Cancer Therapy: Preclinical

Discordant In Vitro and In Vivo Chemopotentiating Effects of the PARP Inhibitor Veliparib in Temozolomide-Sensitive versus -Resistant Glioblastoma Multiforme Xenografts

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Abstract

Purpose: Effective sensitizing strategies potentially can extend the benefit of temozolomide (TMZ) therapy in patients with glioblastoma (GBM). We previously demonstrated that robust TMZ-sensitizing effects of the [poly (ADP-ribose) polymerase] (PARP) inhibitor veliparib (ABT-888) are restricted to TMZ-sensitive GBM xenografts. The focus of this study is to provide an understanding for the differential sensitization in paired TMZ-sensitive and -resistant GBM models.

Experimental Design: The impact of veliparib on TMZ-induced cytotoxicity and DNA damage was evaluated in vitro and in vivo in models of acquired TMZ resistance (GBM12TMZ-mgmtHigh, GBM12TMZ-mgmtLow, and U251TMZ), inherent TMZ resistance (T98G), and TMZ-sensitive (U251 and GBM12). In vivo drug efficacy, pharmacokinetics, and pharmacodynamics were analyzed using clinically relevant dosing regimens.

Results: Veliparib enhanced TMZ cytotoxicity and DNA-damage signaling in all GBM models in vitro with more pronounced effects in TMZ-resistant lines at 3 to 10 μmol/L veliparib. In vivo, combined TMZ/veliparib, compared with TMZ alone, significantly delayed tumor growth and enhanced DNA-damage signaling and γH2AX levels in the sensitive GBM12 xenograft line but not in the resistant GBM12TMZ lines. The pharmacokinetic profile of veliparib was similar for GBM12 and GBM12TMZ tumors with Cmax (~1.5 μmol/L) in tissue significantly lower than concentrations associated with optimal in vitro sensitizing effects for resistant tumors. In contrast, robust suppression of PARP-1 expression by shRNA significantly increased TMZ sensitivity of U251TMZ in vitro and in vivo.

Conclusions: In vitro cytotoxicity assays do not adequately model the therapeutic index of PARP inhibitors, as concentrations of veliparib and TMZ required to sensitize TMZ-resistant cancer cells in vivo cannot be achieved using a tolerable dosing regimen. Clin. Cancer Res; 20(14); 3730–41. ©2014 AACR.

Introduction

Temozolomide (TMZ) is an important component of conventional chemotherapy for glioblastoma (GBM) multiforme, but inherent and acquired resistance significantly limits its therapeutic efficacy (1–3). The main cytotoxic lesions induced by TMZ are N7-methylguanine (N7-MeG), N3-methyladenine (N3MeA), and O6-methylguanine (O6-MeG). N7-MeG and N3MeA are repaired by base-excision repair (BER), whereas the O6-MeG lesion is repaired by O6-methylguanine-DNA-methyl transferase (MGMT; refs. 3–7). Disruption of either repair process can sensitize tumors to TMZ cytotoxicity, but BER generally is robust in cells and is a minor determinant of overall TMZ responsiveness. In contrast, MGMT expression is silenced by promoter methylation in approximately one third of GBM, and high expression of MGMT is a common mechanism of inherent TMZ resistance and likely contributes to mechanisms of acquired resistance (7–9). In tumors lacking MGMT, unrepaired O6-MeG mispairs with thymidine and triggers futile cycles of mismatch repair (MMR) during replication, resulting in replication fork-associated DNA double-strand breaks (DSB) and cytotoxicity (7, 10). Although uncommon in untreated tumors, defects in MMR are an important mechanism of acquired TMZ resistance in recurrent GBM (11–13). Collectively, MGMT overexpression and MMR deficiency contribute to TMZ resistance in GBM, and defining strategies to sensitize resistant tumors to TMZ could significantly extend the survival gains associated with TMZ therapy.

