The Efficacy of the Wee1 Inhibitor MK-1775 Combined with Temozolomide Is Limited by Heterogeneous Distribution across the Blood–Brain Barrier in Glioblastoma

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Abstract

Purpose: Wee1 regulates key DNA damage checkpoints, and in this study, the efficacy of the Wee1 inhibitor MK-1775 was evaluated in glioblastoma multiforme (GBM) xenograft models alone and in combination with radiation and/or temozolomide.

Experimental Design: In vitro MK-1775 efficacy alone and in combination with temozolomide, and the impact on DNA damage, was analyzed by Western blotting and γH2AX foci formation. In vivo efficacy was evaluated in orthotopic and heterotopic xenografts. Drug distribution was assessed by conventional mass spectrometry (MS) and matrix-assisted laser desorption/ionization (MALDI)-MS imaging.

Results: GBM22 (IC50 = 68 nmol/L) was significantly more sensitive to MK-1775 compared with five other GBM xenograft lines, including GBM6 (IC50 >300 nmol/L), and this was associated with a significant difference in pan-nuclear γH2AX staining between treated GBM22 (81% cells positive) and GBM6 (20% cells positive) cells. However, there was no sensitizing effect of MK-1775 when combined with temozolomide in vitro. In an orthotopic GBM22 model, MK-1775 was ineffective when combined with temozolomide, whereas in a flank model of GBM22, MK-1775 exhibited both single-agent and combination activity with temozolomide. Consistent with limited drug delivery into orthotopic tumors, the normal brain to whole blood ratio following a single MK-1775 dose was 5%, and MALDI-MS imaging demonstrated heterogeneous and markedly lower MK-1775 distribution in orthotopic as compared with heterotopic GBM22 tumors.

Conclusions: Limited distribution to brain tumors may limit the efficacy of MK-1775 in GBM.

Introduction

The prognosis for patients with glioblastoma multiforme (GBM) remains dismal despite aggressive therapy with surgical resection followed by high-dose radiation and concomitant and adjuvant temozolomide. Temozolomide induces methylation of purine bases within DNA, including O6-methylguanine (O6MG). If this adduct is not removed by the DNA repair protein O6-methylguanine methyltransferase (MGMT), O6MG mispairs with thymidine during replication and subsequently triggers futile cycles of mismatch repair, stalled replication forks, and replication-associated DNA breaks. Radiation similarly induces DNA breaks, and failure to repair these lesions ultimately leads to cell death. Although both radiation and temozolomide provide proven survival gains for patients with GBM, their efficacy is limited by inherent and/or acquired resistance to the DNA-damaging effects of these cytotoxic therapies. Therefore, identification of effective strategies that disrupt the cellular response to DNA damage may provide significant survival benefits in this devastating disease.

Occurrence of DNA double-strand breaks within the genome triggers a highly orchestrated cellular response to damage. DNA damage sensing protein complexes accumulate at DNA breaks and trigger the activation of ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases (1, 2). These kinases coordinate relaxation of chromatin, recruit DNA repair complexes to regions of damaged DNA, and aid in initiation of cell-cycle arrest until the integrity of DNA is restored. The cell-cycle checkpoint functions are effected by signaling pathways that ultimately control the kinase activities of cyclin-dependent kinases (CDK), in association with Cyclin partners, to prevent progression through
Translational Relevance
Disruption of checkpoint signaling by targeting Wee1 causes premature mitosis and cytotoxicity in cells exposed to genotoxic stress. In this study, a panel of patient-derived glioblastoma multiforme (GBM) xenograft models was screened for single-agent and combinatorial efficacy of MK-1775. These studies identified GBM22 as being highly sensitive to Wee1 inhibition in vitro. Although MK-1775 was quite effective alone or in combination with temozolomide in GBM22 flank tumors, MK-1775 was ineffective in orthotopic tumors. This lack of efficacy was associated with restricted delivery of MK-1775 into normal brain and highly heterogeneous delivery into orthotopic tumors with significant regions of tumor being exposed to drug levels similar to normal brain. These data suggest that heterogeneous delivery of drugs with poor distribution to the brain may limit the efficacy of drug treatment.

