Inhibition of radiation-induced glioblastoma invasion by genetic and pharmacological targeting of MDA-9/Syntenin

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Glioblastoma multiforme (GBM) is an intractable tumor despite therapeutic advances, principally because of its invasive properties. Radiation is a staple in therapeutic regimens, although cells surviving radiation can become more aggressive and invasive. Subtraction hybridization identified melanoma differentiation-associated gene 9 [MDA-9/Syntenin; syndecan-binding protein (SDCBP)] as a differentially regulated gene associated with aggressive cancer phenotypes in melanoma. MDA-9/Syntenin, a highly conserved double-PDZ domain-containing scaffolding protein, is robustly expressed in human-derived GBM cell lines and patient samples, with expression increasing with tumor grade and correlating with shorter survival times and poorer response to radiotherapy. Knockdown of MDA-9/Syntenin sensitizes GBM cells to radiation, reducing postradiation invasion gains. Radiation induces Src and EGFRvIII signaling, which is abrogated through MDA-9/Syntenin down-regulation. A specific inhibitor of MDA-9/Syntenin activity, PDZ1i (113B7), identified through NMR-guided fragment-based drug design, inhibited MDA-9/Syntenin binding to EGFRvIII, which increased following radiation. Both genetic (sh*mda-9*) and pharmacological (PDZ1i) targeting of MDA-9/Syntenin reduced invasion gains in GBM cells following radiation. Although not affecting normal astrocyte survival when combined with radiation, PDZ1i radiosensitized GBM cells. PDZ1i inhibited crucial GBM signaling involving FAK and mutant EGFR, EGFRvIII, and abrogated gains in secreted proteases, MMP-2 and MMP-9, following radiation. In an in vivo glioma model, PDZ1i resulted in smaller, less invasive tumors and enhanced survival. When combined with radiation, survival gains exceeded radiotherapy alone. MDA-9/Syntenin (SDCBP) provides a direct target for therapy of aggressive cancers such as GBM, and defined small-molecule inhibitors such as PDZ1i hold promise to advance targeted brain cancer therapy.

MDA-9/Syntenin | glioblastoma multiforme | radiation | EGFR | PDZ1 inhibitor

Despite advances in surgical, pharmacological, and radiation therapeutic approaches, glioblastoma multiforme (GBM) remains a particularly aggressive and ultimately an invariably fatal tumor, with a median survival of less than 15 mo and 5-y survival at 5% (1, 2). The current standard of care includes maximal surgical resection followed by radiation and temozolomide chemotherapy. However, inevitable recurrence occurs near resection margins and within the high-dose radiation field, implying that intrinsic invasiveness and radioresistance contribute significantly to relapse (3, 4). Sublethal radiation has repeatedly been shown to induce invasion and migration in surviving tumor cells, enhancing the very property that makes curative treatment so difficult (5, 6). A contributing factor to tumor relapse and recurrence is the ability of tumor cells to escape from the primary tumor mass (7), which underscores the importance of developing antiinvasive therapies that complement, and ideally enhance, conventional therapeutic approaches (8).

Melanoma differentiation-associated gene 9 (*mda-9*), also known as *syntenin* (syndecan-binding protein; SDCBP), is involved in invasion and metastatic signaling in multiple cancer settings (9–11). MDA-9/Syntenin serves critical roles in signal transduction, as well as in cell–cell and cell–matrix adhesion (12, 13). In addition to its roles in melanoma metastasis and tumor progression, MDA-9/Syntenin is highly expressed and involved in breast, gastric, and urothelial cell cancers (10, 13). MDA-9/Syntenin is an important regulator of GBM invasion, angiogenesis, and tumor progression (14), and inhibiting MDA-9/Syntenin expression decreases GBM invasion (14, 15) and enhances survival. Moreover, MDA-9/Syntenin is also overexpressed in glioma stem cells (GSCs), and is a regulator of GSC survival and stemness (16). Gene

Significance

In the setting of glioblastoma multiforme (GBM), invasion of cells into normal brain and the unlikeliness of complete surgical removal contributes to GBM lethality and recurrence. "Gold standard" GBM treatment includes adjuvant radiotherapy. Unfortunately, cells surviving radiation demonstrate increased invasion and therapeutic resistance. Melanoma differentiation-associated gene 9 (MDA-9/Syntenin) expression is elevated in patient-derived tumors and GBM cell lines, which correlates with decreased survival and poor response to radiation. Genetic suppression of MDA-9/Syntenin sensitizes GBM to radiation by inhibiting radiation-induced invasion gains and signaling changes. Additionally, intraperitoneal administration of a small-molecule MDA-9/Syntenin inhibitor, PDZ1i, developed using innovative fragment-based drug design and NMR approaches, improved survival of brain tumor-bearing mice. Survival was enhanced further when used with radiation, supporting MDA-9/Syntenin as a therapeutic target for this deadly disease.

