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The Mitotic Kinesin KIF11 is a Central Driver of Invasion, Proliferation, and Self Renewal in Glioblastoma

Monica Venere^{1,2}, Craig M. Horbinski³, James F. Crish¹, Xun Jin², Amit Vasanji⁴, Jennifer Major¹, Amy Burrows¹, Cathleen Chang², John Prokop², Quilian Wu², Peter A. Sims^{5,6}, Peter Canoll⁷, Matthew K. Summers¹, Steven S. Rosenfeld^{1,*}, and Jeremy N. Rich^{2,*}

¹Department of Cancer Biology, Cleveland Clinic Foundation, Cleveland, OH 44195

²Department of Stem Cell Biology and Regenerative Medicine, Cleveland Clinic Foundation, Cleveland, OH 44195

³Department of Pathology and Laboratory Medicine, University of Kentucky College of Medicine, Lexington, KY, 40506

⁴ImageIQ, Inc., Cleveland, OH 44128

⁵Department of Systems Biology, Columbia University Medical Center, New York, NY 10032

⁶Department of Biochemistry & Molecular Biophysics, Columbia University Medical Center, New York, NY 10032

⁷Department of Pathology & Cell Biology, Columbia University Medical Center, New York, NY 10032

Abstract

The proliferative and invasive nature of malignant cancers drives lethality. In glioblastoma, these two processes are presumed mutually exclusive and hence termed “go or grow”. Here, we identified a molecular target that shuttles between these disparate cellular processes—the molecular motor KIF11. Inhibition of KIF11 with a highly specific small molecule inhibitor stopped the growth of both the more treatment resistant glioblastoma tumor initiating cells (TICs, or cancer stem cells) as well as non-TICs and impeded tumor initiation and self-renewal of the TIC population. Targeting KIF11 also hit the other arm of the “go or grow” cell fate decision by reducing glioma cell invasion. Administration of a KIF11 inhibitor to mice bearing orthotopic glioblastoma prolonged their survival. In its role as a shared molecular regulator of cell growth and motility across intratumoral heterogeneity, KIF11 is a compelling target for glioblastoma.

*To whom all correspondence should be addressed at: rosenfs@ccf.org (for SSR) and richj@ccf.org (for JNR).

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Introduction

The prognosis for patients afflicted with glioblastoma (GBM) has remained grim, despite decades of translational and clinical investigation. Several features contribute to the malignant phenotype of this disease. GBM has a high proliferative capacity that is supported by a highly pro-angiogenic microenvironment (1). In addition, although GBM rarely metastasizes outside the central nervous system (CNS), it is capable of widely disseminating within the brain—a feature that severely limits the efficacy of surgery and radiotherapy (2, 3). Each of these features is augmented in a subset of GBM cells that have stem cell-like properties and are referred to as glioblastoma tumor initiating cells (TICs). TICs are resistant to radiotherapy and alkylating chemotherapy, drive angiogenesis, and are highly invasive (4). These features have led to efforts in multiple laboratories to find points of vulnerability for the TIC population. However, under some circumstances, the non-TIC subpopulation can assume TIC properties (5, 6). This implies that effective GBM therapy will require use of either two classes of drugs—one to target TICs and another to target the non-TIC population—or one class that targets both populations. Such a target would therefore be expected to play several essential roles in maintaining the GBM phenotype. First, it would drive mitosis in order to support tumor cell proliferation. Second, it would be needed for cell motility, which underlies tumor cell dispersion. Finally, it would be beneficial to block such a target with highly specific, high affinity small molecule inhibitors.

Mitosis and cell motility require the microtubule-based cytoskeleton, and these cellular physiologies are important not only for GBMs, but for a number of other highly aggressive malignancies as well. Several classes of drugs that inhibit microtubule dynamics, including the taxanes, vinca alkaloids, and epothilones, have been used successfully in treating hematologic and solid malignancies (7). However, the microtubule-based cytoskeleton is crucial for CNS function, including axonal transport; and neurotoxicity is the dose-limiting side effect of many of these drugs (8). This has spurred efforts to identify and target microtubule-associated proteins (MAPs) whose inhibition would block mitosis without producing neurotoxicity. One class of MAPs that appear to satisfy these requirements are a group of molecular motors, the mitotic kinesins, that orchestrate a number of steps in the mitotic process, including chromosome congression, formation of the mitotic spindle, kinetochore microtubule dynamics, and cytokinesis (9). Highly specific small molecule inhibitors directed against several of these have been developed in both preclinical models and in clinical trials (10), and as expected, these drugs have not produced the neurotoxicity seen with microtubule poisons. Furthermore, an inhibitor of one of these, KIF11 (also known as EG5 or Kinesin-5), is accruing patients in multiple Phase II trials in recurrent multiple myeloma with plans for a Phase III trial in the near future (11).

KIF11 is a plus end directed kinesin required for formation of the bipolar spindle in metaphase, where it opposes the action of minus end directed molecular motors (12). It is the target for over twenty high-affinity, specific small molecule inhibitors that all bind to the same structural motif in the catalytic domain (13). Suppression of KIF11 function results in either prolonged mitotic arrest leading to cell death in mitosis or inappropriate progression through mitosis that is subsequently followed by cell death (14). Interestingly, KIF11 also appears to have non-mitotic functions as well. It has been shown to regulate axonal

branching and growth cone motility and, more recently, was shown to be involved in cell motility (15–20). Although the importance of KIF11 in the malignant behavior of GBM cells has never been explored, the evidence that it is required for mitotic progression as well as for cell motility suggests that it may play an especially important role. We therefore sought to determine if KIF11 is indeed an essential driver of both the proliferative and invasive behaviors that are characteristic of GBM.