Materials and Methods
Cell culture and drugs
Short-term explant cultures from xenograft lines were grown in DMEM (VWR) supplemented with 10% FBS (Atlanta Biologicals) or in serum-free media (StemPro NSC SFM; Invitrogen) at 37°C in 5% CO2. Cyquant and neurosphere formation assays were performed as described (14). Temozolomide (Sigma) and MK-1775 (Merck) were dissolved in DMSO, stored at −20°C, and diluted in culture medium for in vitro assays. For in vivo studies, temozolomide (Mayo Clinic Pharmacy) was suspended in Ora-plus (Perrigo) and MK-1775 in 0.5% Methocel (DOW Chemicals), and both were administered orally. Antibodies in use were phospho-S345-Chk1, phospho-T68-Chk2, phospho-Y15-CDK1 (Cell Signaling); CDK1 and β-actin (Thermo-Pierce); yH2AX, Chk1, and Chk2 (Millipore); Wee1, phospho-S824-KAP1 (Abcam), and KAP1 (Santa Cruz Biotechnology).

Immunofluorescence and Western blotting
Immunofluorescence for yH2AX was performed as described (15, 16). Briefly, cells plated on coverslips were treated with 0 or 300 nmol/L MK-1775 and fixed in methanol. Cells were stained with anti-human mouse monoclonal antibody to yH2AX, a secondary goat anti-mouse IgG conjugated to Alexa Fluor 488 (Jackson ImmunoResearch), counterstained with DAPI and mounted with ProLong Gold Antifade (Invitrogen). Immuno-stained cells were analyzed by fluorescent microscopy (Leica DMi6000B; ×40 objective) and nuclei positive for foci (>20 foci) or pan-nuclear staining were quantified. For Western blotting, cells or tissues were processed for protein extraction and subsequent SDS-poly acrylamide gel electrophoresis as described (15).

In vivo efficacy studies
Studies were approved by Mayo Animal Care and Use Committee (Rochester, MN). Xenografts were established in athymic mice (Harlan) as described (17). Mice with established tumors were randomized into treatment groups. Flank tumors were measured thrice weekly, and mice were euthanized when tumor volume exceeded 2,000 mm³. Mice with intracranial xenografts were observed daily and euthanized upon reaching a moribund state.

Blood and tissue bioanalysis of MK-1775
Mice were treated with a single dose of MK-1775 (50 mg/kg), euthanized at indicated times, and whole blood and brain were collected for analysis. Pharmacokinetics blood samples were collected by tail clip and 10 μL of whole blood mixed with 30 μL of 0.1 mol/L sodium citrate. Brain tissues were flash frozen and homogenized in three volumes per weight of water for analysis. Blood and brain concentrations of MK-1775 were determined by protein precipitation followed by LC/MS-MS. Blood pharmacokinetic parameters were calculated using established noncompartmental methods.

Matrix-assisted laser desorption/ionization mass spectrometric imaging analyses
Mice with established tumors received a single MK-1775 dose (200 mg/kg), and tumors were harvested 2 hours later and frozen in Optimal Cutting Medium (Tissue-Tek) on dry ice. Cryo-sections were thaw mounted onto optical slides for hematoxylin and eosin (H&E) staining and ITO-coated glass slides (Bruker Daltonics) for matrix-assisted laser desorption/ionization mass spectrometric imaging MALDI-MSI. Matrix CHCA (5 mg/mL solution in ACN/0.2% TFA 60:40 vol/vol) was deposited using an ImagePrep (Bruker Daltonics) as described (18). Mass spectra were acquired using an UltraFlexXtreme MALDI-TOF/TOF (Bruker Daltonics) equipped with a 1-kHz smartbeam...
laser. MALDI-MSI experiments were acquired with a pixel step size for the surface raster set to 75 μm for brain sections and 50 μm for tumor flank sections in FlexImaging 4.0 software. Spectra were externally calibrated using a small-molecule calibration standard solution. Spectra were acquired in positive ion mode from 1,000 laser shots accumulated at each spot for a mass range of m/z 0 to 3,300. The laser intensity was set to 50% with a frequency of 1,000 Hz. The MALDI images were displayed using the software FlexImaging 4.0. The permeability of MK-1775 through the blood vessel is visualized following the signal of the drug (m/z 501.2 ± 0.2) and heme as a biomarker of the vasculature (m/z 616.2 ± 0.2) as described (19).