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expression analysis of The Cancer Genome Atlas (TCGA) database revealed that patients whose tumors express high levels of MDA-9/Syntenin have a poorer prognosis and reduced survival compared with low-expressing MDA-9/Syntenin tumors (11). *mda-9/syntenin* expression correlates positively with astrocytoma grade, as analyzed through tissue samples and gene expression databases (11), and is most highly expressed in GBM. In both melanoma and glioma, MDA-9/Syntenin is involved in NF-κB activation through a proto-oncogene tyrosine-protein kinase Src (c-Src)/p38 MAPK signaling pathway (13, 15). Inhibiting MDA-9/Syntenin expression reduces NF-κB target gene expression such as matrix metalloproteinase-2 (MMP-2), a critical secreted metalloproteinase involved in GBM invasion.

A vital characteristic of MDA-9/Syntenin is the inclusion of two tandem PDZ domains, so named for their discovery in PSD-95/SAP90, DLGA, and ZO-1 (15). PDZ domains are common to a number of scaffolding proteins, and are critical for facilitating protein–protein interactions throughout various regions of the cell. MDA-9/Syntenin uses these motifs to successfully facilitate the interaction of c-Src–focal adhesion kinase (FAK) kinase complexes, noted for involvement in proinvasive signaling in cancer (13, 15). In these contexts, inhibiting the interaction between MDA-9/Syntenin and its targets in GBM might provide a strategy to inhibit invasive functions of this gene. Fragment-based lead design, or fragment-based drug design (FBDD), is an emerging and useful strategy for developing biologically active compounds (17). Using an NMR-guided FBDD approach (18) combined with in silico modeling and synthetic medicinal chemistry, we developed a PDZ1 MDA-9/Syntenin–binding small molecule, represented by compound 113B7 (herein referred to as PDZ1i), that binds with micromolar affinity to the PDZ1 domain of MDA-9/Syntenin (Fig. S1). PDZ1i does not bind appreciably to the PDZ2 domain of MDA-9/Syntenin (Fig. S1), and is long-lived in vivo after both i.v. and i.p. administration in mice (Table S1). These data indicated that PDZ1i was a suitable potential pharmacological tool to investigate the role of the PDZ1 domain of MDA-9/Syntenin in cellular mechanistic and in vivo efficacy studies.

Here we demonstrate the efficacy of supplementing radiotherapy by targeting, either genetically [small hairpin RNA for *mda-9/syntenin* (sh*mda-9*)] or pharmacologically (PDZ1i), MDA-9/Syntenin in GBM. By counteracting gains in Src, FAK, EPH receptor A2 (EphA2), and epidermal growth factor receptor (EGFR) signaling, MDA-9/Syntenin inhibition can reduce radiation-induced invasion as well as radiosensitize GBM cells, ideal properties to complement radiation treatment.

Results

MDA-9/Syntenin Inhibition Leads to Radiosensitization and Inhibition of Radiation-Induced Invasion. MDA-9/Syntenin was shown previously to be a valuable target in addressing one of the deadliest aspects of GBM, its propensity to invade (14). Because radiation therapy has been demonstrated to induce invasion in GBM cells, targeting MDA-9/Syntenin could be a useful approach to complement conventional treatment. Gliomas with higher expression of MDA-9/Syntenin are more likely to be high-grade, and patients whose tumors had high levels of MDA-9/Syntenin had a worse prognosis (14). With this in mind, we explored possible connections between MDA-9/Syntenin expression and radiotherapy in glioma using the Repository for Molecular Brain Neoplasia Data (REMBRANDT) database, a publicly available dataset with information on tumor gene expression, treatment history, and survival (https://gdoc.georgetown.edu/gdoc/). In glioma patients with no history of radiation who then underwent subsequent radiotherapy, we probed whether survival time correlated with MDA-9/Syntenin expression. Patients whose tumors had higher expression of MDA-9/Syntenin had significantly shorter survival (Fig. 1*A*; $P < 0.01$). Median survival was reduced nearly threefold in patients with gliomas expressing high levels of MDA-9/Syntenin (15.2 mo) compared with others (43.6 mo).

We then asked whether inhibiting the expression of MDA-9/Syntenin would result in changes in radiosensitivity in vitro. Using GBM cells expressing a control shRNA, U1242-shcon, or shRNA targeting