Results

Mitotic kinesins, including KIF11, are upregulated in GBM

As proliferation depends on mitotic kinesins, we sought to characterize their expression in human GBM. We queried the expression of 17 kinesins in a dataset generated by RNA-seq of MRI-localized biopsies of human GBM (21). These included samples from the highly cellular, contrast enhancing core (CE) and the non-enhancing (NE) margins of GBM, which harbor the infiltrating glioma cells that often escape surgery and give rise to recurrence. Previous analysis of this dataset showed that GBM subtypes, as defined by the Verhaak classifier, differed in the expression of genes associated with proliferation (21, 22). We therefore performed differential expression analysis (DEseq) to determine which kinesins are significantly increased or decreased in each GBM subtype compared to non-neoplastic brain samples (Fig. 1A). This analysis revealed that most mitotic kinesins, including KIF11 for which clinically relevant small molecule inhibitors exist, were elevated in the CE samples across subtypes with the highest expression in the proneural subtype—a pattern that was most pronounced when examining the samples from the NE margins of GBM. In an effort to understand the potential impact of KIF11 inhibition on nonneoplastic cells as well as tumor cells, we calculated the Spearman correlation between *KIF11* and two sets of cell type-specific genes: those expressed by TICs and OPC-like proneural glioma cells, and those expressed by the major lineages found in brain tissue, including astrocytes, microglia, oligodendrocytes and neurons. Results show that *KIF11* expression is highly correlated with markers of TIC and OPC/proneural glioma cells in both the CE core and NE margins of GBM (Fig 1B). *KIF11* expression does not correlate with markers of oligodendrocytes or neurons despite the abundance of these cell types in the margins. This is important because the infiltrating tumor cells that reside in the margins are the target for post-surgical chemotherapy. These findings suggest that ispinesib would selectively target tumor cells and not neurons or oligodendrocytes. To understand the genes most strongly correlated with *KIF11* expression in the CE core and NE margin, we performed an unsupervised gene ontology analysis (iPAGE) with the resulting highly enriched ontologies associated with mitotic cell cycle and DNA replication genes (fig S1). Together, these results suggest that mitotic kinesins in general, and *KIF11* in particular, are expressed in proliferating and migrating cells in glioma.

We also compared three matched sets of TICs and non-TICs from patient-derived xenografts that span the spectrum of GBM subtypes for the differential expression of a group of mitotic regulators using quantitative PCR (fig S2). Of these, only *KIF11* was consistently elevated above two-fold (Fig. 1C). Prospective isolation of TICs and non-TICs was used so that mRNA could be acutely generated with minimal exposure of the cells to tissue culture

conditions and potential changes in expression profiles. The glycosylated cell-surface epitope CD133 was used to separate TICs from non-TICs. TICs were functionally validated for their ability to self-renew and form tumors in immunocompromised mice (23–25). These data establish upregulation of mitotic kinesins as a hallmark of GBM and implicate KIF11 as a putative therapeutic target in GBM. Furthermore, although KIF11 inhibitors have been extensively studied clinically, they have never been examined in GBM (13). This motivated us to examine the roles of KIF11 in the GBM phenotype in order to determine if it would be a compelling therapeutic target for treatment of this disease.

KIF11 protein levels are elevated in TICs due in part to attenuated protein turnover

Having established KIF11 as a potential target in GBM, we then wanted to further explore the expression differences of KIF11 between TICs and non-TICs. We isolated matched TICs and non-TICs from patient-derived xenografts and evaluated KIF11 levels by Western blot. TICs and non-TICs were grown in the same culture conditions for all experiments. We have previously used this approach to maintain the TIC or non-TIC phenotypes while also providing mitogenic signals that produce similar cell cycle profiles and proliferation rates (fig. S3A) (26, 27). In all three TIC/non-TIC pairs tested, KIF11 protein levels were higher in the TICs (Fig. 2A). To insure that the TIC and non-TIC phenotypes were maintained after sorting, we probed for OLIG2, a marker for TICs, and for GFAP, a marker for more differentiated non-TICs. We found a higher level of OLIG2 protein in the TICs, and conversely, a higher level of GFAP protein in the non-TICs (Fig. 2A).

In non-transformed cells, levels of KIF11 fluctuate throughout the cell cycle and are regulated by targeted protein destruction by means of ubiquitination (28, 29). The anaphase promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase that is a central mediator of the ubiquitin-mediated degradation of mitotic proteins. APC/C substrate specificity is dictated by its association with the substrate adaptors Cdc20 (APC/C^{Cdc20}; active in early mitosis) or Cdh1 (APC/C^{Cdh1}; active in late mitosis and during G1). KIF11 is ubiquitinated and degraded in an APC/C^{Cdh1}-dependent manner to reduce its levels in G1 (28, 29). To determine whether KIF11 turnover differs between TICs and non-TICs and whether this contributes to the differences in protein levels, we utilized a double-thymidine block to arrest TICs and matched non-TICs from two human GBM xenografts. The cells were then released from the arrest and samples were harvested for protein lysates every two hours over a 10-hour time course for specimen 3691 and over a 24-hour time course for specimen 08-387, and the resulting lysates were probed for KIF11 by Western blot. Flow cytometry was performed to validate the degree of cell synchronization (fig. S3A). While KIF11 levels in non-TICs rose in G2/M and then fell once mitosis was complete, as previously reported for non-transformed cells (29), TICs maintained high levels of KIF11 throughout the cell cycle (Fig. 2B; fig. S3B). Since APC/C^{Cdh1} targets KIF11 for destruction, this finding suggests that there is a defect in APC/C^{Cdh1} activity in TICs. In order to confirm this, we examined the levels of an additional APC/C^{Cdh1} target, CDC20 in TICs and non-TICs. We found that as in the case of KIF11, levels of CDC20 failed to drop in G1 in TICs (fig. S3C). These results imply that APC/C^{Cdh1}, a central component in cell cycle regulation and tumor suppression, is dysfunctional in TICs (30). To gain additional insight into this postulate, we treated TICs and non-TICs with nocodazole to arrest cells at the start of M phase.