References

Translational Relevance

Numerous preclinical studies have suggested that [poly (ADP-ribose) polymerase] (PARP) inhibitors, including veliparib, can enhance the efficacy of temozolomide (TMZ) in both sensitive and resistant tumor models. However, we previously demonstrated that the in vivo TMZ-sensitizing effects of veliparib were restricted exclusively to TMZ-sensitive glioblastoma xenograft models. Using paired sensitive and derivative resistant models, robust sensitization was observed in vitro for TMZ-resistant models at supratherapeutic drug concentrations. In contrast, using the maximally tolerable in vivo regimen that approximates clinically achievable drug exposures, robust sensitizing effects with veliparib only were observed in TMZ-sensitive models. Although these results have implications for understanding the spectrum of tumors that may respond to this novel therapeutic strategy, they also highlight the importance of evaluating PARP inhibitors or other novel chemosensitizing strategies in the context of clinically achievable drug concentrations.

Inhibitors of poly (ADP-ribose) polymerase 1 and 2 (PARP1/2) are potent TMZ-sensitizing agents being studied in clinical trials for GBM and other solid tumors (14, 15). PARP1/2 enzymes are responsible for poly ADP-riboseylation (PARylation) of numerous proteins and play crucial roles in modulating DNA repair. However, in context of BER, PARP1 also functions as a scaffold that recruits XRCC1 and DNA polymerase-β to apurinic sites, and the TMZ-sensitizing effects of PARP inhibition mainly have been ascribed to this function (4, 16–19). Consistent with this concept, genetic or pharmacologic inhibition of BER or PARP1/2 can significantly sensitize TMZ-resistant tumors in vitro (16, 17, 20–26). Contrary to these observations, we previously reported robust in vivo TMZ-sensitizing effects with the PARP inhibitor, veliparib that were limited to TMZ-sensitive primary GBM xenograft lines and these effects were lost in derivative TMZ-resistant xenograft models (27). The focus of this study is to evaluate both in vitro and in vivo chemosensitizing effects of veliparib in GBM models with differential TMZ sensitivities and to provide an understanding for the lack of sensitizing effects of veliparib in TMZ-resistant GBMs in vivo.

Materials and Methods

Cell culture, drugs, and antibodies

Short-term explant cultures (at passage 2–5) of GBM12 and derivative GBM12TMZ sublines were grown in neurobasal media (Life Technologies; refs. 9, 28). The generation of the TMZ-resistant GBM12 sublines with differential MGMT expression, GBM12TMZ-mgmt-low (#5476 or #5920) and GBM12TMZ-mgmt-high (#3080), were reported previously (9). U251 and T98G cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated by short tandem repeat analysis (29) performed by the ATCC in November 2013. The U251TMZ model was reported previously (28). U251, U251TMZ, and T98G malignant glioma cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Cyquant and neurosphere formation assays were performed as described previously (9). TMZ was purchased from the Mayo Clinic Pharmacy and veliparib obtained from the Cancer Therapy Evaluation Program at NCI, Bethesda, MD. For in vivo studies, TMZ was suspended in Ora-plus (Perrigo) and veliparib diluted in saline, and both were administered orally. Antibodies for phospho-S345-Chk1, phospho-T68-Chk2, γH2AX, Histone H3, β-actin, and PARP1 were obtained from Cell Signaling Technology; Chk1, Chk2, and replication protein-A (RPA) from Millipore; phospho-S824-KAP1 from Abcam; PAR from Trivigen; KAP1 and GAPDH from Santa Cruz Biotechnology; and MGMT from R&D Systems.

Lentivirus production and cell transduction

Lentivirus (pGIPZ) encoding shRNA for PARP1 and a nontargeting (NT) control obtained from the Mayo RNA Interference Shared Resource and packaged in HEK293 T cells. Transduction was performed in the presence of 5 µg/ml polybrene (Millipore) for 24 hours with subsequent selection with puromycin.

Western blotting

Cells were processed for protein extraction and subsequent SDS-poly acrylamide gel electrophoresis as previously described (9, 28). Acid extraction of nuclear proteins was performed to analyze γH2AX and Histone H3; cells were suspended in ice-cold PBS/0.5% TritonX100 for 10 minutes, centrifuged and supernatant discarded. Remaining nuclei were extracted with 0.2 N hydrochloric acid and soluble nuclear proteins were recovered.