Statistical analyses

Unless stated otherwise, all in vitro data presented are the mean ± SEM from three or more experiments. Statistical differences were evaluated using the Student t test and P<0.05 considered statistically significant. Calculations for IC_{50} were performed by fitting the experimental data to a sigmoidal curve using GraphPad software. Distribution of survival and tumor progression beyond 1,500 mm³ was estimated using the Kaplan–Meier method and compared by the log-rank test.

Results

Single-agent efficacy of MK-1775 in a panel of primary GBM xenografts

The expression of Wee1 in flank tumors from 12 GBM xenograft lines was evaluated by Western blotting and qRT-PCR. There was a wide range of Wee1 transcript levels ranging from 75 ± 38% (GBM22), relative to internal control, to 480 ± 127% (GBM6; Supplementary Fig. S1), and a similar range in protein expression levels (Fig. 1A). A subset of these lines was selected for evaluation of in vitro sensitivity to MK-1775 in CyQuant and neurosphere formation assays. In both assays, GBM6 was significantly more resistant to single-agent MK-1775 than GBM22 (Fig. 1B and C); in the neurosphere assay, the IC_{50} for MK1775 for GBM6 and GBM22 was 695 and 12 nmol/L (P = 0.0001), respectively. Using phosphorylation of Cdk1 on Tyr-15 as a surrogate measure of MK-1775 activity, treatment of GBM6 cells with graded concentrations resulted in significant suppression of phosphorylation only at 300 nmol/L MK-1775, whereas Cdk1 phosphorylation was reduced even at 10 nmol/L MK-1775 and completely suppressed at 100 nmol/L MK-1775 in GBM22 (Fig. 1D).

Previous studies have suggested that MK-1775 can induce DNA damage (7, 20–22), and consistent with the differential sensitivity observed, treatment of GBM6 cells with 300 nmol/L MK-1775 had a modest impact on the total number of cells staining positive for γH2AX (Fig. 2A and C; 3.8 ± 1.1% DMSO control vs. 20.5 ± 6.5% MK-1775 treated; P = 0.04) and had no significant impact on cells exhibiting pan-nuclear γH2AX staining (0% untreated vs. 2.6 ± 2.2% treated; P = 0.22). Although pan-nuclear staining was uncommon in GBM6 cells, the majority of GBM22 cells with γH2AX staining exhibited pan-nuclear staining, and the fraction of cells with pan-nuclear staining significantly increased after MK-1775 treatment (72.2 ± 4.5%) as compared with controls (30.2 ± 6.1%; P = 0.0007) (Fig. 2B and C). Similar results were observed 48 hours after treatment (Supplementary Fig. S2A–S2C). These data suggest that differences in cytotoxicity observed with MK-1775 treatment may relate to induction of DNA damage.

Figure 1. Initial in vitro evaluation of MK-1775 in a panel of primary GBM lines. A, Western blot analysis of flank tumor GBM xenografts. Pooled lysate from three biologic replicates of a tumor line were run in each lane for the indicated xenograft models. B, effects of graded concentrations of MK-1775 were assessed by Western blotting. Because of low basal phosphorylation, GBM22 was highly resistant to temozolomide and MK-1775, whereas Cdk1 phosphorylation was reduced even at 10 nmol/L MK-1775 and completely suppressed at 100 nmol/L MK-1775 in GBM22 (Fig. 1D).