Mitotically arrested cells were then released from the nocodazole block. Four hours later, at a time when the cells were in late M/G1 and where APC/C^{Cdh1} should be active, cell extracts were generated. The activity of APC/C^{Cdh1} was directly tested by introducing hemagglutinin (HA)-tagged Securin, an APC/C^{Cdh1} substrate, into the lysates. The levels of HA-Securin were evaluated over time by Western blot to monitor the extent of protein turnover during the time course between TICs and non-TICs. HA-Securin exhibited greater stability in extracts of G1 TICs indicating that the activity of APC/C^{Cdh1} is attenuated in TICs compared to non-TICs (Fig. 2, C and D). These results indicate that chronic expression of central cell cycle regulators such as KIF11 and CDC20 is a key feature in TICs and establishes attenuated ubiquitin-mediated proteolysis as a contributing factor to their differential protein expression between TICs and non-TICs.

KIF11 inhibition targets TICs and compromises their ability to self-renew

In order to examine the importance of KIF11 in GBM pathophysiology, we utilized ispinesib, a cell permeable and highly specific small molecule inhibitor to KIF11 (31). We first determined the dose response relationships for cytotoxicity *versus* ispinesib concentration for matched TICs and non-TICs from 5 independent xenograft specimens after 72 hours of ispinesib exposure. The mean EC₅₀ of ispinesib for these 5 TIC samples (1.15 ± 0.35 nM) was slightly but significantly lower than that for the matched non-TICs from the same tumor (1.79 ± 0.22 nM, $p = 0.0085$) (Fig. 3A). We next exposed TICs and non-TICs for 96 hours to 3 nM ispinesib and used flow cytometry to measure the subG1 population, a surrogate marker for apoptosis. TICs demonstrated a higher subG1 fraction over non-TICs in the three xenograft specimens over the four days of observation (Fig. 3B). To confirm these findings, an ATP-based viability assay was used to monitor the impact of exposure to 3 nM ispinesib over a five-day time course for two matched TICs and non-TICs. Both populations demonstrated sensitivity to KIF11 inhibition with the TICs showing a slight increased sensitivity over the non-TICs at earlier time points (Fig. 3C). These data indicate that while TICs and non-TICs may have slightly different sensitivities to ispinesib, proliferation of both cell populations is effectively inhibited by this drug.

We also examined the effect of transient KIF11 inhibition on TIC self-renewal as measured by the ability of a single cell to form a tumorsphere in an *in vitro* limiting dilution assay (32). We sorted TICs from 3 different patient-derived xenografts (3 independent tumors per specimen), and allowed them to recover overnight before pretreatment for 18 hours with 3 nM ispinesib or vehicle control. Cells were then released from drug inhibition and plated using flow cytometry at a range of 1 cell per well to 50 cells per well and scored for tumorsphere formation ten days later. In all specimens tested, stem cell frequency was compromised in TICs pre-exposed to ispinesib ($p = 1.37\text{E-}23$ for 3565; $p = 1.12\text{E-}16$ for 3691, and $p = 9.96\text{E-}26$ for 08-387) (Fig. 3D; fig. S4). Together these data support KIF11 as a robust target that compromises viability of both TICs and non-TICs with the ability to also impact TIC self-renewal.

KIF11 is required for the motility and morphogenesis of TICs and affects microtubule polymerization

While KIF11 has been considered to be an essential component in mitosis, there have been a number of reports suggesting that it also has a role outside of the mitotic cycle, including in the regulation of cell motility, axonal branching, and angiogenesis (15–20, 33, 34). A potential caveat to the interpretation of some of these studies, however, is that cell motility ceases during mitosis, when both the microtubule- and actin-based cytoskeletons are recruited to form mitotically important structures, including the spindle and the cytokinetic ring. Hence, studying a putative extra-mitotic role for KIF11 in cell motility or morphogenesis requires ensuring that any effects of KIF11 inhibition on these processes occur in cells that are outside of mitosis. In order to accomplish this, we treated TICs with a double-thymidine block in order to arrest them at the G1/S boundary. Cells were then released from the arrest and allowed to reenter the cell cycle in a synchronized manner. Their cell cycle state was monitored by flow cytometry to determine when the cells were enriched in G1 (fig. S5). Approximately twelve hours after release from the thymidine block, when the cells had fully recovered from the arrest and had begun to enter G1 (fig. S5), they were plated in a 3 μ m Transwell assay in the presence of increasing concentrations of ispinesib (Fig. 4A). After 8 hours, while the cells were still largely outside of G2/M, they were fixed and nuclei stained with DAPI for visualization and quantification of cells that had migrated through the membrane. Ispinesib blocked Transwell migration under these conditions with an EC₅₀ of 67.2 nM (Fig. 4B). In the presence of 200 nM ispinesib, approximately 6-fold fewer cells migrated per field through the Transwell compared to vehicle control (Fig. 4C). We next examined the effect of ispinesib on glioma dispersion within brain tissue using a rodent GBM model produced by intracranially injecting a bicistronic retrovirus encoding for PDGF and GFP in 3 day old rat pups (Fig. 4D). As we have previously shown, diffusely infiltrating tumors with the histological features of GBM develop robustly in this rodent model within 10 days post injection (35). The retrovirus-infected cells are highly migratory, proliferative and express the OPC/proneural markers OLIG2, PDGFR α and NG2 (35). Furthermore, although TICs are thought to represent a relatively small subpopulation, SOX2 and OLIG2 are seen in the vast majority of cells in Proneural GBM, and represent the predominant proliferating population in these tumors (21, 36). We generated 300 μ m thick sections through the tumor-bearing portion of the brain and monitored the effect of ispinesib (200 nM) or vehicle (DMSO) on the migration of the fluorescent tumor cells using time-lapse microscopy. Slices were counterstained with rhodamine-labeled isolectin b4 in order to fluorescently label tumor-associated microglial cells and allow for visualization of the interface between tumor and the surrounding non-neoplastic, microglial-rich interface. Individual green fluorescent tumor cells (n = 50) from the treatment and control groups from 6 separate recordings were tracked over the course of 9.5 hours. Representative videos of ispinesib and vehicle-treated brain slices are shown in Supplement Movies 1 and 2. Cell tracks were transposed to a common origin to generate Wind Rose plots for ispinesib and vehicle-treated samples (Fig. 4E). These demonstrate a reduction in tumor dispersion, as illustrated by a plot of mean squared displacement (MSD) *versus* time (Fig. 4F). Data were fit to a Persistent Random Walk model (37), which relates MSD to time by the equation:

$$MSD=2S^2P \bullet [t-P(1-\exp(-t/P))]$$

where S is cell velocity and P is the persistence time (average length of time a cell moves in a single direction). This analysis reveals that ispinesib reduces cell velocity by approximately two-fold ($21.9 \pm 0.1 \mu\text{m/hr}$ for control, versus $11.3 \pm 0.2 \mu\text{m/hr}$ for ispinesib treated) but has little effect on persistence time (1.9 ± 0.02 hrs for control *versus* 2.3 ± 0.1 hrs for ispinesib treated). Since MSD varies as the square of velocity, a two-fold reduction in the latter corresponds to a four-fold reduction in the former.

Microtubules play important roles in cell motility. Several mitotic kinesins, including KIF11, are known to affect tubulin polymerization at the dynamic, plus end, which in interphase cells is located near the cell periphery at the leading edge (15–17). This implies that KIF11 inhibitors, such as ispinesib, might inhibit cell motility by altering microtubule lengthening at the leading edge of migrating cells. To test this hypothesis, we first seeded TICs enriched in interphase via cell synchronization (fig. S5) at a low density in the presence of vehicle or 200 nM ispinesib and monitored the kinetics of cytoplasmic process formation in these cells. Process formation in TICs was appreciably attenuated by drug treatment ($p < 0.0001$) in a time-dependent manner (Fig. 5, A and B). Next, we examined the effect of ispinesib on microtubule content in interphase-enriched TICs (fig. S5). We plated TICs in a wound assay in order to induce cell polarization toward the cell free zone. Cells were treated with either vehicle or 200 nM ispinesib for 6 hours, fixed, permeabilized and stained with anti-tubulin primary antibody followed by a secondary antibody conjugated to AlexaFluor488. While vehicle treated cells at the wound edge displayed organized arrays of microtubules, ispinesib treatment reduced their appearance, and few cells had developed processes (Fig. 5C). We measured the intensity of the AlexaFluor488 signal with confocal microscopy, and this revealed that ispinesib treatment significantly ($p < 0.0001$) decreased polymerized tubulin in the TICs (Fig. 5D). These findings highlight KIF11 as a mediator of the TIC invasive phenotype that can be attenuated with small molecule inhibition.

KIF11 inhibition impacts TICs *in vivo*

Our results so far show that targeting KIF11 blocks both invasion and proliferation in both TICs and non-TICs, and therefore suggest that KIF11 may be a very compelling therapeutic target. We therefore next examined how ispinesib affects the defining features of TICs—tumor initiation and propagation. We started with a flank tumor model where GBM cells (100,000/mouse) were injected into NSG mice. When tumors reached 0.12 cm^3 , mice were randomized into either vehicle (DMSO) or ispinesib (10 mg/kg) treated groups with drug or vehicle given daily for 7 consecutive days via intraperitoneal injection. Flank tumor volume was measured daily. Two hours after the last administration, tumors were harvested and weight and volume measured with ispinesib treatment leading to significant reduction of both (Fig. 6, A and B). We then evaluated the excised tumors by immunocytochemistry for the marker SOX2, which is expressed both by TICs and by transformed OPCs. Ispinesib treated tumors showed no SOX2 positive cells (Fig. 6C). These findings support *in vivo* cell killing of GBM cells by ispinesib, including the TIC subpopulation.

Tumor initiation is a defining characteristic of TICs. To determine whether ispinesib treatment alters tumor initiation and survival in orthotopic xenograft models, we pretreated TICs *in vitro* for 18 hours with either 3 nM ispinesib or an equal volume of vehicle (DMSO). Cells were then intracranially implanted at 2,500 or 25,000 cells per mouse (n = 10 mice per group for vehicle, n = 7 for ispinesib). Mice were monitored daily for weight loss and neurological signs indicative of brain tumor development. TICs that had been treated with ispinesib were significantly impaired in the ability to form secondary tumors, further confirming a compromised stem cell phenotype (Fig. 6D). At the first sign of neurologic impairment within the vehicle group, mice were also harvested from the ispinesib group to evaluate tumor burden. Representative hematoxylin and eosin stained brains from these mice indicate tumor burden only within the vehicle group, supporting a delay in tumor development for drug treated TICs (Fig. 6D). In an effort to evaluate the impact of *in vivo* exposure to ispinesib on tumor initiation, we treated mice with established subcutaneous tumors for 3 days with 10 mg/kg ispinesib or with vehicle. The tumors were then dissociated, and FACS isolated CD133-positive TICs from each group were intracranially injected at 1,000 or 10,000 cells per mouse (n = 10 mice per group). In line with *in vitro* exposure to ispinesib, *in vivo* targeting of TICs also reduced their tumor initiating capability (Fig. 6E). Altogether, these data indicate that TICs, which are well appreciated to resist alkylating chemotherapy and radiation therapy, are highly sensitive to a KIF11 inhibitor *in vivo*.

