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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Running Title: Efficacy of MK-1775 in PDX models of GBM

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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. While MK-1775 was quite effective alone or in combination with TMZ 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.
ABSTRACT

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

Experimental design: In vitro MK-1775 efficacy alone and in combination with TMZ, 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 nM) was significantly more sensitive to MK-1775 compared to 5 other GBM xenograft lines including GBM6 (IC₅₀ >300 nM), 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 TMZ in vitro. In an orthotopic GBM22 model, MK-1775 was ineffective when combined with TMZ, while in a flank model of GBM22, MK-1775 exhibited both single agent and combinatorial activity with TMZ. 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 to 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 (TMZ). TMZ 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. While both radiation and TMZ 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 (CDKs), in association with Cyclin partners, to prevent progression through the cell cycle (3, 4). Specifically within S and G2, inhibitory phosphorylation of Cdk1 at Thr-14 and Tyr-15 by Wee1 and Myt1 kinases, and coordinated sequestration of the Cdc25 phosphatases that dephosphorylate these residues, prevents cell cycle progression in an ATM- and ATR-dependent manner. Disruption of these tightly controlled checkpoints can lead to premature entry into mitosis and may be associated with significant cytotoxicity. Thus, inhibitors of the S- and G2/M checkpoint pathways may be useful as chemo- or radio-sensitizing agents.
MK-1775 is a selective inhibitor of Wee1 kinase with effective checkpoint inhibitory activities. Preclinical studies have demonstrated potent chemo-sensitizing activities when MK-1775 is combined with S-phase toxins, such as DNA cross-linking agents (mitomycin C, carboplatin and cisplatin), nucleoside analogs or inhibitors of DNA metabolism (pemetrexed, cytarabine, 5-fluorouracil, gemcitabine) or topoisomerase poisons (SN38, doxorubicin, camptothecin) (5-10). Specifically in GBM models, MK-1775 modestly enhanced the efficacy of radiation in established and primary glioma cell lines in vitro and in vivo (11, 12), and similar effects were observed with an unrelated Wee1 inhibitor, PD0166285, when combined with either radiation or TMZ (13). MK-1775 is now progressing through clinical trial development, and Phase I toxicity studies suggest that MK-1775 can be safely combined with a variety of chemotherapy agents in solid tumors. With features relatively favorable for brain penetration (MW 501.2 Da, LogP of 2.8 and unbound fraction of 23%), there is significant interest in developing MK-1775 as a sensitizing agent for patients with GBM. In this manuscript, the in vitro and in vivo activities of MK-1775 given alone and in combination with TMZ were studied in several patient derived GBM xenograft models. While MK-1775 combined with TMZ was highly effective in a flank tumor model, MK-1775 has poor penetration into normal brain, and the combination was ineffective in a more clinically relevant orthotopic model.

MATERIALS and METHODS

Cell culture and drugs: Short-term explant cultures from xenograft lines were grown in DMEM (VWR) supplemented with 10% fetal bovine serum (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). TMZ (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, TMZ (Mayo Clinic Pharmacy) was suspended in Ora-plus (Perrigo) and MK-1775 in 0.5% Methocel (DOW Chemicals), and both were administered orally. Antibodies used were phospho-S345-Chk1,
phospho-T68-Chk2, phospho-Y15-CDK1 (Cell Signaling); CDK1 and β-actin (Thermo-Pierce); γH2AX, Chk1 and Chk2 (Millipore); Wee1, phospho-S824-KAP1 (Abcam) and KAP1 (Santa Cruz).

**Immunofluorescence and Western blotting:** Immunofluorescence for γH2AX was performed as described (15, 16). Briefly, cells plated on coverslips were treated with 0 or 300 nM MK-1775 and fixed in methanol. Cells were stained with anti-human mouse monoclonal antibody to γH2AX, a secondary goat anti-mouse IgG conjugated to Alexa-Fluor-488 (Jackson ImmunoResearch), counterstained with DAPI and mounted with ProLong Gold Antifade (Invitrogen). Immunostained cells were analyzed by fluorescent microscopy (Leica DMI6000B; 40X 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. 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 2000 mm$^3$. Mice with intracranial xenografts were observed daily and euthanized upon reaching a moribund state.

**Blood and tissue bio-analysis 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 M sodium citrate. Brain tissues were flash frozen and homogenized in 3 volumes per weight of water for analysis. Blood and brain concentrations of MK-1775 were determined by protein
precipitation followed by liquid chromatography – tandem mass spectrometry. Blood pharmacokinetic parameters were calculated using established non-compartmental methods.

**Matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI–MSI) 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 staining and ITO-coated glass slides (Bruker Daltonics) for 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 UltrafleXtreme 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 1000 laser shots accumulated at each spot for a mass range of m/z 0-3300. The laser intensity was set to 50% with a frequency of 1000 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 ± standard error of the mean (SEM) from 3 or more experiments. Statistical differences were evaluated using Student’s T-test and p-values <0.05 considered statistically significant. Calculations for IC₅₀ were performed by fitting the experimental data to a sigmoidal curve using GraphPad software. Distribution of survival and tumor progression beyond 1500 mm³ were estimated using the Kaplan-Meier method and compared by 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 quantitative RT-PCR. There was a wide range of Wee1 transcript levels ranging from 75 ± 38% (GBM22), relative to internal control, to 480 ± 127% (GBM6) (Supplementary Figure S1), and a similar range in protein expression levels (Figure 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 (Figure 1B and C); in the neurosphere assay, the IC_{50} for MK1775 for GBM6 and GBM22 was 695 nM and 12 nM (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 nM MK-1775, while Cdk1 phosphorylation was reduced even at 10 nM MK-1775 and completely suppressed at 100 nM MK-1775 in GBM22 (Figure 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 nM MK-1775 had a modest impact on the total number of cells staining positive for γH2AX (Figure 2A and B; 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). While 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 to controls (30.2 ± 6.1%; p=0.0007). Similar results were observed 48 hours after treatment (Supplementary Figure S2A-C). These data suggest that differences in cytotoxicity observed with MK-1775 treatment may relate to induction of DNA damage.
**Lack of synergy between temozolomide and MK-1775 in vitro**

Disruption of DNA damage induced checkpoint signaling after Wee1 inhibition may be a potential TMZ-sensitizing strategy in GBM. Therefore, the combination of TMZ 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 TMZ and MK-1775, and there was no apparent combinatorial effect on cytotoxicity (Figure 3A and Supplementary Figure S3A). GBM22 was highly sensitive to treatment with TMZ or MK-1775, but similar to GBM6, there was no apparent combinatorial interaction (Figure 3B and Supplementary Figure S3B). Similarly, in GBM12, which can only be grown under neurosphere forming conditions *in vitro*, there was no obvious interaction between TMZ and MK-1775 (Supplementary Figure 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 TMZ 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 TMZ 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. While 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 (Figure 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 TMZ than GBM22 by virtue of high level MGMT expression (23), and consistent with this, phosphorylation of KAP1 was not influenced by TMZ treatment in GBM6 cells, while both P-KAP1 and P-Chk1 were elevated in GBM22, especially 48 hours after treatment (data not shown). Consistent with the cytotoxicity data, co-treatment was not associated with increased DNA damage beyond that seen.
with TMZ or MK-1775 monotherapy. Collectively, the cytotoxicity and western blotting results both are consistent with a lack of synergy between TMZ and MK-1775 in vitro.

**Evaluation of MK-1775 efficacy in orthotopic tumors**

The combination of MK-1775 with radiation (RT) and/or TMZ was evaluated in pre-clinical 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 versus 34 days, respectively; p=0.15; Figure 4A), and the combination of MK-1775 with fractionated radiation (2 Gy x 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 co-treated with RT and TMZ was not significantly extended in combination with MK-1775 (median survival of 441 versus 384 days, respectively, p=0.40), although 2 mice in the triple combination therapy died early presumably from toxicity (Figure 4B). Finally, compared to treatment with TMZ alone, MK-1775 given concurrently with TMZ had no impact on survival (median survival 169 days versus 251 days, respectively; p=0.19; Figure 4C). Similar results were observed in GBM12 orthotopic xenografts with no impact of MK-1775 on survival when given alone or in combination with TMZ and/or RT (Supplementary Figure S4A and B). These data demonstrate in 2 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 (Figure 5A). Peak concentrations in brain (0.31 µM ± 0.16 µM) and whole blood (7.78 µM ± 2.15 µM) were achieved at 1 to 2 hours after dosing. While 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 to 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 2-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, while no equivalent peak was observed in placebo treated tumor (Supplementary Figure S5A and B). Tandem MS-MS analysis of this peak confirmed the expected fragmentation products for MK-1775 when compared to the standard (Supplementary Figure 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 MK1775 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 (Figure 5B). In contrast, drug delivery into flank tumors was more homogeneous (Figure 5C). These data demonstrate that the distribution of MK-1775 within orthotopic GBM22 is heterogeneous and overall lower in exposure level in comparison to flank tumors.