Immunocytochemistry

Cells cultured on coverslips were fixed, permeabilized and immunostained with replication protein A and/or γH2AX (Cell Signaling Technology) antibodies in 1% bovine serum albumin at 4°C overnight. Cells were washed and then incubated with anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 conjugated IgG (BD Biosciences) before mounting with Slowfade/DAPI (Invitrogen). Immunostained cells were analyzed by confocal microscopy (Zeiss LSM510; 63× objective lenses) and the nuclei positive for foci were quantified.

Efficacy studies in vivo

Studies were reviewed and approved by the Mayo Institutional Animal Care and Use Committee. Subcutaneous xenografts were established by injecting the flank of athymic nude mice with 1 × 10^6 cells suspended in Matrigel/PBS. Mice with established tumors of approximately 100−15-mm^3 size were randomized and treated with placebo, TMZ (50 mg/kg/d) with or without veliparib (25 mg/kg/d,
delivered in two divided doses) for 5 days every 28 days for three cycles. Tumor volume was measured thrice weekly until euthanasia. Intracranial xenografts were established from virally transduced U251TMZ cells as previously described (9); one week after implantation, mice were treated as above, observed daily and euthanized upon reaching a moribund state. For pharmacokinetic analysis, mice with tumors were treated with TMZ and veliparib for 5 days, and tumor harvested 30 minutes, 2, 4, or 6 hours after the last dose. For pharmacodynamic assessment, tumors were harvested at 2 or 72 hours after the last drug dose.

**Tissue analysis of veliparib concentrations**

Tumor samples were homogenized, extracted with chilled acetonitrile, and chromatographic separations were achieved on an Aquasil C18 5-μm (250 × 4.6 mm) column (Thermo Fisher Scientific) using an acetonitrile/water mixture containing 0.1% formic acid delivered at a flow rate of 1.2 mL/min. The fractionated column effluent was monitored using fluorescence detection with an excitation and emission of 320 and 390 nm, respectively. Veliparib concentrations were determined using standard curves. Pharmacokinetics was analyzed using Phoenix WinNonLin software (Certara).

**Statistical analyses**

*In vitro* data presented are the mean ± SE from three or more experiments. Two-tailed Student *t* tests were used to measure statistical differences. The IC50 values were calculated by fitting the experimental data to a sigmoidal curve using GraphPad software or by linear regression specifically for GBM12TMZ-mgms<sup>High</sup>. Statistical analysis of animal survival and tumor progression was performed using the log-rank test.

**Results**

**Veliparib sensitizes GBM cells to TMZ**

The dose–response for inhibition of PARP activity was initially assessed in T98G cells. Using hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to maximally stimulate PARP, treatment with veliparib resulted in marked suppression of H<sub>2</sub>O<sub>2</sub>-induced poly ADP-ribosylation (PARylation) at concentrations greater than 1 μmol/L (Fig. 1A, top). At this concentration, veliparib effectively suppressed PARP activity for up to 6 days, which suggests that the drug is highly stable in culture media (Supplementary Fig. S1A). Therapeutically achievable concentrations of TMZ (100 μmol/L; Ref. 30) induced PARP activity in T98G cells with a steady increase in PARylation up to 24 hours (Fig. 1A, bottom). Consistent with these data, veliparib alone had minimal growth inhibitory effects, whereas combined veliparib/TMZ treatment significantly decreased the growth of T98G cells (Fig. 1B). At the lowest concentration of veliparib tested (1 μmol/L), no enhanced TMZ cytotoxicity was observed except at 300 μmol/L TMZ, and only a marginal increase in growth inhibition was observed with repeated coadministration of 100 μmol/L TMZ (Supplementary Fig. S1B). In contrast, significantly enhanced cytotoxicity was seen with 30 μmol/L veliparib at all concentrations of TMZ (Fig. 1B). The effects of combination therapy also were evaluated in the U251 cell line and the derivative TMZ-resistant U251TMZ line developed by our laboratory (28). Combining veliparib with TMZ showed enhanced effects in U251 cells and U251TMZ line (Fig. 1C). In combination with 10 μmol/L veliparib, the difference in cytotoxicity for 100 μmol/L TMZ was more profound in the TMZ-resistant U251TMZ cell line (relative fluorescence 0.95 ± 0.04 for TMZ alone vs. 0.14 ± 0.01 for the combination, *P* < 0.01) and T98G (1.15 ± 0.02 vs. 0.38 ± 0.02, respectively, *P* < 0.01) as compared with U251 cells (0.23 ± 0.02 vs. 0.15 ± 0.01, respectively, *P* < 0.01). Similarly, veliparib enhanced TMZ-induced apoptosis in all three lines with more pronounced effects seen in TMZ-resistant T98G and U251TMZ (Supplementary Fig. S2). Taken together, these data suggest that inhibition of PARP by veliparib effectively sensitizes both sensitive and resistant GBM cells to TMZ, and at high concentrations of veliparib, the sensitizing effects are more evident in TMZ-resistant models.