Lack of synergy between temozolomide and MK-1775 in vitro

Disruption of DNA damage-induced checkpoint signaling after Wee1 inhibition may be a potential temozolomide-sensitizing strategy in GBM. Therefore, the combination of temozolomide and MK-1775 was assessed in CyQuant and/or neurosphere formation assays in GBM6, 12, and 22. In both assays, GBM6 cells were highly resistant to temozolomide and MK-1775, and there was no apparent combinatorial effect on cytotoxicity (Fig. 3A and Supplementary Fig. S3A). GBM22 was highly
sensitive to treatment with temozolomide or MK-1775, but similar to GBM6, there was no apparent combinatorial interaction (Fig. 3B and Supplementary Fig. S3B). Similarly, in GBM12, which can only be grown under neurosphere forming conditions in vitro, there was no obvious interaction between temozolomide and MK-1775 (Supplementary Fig. S3C). Analysis of synergy using the MacSynergy II program confirmed the subjective impression that there was no evidence for a synergistic interaction between MK-1775 and temozolomide for any of the lines tested (data not shown). In summary, the results from cytotoxicity assays and drug–drug interaction analyses suggest a possible additive effect for the combination of temozolomide and MK-1775 in the lines tested.

The impact of combinatorial therapy on DNA damage signaling in both GBM6 and GBM22 cells was assessed by Western blotting for ATM- and ATR-dependent phosphorylation of downstream targets KAP1, Chk1, and Chk2. Although treatment with MK-1775 for 24 hours induced KAP1 phosphorylation in both GBM6 and GBM22, the extent of MK-1775–induced phosphorylation was greater in GBM22 (Fig. 3C). Treatment with MK-1775 also consistently induced P-Chk1 in both GBM lines; taking into account the differences in total Chk1 levels, the extent of induction is subjectively similar between GBM6 and GBM22. GBM6 is significantly more resistant to temozolomide than GBM22 by virtue of high-level MGMT expression (23), and consistent with this, phosphorylation of KAP1 was not influenced by temozolomide treatment in GBM6 cells, while both P-KAP1 and P-Chk1 were elevated in GBM22, especially 48 hours after treatment (data not shown).
not shown). Consistent with the cytotoxicity data, cotreatment was not associated with increased DNA damage beyond that seen with temozolomide or MK-1775 monotherapy. Collectively, the cytotoxicity and Western blotting results both are consistent with a lack of synergy between temozolomide and MK-1775 in vitro.

Evaluation of MK-1775 efficacy in orthotopic tumors

The combination of MK-1775 with radiation (RT) and/or temozolomide was evaluated in preclinical studies with GBM12 and GBM22 orthotopic xenografts using clinically relevant dosing schedules. In GBM22, there was no difference in survival for placebo versus single-agent MK-1775 treatment (median survival of 36 vs. 34 days, respectively; \( P = 0.13 \); Fig. 4A), and the combination of MK-1775 with fractionated radiation (2 Gy \times 10 fractions) had no impact on survival relative to RT alone (median survival for both groups 53 days; \( P = 0.81 \)). Similarly, the survival for mice cotreated with RT and temozolomide was not significantly extended in combination with MK-1775 (median survival of 441 vs. 384 days, respectively; \( P = 0.40 \)), although 2 mice in the triple combination therapy died early presumably from toxicity (Fig. 4B). Finally, compared with treatment with temozolomide alone, MK-1775 given concurrently with temozolomide had no impact on survival (median survival 169 days vs. 251 days, respectively; \( P = 0.19 \); Fig. 4C). Similar results were observed in GBM12 orthotopic xenografts with no impact of MK-1775 on survival when given alone or in combination with temozolomide and/or RT (Supplementary Fig. S4A and S4B).

These data demonstrate in two different orthotopic xenograft models a lack of efficacy for MK-1775 alone or in combination with standard therapies.

Pharmacokinetics and distribution of MK-1775 in normal brain and orthotopic tumors

The lack of MK-1775 efficacy in the orthotopic survival studies prompted an evaluation of the pharmacokinetics in brain versus whole blood after oral administration. Nude mice were treated with a single 50 mg/kg dose of MK-1775 and brain and whole blood samples harvested up to 12 hours later (Fig. 5A). Peak concentrations in brain (0.31±0.16 \( \mu \)mol/L) and whole blood (7.78±2.15 \( \mu \)mol/L) were achieved at 1 to 2 hours after dosing. Although this peak concentration in brain is significantly above the in vitro IC_{50} for GBM22, the exposure at this concentration is short lived with a half-life in whole blood and brain of 112 minutes and 114 minutes, respectively. Consistent across all time points, the brain to whole blood ratio was 4% to 5%. These data demonstrate limited distribution of MK-1775 to normal brain with an intact blood-brain barrier (BBB).