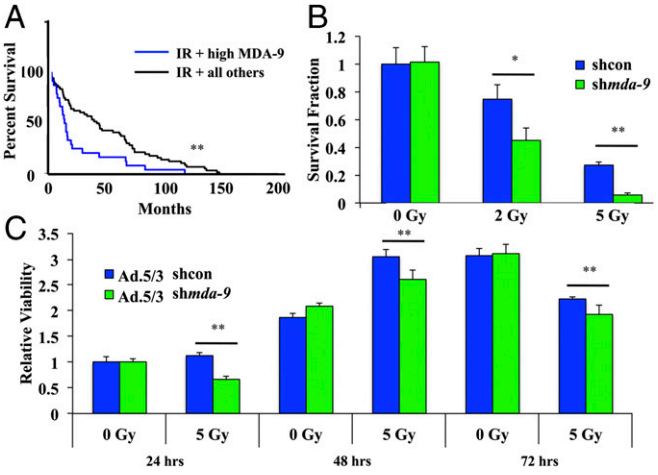

Fig. 1. MDA-9/Syntenin is involved in radiosensitivity. (*A*) The REMBRANDT database was mined for glioma patients with no prior radiation treatment who then underwent radiation therapy. These were stratified by *SDCBP* (*mda-9/syntenin*) expression (high was set at >1.5-fold overexpression). High tumor MDA-9/Syntenin led to a worse prognosis in those undergoing radiotherapy. (*B*) U1242-shcon and U1242-sh*mda-9* cells were analyzed for colony formation 14 d postradiation treatment. (*C*) U1242 cells treated with either Ad.5/3-shcon or Ad.5/3-sh*mda-9* were analyzed for viability at the indicated time points via MTT assay. Error bars indicate ±SD. $*P < 0.05$, $**P < 0.01$.

mda-9/syntenin, U1242-sh*mda-9*, we found that low levels of MDA-9/Syntenin radiosensitized GBM in a colony-formation assay (Fig. 1*B*). Furthermore, proliferation was inhibited in cells transiently reduced in MDA-9/Syntenin expression via adenoviral vector Ad.5/3-sh*mda-9* (Fig. 1*C*), indicating that MDA-9/Syntenin may play a central role in cell survival following radiation exposure.

Radiation consistently increases motility and invasion in GBM cells (5, 6, 19). Four cell lines were exposed to radiation after treatment with control adenovirus, Ad.5/3-shcon, or Ad.5/3-sh*mda-9*. Each of the cell lines received a range of radiation doses, and the dose that demonstrated maximal radiation gains was used for MDA-9/Syntenin knockdown studies. As anticipated, knockdown of MDA-9/Syntenin levels reduced invasion with no radiation exposure. After irradiation, each GBM cell line exhibited at least a twofold invasion gain, whereas MDA-9/Syntenin knockdown significantly curtailed these gains (Fig. S2). GBM invasion following radiation was reduced with MDA-9/Syntenin knockdown to about 33% of levels observed in controls, representing a substantial reduction to an undesirable side effect of conventional radiotherapy.

Knockdown of *mda-9/syntenin* Inhibits Src and EphA2 Activation Postradiation. MDA-9/Syntenin amplifies Src and NF-κB signaling in melanoma and glioma (13, 14, 20). Src is a recognized enhancer of invasive signals in cancer settings, and has demonstrated involvement in postradiation signaling (19). Whereas a modest increase in MDA-9/Syntenin levels 24 h postradiation was observed, we detected a significant increase in activated levels of Src (Fig. 2). MDA-9/Syntenin knockdown reduced those activation levels in each of the GBM cell lines tested. EphA2 is a member of the Eph receptor family, the largest subfamily of receptor tyrosine kinases, and is involved in numerous cellular processes, including angiogenesis and motility (21). EphA2 overexpression correlates with poor prognosis and increased metastasis, and is implicated in the pathogenesis of numerous tumors, including those of the brain (22). EphA2 has been shown to interact with Src in invasion signaling (23), is up-regulated following radiation, and enhances the malignant phenotype in melanoma (24). Both total and activated EphA2 levels increased after radiation exposure, yet MDA-9/Syntenin inhibition effectively reduced these gains (Fig. 2). Because MDA-9/Syntenin inhibition negated the gains in Src and

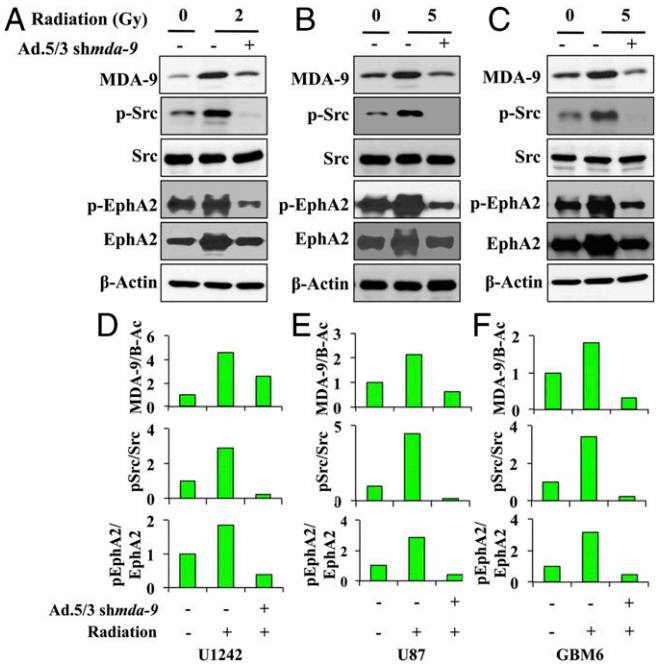

Fig. 2. Inhibition of MDA-9/Syntenin impairs Src/EphA2 signaling. (*A–C*) Immunoblot analysis of GBM cells treated with either Ad.5/3-shcon or Ad.5/3-sh*mda-9* and irradiated 48 h later. Cell lysates were collected 24 h postradiation. (*D–F*) Changes in protein levels were quantified. β-Actin is the protein loading control.