**Veliparib promotes TMZ-mediated DNA damage in GBM cells**

The impact of veliparib on TMZ-induced DNA-damage signaling was evaluated *in vitro*. Treatment with 100 μmol/L TMZ alone resulted in reproducible increased phosphorylation of Chk1, Chk2, and KAP1 only in U251 cells, whereas monotherapy with veliparib in U251 cells and veliparib or TMZ in U251TMZ resulted in nominal changes in DNA-damage signaling. In contrast, cotreatment with TMZ and 10 μmol/L veliparib resulted in markedly elevated phosphorylation of these proteins in all three tumor lines after 24 hours of treatment. The phosphorylation signals were maintained 72 hours after treatment in U251 and U251TMZ cells and reduced in intensity in T98G cells (Fig. 1D). As a second measure of DNA damage, the effects of drug treatment on induction of stalled replication forks (RPA foci) and DNA DSBs (γH2AX foci) were evaluated by immunofluorescence. As seen in Fig. 2, treatment with TMZ in U251 and TMZ/veliparib in all three cell lines induced γH2AX and RPA foci. Although TMZ treatment induced RPA and γH2AX foci in all lines tested, the fraction of cells with >20 γH2AX foci 24 hours after treatment was markedly lower in the resistant U251TMZ and T98G as compared with U251 cells. Cotreatment with veliparib and TMZ, compared with TMZ alone, resulted in a modest increase in the fraction of U251 cells with γH2AX foci 24 hours after treatment (90.6 ± 1.9% vs. 78.1 ± 7.6%, respectively; *P* = 0.042), whereas the combination had more profound effects in both resistant models: U251TMZ (85.0 ± 1.8% vs. 24.7 ± 4.2%, respectively; *P* < 0.002), and T98G (89.3 ± 3.6% vs. 20.7 ± 2.3%, respectively; *P* < 0.001). Similar results were observed for γH2AX 72 hours after treatment, and evaluation of RPA foci resulted in a highly similar result at both time points. Collectively, the differences in cell survival and the extent of DNA damage induced by TMZ alone versus the TMZ/veliparib combination are greater in...
the TMZ-resistant glioma lines as compared with a TMZ-sensitive line.