The integrity of the BBB is heterogeneous in GBM and might influence the delivery of MK-1775 for orthotopic tumors as compared with heterotopic tumors that are devoid of a BBB. Therefore, drug distribution to tumors was compared between heterotopic and orthotopic xenografts by analyzing drug levels in histologic sections by two-dimensional MALDI-MSI. Mice with established intracranial or flank tumors were treated with a single dose of placebo or MK-1775 and processed for frozen sectioning. The expected molecular mass for MK-1775 by MS is 501.2, and in the MK-1775–treated tumors analyzed by MALDI-MSI, a peak was observed at 501.28, whereas no equivalent peak was observed in placebo-treated tumor (Supplementary Fig. S5A and S5B). Tandem MS-MS analysis of this peak confirmed the expected fragmentation products for MK-1775 when compared with the standard (Supplementary Fig. S5C). This enabled the measurement of relative MK-1775 levels across tissue sections at 50 to 75 micron spatial resolution, with the pixel intensity used as a measure of relative MK-1775 exposure. The pixel intensity across the entire orthotopic tumor was much lower than the level across the flank tumor, which exhibited higher pixel intensity. The orthotopic tumor also had a more heterogeneous distribution of MK-1775, with some areas exhibiting MK-1775 exposure levels similar to normal brain (Fig. 5B). In contrast, drug delivery into flank tumors was more homogeneous (Fig. 5C). These data demonstrate that the distribution of MK-1775 within orthotopic GBM22 is heterogeneous and overall lower in exposure level in comparison with flank tumors.

Efficacy of MK-1775 in heterotopic tumors

On the basis of the MALDI-MSI analyses, the efficacy of MK-1775 was evaluated in flank GBM22 tumors using a dosing schedule similar to the orthotopic studies. When administered
on days 1 to 5 of a 28-day cycle, MK-1775 monotherapy resulted in a modest but statistically significant increase in median time to exceed endpoint (tumor size of 1,500 mm³) as compared with placebo treatment (38 days vs. 30 days, \(P = 0.01\); Fig. 6A). An additional cohort of animals was treated with extended MK-1775 dosing delivered Monday-Friday every week for the duration of three cycles, and this treatment regimen further extended the benefit of single-agent MK-1775 (median time to endpoint 42 days, \(P = 0.0004\) relative to placebo; Fig. 6B). In combination with temozolomide, MK-1775 given only concurrently with temozolomide extended time to endpoint compared with temozolomide only (median time 144 days vs. 91 days; \(P = 0.15\)) but did not reach statistical significance due to two temozolomide-treated tumors that did not recur, which is highly unusual for this model (14). Extended MK-1775 treatment combined with the same cyclical temozolomide regimen had an even more pronounced effect on efficacy (median not reached with 5 mice without tumor regrowth at 240 days, \(P = 0.02\) relative to temozolomide only). Thus unlike the orthotopic model, MK-1775 alone and in combination with temozolomide resulted in significant tumor control benefits.

A similar study was performed in GBM12 using the standard MK-1775 dosing regimen. In the MK-1775–resistant GBM12 model, median time to exceed 1,500 mm³ for MK-1775 treatment alone was not significantly longer than in placebo group (47 vs. 45 days; \(P = 0.98\); Supplementary Fig. S4C). GBM12 is highly sensitive to temozolomide, and compared with placebo, temozolomide treatment significantly extended the time to endpoint (\(P = 0.03\)), but the addition of MK-1775 to temozolomide did not.
Discussion

DNA damage-inducible cell-cycle checkpoints ensure repair of potentially cytotoxic lesions before cells progress through the cell cycle. These mechanisms are essential for maintaining genetic integrity, and disruption of checkpoint signaling potentially can be used to enhance the efficacy of genotoxic chemotherapies. MK-1775 specifically targets the Wee1 kinase, which maintains inhibitory phosphorylation of CDK1 and CDK2 to prevent premature progression through S-phase and entry into mitosis. Extending a previous study of MK-1775 combined with radiation (12), here the single agent and combinatorial effects with temozolomide or RT were evaluated in three GBM patient-derived xenograft models. Significant single-agent activity was observed in the GBM22 model as compared with relative resistance in two other GBM models. Although there was no evidence for significant interactions in vitro between the two drugs, there was some enhanced efficacy with the combination in vivo in heterotopic but not orthotopic tumor models. Consistent with this observation, accumulation of MK-1775 was markedly lower in orthotopic xenografts as compared with heterotopic tumors. These data suggest that heterogeneous accumulation within brain tumors may limit the efficacy of MK-1775 in GBM.