EphA2 signaling postradiation, it can be considered a valuable target in controlling radiation-induced pathogenesis.

A Small Molecule Targeting MDA-9/Syntenin Inhibits Invasion and Radiosensitizes GBM. FBDD is an important technique used with success in efforts to develop small-molecule inhibitors of challenging drug targets, including those involved in protein–protein interactions (18). NMR-based screening of an in-house assembled fragment library of about 5,000 compounds using [^{15}N,^{1}H] heteronuclear single-quantum coherence spectroscopy (HSQC) spectra with a ^{15}N-labeled PDZ1/2 tandem domain from MDA-9/Syntenin identified two hit compounds (Fig. S1). Chemical shift mapping studies revealed that these two molecules interacted mainly with the PDZ1 domain and in a region at the interface between the domains, whereas no viable fragment hits were found binding to the PDZ2 domain (Fig. S1). A combination of molecular docking studies and structure–activity relationship studies led to the synthesis of the bidentate molecule 113B7 (PDZ1i) (Fig. S1). The proposed docked structure of PDZ1i in the PDZ1 domain and interdomain of MDA-9/Syntenin is shown in Fig. S1, and the chemical structure of PDZ1i is also shown in Fig. S1. In agreement with the data with the individual fragments that compose 113B7, the molecule does not appreciably bind to the PDZ2 domain of MDA-9/Syntenin, indicating selectivity (Fig. S1). Larger-scale amounts of the compound (>500 mg) were synthesized and used for cellular and in vivo characterizations, including pharmacokinetics (PK) and efficacy studies in mice (Table S1).

Initial studies revealed that PDZ1i effectively inhibited invasion in T98G and U87 cells (Fig. S3). Moreover, it inhibited MDA-9/Syntenin–induced invasion following MDA-9/Syntenin overexpression (Fig. S3). We then tested whether PDZ1i treatment would radiosensitize glioma cells by exposing immortalized primary human fetal astrocytes (Im-PHFAs) and the GBM cell line U87 to 0-, 2-, and 5-Gy radiation after a 2-h pretreatment with PDZ1i. Normal astrocytes showed significant radiosensitivity following radiation exposure, yet PDZ1i treatment did not further radiosensitize these cells (Fig. 3*A*). However, U87 cells showed markedly more radiosensitivity when combined with PDZ1i treatment. Proliferation following radiation was also significantly decreased in U87 cells that combined radiation and PDZ1i compared with radiation with control DMSO treatment (Fig. 3*B*). Finally, U87 cells were exposed to 2 and 5 Gy of radiation, with or without PDZ1i pretreatment, which increased the ability of these cells to invade, whereas PDZ1i pretreatment abolished these invasion gains (Fig. 3 *C* and *D*).

PDZ1i Inhibits EGFRvIII-Driven Signaling in GBM and Reduces MMP Secretion. EGFR amplification and mutation are both a common and important alteration in GBM (25, 26). A particular mutation of this receptor, EGFR variant III (EGFRvIII), is often coexpressed along with EGFR amplification in GBM. EGFRvIII and FAK interact as part of a complex to mediate EGFRvIII-mediated MAPK activation (27). Given the demonstrated role of MDA-9/Syntenin in enhancing downstream signaling of the FAK–Src complex in melanoma (13), we determined whether PDZ1i could inhibit EGFRvIII signaling in GBM. We used U87 cells stably expressing EGFRvIII, U87-EGFRvIII (Fig. 4*A*), and treated them with PDZ1i before radiation. In both radiated and nonradiated cells, phospho-EGFR was significantly reduced in the presence of PDZ1i. FAK activation was enhanced after radiation, and this activation was nullified by PDZ1i treatment (Fig. 4*B*). Downstream, we observed that NF-κB activation, enhanced following radiation, was reduced in the presence of PDZ1i. Additionally, treatment with the PDZ1i disrupted MDA-9 interactions with EGFR in both radiated and nonradiated cells (Fig. 4*C*). From these findings, we propose that PDZ1i disrupts EGFRvIII–FAK signaling, which ultimately reduces the observed postradiation gains in NF-κB activation.