**Veliparib potentiates cytotoxic effects of TMZ in primary xenograft GBM lines in vitro**

Combination therapy was evaluated further in a patient-derived xenograft line that faithfully maintains the molecular features of the derivative patient tumor (31) and in two derivative TMZ-resistant sublines we developed by treating GBM12 tumors with a clinically relevant TMZ dosing regimen (9). The effects of veliparib on TMZ cytotoxicity were evaluated in neurosphere cultures, which maintain a pluripotent phenotype (Supplementary Fig. S3). Parental GBM12 was markedly sensitive to even 10 μmol/L TMZ with only 20.5% ± 2.3% neurosphere formation compared with control and no surviving neurospheres after 100 μmol/L TMZ. In contrast, GBM12TMZ-mgmtLow and GBM12TMZ-mgmtHigh sublines were significantly resistant to 100 μmol/L TMZ (39.4% ± 1.4% and 95.7% ± 3.1% relative neurosphere formation, respectively; Fig. 3A). The addition of veliparib significantly enhanced the efficacy of TMZ in all three xenograft lines with more pronounced effects observed at higher concentrations of TMZ and/or veliparib (Fig. 3A and Supplementary Fig. S4A). At each concentration tested, veliparib treatment was associated with a reduced IC50 for TMZ in all three xenograft lines. The differences in IC50 with and without veliparib were more pronounced in the TMZ-resistant sublines (Fig. 3B), although at a concentration of 1 μmol/L veliparib, the TMZ IC50 values for the GBM12TMZ-mgmtLow (38 μmol/L) and GBM12TMZ-mgmtHigh (207 μmol/L) are significantly higher than the IC50 value for TMZ only in GBM12 (6.9 μmol/L). Similar chemopotentiating effects of veliparib also were observed in other xenograft lines with differential TMZ sensitivities.
Figure 2. Veliparib promotes TMZ-induced γH2AX and RPA foci formation in GBM cell lines. U251 (A), U251TMZ (B), and T98G (C) cells cultured on glass cover slips were treated with DMSO, 10 μmol/L veliparib for 30 minutes and subsequently with or without 100 μmol/L TMZ for 24 or 72 hours and immunostained for RPA32 and γH2AX and counterstained with DAPI. Top, representative images (bar, 10 μm), and graphs depicted below present the mean ± SEM from three independent experiments for the percentage of cells with >20 foci/nuclei for each treatment; *, P < 0.05.
Figure 3. Veliparib sensitizes GBM12 derivative TMZ-resistant xenograft lines in vitro. A and B, cytotoxicity assay: The sensitive GBM12 and derivative TMZ-resistant sublines, GBM12-TMZ-mgmtLow and GBM12-TMZ-mgmtHigh, were analyzed for neurosphere growth following treatment with graded concentrations of veliparib and/or TMZ. A, the relative neurosphere counts for the indicated treatments are graphed as the mean ± SEM from three independent experiments. B, the calculated IC50 value for TMZ at different concentrations of veliparib is plotted as the mean ± SD from three independent experiments. C, DNA-damage signaling: GBM12 and derivative sublines were treated for 24 hours with the indicated doses of veliparib and/or TMZ and then processed for immunoblotting with the indicated antibodies. D, cells were analyzed for γH2AX foci as in Fig. 2 after drug treatment for 24 hours. Results are plotted as the mean ± SEM from a minimum of three independent experiments; *, P < 0.05; NS, P > 0.05; MW, molecular weight.
Veliparib enhances TMZ-induced DNA-damage signaling

The impact of graded veliparib concentrations on TMZ-induced damage signaling was evaluated in vitro for the GBM12 sublines. TMZ induced robust activation of PARP activity, and veliparib was equally effective at suppressing PAR levels in all three sublines with complete suppression of detectable PAR at concentrations of 0.3 μmol/L veliparib (Fig. 3C). A similar concentration range (1–3 μmol/L) combined with TMZ was associated with reproducibly increased phosphorylation of Chk1, Chk2, and KAP1 as compared with TMZ alone in all three xenograft lines. Furthermore, veliparib equally enhanced TMZ-induced DNA-damage signaling in undifferentiated (neurospheres) or differentiated (FBS supported monolayer) cell cultures of each xenograft line (Supplementary Fig. S5). Treatment with TMZ alone resulted in significantly increased γH2AX foci only in GBM12 cells. Cotreatment resulted in significantly increased γH2AX foci compared with TMZ alone in all three lines: GBM12 (74.2% ± 2.1% cell with >20 foci vs. 59.4% ± 2.0% with TMZ alone; P = 0.002), GBM12TMZ-mgmtLow (77.3% ± 2.8% vs. 17.6% ± 3.7%, respectively; P = 0.0005), and GBM12TMZ-mgmtHigh (64.2% ± 3.7% vs. 12.9% ± 1.9%, respectively; P = 0.0009; Fig. 3D). Taken together, these results indicate that veliparib enhances TMZ-induced DNA-damage signaling response in each of the xenograft lines regardless of their differential sensitivity to TMZ.

Differential TMZ-sensitizing effects of veliparib in vivo

The efficacy of combined veliparib and TMZ was assessed in the GBM12 and derivative sublines as subcutaneous xenografts to eliminate issues of drug delivery across the blood–brain barrier. Consistent with observed chemosensitization in vitro, the combination of veliparib and TMZ resulted in significant delay in tumor progression in parental GBM12 as compared with treatment with TMZ alone (difference in median time to endpoint 68 days for TMZ vs. TMZ/veliparib, P = 0.003; Fig. 4A and Supplementary Fig. S6). However, tumor progression in both GBM12TMZ sublines was unaffected by the combination as compared with TMZ alone (difference in median time to end point –4 days for GBM12TMZmgmtLow (P = 0.85) and +1 day for GBM12TMZmgmtHigh (P = 0.67; Fig. 4A and Supplementary Fig. S6). These data demonstrate a marked discordance between the profound sensitizing effects observed in vitro compared with a lack of sensitizing effect in vivo for the resistant lines.