High KIF11 portends poor patient prognosis and targeting improves survival in a preclinical model

To evaluate the efficacy of KIF11 inhibition in a clinically relevant system, we utilized a TIC-derived orthotopic xenograft model to examine the effect of systemically delivered ispinesib on survival. We first validated that ispinesib reaches an intracranial tumor by administering a single 10 mg/kg dose to an orthotopic tumor-bearing mouse and harvesting the tumor 5 hours later. The tumor was sectioned and probed for tubulin to evaluate for the presence of mono-astral spindles, a histological hallmark of KIF11 inhibition (38). The ispinesib-treated tumor contained numerous cells with mono-astral spindles (Fig. 7A) which prompted us to evaluate the effect of systemically administered ispinesib on survival in an orthotopic GBM model, in which 10,000 luciferase-expressing TICs were injected intracranially in each of a group of 20 NSG mice. After waiting 7 days, mice were then randomized into two groups of 10, with one group receiving vehicle (DMSO) and the other ispinesib (10 mg/kg, administered every 4 days for 6 doses), both administered by intraperitoneal injection. We monitored each cohort for weight loss and neurological signs indicative of brain tumor development. Mice treated with ispinesib demonstrated a significant survival advantage over those treated with vehicle ($p < 0.001$) with a median survival of 36 days versus 24 days for the DMSO vehicle cohort (Fig. 7B). These data therefore demonstrate that KIF11 inhibition is effective in a preclinical model, where it appears to prolong tumor latency and survival.

We probed a new, well-annotated tissue micro-array from normal and glioma patients and found that KIF11 protein levels correlate positively with WHO grade ($p = 0.0001$; Fig. 7C), and in the case of grade III and grade IV glioma, with patient survival ($p = 0.047$; Fig. 7D;

table S1). Within GBM (WHO grade IV), KIF11 protein levels were markedly elevated over normal brain, with the specificity of the KIF11 antibody validated by localization to the spindles of mitotically active cells (Fig. 7, E and F). These data establish that KIF11 is consistently upregulated in GBM at the protein level, and they implicate KIF11 as a putative therapeutic target.

Discussion

Proliferation and dispersion are hallmarks of the malignant phenotype and both contribute to the grim prognosis that characterizes glioblastoma (2). Thus, an ideal therapeutic target would be a cellular component that drives both proliferation and dispersion in both TICs and non-TICs. The cytoskeleton is an essential component for both mitosis, which drives tumor proliferation, and cell motility, which drives invasion. Microtubules play important roles in cell motility, including membrane vesicle transport, signal transduction, and stabilization and cross-linking of actin microfilaments; and their role in generating the mitotic spindle is well known (39). They can be targeted with a variety of clinical grade drugs that block microtubule dynamics, including the vinca alkaloids and taxanes. These drugs have been very effective in treating a variety of malignancies (7). However, microtubules are essential for CNS function, which means that therapeutic levels of these microtubule inhibitors in the CNS would be expected to produce severe neurotoxicity, a common issue for the peripheral nervous system after systemic administration of these drugs (8).

These considerations have served as the rationale for developing small molecule inhibitors directed at the mitotic kinesins that are largely expressed in mitosis and are involved in forming the mitotic spindle (10). Such a target would be expected to be markedly upregulated in a mitotically active CNS tumor, such as GBM, compared to the relatively post-mitotic brain. The most intensively investigated of these mitotic motors is KIF11, a member of the kinesin 5 family, for which well over twenty inhibitors have been synthesized (13). Unlike the vinca alkaloids and taxanes, KIF11 inhibitors are not neurotoxic—a point of direct relevance for a possible GBM therapy (10). However, clinical trials with some of these drugs, none of which were used in GBM, have been disappointing (40). These inhibitors have a short half-life and have largely been used in tumors with a low proliferation index. This mismatch between pharmaco- and cell cycle kinetics means that it is unlikely adequate levels of drug can be sustained long enough to kill large numbers of tumor cells when they are most vulnerable—in the mitotic cycle. However, more recent clinical trials with other KIF11 inhibitors that have a long half life (ARRY-520) or can be given by oral metronomic dosing (4SC-205) have been much more encouraging, particularly when combined with other drugs that synergize (41, 42). Unlike many other solid tumors, GBM has a high proliferation index, and it rarely spreads outside the CNS, so optimizing regional delivery of a KIF11 inhibitor to a largely post-mitotic tissue, such as brain, should improve efficacy and reduce toxicity. Consequently, we began our investigation of KIF11 by examining its expression in GBM at both the RNA and protein levels. Our annotated tissue microarray data clearly demonstrated that KIF11 protein expression correlates with both glioma grade and with patient outcome. This finding is complemented by our DEseq expression analysis, which shows that mitotic kinesins have the greatest increase in the proneural subtype of GBM, and this pattern is most pronounced in samples taken from the

non-enhancing margins of the tumor. KIF11 expression is highly correlated with genes that are expressed in TIC and OPC-like proneural glioma cells. While TICs are thought to represent a relatively small subpopulation, SOX2 and OLIG2 are highly expressed by OPC-like glioma cells, which account for the vast majority of cells in Proneural GBM, and represent the predominant proliferating population in these tumors (21, 36). Similarly, an unsupervised gene ontology analysis (iPAGE) showed KIF11 expression is most highly correlated with genes associated with mitotic cell cycle and DNA replication. These results show that KIF11 expression is increased in proliferating populations in GBM, both in the contrast-enhancing core and in the non-enhancing margins, which harbor the infiltrating glioma cells that give rise to recurrence.