Although typically associated with control of the G2–M checkpoint, Wee1 also plays an important role in regulation of S-phase. Wee1 activity suppresses replication stress by modulating Cdk2/CyclinE and Cdk2/CyclinA activities within S-phase, and suppression of Wee1 results in increased rates of replication origin firing, increased rates of DNA synthesis, a corresponding depletion of nucleoside pools, and ultimately slowing and stalling of replication forks (24). Endonuclease-mediated cleavage of chicken-foot DNA structures associated with stalled replication forks then leads to replication-associated DNA double-strand breaks (25). Consistent with this model, MK-1775 monotherapy is associated with induction of pan-nuclear γH2AX staining in this current report and several other studies, and a similar phenotype is seen with Wee1 siRNA (7, 20–22). Interestingly, Myc overexpression is associated increased replicative stress (26, 27), and the highly sensitive GBM22 xenograft line from this study harbors high-level Myc amplification (unpublished data). Consistent with the presence of replicative stress in this model, GBM22 cells are highly aneuploid and exhibited marked basal elevation in γH2AX staining that was further accentuated with MK-1775 treatment. In a neurosphere assay, which provides results similar to a clonogenic assay and is generally more sensitive than the CyQuant assay for assessment of cytotoxicity, GBM22 cells (IC50 of 12 nmol/L; Fig. 1C) were exclusively sensitive to single-agent MK-1775 as compared with previously published studies in sarcoma and neuroblastoma with IC50 ranging from 100 nmol/L to over 1,000 nmol/L (7, 9, 22). In a transgenic model of neuroblastomas, homoygous expression of the MYCN oncogene was associated with an almost 3-fold reduction in IC50 (62 nmol/L) as compared with heterozygous expression of MYCN (161 nmol/L; ref. 9). On the basis of these observations, we speculate that the profound sensitivity of GBM22 to MK-1775 may be linked to Myc overexpression. Alternatively, the exquisite sensitivity to Wee1 inhibition may be related to the very low expression levels of Wee1 in GBM22 relative to other tumor lines. Future studies could address these hypotheses to facilitate the clinical development of biomarkers of response to MK-1775 using relevant tumor models.

Wee1 inhibition is a promising chemo- and radiosensitizing strategy in solid and hematologic malignancies. On the basis of promising preclinical testing in cell culture and/or animal models, there are several phase I clinical trials evaluating the safety and tolerability of MK-1775 combined with cisplatin, carboplatin, 5-fluorouracil, gemcitabine, temozolomide and/or radiation in solid malignancies (from ClinicalTrials.gov). Although preclinical MK-1775 combinations with temozolomide have not been previously reported, incubation of glioma cells with the Wee1 inhibitor PD0166285 further reduced the viability of cells cotreated with temozolomide (13). Similarly, we observed decreased neurosphere formation when temozolomide was combined with MK-1775 in both GBM12 and GBM22. Interestingly, for the combination of MK-1775 with temozolomide in a flank xenograft model, only the sensitive GBM22 line demonstrated significant enhancement in treatment efficacy. Although the reason for the discrepancy in the combinatorial effect observed in vitro versus flank tumors in GBM12 is unclear, we have described a similar discrepancy for the combination of the PARP inhibitor veliparib combined with temozolomide in our primary GBM xenograft models that was attributed to significant differences in drug exposure in vitro versus in vivo (15). Although several studies have suggested that the chemo- or radiosensitizing effects of Wee1 inhibition are linked to a lack of p53 function (5, 8, 28–30), both GBM22 and GBM12 harbor TP53 mutations, so the p53 status is not an adequate explanation for the lack of efficacy in GBM12. Although MK-1775 specifically did not provide a benefit in orthotopic tumors, the flank tumor data from GBM22 are consistent with at least an additive effect and support the concept of combining brain-penetrant Wee1 inhibitors with temozolomide in selected GBM or other solid malignancies, but significant work remains to understand the molecular features associated with sensitivity or resistance to this strategy.