Pharmacodynamic effects were evaluated in the primary GBM12 line and the GBM12TMZ-mgmtHigh subline to compare the effects of veliparib on TMZ-induced damage signaling after 5 days of therapy. As expected, veliparib alone or in combination with TMZ efficiently suppressed PARP activity 2 hours, but not as effectively at 72 hours, after the last drug treatment. In the GBM12 xenografts, there was robust induction of phospho-KAP1, phospho-Chk1, and phospho-Chk2 associated with TMZ treatment with or without veliparib at both time points (Fig. 4B). In contrast, discrete but marginal increases in DNA damage signaling were observed in the GBM12TMZ-mgmtHigh subline only 2 hours after the last dose of TMZ/veliparib combination for phospho-KAP1 and phospho-Chk1 with no evidence of persistent activation at 72 hours (Fig. 4B). Analysis of nuclear extracts demonstrated enhanced γH2AX levels with combination TMZ/veliparib, as compared with TMZ alone in GBM12 samples harvested 72 hours after drug treatment. In contrast, TMZ alone or TMZ/veliparib had a marginal increase in γH2AX signal 2 hours but not 72 hours after the last drug dose in GBM12TMZ subline (Fig. 4B). Collectively, these data demonstrate that lack of sensitization in vivo by veliparib in the TMZ-resistant GBM12TMZ-mgmtHigh subline is associated with nominal induction of DNA damage.

Pharmacokinetic evaluation

The tumor tissue concentration–time profile of veliparib was determined to evaluate whether lack of tumor response in the resistant tumor model was associated with differences in achievable drug levels. For GBM12, tumors harvested at 30 minutes (Tmax determined previously) revealed a tumor tissue Cmax of 1.23 ± 0.11 μmol/L and an AUC0–6 h value of 3.04 μmol/L·h (Fig. 4C). The tumor tissue concentration–time profile in GBM12TMZ-mgmtHigh was similar with a slightly higher Cmax of 1.36 ± 0.28 μmol/L (P = 0.55 vs. Cmax for GBM12) and AUC0–6 h of 4.4 μmol/L·h (P = 0.10 vs. AUC for GBM12). The half-life values of the drug in GBM12 and GBM12TMZ-mgmtHigh tumors were 2.6 and 2.3 hours, respectively (P = 0.60; Fig. 4C). In comparison with the in vitro concentration–response curves for the GBM12 sublines, the maximum concentration of veliparib (1.2–1.4 μmol/L) in vivo is much lower than optimal concentrations associated with robust TMZ-sensitizing effects in vitro.

PARP knockdown potentiates TMZ response in the TMZ-resistant model

Results from our pharmacologic analyses implied that lack of efficient chemosensitization in resistant tumors may be related to the biologically achievable/tolerable concentrations of veliparib. To test this possibility, the effects of PARP1 knockdown were assessed in the U251TMZ model. As seen in Fig. 5A, effective PARP1 knockdown was achieved using three different lentiviral shRNA constructs (D2, F6, and F10), and PARP1 knockdown significantly suppressed PAR accumulation in response to H2O2 as compared with nontransduced or cells transduced with a nontargeted shRNA (Fig. 5A). In a cell growth assay, PARP1 knockdown (1.2–1.4 μmol/L) in vivo is much lower than optimal concentrations associated with robust TMZ-sensitizing effects in vitro.
with TMZ alone was as effective as TMZ/veliparib. These data demonstrate that PARP1 knockdown in U251TMZ cells can provide equivalent TMZ-sensitizing effects as an optimal concentration of veliparib in vitro.

