sunitinib treatment, cells were scraped down the middle of the Petri dish using a 1 ml pipette to induce the resemblance of a 'wound'. The scraped off cells were washed out ($1\times$) using serum-free media and fresh media was replenished containing 20 ng/ml PDGF-BB and supplemented with the same concentration of sunitinib. Images of 6–8 random fields in the scraped 'wound' were taken at the time of 'wound' induction (0 h) and again 24 h after. The area of the 'wound' in these fields was traced and measured in μM^2 using Axiovision systems. The average area in μM^2 of the 'wound' 24 h after induction was subtracted from that at time of induction (0 h) and graphed using Microsoft excel.

Cell viability by counting

Cells were harvested by trypsinizing and resuspended in 2 ml serum-free media. 10 μ l of the cell suspension was stained with 10 μ l trypan blue (Lonza, Basel, Switzerland) and counted on a hemocytometer. Experiments conducted in triplicates and the mean of all three experiments was calculated and plotted $\pm$ the standard error of the means. To determine whether cell count was affected by sunitinib treatment, serum-deprived cells were treated with vehicle or 0.2 μ M sunitinib for 1 h then stimulated to undergo proliferation using 20 ng/ml PDGF-BB. Cells were counted on a hemocytometer at 0, 24, 48, and 72 h after sunitinib treatment to assess the rate of proliferation compared to untreated controls.

XTT cell viability assay

Viability was determined by the colorimetric absorbance of the XTT assay at 0, 24, 48, 72, 96, and 192 h post increasing concentrations of sunitinib treatment in PS125 mouse medulloblastoma cells compared to untreated controls. Cells (5×10^3) were plated into a 96-well plate and treated with vehicle or 0.1, 0.2, 0.4, and 0.8 μ M sunitinib for the indicated time points. The average absorbance at 405 nm of each group, run in six replicates, was plotted on a line graph using Microsoft Excel at the indicated time points. The mean $\pm$ the standard error of the mean of multiple experiments was graphed and a Student's *t* test used to determine statistical significance of the means between groups.

Statistical analysis

Experiments were conducted in triplicates and the mean $\pm$ SE of all three experiments was calculated and plotted. A two-sided Student's *t* test was used to determine statistical significance between groups. One-way ANOVA test was used to test for significant differences among means of multiple groups obtained from three or more independent experiments. The mean $\pm$ SE of three or more experiments was derived and graphed using Microsoft Excel.

Acknowledgments

Sunitinib was provided by Pfizer Pharma. Supported by NIH R01 grant CA111835 (T.J.M.).

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Fig. 1. Sunitinib treatment inhibits PDGFR cell signaling in a RAS-independent manner. **a** Representative western blot of whole cell lysates demonstrating dose-dependent inhibition of PDGFRβ, PI3K, AKT and ERK phosphorylation in response to PDGF-BB (10 ng/ml) following increasing concentrations of 1 h sunitinib pre-treatment of Daoy cells. **b** Densitometric analysis of multiple western blots indicating significant reduction (* $P < 0.05$) in the phosphorylation levels of PDGFRβ (upper panel) and PI3K (lower panel) in response to PDGF-BB (10 ng/ml) in Daoy and D556 cells pre-treated for 1 h with 0.2 μM sunitinib. **c** Representative western blot of Daoy cell lysates (upper panel) and results of multiple pull-down assays in Daoy and D556 cells (lower panel) demonstrating no change in total RAS protein or RAS-GTP activity levels, respectively, in response to PDGF-BB (10 ng/ml) following sunitinib (0.2 μM) pre-treatment for 1 h. **d** Representative western blot demonstrating inhibition of PDGFRβ, AKT and ERK phosphorylation in response to 10% serum following 1 h sunitinib pre-treatment (0.2 μM) of Daoy cells.