**Efficacy of MK-1775 in heterotopic tumors**

Based on 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 1-5 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 1500 mm$^3$) as compared to placebo treatment (38 days versus 30 days, p=0.01; Figure 6A). An additional cohort of animals was treated with extended MK-1775 dosing delivered Monday-Friday every week for the duration of 3 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; Figure 6B). In combination with TMZ, MK-1775 given only concurrently with TMZ extended time to endpoint compared to TMZ only (median time 144 days versus 91 days; p=0.15) but did not reach statistical significance due to two TMZ-treated tumors that did not recur, which is highly unusual for this model (14). Extended MK-1775 treatment combined with the same cyclical TMZ 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 TMZ only). Thus unlike the orthotopic model, MK-1775 alone and in combination with TMZ 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 1500 mm$^3$ for MK-1775 treatment alone was not significantly longer than in placebo group (47 versus 45 days; p=0.98; Supplementary Figure S4C). GBM12 is highly sensitive to TMZ, and compared to placebo, TMZ treatment significantly extended the time to endpoint (p=0.03), but the addition of MK-1775 to TMZ did not impact tumor regrowth compared to TMZ alone (median time to endpoint 111 days versus 101 days, p=0.86; Supplementary Figure S4C). Thus, similar to in vitro data, GBM12 was highly resistant to MK-1775 and there was no evidence for an interaction with TMZ.

The impact of TMZ and/or MK-1775 on DNA damage signaling in the sensitive GBM22 flank model was investigated further to understand the in vivo damage kinetics of TMZ and MK-1775 treatment. Mice with established GBM22 flank xenografts were treated with TMZ at 50 mg/kg/day on Days 1-5 and/or MK-1775 at 50 mg/kg/day BID Days 1-5 and 8-12. Tumors were harvested on Days 5, 8 or 12, 1 hour after the first dose of the day (Figure 6C). Treatment with either TMZ or MK-1775 induced phosphorylation of KAP1 on day 5, although KAP1 phosphorylation was not sustained on day 8 in the MK-1775 treated mice (no drug treatment on days 6-7), while KAP1 phosphorylation
actually increased in this time-frame for animals treated with TMZ alone. Interestingly, even though KAP1 phosphorylation after treatment with MK-1775 on day 12 was greater than on day 5, there was no evidence for enhanced combinatorial effects of TMZ combined with MK-1775 at any time-point for the DNA damage signaling markers evaluated.

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 genomic integrity, and disruption of checkpoint signaling potentially can be employed 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 TMZ or RT were evaluated in 3 GBM patient-derived xenograft models. Significant single agent activity was observed in the GBM22 model as compared to relative resistance in 2 other GBM models. While there was no evidence for significant interactions in vitro between the 2 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 to 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 (IC\textsubscript{50} of 12 nM; Figure 1C) were exquisitely sensitive to single agent MK-1775 as compared to previously published studies in sarcoma and neuroblastoma with IC\textsubscript{50}s ranging from 100 to over 1000 nM (7, 9, 22). In a transgenic model of neuroblastomas, homozygous expression of the MYCN oncogene was associated with an almost 3 fold-reduction in IC\textsubscript{50} (62 nM) as compared to heterozygous expression of MYCN (161 nM) (9). Based on these observations, we speculate that the profound sensitivity of GBM22 to MK-1775 may be linked to Myc over-expression. 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 radio-sensitizing strategy in solid and hematologic malignancies. Based on promising pre-clinical 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 pre-clinical MK-1775 combinations with TMZ have not been previously reported, incubation of glioma cells with the Wee1 inhibitor PD0166285 further
reduced the viability of cells co-treated with TMZ (13). Similarly, we observed decreased neurosphere formation when TMZ was combined with MK-1775 in both GBM12 and GBM22. Interestingly, for the combination of MK-1775 with TMZ in a flank xenograft model, only the sensitive GBM22 line demonstrated significant enhancement in treatment efficacy. While 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 TMZ in our primary GBM xenograft models that was attributed to significant differences in drug exposure in vitro vs. in vivo (15). While several studies have suggested that the chemo- or radio-sensitizing 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 TMZ 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 blood brain barrier can be a significant obstacle to delivering small molecules into the brain. While 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 $^{14}$C-labelled 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. Based on heterogeneous accumulation of MK-1775 coupled with the limited penetration of MK-1775 into normal brain, we hypothesize the lack of...
efficacy for MK-1775 combined with TMZ 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 TMZ in GBM12 as a flank tumor model but not an orthotopic model (Kizilbash et. al., manuscript under preparation), while the combination of TMZ with the brain penetrant PARP inhibitor veliparib is effective in both flank and orthotopic models (15, 33). While 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 chemo-sensitizing agents across the BBB is critical to achieve optimal TMZ-sensitizing effects.

There are 2 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 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 prior to 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.