The U251TMZ-NT and U251TMZ-D2 sublines then were used in an intracranial tumor model to evaluate the effects of PARP1 knockdown on TMZ responsiveness in vivo. As expected the U251TMZ-NT subline was highly resistant to TMZ therapy with no significant gain in survival compared with placebo treatment ($P = 0.30$), and similar to previous experience with GBM12TMZ lines, the combination of veliparib with TMZ was equally ineffective ($P = 0.81$ compared with TMZ alone, and $P = 0.09$ compared with placebo; Fig. 5C). In contrast, the U251TMZ-D2 subline
was remarkably sensitive to TMZ compared with placebo with a significant extension in survival (median survival 26 days vs. 9 days, \( P < 0.001 \); Fig. 5D). These results suggest that effective PARP suppression in TMZ-resistant tumors may provide significant gain in TMZ sensitivity similar to that seen \textit{in vitro} with the combination of TMZ and veliparib.

**Discussion**

The combination of PARP inhibitors with specific chemotherapies can provide significant sensitizing effects in preclinical models, and there is intense interest in moving the most promising regimens into clinical trials. The data presented here demonstrate profound \textit{in vitro} TMZ-sensitizing effects of veliparib in four GBM models of acquired (U251TMZ, GBM12TMZ-mgmt\textsuperscript{High} or GBM12TMZ-mgmt\textsuperscript{Low}) or inherent (T98G) TMZ resistance as compared with more modest sensitizing effects in TMZ-sensitive models (U251 and GBM12). Multiple other studies have observed a similar phenomenon with more pronounced \textit{in vitro} TMZ-sensitizing effects of PARP inhibitors observed in TMZ-resistant models (14, 25, 32). Although direct comparison of \textit{in vitro} and \textit{in vivo} results for sensitive versus resistant lines have not been published previously, the \textit{in vivo} sensitizing effects of PARP inhibition generally are limited in TMZ-resistant models and more profound in TMZ-sensitive models (14, 15, 18, 22, 33–39). Clear understanding of the basis for this striking difference between \textit{in vitro} and \textit{in vivo} sensitizing effects has important implications for understanding the spectrum of tumors that may respond to this novel therapeutic strategy.

The discordance between cell culture and animal studies at least partially can be explained by examining \textit{in vitro} sensitizing effects relative to tolerable drug concentrations in animals or humans. TMZ is clinically dosed at 150 to 200 mg/m\textsuperscript{2} to achieve peak concentrations approximating 30 to 100 \( \mu \)mol/L in humans, and the dosing schedule used in the animal studies reported here provide a similar pharmacokinetic profile in mice as in humans (30). Conversely,
hematologic toxicities limit the maximally tolerated dose of veliparib in humans to 40 mg/kg twice daily when given concurrently with TMZ. This dose of veliparib is associated with peak plasma concentrations of less than 1.5 μmol/L in humans, which is similar to the drug concentrations achieved in mice in these studies (Fig. 4C; refs. 33, 40). The initial dose–response evaluation presented in Fig. 1B demonstrates that effective sensitization of T98G cells with 1 μmol/L veliparib was only achieved at supratherapeutic concentrations of 300 μmol/L TMZ, and the more profound sensitizing effects within the clinically achievable TMZ concentration range were limited to supratherapeutic levels of veliparib. A similar effect was seen in the primary GBM12 xenograft line and derivative resistant models in which the sensitizing effects were nominal at 1 μmol/L veliparib (Fig. 3B). In this same context, the profound TMZ-sensitizing effects of PARP inhibitors in TMZ-resistant models shown in multiple studies mostly are limited to clinically unachievable concentrations of either TMZ and/or the PARP inhibitor (14, 18, 25, 32, 38, 41). These data highlight the importance of interpreting in vitro results in the context of clinically relevant drug concentrations.