Secreted factors significantly impact on cancer cell invasion, and MDA-9/Syntenin affects the secretion of important factors such as MMP-2 and VEGF in glioma (14). Radiation enhances the release of important enzymes, including those involved in invasion, such as the matrix metalloproteinase family (19). We determined whether PDZ1i could affect the secretion of invasion-related proteins following radiation therapy. U1242 cells were treated for 2 h before radiation therapy, and media were collected after 48 h. Expression of several MMP family members significantly increased following radiation therapy, including MMP-2 and MMP-9. PDZ1i treatment reduced the levels of these enzymes following radiation therapy (Fig. S4), an important aspect of reducing radiation-induced invasion gains. Additionally, radiation therapy increased expression of several cathepsin family members as well as

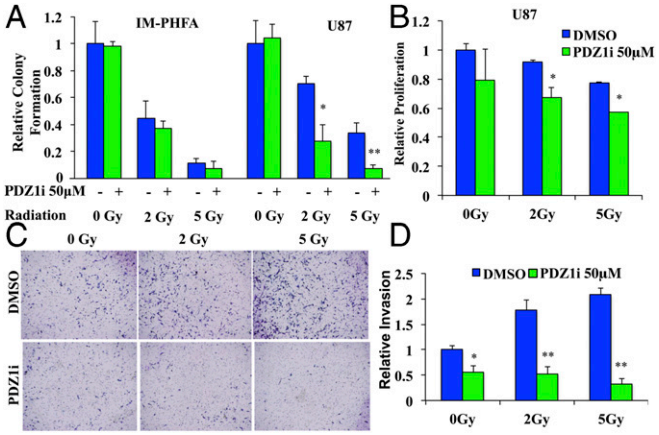

Fig. 3. PDZ1i produces similar effects to *mda-9/syntenin* knockdown postradiation. (*A*) Im-PHFAs and U87 cells were treated with 50 μM PDZ1i 2 h before radiation and analyzed for colony formation after 14 d. (*B*) U87 cells were treated with 50 μM PDZ1i 2 h before radiation and analyzed via MTT assay after 24 h. (*C*) U87 cells were pretreated with 50 μM PDZ1i 2 h before radiation and subsequently seeded in a Transwell Matrigel invasion assay and stained after 18 h. (*D*) Invasion was quantified from five random fields. Error bars indicate ±SD. *$P < 0.05$, **$P < 0.01$.

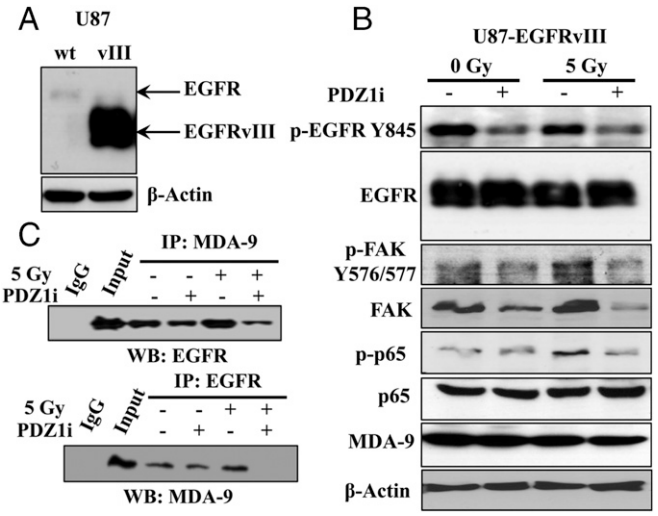

Fig. 4. PDZ1i treatment impairs EGFRvIII and FAK signaling as well as EGFRvIII–MDA-9 interaction. (*A*) Immunoblot analysis showing expression of mutated EGFR in U87 or U87-EGFRvIII cells. (*B*) Cells were treated with either DMSO or PDZ1i 2 h before radiation. Cells were subsequently seeded on fibronectin-coated plates and cell lysates were collected after 1 h and analyzed for protein expression. (*C*) Lysates from cells treated with and without radiation or PDZ1i as indicated were analyzed via immunoprecipitation (IP). Anti–MDA-9 (*Top*) and anti-EGFR (*Bottom*) were used to pull down associated complexes, and Western blotting (WB) used the indicated antibodies. (*Left*) IgG and control input samples.

ADAM9, whereas PDZ1i eliminated these gains (Fig. S4). Cathepsin family proteases modulate tumor invasion and metastasis in a variety of malignancies, including melanoma and breast cancer metastatic to the brain. Notably, the expression of this family is important in microenvironment-mediated chemo- and radioresistance and in facilitating supportive tumor stroma (28).