The TIC subpopulation contains the most treatment-resistant subset of cells that constitute a GBM. We find that KIF11 expression is upregulated in TICs compared to non-TICs at both the RNA and protein levels. While increased transcription might account for some of this increased KIF11 expression, our data also clearly demonstrated that increased KIF11 in TICs also reflects a defect in protein degradation at the level of the APC/C^{Cdh1}. The delayed kinetics of KIF11 protein turnover likely overlaps with cell cycle regulated transcription of KIF11 at the G1 to S-phase transition, maintaining KIF11 at a chronically higher level in TICs. Furthermore, failed protein turnover of KIF11 by APC/C^{Cdh1} has been reported to impair clustering of supernumerary centrosomes, a common feature of cancer cells (28). Thus, TICs might maintain their viability by balancing genomic instability caused by the reported persistent DNA damage and/or by the loss of APC/C^{Cdh1}, a key regulator of genomic stability, with altered cell cycle regulation (23, 27, 30). Additionally, KIF11 has been reported to play a role in polypeptide synthesis in interphase and the differential levels of KIF11 between TICs and non-TICs could alter the kinetics and fidelity of protein translation (43).

Both TICs and non-TICs are exquisitely sensitive to nanomolar concentrations of ispinesib although our results support increased sensitivity for TICs. These data could indicate an increased dependence on KIF11 for TICs and/or an increased sensitivity to perturbation of mitosis. The latter hypothesis is supported by our own data regarding altered APC/C^{Cdh1} activity as well as recent findings that TIC viability is compromised when other key mitotic regulators, such as CDC20 and BUBR1, are targeted (44, 45, 46).

Beyond viability, the key stem cell phenotypes of self-renewal and tumor initiation are abrogated following KIF11 inhibition. Furthermore, systemic administration of ispinesib to mice bearing orthotopic, TIC-derived GBMs produces the expected phenotype of monopolar spindles, as well as a prolongation in survival, implying that sufficient amounts of drug reaches the intracranial tumors to exert a therapeutic effect. Taken together, our results indicate that ispinesib has a broad-spectrum anti-proliferative activity against the major subpopulations that constitute a GBM. While it is well appreciated that the TIC subpopulation can generate non-TICs, recent evidence suggests that under at least some conditions, the reverse is also true, implying that there is dynamic interchange between the TIC and non-TIC populations (5, 6). This suggests that therapies that are only active against TICs may ultimately fail, if the remaining non-TICs can repopulate the TIC niche. Thus, our results with KIF11 provide an example of a cellular component whose inhibition targets

both TICs and non-TICs, and which may therefore prevent the inevitable recurrence that is seen in humans afflicted with GBM.

Inhibition of KIF11 induces axonal lengthening and branching, suggesting that this motor acts as a “brake” on microtubule sliding and regulates microtubule entry into neuronal dendrites (16, 17). In human foreskin fibroblasts, inhibition of KIF11 blocks cell motility and membrane ruffling on a 2 dimensional surface when non-muscle myosin II is inhibited (47) suggesting that KIF11 can act as an “accessory” motor in driving cell motility in the absence of myosin II. These earlier studies motivated us to determine if ispinesib also affects morphology and cell motility in TICs. An *in vitro* Transwell assay demonstrated that TIC invasion could be abolished by ispinesib, under conditions where we could rule out any appreciable effect from a block in G2/M, when cells normally become immobile. We further confirmed that ispinesib also reduced GBM dispersion through brain tissue using an *ex vivo* slice assay. Fitting to a persistent random walk model reveals that ispinesib reduces tumor cell velocity and not directional persistence. Of the 8 residues in KIF11 that interact with ispinesib, all are conserved in the KIF11 sequences from human, rat and mouse making it highly likely that the effects of ispinesib that we see in the rat *ex vivo* slice assay reflect direct inhibition of KIF11 (31). Associated with this block in dispersion is a change in tubulin polymerization and cell polarization with ispinesib. A number of studies have shown that microtubules contribute to cell motility by regulating actin polymerization, transporting vesicles to the leading edge, and controlling the localization of focal adhesions (48). Furthermore, there also is evidence that KIF11 can enhance elongation of tubulin protofilaments at the “+”, dynamic end, acting in essence as a tubulin polymerase (49).

We note that the EC₅₀ for ispinesib inhibition of Transwell migration by TICs is 30–50-fold larger than that for cytotoxicity. We believe that this difference is not likely due to an off target effect of the drug for two reasons. First, the viability dose response experiments involved prolonged exposure to ispinesib for 72 hours, while exposure in the Transwell assay was for only 8 hours. Prolonged exposure could allow for cumulative toxic effects that would not necessarily be evident by briefer exposure times. Second, the anti-mitotic effects of ispinesib are on the mitotic spindle—a structure in which KIF11 generates sustained force to oppose cytoplasmic dynein and non-claret disjunctional (ncd). Thus, even a small degree of KIF11 inhibition would lead to an imbalance of forces within the spindle, implying that a low dose of inhibitor over a sustained period of time may be sufficient to produce mitotic arrest. By contrast, if KIF11 acts more as a supplementary driver of cell motility, it might be expected that higher doses of ispinesib would be needed to detect an anti-migratory phenotype.