The in vitro veliparib/TMZ combination data analysis suggests that the limited in vivo efficacy observed in TMZ-resistant models may be related to inadequate suppression of PARP activity in animals. Consistent with this idea, PARP1 knockdown effectively sensitized the U251TMZ subline to TMZ in vitro to an extent similar as supraphysiologic levels (10 μmol/L) of veliparib. These data suggest that inhibition of PARP1 specifically may be important for sensitizing effects at these drug concentrations, and the residual PAR modifications seen in Fig. 5A for the shPARP1-treated cells may reflect basal PARP2 activity. Similar studies in HeLa and B16 tumor cell lines have demonstrated robust TMZ-sensitizing effects in vitro and in vivo following PARP1 knockdown without additional sensitizing effects when cells were cotreated with the PARP inhibitor GPI15427 (26). These data exclude veliparib-mediated PARP trapping onto DNA as a probable mechanism of the in vitro sensitizing effects observed here (42). Similar to the in vitro results, the PARP knockdown in U251TMZ-shPARP-D2 xenografts significantly sensitized this highly resistant model to TMZ, whereas veliparib had no impact on TMZ efficacy in the corresponding nontargeted shRNA subline (Fig. 5D). Although the biochemical effects of shRNA knockdown versus pharmacologic inhibition are distinct, the shRNA approach allowed robust suppression of PARP1 activity without associated systemic toxicity when combined with TMZ. Considering that tolerable dosing of veliparib with TMZ limits the exposure to approximately 1 μmol/L veliparib (Fig. 4C), the shRNA data presented suggest that the achievable drug levels provide insufficient suppression of PARP1 activity to effectively sensitize resistant tumor models to TMZ.

The impact of veliparib on various PARP-mediated DNA repair processes, and the mechanism of TMZ sensitization at clinically achievable concentrations remains unclear. PARP1/2 facilitates the recruitment of BER proteins to abasic sites, and PARP1 knockdown or robust inhibition can efficiently suppress BER (16). Because more than 80% of TMZ-induced lesions are repaired by BER and disruption of BER significantly enhances the lethality of TMZ, the disruption of BER by PARP inhibition is widely viewed as the likely mechanism of sensitization (43). Consistent with this notion, robust sensitization was observed in the TMZ-resistant tumor lines at high concentrations of veliparib. However, at clinically relevant concentrations of ≤1 μmol/L veliparib, the efficacy of TMZ therapy was enhanced only in TMZ-sensitive models (Fig. 4A), and the extent of in vitro enhancement was modest compared with more profound sensitizing effects observed in vivo (Figs. 3A and 4A). In comparing the extent of DNA damage induction in vivo (Fig. 4B), TMZ with or without veliparib did not induce significant DNA-damage signaling in the MGMT-overexpressing GBM12TMZ subline, whereas the same treatment induced robust damage signaling in the parental GBM12 line. MGMT directly removes O6MeG lesions, which otherwise mispair with thymidine, trigger futile cycles of MMR, and ultimately lead to replication-associated DNA DSBs. Recent studies have demonstrated a role for PARP in preventing MRE11-mediated degradation of stalled replication forks (9, 44), and we hypothesize that the effects of veliparib on the processing of O6MeG-induced stalled replication forks may be an important contributor to the sensitizing effects of this drug in vivo.

The results of this study provide critical insight into the design of clinical trials with veliparib and possibly other PARP inhibitors. The lack of efficacy of veliparib combined with TMZ in resistant models suggests that this treatment regimen is unlikely to be useful in patients that have progressed on a TMZ-based therapy or in tumors that are inherently resistant to TMZ. On the basis of these and other data, the Alliance cooperative group is launching a randomized phase II/III clinical trial to test the efficacy of veliparib combined with TMZ in newly diagnosed patients with GBM. Using MGMT promoter hypermethylation as a biomarker for TMZ-sensitive tumors, this trial only will enroll patients with tumor MGMT promoter hypermethylation. Our data would suggest that robust TMZ-sensitizing effects could be achieved in either TMZ-sensitive or -resistant tumors if PARP activity can be sufficiently suppressed. This is especially interesting in the context of the next generation of highly potent and specific PARP inhibitors (45–47). Combinations of TMZ with these more potent PARP inhibitors are less well tolerated in animals than veliparib and require significant dose reductions of either TMZ or the PARP inhibitor (unpublished data). Thus, similar to previous clinical studies with the MGMT inhibitor O6-benzylguanine, successful clinical integration of these more potent PARP inhibitors with TMZ may be limited by a narrow therapeutic window (48). These observations reinforce the importance of evaluating the potential clinical benefit for specific PARP inhibitor/TMZ combinations in the context of clinically achievable concentrations of the combination partners.
Disclosure of Potential Conflicts of Interest
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Authors' Contributions
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