Stability in the circulation and bioavailability in vivo are critical properties of a potential therapeutic agent. PK studies were performed administering 3.0 mg/kg (i.v.) and 30.0 mg/kg (i.p.) in mice ($n = 3$), and the compound concentration in serum was measured at various times (15 min to 24 h after drug administration). Noteworthy were the lack of adverse signs of toxicity during the experiment, the very slow clearance with $T_{1/2}$ >9 h in each route, and the 80% bioavailability for the compound administered i.p. vs. i.v. (Table S1). Based on these data, we anticipated that one to three weekly doses of the drug i.p. at 30 mg/kg would result in an effective dose for achieving a constant inhibition of the target, despite the relatively low affinity (micromolar) of the compound for the target. We did not anticipate issues inhibiting brain exposure, because mice with GBM have a leaky blood–brain barrier (29). Before in vivo efficacy studies, we evaluated the ability of the compound to cross the blood–brain barrier using a target-based surrogate cellular assay. This method is preferred to direct in vivo measurements of compound concentration in the brain because of low sensitivity of the detection methods and because of the presence of drug in various vascular spaces, namely the residual blood of the brain (30). Accordingly, we used human brain microvascular endothelial cells (HBMECs) (30) seeded with PDZ1i in the upper chamber of Transwell inserts placed on top of wells containing GBM6 cells. After 24 h, the invasive ability of the treated GBM6 cells was compared with pretreated controls. PDZ1i effectively crossed the HBMEC barrier to inhibit invasion in GBM6 cells comparably to the pretreated control with no barrier (Fig. S5). Taken together, these results suggest that PDZ1i is a potent inhibitor of invasion with radiosensitizing effects and favorable properties for CNS treatment.

PDZ1i Is Effective in Reducing Tumor Invasion in an Animal Model of GBM. Orthotopic xenograft models with GBM6 cells were pretreated with either DMSO or PDZ1i and injected intracranially to form tumors. Seven days postinjection, tumor and brain tissue were isolated for sectioning. Tumor cells treated with PDZ1i developed noticeably smaller and more demarcated neoplasms compared with controls (Fig. 5*A*). Separately, untreated cells were injected and tumors were established, and then mice were treated i.p. with either DMSO or PDZ1i (30 mg/kg) three times per wk for 2 wk. Survival was significantly increased in treated mice compared with controls (Fig. 5*C*), and tumors were well-circumscribed and less infiltrative on analysis than those of control-treated mice (Fig. 5*B*). Similar to previous studies knocking down MDA-9/Syntenin expression in an in vivo model (14), PDZ1i was effective in reducing tumor invasion and extending survival in an animal model of GBM.

PDZ1i Combined with Radiation Extends Survival and Reduces GBM Invasion in Vivo. We investigated the combination of PDZ1i with radiotherapy in vivo. U1242-luc cells were injected intracranially under anesthesia and formed tumors, confirmed by imaging at 7 d postinjection. At this point, mice were randomized to four groups: DMSO treatment, PDZ1i treatment (30 mg/kg), DMSO + ionizing radiation (IR), and PDZ1i + IR. Treatment began at 11 d postinjection, and mice receiving radiation were treated with 2.5 Gy for 4 consecutive days. PDZ1i was administered 2 h before radiation treatment on each of the 4 treatment days (Fig. 6*A*). Control-treated mice had an average survival of 41.3 d, whereas PDZ1i treatment extended that to 54.7 d. Radiation alone extended survival to 62.8 d, whereas PDZ1i treatment + IR therapy led to a survival of 78.8 d (Fig. 6*B*). To investigate whether tumor morphology and invasion pattern changed in treatment groups, brain tissue sections were collected and H&E stains were analyzed. Indeed, U1242-luc cells developed diffusely infiltrating tumors in control groups. Tumor margins were slightly more demarcated in groups treated with PDZ1i (Fig. 6*C*). Radiation treatment led to generally smaller, yet still diffuse, tumor patterns, which frequently crossed the midline of the brain with invasive outgrowths. The combination of PDZ1i with radiation led to tumors with markedly more circumscribed margins, less invasive outgrowths, and less spread to the leptomeninges (Fig. 6*C*). Analysis of tumor sections from these treatment groups indicated that activated forms of EGFR, FAK, and Src correlated with PDZ1i treatment status (Fig. S6). Those mice treated with only radiation showed marked increases in activation of these proteins, whereas groups that underwent treatment with PDZ1i showed decreased signal intensity compared with both control- and

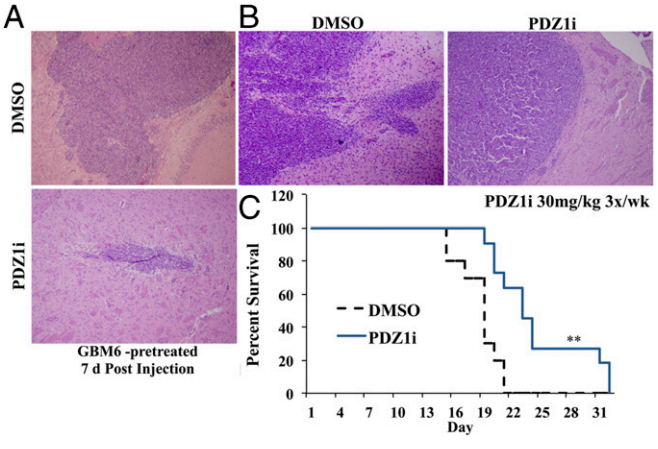

Fig. 5. Effect of PDZ1i on survival in an in vivo model of glioma. (*A*) GBM6 cells were pretreated for 2 h before intracranial injection. After 7 d, brain tissue was isolated and sectioned at the injection site. (*B*) Seven days after tumor implantation, mice received either vehicle or PDZ1i (30 mg/kg) three times per wk for 2 wk. Brain tissue was isolated and analyzed via H&E stain. (*C*) Kaplan–Meier curves of these groups based on animal survival. $^{**}P < 0.01$.