Fig. 2. Sunitinib treatment increases PTEN expression in PDGF-treated medulloblastoma cells. Representative western blot of Daoy cell lysates (*upper panel*) demonstrating a relative reduction in the level of total PTEN in response to PDGF-BB (10 ng/ml) and a relative increase in the expression of total PTEN in response to PDGF-BB when cells are pre-treated for 1 h with 0.2 μ M sunitinib. Densitometry analysis of multiple western blots of Daoy cell lysates (*lower panel*) demonstrates a significant increase (* $P < 0.05$) in total PTEN expression in PDGF-stimulated Daoy cells pre-treated with sunitinib compared to untreated, PDGF-stimulated control cells

Fig. 3. Sunitinib treatment inhibits PDGF-mediated EGFR transactivation in medulloblastoma cells. **a** Representative western blot of Daoy whole cell lysates demonstrating that 1 h pre-treatment with 0.2 μ M sunitinib abolishes PDGF-BB (10 ng/ml)-mediated transactivation (phosphorylation) of EGFR (*left panel*) but does not alter EGF (50 ng/ml)-mediated activation of EGFR. **b** Densitometric analysis of multiple western blots of Daoy (*upper panel*) and D556 (*lower panel*) whole cell lysates demonstrating that sunitinib pre-treatment completely abrogates (* $P < 0.05$) PDGF-BB mediated, but not EGF-mediated, phosphorylation of EGFR

Fig. 4.

Sunitinib treatment inhibits medulloblastoma cell migration. **a** Results of multiple Boyden chamber 4 h migration assays demonstrating that the relative fold-change in PDGF-BB-mediated (20 ng/ml) migration over basal migration is significantly inhibited by 1 h sunitinib (0.2 μM) pre-treatment of Daoy and D556 cells (* $P < 0.01$ and * $P < 0.05$, respectively). **b** Representative photomicrographs demonstrating that in longer term 24 h wound healing migration assays, PDGF-BB (20 ng/ml)-mediated cell migration of Daoy (*upper panel*) and D556 (*lower panel*) cells is inhibited after 1 h sunitinib (0.2 μM) pre-treatment. **c** Results of multiple wound healing migration assays demonstrating that the calculated area (micron²) of wound closure by sunitinib-treated cells is significantly reduced (* $P < 0.005$) in response to both 20 ng/ml PDGF-BB (*upper panel*) and 10% serum (*lower panel*)

Fig. 5. Sunitinib induces cell detachment and inhibits viability of D556 cells at 48 h. **a** Representative photomicrograph demonstrating dose-dependent detachment of D556 cells at 48 h in response to increasing concentrations of 1 h sunitinib pre-treatment. **b** Results of multiple cell counting assays demonstrating significant inhibition (* $P < 0.05$) of D556, but not Daoy, cell viability in response to PDGF-BB (20 ng/ml) 48–72 h following 1 h pre-treatment with 0.2 μ M sunitinib

Fig. 6. Sunitinib treatment inhibits PS125 primary murine medulloblastoma cell migration and PDGFR signaling. **a** Representative photomicrographs demonstrating that in a long term 24 h wound healing migration assays, PDGF-BB (20 ng/ml)-mediated cell migration of PS125 cells is inhibited after 1 h sunitinib (0.2 μM) pre-treatment. Results of multiple wound healing migration assays demonstrating that the calculated area (micron²) of wound closure by sunitinib-treated cells is significantly reduced (* $P < 0.005$) in response to both 20 ng/ml PDGF-BB and 10% serum. **b** Representative western blot of whole cell lysates demonstrating inhibition of PDGFRB, SHP2, AKT and ERK phosphorylation and increase in PTEN expression with no change in total RAS expression in response to PDGF-BB (10 ng/ml) following 1 h sunitinib pre-treatment of PS125 cells

Fig. 7. Sunitinib inhibits viability of PS125 primary murine medulloblastoma cells. *Graph* representing the average rate of proliferation in PS125 mouse medulloblastoma cells treated with increasing doses of sunitinib (0.1, 0.2, 0.4, and 0.8 μ M, however, only 0.1 and 0.2 μ M doses were plotted as there was no significant difference in response between the 0.2 μ M dose and the higher doses) for 1, 2, 3, 4, 5, and 8 days compared to untreated controls. There was no significant change in cell viability as determined by the XTT colorimetric absorbance in all treatment groups at 24 and 48 h post treatment compared to untreated controls. However, by 72 h post treatment at all concentrations of drug tested led to a significant ($P < 0.05$) reduction in the viability of the treated cells compared to the untreated controls. Results represent the mean $\pm$ SE of mean of multiple experiments