The BBB can be a significant obstacle to delivering small molecules into the brain. Although the BBB is disrupted in essentially all GBM, as evidenced by the accumulation of radiographic contrast within regions of tumor, the extent of contrast enhancement across
a tumor is heterogeneous, with some regions of dense tumor exhibiting no contrast accumulation (31). Similarly, analysis of brain tumor cross sections for MK-1775 accumulation within GBM22 orthotopic xenografts by MALDI-MS imaging demonstrated limited and heterogeneous drug distribution across the tumor region at levels much lower than flank tumors. Similar heterogeneity has been described using 14C-labeled lapatinib in an orthotopic breast brain metastatic model (32). GBM cells also are highly invasive and essentially all GBM patient tumors have single cells invading into normal brain parenchyma, which have an intact BBB. On the basis of heterogeneous accumulation of MK-1775 coupled with the limited penetration of MK-1775 into normal brain, we hypothesize that the lack of efficacy for MK-1775 combined with temozolomide in orthotopic GBM22 reflects a failure to effectively deliver MK-1775 to 100% of the tumor cells. Consistent with these data, a PARP inhibitor (AG014669) with poor brain penetration is only effective in combination with temozolomide in GBM12 as a flank tumor model but not in an orthotopic model (Kizilbash et al.; manuscript under preparation), whereas the combination of temozolomide with the brain penetrant PARP inhibitor veliparib is effective in both flank and orthotopic models (15, 33). Although MK-1775 combined with radiation was effective in orthotopic glioma xenografts established from cell lines in previous studies (11, 13), the radiation schedule was quite different and the BBB for these tumors may be more highly compromised than those seen in the primary GBM xenograft models used here. Collectively, the data presented in this study suggest that penetration of chemosensitizing agents across the BBB is critical to achieve optimal temozolomide-sensitizing effects.

There are two clinical trials currently evaluating the combination of MK-1775 with radiation and/or chemotherapy in brain tumors. MK-1775 combined with radiation is being tested in pontine gliomas (ClinicalTrials.gov Identifier: NCT01922076); these childhood tumors have a grave prognosis and over half do not have significant contrast enhancement on MR imaging (34–36). This latter finding suggests that many of these tumors have a relatively intact BBB, which, in conjunction with our observation that MK-1775 has limited penetration into normal brain, might suggest that only a subset of patients with a more open BBB may benefit from therapy. However, as demonstrated by increased contrast enhancement on MRI, there is evidence that fractionated radiation can increase the disruption of the BBB in high-grade gliomas (37). Further work will be required to understand whether these radiation-induced changes in the BBB result in clinically meaningful improvements in drug accumulation within brain tumors. A second trial is evaluating MK-1775 in combination with radiation and temozolomide in GBM (NCT01849146). A subset of patients with recurrent GBM on this trial, on a separate phase 0 only trial (NCT02207010), will be treated with MK-1775 alone before surgical resection of their tumor, and through sampling of multiple regions within the tumor, the potential heterogeneity of drug delivery within the tumor will be evaluated. These innovative phase 0 clinical trials should provide important insight into how heterogeneity of the BBB affects drug delivery in patients, and our ongoing studies will address whether improving delivery of MK-1775 or other sensitizers across the BBB can improve therapeutic efficacy.

Disclosure of Potential Conflicts of Interest
D. Calligaris is a consultant/advisory board member for Bayview, Genentech, Novartis, and Pfizer. N.Y.R. Agar is the founder of BayviewDX and is a consultant/advisory board member for BayviewDX and inviCRO. J.N. Sarkaria reports receiving commercial research grants from Basilea, Beigene, Eli Lilly, Genentech, Merck, and Sanofi Aventis. No potential conflicts of interest were disclosed by the other authors.

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