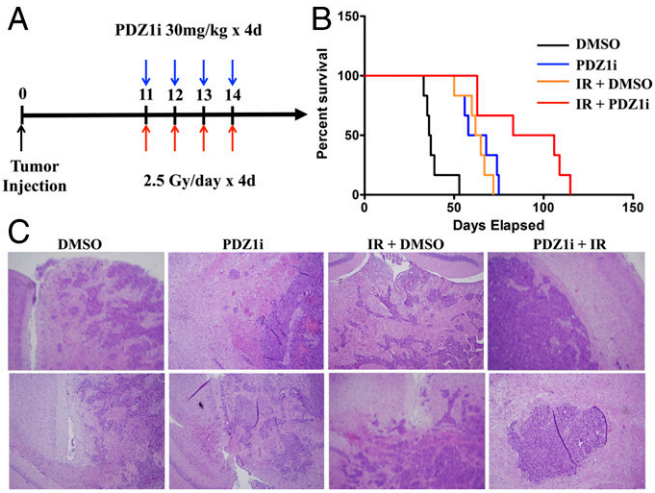

Fig. 6. PDZ1i treatment combined with radiation in an in vivo model of GBM. (*A*) U1242-luc cells were injected intracranially into nude mice. After 7 d, mice were randomized to four groups, and mice receiving therapy were treated on days 11 to 14 as pictured. (*B*) Kaplan–Meier survival curves for each treatment group. Median survival is as listed. (*C*) Brain tissue was isolated and sectioned, and H&E staining is shown.

radiation-treated groups. Collectively, these data support the hypothesis that targeting MDA-9/Syntenin is a promising approach to enhancing conventional radiation treatment.

Discussion

We demonstrate that MDA-9/Syntenin is an important mediator of the postradiation signaling process, eliminating invasion enhancement induced by radiation. Database analysis of REMBRANDT clinical samples supports the idea that high MDA-9/Syntenin expression can lead to a more radioresistant phenotype compared with tumors with lower MDA-9/Syntenin expression. Interesting links in glioma to MDA-9/Syntenin–related signaling have emerged recently. Database expression profiles comparing long-term survivors (LTSs) of GBM (>48 mo) with short-term survivors (<12 mo) revealed a 40% decrease in IGFBP-2, a noted downstream product of MDA-9/Syntenin signaling (9), in LTS tumors (31). Additionally, identification of a radiosensitive gene signature via expression data from the NCI-60 cancer cell panel revealed that a recognized MDA-9/Syntenin binding partner, integrin-linked kinase (32), was significantly down-regulated in radiosensitive cells (33). An ideal complement to radiotherapy would sensitize tumor cells to the cytotoxic effects of radiation while abrogating the unwanted proinvasive effects. We now establish MDA-9/Syntenin as a promising therapeutic target by showing that knockdown potentiates radiosensitivity and abolishes radiation-induced invasion.

MDA-9/Syntenin activates multiple signaling pathways, including enhancing Src signaling. Src is involved in numerous cancer-related processes, including survival, motility, and invasion (34). Especially relevant for GBM, Src interacts with EGFR, including wild-type and mutant versions, activating EGFR through phosphorylation (34, 35). Notably, radiation induces Src-dependent activation of EGFR in GBM, leading to MMP-2 expression and invasion (19), supporting our hypothesis that targeting MDA-9/Syntenin could inhibit radiation-induced invasion signaling. Although extensive preclinical data support the targeting of Src directly for cancer therapy, the results from clinical trials have overall been disappointing (34). Low or no response rates as a single agent or in combination were reported in breast cancer and GBM (34), and GBM patients progressing while on bevacizumab also did not respond on Src inhibitors (36). A proposed method of escape and resistance to Src inhibitors involves up-regulation of IGFBP-2/FAK signaling (37). High levels of IGFBP-2 correlated with resistance, and exogenous IGFBP-2 rendered cells less sensitive to dasatinib. Additionally, FAK activation has been linked to radioresistance (37–39). Targeting MDA-9/Syntenin reduces FAK phosphorylation as well as IGFBP-2 production in addition to Src activation. Therefore, our approach of inhibiting the action of MDA-9/Syntenin may preempt resistance stemming from Src inhibition.

Recent efforts reveal novel mechanisms by which cells evade the cytotoxic effects of radiation therapy. Exosome signaling is a potential pathway by which cancer cells survive under the stress of radiation damage (40, 41). Among exosome cargoes are full-length protein receptors, ligands, RNA, and DNA, including oncogenes (40). Radiation therapy induces greater exosome release from both GBM cells and normal astrocytes. These exosomes contain much higher levels of IGFBP-2 and enhance migration in recipient cells. Furthermore, radiation increases cellular uptake of exosomes (41), and uptake following radiation promotes FAK and Src activation in target cells (40). This is relevant to our work, because MDA-9/Syntenin gain enhances exosome yield whereas MDA-9/Syntenin knockdown reduces exosome release (42). MDA-9/Syntenin targeting is an effective inhibitor of Src activation in glioma and reduces the release of MMP family proteins. Thus, PDZ1i (113B7) could prove useful in reducing both the efficiency of exosome release as well as their effect on the ECM in recipient cells.