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REFERENCES


FIGURE LEGENDS

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 3 biological 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 in a CyQuant assay for 5 xenograft lines. Results shown are the mean ± SEM from three independent experiments. Statistically significant differences in fluorescence following a given treatment relative to control for each line are shown with * denoting p-value < 0.05, C) The sensitivity of GBM6, GBM12 and GBM22 to MK-1775 was assessed in a primary neurosphere assay. The number of neurospheres following drug treatment in 3 independent experiments is shown as the mean ± SEM. Statistically significant differences are noted comparing GBM22 vs. GBM12 and GBM22 vs. GBM6 at 100 and 300 nM. D) The impact of a 24 hour pre-treatment of MK-1775 on p-Cdk1 was assessed by western blotting. Because of low basal phosphorylation, GBM22 was irradiated (RT) with 10 Gy.

Figure 2: DNA damage analysis in GBM6 and GBM22 after MK-1775 treatment. A) γH2AX foci formation in GBM6 and GBM22 were assessed 24 h after a single treatment of 300 nM MK-1775. B) All cells with γH2AX staining (>20 foci/nuclei or pan-nuclear) or just those only with pan-nuclear γH2AX staining were quantitated and shown as the mean ± SEM from 3 independent experiments, magnification bar is 25 µm and * indicates p<0.05 compared to controls.

Figure 3: *in vitro* combination of MK-1775 and TMZ in GBM6 and GBM22. A) GBM6 and B) GBM22 cells were treated simultaneously with graded concentrations of TMZ and MK-1775 and then analyzed in a CyQuant assay. C) Western blot evaluation of GBM6 and GBM22 short-term explant cultures 24 hours after treatment with either MK-1775 or TMZ or the combination. Results are
representative of 3 independent experiments, * indicates p<0.05 for a given concentration of MK-1775 relative to TMZ/control alone.

**Figure 4: Efficacy of MK-1775 in combination with RT and TMZ in brain tumors.** Mice with established orthotopic GBM22 tumors treated with radiation (RT), TMZ and/or MK-1775 in a single experiment and survivals are presented in 3 graphs. A) The combination of MK-1775 (50 mpk twice daily Days 1-5 and 8-12) alone or concurrent with RT (2 Gy/day, Days 1-5 and 8-12). B) The same dosing regimen with TMZ (20 mpk/day given Days 1-5 and 8-12). C) Treatment with TMZ alone (50 mpk daily Days 1-5, 29-33 and 57-61) with MK-1775 (50 mpk twice daily Days 1-5, 29-33 and 57-61).

**Figure 5. Evaluation of MK-1775 distribution in brain and tumor tissues.** A) Mice received a single oral dose of MK-1775 (50 mg/kg) and blood and normal brain were collected at the indicated time-points. Results are the mean ± SD at each time-point (n=3 mice per point). Mice with established GBM22 B) orthotopic or C) flank tumors were euthanized two hours after a single dose of MK-1775 (200 mg/kg) and processed for MALDI-MSI. Red and green color intensities indicate relative levels of heme (m/z 616.2 ± 0.2) and MK-1775 (m/z 501.2 ± 0.2), respectively. Black dotted lines delineate tumor tissue in H&E stained sister sections. Results are representative of 3 mice in each condition.

**Figure 6: MK-1775 and TMZ efficacy evaluation in GBM22 flank xenografts.** A) Mice with established GBM22 flank xenografts were treated with TMZ 50 mpk with or without MK-1775 50 mpk twice daily on Days 1-5, 29-31 and 57-61. B) In the same experiment, extended dosing of MK-1775, given 5 days a week from Day 1 until Day 110, was evaluated alone and in combination with the same TMZ dosing regimen used in A. C) Animals with established GBM22 flank xenografts were treated for days 1-5 with TMZ 50 mg/kg/day, with or without MK-1775 dosed at 50 mg/kg twice daily.
for days 1-5 then days 8-12, and harvested on the days listed. Equal amounts of protein from 3 biological replicates were pooled and run in an individual lane as indicated.
Figure 1

A. GBM# 6 8 10 12 14 22 26 28 38 39 43 44

Wee1

β-Actin

B. MK-1775

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C. Neurosphere counts

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D. GBM6

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</table>

RT, 10 Gy
Figure 5

A. 

![Graph showing MK-1775 (µM) over time for Whole blood and Brain](image)

B. 

![Images showing Heme and MK-1775](image)

C. 

![Images showing Overlay of Heme and MK-1775](image)
Clinical Cancer Research

The efficacy of the Wee1 inhibitor MK-1775 combined with temozolomide is limited by heterogeneous distribution across the blood-brain barrier in glioblastoma

Jenny L Pokorny, David Calligaris, Shiv K Gupta, et al.

Clin Cancer Res  Published OnlineFirst January 21, 2015.

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