While the power of shRNA/siRNA transfection is well established, their use in determining if KIF11 has a separate role in cell motility is problematic. Gene knockdown with these tools generally requires several days, and during this time, it would be expected that the gradual loss of KIF11 would lead to a gradual mitotic block at the G2/M boundary, when cells normally become non-motile. Interrogating the role of KIF11 in tumor invasion requires instead a way of rapidly blocking its function intracellularly and under controlled conditions. Ispinesib is freely cell permeable, and we have shown that it binds to and inhibits KIF11 within several seconds (31). The high specificity and affinity of this drug for its

target and its rapid onset of action means that interrogating the role of KIF11 in cell motility can be most directly accomplished through the use of highly specific small molecule inhibitors, such as ispinesib.

One major limitation to this study is that our pre-clinical model did not allow for interrogation of the impact of KIF11 inhibition on invasion in an *in vivo* system. This limitation was due to the study being designed to assess the impact on survival hence the time between the last administration of ispinesib and end-point of the mice was too great to evaluate this factor. Despite this limitation, we show that ispinesib targets both TICs and non-TICs and inhibits both the proliferative and invasive phenotypes that characterize GBM. Another limitation is balancing efficacy while avoiding the dose limiting bone marrow suppression seen with systemic administration. This raises the possibility of administering KIF11 inhibitors intracerebrally with technologies that are being used clinically, such as convection-enhanced delivery (CED), which would provide for prolonged intracerebral CED using a subcutaneous implantable infusion pump (50). These considerations make clear that KIF11 inhibitors may be relevant therapies for GBM, particularly when combined with existing treatments.

Materials and Methods

Detailed materials and methods can be found in supplementary materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1. Expression of mitotic kinesins, including *KIF11*, is upregulated in GBM
(A) Differential expression analysis by RNA-Seq from radiographically-localized biopsies of non-enhancing (NE) and contrast-enhancing (CE) regions of human GBM surgical specimens (B) Correlation of cell type-specific marker genes with *KIF11* expression (C) qPCR was performed for the listed mitotic regulators on RNA isolated from TICs and matched non-TICs from three patient derived xenografts.

Fig 2. KIF11 is elevated in TICs due to attenuated protein turnover

(A) Whole cell lysates from matched TICs and non-TICs from three patient derived xenografts were probed for KIF11, OLIG2 and GFAP. (B) TICs and non-TICs from xenograft specimen 08-387 were synchronized at G1/S using a double-thymidine block. Following release, whole cell lysates were made every 2 hours over a 24-hour time course. Asynchronous (A) lysates were also harvested. Resulting lysates were probed for KIF11. (C) TICs and non-TICs from xenograft specimen 3691 were synchronized at M using a nocodazole block. Lysates were made 4 hours following release at late-M early G1 and APC^{Cdh1} activity was evaluated using exogenous HA-Securin as a substrate. Samples for immunoblot analysis were taken every 30 minutes over a 90 minute time course and probed for HA. For all immunoblots, βactin served as a loading control. Molecular weight (MW) of resulting bands is given in kilodaltons (kD). (D) Quantification of band intensity, normalized to actin.

Fig 3. KIF11 inhibition targets viability and self-renewal

(A) Matched TICs and non-TICs from patient-derived xenografts (n = 5, 3 technical replicates per specimen) were exposed to increasing concentrations of ispinesib (0 to 32 nM) for 72 hours, followed by analysis of cell viability using an ATP based assay. Error bars represent s.d., p = 0.0085, two tailed t-test (B) Matched TICs and non-TICs from 3 patient derived xenografts were evaluated for the percent of subG1 (apoptotic) cells over a 4 day time course following a single exposure to vehicle (DMSO) or 3 nM ispinesib at Day 0. (C) Matched TICs and non-TICs from 2 patient derived xenografts were monitored for cell viability via an ATP based assay over a 5 day time course following a single exposure to

vehicle (DMSO) or ispinesib. Error bars represent s.d., $n = 3$ biological replicates (with 3 technical replicates/biological), $p < 0.01$ or $p < 0.0001$, One-way ANOVA with a Bonferroni post-test. **(D)** TICs were isolated from xenografts 3565, 3691, and 08-387 (3 biological replicates for each xenograft) and plated in a limiting dilution from 50 down to 1 cell per well. Wells were scored 10 days later for the presence of a tumorsphere. Representative results from the third xenograft harvested for each specimen are shown.

Fig 4. KIF11 inhibition blocks glioma cell motility and brain invasion

(A) Schematic of Transwell assay. TICs from patient derived xenograft 08-387 were enriched within interphase using a thymidine arrest and release paradigm. 125,000 interphase-enriched cells were plated per well of a Matrigel coated Transwell and given 8 hours to migrate in the presence of increasing concentrations of isipinesib (0 to 200 nM) before fixation and staining of the nuclei with DAPI. (B) Resulting membranes were scored for the movement of nuclei through the transwell membrane. Error bars represent s.d., n = 3 biological replicates (with 3 technical replicates/biological). (C) The Transwell assay was run as above with vehicle (DMSO) or 200 nM isipinesib with data represented as the number of migrated cells per field. Error bars represent s.d., n = 3 biological replicates (with 3 technical replicates/biological), p = 0.0009, unpaired t-test. (D) Schematic of slice culture assay. A PDGF-IRES-GFP retrovirus was used to generate tumors in a rat glioma model. Resulting tumor bearing brains were isolated to generate slice cultures. GFP-positive tumor cells were monitored by time-lapse video microscopy over a 10-hour time course. (E) Wind-Rose plots of tumor cell dispersion (55 cells/group). Individual cell tracks from time-lapse microscopy were plotted to a common origin to generate Wind-Rose plots for displaying the

dispersion of tumor cells in the presence of DMSO vehicle (left, black) or 200 nM ispinesib (right, red). (F) Mean squared displacement (MSD) *versus* time for vehicle (black) and 200 nM ispinesib (red) conditions. MSD was calculated for 55 cells from control and ispinesib conditions for each time interval over 9.5 hours of observation. Black and red curves depict MSD (± 1 SD) *versus* time, and superimposed white curves depict fitting of the data to Equation 1 using a non-linear least-squared regression.