The combination of PDZ1i and radiotherapy in a model of GBM showed improvement over each method alone. In vivo, important interactions occur within the tumor microenvironment, including extracellular communication between tumor cells, but also between tumor cells and normal cells, such as endothelial cells. Recent work suggests a mechanism of radioresistance through endothelial cell FAK–NF-κB signaling (43). In normal endothelial cells within the tumor vasculature, DNA-damaging therapy induced FAK activation and NF-κB translocation and activation, leading to secretion of protective cytokines and the development of a sheltering perivascular niche following radiation (43). Therefore, our PDZ1i has the potential to have positive therapeutic effects on both tumor and normal cells within the tumor microenvironment.

Overall, we now show that MDA-9/Syntenin targeting is a viable approach for combating radiation-induced invasion and can radiosensitize GBM. A targeted inhibitor leads to a reduction in phenotypes enhanced by MDA-9/Syntenin, and can be combined effectively with conventional radiotherapy in vivo. This study highlights an MDA-9/Syntenin PDZ1 inhibitor with profound antiinvasive effects, good stability without overt toxicity in vivo, a potential ability to pass the blood–brain barrier, and the capacity to both radiosensitize and block invasion gains of radiation in aggressive GBM cells in vivo. Because MDA-9/Syntenin is involved in many important cancer-related cellular processes and signaling events, further investigation into the spectrum of activities of PDZ1i will be enlightening, as will the potential further development of this class of inhibitors into potential drugs for potentiating the therapeutic utility of radiation for the therapy of GBM, a currently invariably fatal cancer.

Materials and Methods

Reagents, Plasmids, Adenoviruses, and Stable Cell Lines. The chemical structures of two initial fragment hits, structure of the resulting bidentate molecule 113B7 (PDZ1i), binding curve and representative spectra for the titration of 113B7 against the MDA-9 PDZ1/2 tandem domain, and docked structure of 113B7 in complex with the MDA-9/Syntenin PDZ tandem domain are shown in Fig. S1. Description of the synthesis of 113B7 (PDZ1i) is provided in *SI Materials and Methods*. Small hairpin RNA for *mda-9/syntenin* (sh*mda-9*) and plasmids pAd.5/3-sh*mda-9* and pAd.5/3-shcon were constructed as described (14). Details are in *SI Materials and Methods*.

Cell Lines, Cell Culture, and Treatments. Human malignant glioma cell lines and culture conditions are described in *SI Materials and Methods*. The U1242 human glioma cell line exhibits an invasive and very aggressive phenotype when grown in nude mice (44). Cells were treated with PDZ1i 2 h before irradiation. Irradiations were done using an MDS Nordion Gammacell 40 research irradiator with a ^{137}Cs source delivering a dose rate of 0.896 Gy/min.

Preparation of Whole-Cell Lysates and Western Blotting Analysis. Preparation of whole-cell lysates and Western blotting analysis were performed as described (45). For densitometry evaluation, X-ray films were scanned and analyzed with ImageJ software (NIH).

Extraction of Total RNA and Real-Time PCR. Total RNA was extracted from cells using the QIAGEN miRNeasy Mini Kit. Details are in *SI Materials and Methods*.

Immunohistochemistry. Hematoxylin & eosin staining and immunohistochemistry were conducted using a standard protocol (20). Details are in *SI Materials and Methods*.

Invasion and Migration Assays. Invasion and migration assays were performed following standard procedures. Details are in *SI Materials and Methods*.

Survival and Viability Analysis. Clonogenic radiosurvival experiments were carried out as described (44). Cell viability was assessed through tetrazolium dye, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) reduction analysis as described (46). Details are in *SI Materials and Methods*.

Viral Infections. Viral infection conditions and protocols were performed as described (20). Details are in *SI Materials and Methods*.

Intracranial Implant of Cells in Nude Mice. The Institutional Animal Care and Use Committee at Virginia Commonwealth University School of Medicine approved the animal studies. Intracranial xenograft implantation was performed following standard procedures. Details are in *SI Materials and Methods*.

Database Mining. The REMBRANDT database (now housed as part of the Georgetown Database of Cancer https://gdoc.georgetown.edu/gdoc/) was accessed and mined for all astrocytoma and GBM patients. Details are in *SI Materials and Methods*.

Statistical Analysis. The data are reported as the mean ± SD of the values from three independent determinations, and significance analysis was performed using the Student's *t* test in comparison with corresponding controls. Probability values <0.05 were considered statistically significant. Survival curves were analyzed using Cox proportional hazards survival regression using GraphPad Prism.

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