Fig 5. KIF11 plays a role in cellular process formation

(A) Nascent cellular process formation was monitored for 6 hours via time-lapse microscopy in the presence of vehicle (DMSO) or ispinesib (200 nM) in 08-387 TICs plated on GelTrex and enriched in interphase. Representative images of the two treatment groups are shown at the 6-hour time point. Cell bodies are masked in red and cell processes masked in yellow. Scale bars represent 10 μm . (B) Process length was measured over the time course. Error bars represent s.d., $n > 500$ cells/condition from 3 biological replicates, $p < 0.0001$, Two-way ANOVA with a Bonferroni post-test. (C) Representative images from a modified

scratch wound assay used to drive formation of a leading cellular process using 08-387 TICs enriched in interphase. Just prior to wound formation, media was changed to that containing vehicle (DMSO) or ispinesib (200 nM). 6 hours later, cells were fixed and processed for immunofluorescence to β tubulin. (**D**) Anti-tubulin fluorescence signal from confocal images of the primary cell layer adjacent to the wound was quantified for both treatment groups and presented as signal over area. Horizontal bar represents the mean and error bars represent s.d. $n > 40$ cells/condition, $p < 0.0001$, unpaired t-test.

Fig 6. KIF11 inhibition targets TICs *in vivo* and compromises tumor initiation

(A) Mice were flank injected with 100,000 3691 GBM cells. When tumors reached approximately 0.12 cm³, mice were randomized into one of two treatment groups; vehicle only (DMSO; n = 5) or 10 mg/kg ispinesib (n = 5) with daily administration over a 7 day time course. Arrows indicate day of vehicle or drug administration. Tumor volume was measured daily. Error bars represent s.d., $p < 0.01$, $p < 0.001$ or $p < 0.0001$, One-way ANOVA with a Bonferroni post-test. (B) Tumors were removed at Day 7 from both groups to calculate final weight. Error bars represent s.d. (C) Tumors isolated on Day 7 were processed for immunofluorescence with the stem cell marker, SOX2 (green) with nuclei were counterstained with DAPI (blue). Scale bars represent 20 μ m. (D) TICs from patient derived xenograft 3691 were pretreated *in vitro* for 18 hours with vehicle or 3 nM ispinesib. 2,500 or 25,000 viable cells were intracranially (IC) injected (DMSO, n = 10; ispinesib, n = 7). Mice were monitored for signs indicative of brain tumor development at which time they were sacrificed 3 mice from each group were harvested at the time of first indication of neurological signs amongst the groups, which occurred in the vehicle group. Representative H&E images are shown. White arrowheads indicate tumor. $p = 0.012$ for the 2,500 cohort and $p = 0.020$ for the 25,000 cohort, log-rank test. (E) Mice were flank injected with 100,000

3691 GBM cells. When tumors reached 0.2–0.6 cm³, mice were randomized into one of two treatment groups; vehicle only (DMSO; n = 5) or 10 mg/kg ispinesib (n = 5) with daily administration over a 3 day time course. Tumors were harvested and viable TICs isolated. 1,000 or 10,000 viable cells were intracranially (IC) injected into mice that were then monitored for signs indicative of brain tumor development at which time they were sacrificed. p = 0.0019 for the 1,000 cohort and p = 0.021 for the 10,000 cohort, log-rank test.

Fig 7. KIF11 informs patient prognosis and targeting improves survival in a preclinical model
(A) A mouse bearing an orthotopic tumor was injected with a single dose of 10 mg/kg ispinesib, isolated 5 hours later and processed for immunofluorescence to β tubulin with nuclei counterstained with DAPI. Arrowheads indicate cells with monoastal spindles. a' and a'' represent enlarged regions of interest. Scale bars represent 10 μ m. **(B)** 10,000 TICs from patient derived xenograft 3691 modified to express luciferase were intracranial implanted into mice. 7 days later when positive luminescence signal indicated tumor burden, mice were randomized into one of two treatment groups; vehicle only (DMSO; n = 10) or 10 mg/kg ispinesib (n = 10) administered every 4 days for 6 doses. Arrows indicate day of vehicle or drug administration. Mice were monitored for signs indicative of brain tumor development at which time they were sacrificed to generate a Kaplan-Meier survival curve. $p < 0.001$, log-rank test. **(C)** KIF11 protein levels were scored on a tissue micro-array containing normal brain control (n = 9) and patient specimens from WHO grades II (n = 25), III (n = 22) and IV (n = 47) gliomas. $p = 0.0001$, One-way ANOVA with post-hoc Tukey's test. **(D)** Kaplan-Meier curves of patients with grade III or grade IV gliomas stratified by

low or high levels of KIF11 expression (anti-KIF11 score). $p = 0.047$, log-rank test. **(E)** Representative H & E and immunohistochemistry (IHC) for KIF11 in normal brain and a GBM specimen. Scale bars represent 20 μm . **(F)** Representative signal for KIF11 within the mitotic spindle of GBM cells. Scale bars represent 10 μm .