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Restricted delivery of talazoparib across the blood-brain barrier limits the sensitizing effects of poly (ADP-ribose) polymerase inhibition on temozolomide therapy in glioblastoma

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Abstract

Poly (ADP-ribose) polymerase PARP inhibitors, including talazoparib (TAL), potentiate temozolomide (TMZ) efficacy in multiple tumor types, however TAL-mediated sensitization has not been evaluated in orthotopic glioblastoma (GBM) models. This study evaluates TAL ± TMZ in clinically relevant GBM models. TAL at 1–3 nmol/L sensitized T98G, U251 and GBM12 cells to TMZ, and enhanced DNA damage signaling and G2/M arrest *in vitro*. *In vivo* cyclical therapy with TAL (0.15 mg/kg twice daily) combined with low dose TMZ (5 mg/kg daily) was well tolerated. This TAL/TMZ regimen prolonged tumor stasis more than TMZ alone in heterotopic GBM12 xenografts [median time to endpoint: 76 days vs. 50 days TMZ (p=0.005), 11 days placebo (p<0.001)]. However, TAL/TMZ did not accentuate survival beyond that of TMZ alone in corresponding orthotopic xenografts (median survival 37 vs. 30 days with TMZ (p=0.93), 14 days with placebo, p<0.001). Average brain and plasma TAL concentrations at 2 hours after a single dose (0.15 mg/kg) were 0.49±0.07 ng/g and 25.5±4.1 ng/ml, respectively. The brain/plasma distribution of TAL in Bcrp^{-/-} versus WT mice did not differ, while the brain/plasma ratio in Mdr1a/b^{-/-} mice was higher than WT mice (0.23 vs. 0.02, p<0.001). Consistent with the *in vivo* brain distribution, overexpression of MDR1 decreased TAL accumulation in MDCKII cells. These results indicate that TAL has significant MDR1 efflux liability that may restrict delivery across the blood-brain barrier, and this may explain the loss of TAL-mediated TMZ sensitization in orthotopic versus heterotopic GBM xenografts.

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Keywords

Talazoparib; Temozolomide; Glioblastoma; Blood-brain barrier; Drug efflux

Introduction

Temozolomide (TMZ) is an important component of therapy for patients with glioblastoma (GBM), however emergence of drug resistance leads to inevitable disease recurrence and dismal prognosis despite optimal therapy (1, 2). Mechanisms of TMZ resistance include the direct repair of TMZ induced O⁶-methylguanine (O⁶MeG) lesions by O⁶-methylguanine-DNA-methyltransferase (MGMT) (1, 3, 4) and N⁷-methylguanine (N⁷MeG) and N³-methyladenine (N³MeA) lesions by the base excision repair (BER) (5, 6). Mutations in mismatch repair genes (7–9) and redundancies in DNA repair pathways also contribute towards TMZ-resistance (10). Thus, targeting DNA repair is expected to enhance the therapeutic efficacy of TMZ (11). The poly (ADP ribose) polymerase (PARP) family of enzymes is responsible for the poly ADP-ribosylation (PARylation) of numerous DNA repair proteins; PARP1 also functions as a scaffold to recruit BER components XRCC1 and DNA polymerase-β to apurinic sites (11, 12). Therefore, PARP inhibition is thought to potentiate TMZ efficacy by disrupting BER mediated repair of the TMZ-induced DNA lesions N⁷MeG and N³MeA (13). However, prior studies from our laboratory suggest that the presence of O⁶MeG lesions may be necessary for PARP inhibitor induced TMZ sensitization *in vivo* (14–16). This capacity of PARP inhibitors to sensitize cancer cells to TMZ has emerged as a promising strategy for patients with GBM.

Differences in molecular structure amongst PARP inhibitors greatly influence their biological effects beyond PARP inhibition alone (17). The trapping of PARP1/2 on chromatin by certain PARP inhibitors generates PARP-DNA complexes thought to be more cytotoxic than unrepaired single-strand DNA breaks caused by inhibition of PARP enzymatic activity, and the trapping ability of various PARP inhibitors has been a crucial measure of their potency (17). The most potent PARP trapping agent, talazoparib (TAL) induces a 100-fold greater PARP trapping than olaparib and rucaparib, and is associated with increased cytotoxicity in BRCA deficient tumor models (18–20). Moreover, TAL binds to the variable D-loop of PARP1/2, shorter D-loop in PARP3 and tankyrases cannot accommodate TAL, leading to decreased off-target effects (21). Furthermore, the significant efficacy of TAL when combined with low dose TMZ in multiple tumor models indicates that the cytotoxicity of the combination may be initiated by non-O⁶MeG adducts (N⁷MeG and N³MeA) that promote PARP trapping (22). Although heterotopic GBM models were included in this evaluation, these models may not account for tissue specific constraints including the role of the blood-brain barrier (BBB) associated with GBM therapy. Here we investigated the efficacy of TAL/TMZ in both GBM cell lines and patient-derived xenograft (PDX) models. After determining a tolerable *in vivo* TAL/TMZ regimen, the efficacy was assessed in both heterotopic and orthotopic PDX GBM models. Finally, mechanisms that influence the distribution of TAL across the BBB were investigated.

Materials and Methods

Cell culture and drugs

Short-term explant cultures of the GBM12 PDX line were raised in neural stem cell media (Stem Pro NSC SFM; Invitrogen) (15). U251 (MGMT-low) and T98G (MGMT-high) glioma cell lines obtained from the American Type Culture Collection (ATCC) in the years 1998 and 2012, respectively, and were authenticated by short tandem repeat analysis (23, 24) performed by the ATCC in November 2013. Madin-Darby canine kidney II (MDCKII) cells overexpressing either human multidrug resistance protein 1 (MDR1) or murine breast cancer resistance protein (BCRP1) were gifts from Dr. Piet Borst *circa* (year 1996) and Dr. Alfred Schinkel *circa* (year 2003), respectively (Netherlands Cancer Institute). Cell lines were maintained in DMEM (VWR, Radnor, PA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and 1% penicillin/streptomycin (Fisher Scientific). To ensure quality control, cell cultures were routinely tested for mycoplasma at six months interval using MycoAlert mycoplasma detection kit (cat # LT07-418, Lonza) as per the instruction manual. If necessary, cultures were treated with 50 µg/ml plasmocin (In Vivogen). CyQuant, clonogenic and neurosphere formation assays were performed as previously described (15).

Radiolabeled ³H-vinblastine and ³H-prazosin were procured from Moravsek Biochemicals and PerkinElmer, respectively. For *in vitro* studies, TMZ (Sigma-Aldrich), TAL (Medivation), zosuquidar (MDR1 inhibitor, Lilly) (25) and Ko143 (BCRP inhibitor, Tocris) (26) were dissolved in DMSO and stored at -20°C. For *in vivo* studies, TMZ (Mayo Clinic Pharmacy) was suspended in Ora-plus (Perrigo, Dublin, Ireland) and TAL in 10% dimethylacetamide (Sigma-Aldrich)/5% Solutol HS15 (Sigma-Aldrich)/85% phosphate buffered saline. Both drugs were administered by oral gavage.

Western blotting and Immunofluorescence

Dose-response for PARP inhibition and analysis of TAL/TMZ induced DNA damage signaling were evaluated as previously described (15). Briefly, cells were treated with the indicated concentrations of TAL, TMZ or their combination for 24–48 hours. Extracted proteins were analyzed by western blotting using the antibodies phospho-S345-Chk1, phospho-T68-Chk2, β-actin, PARP1, Histone H3 (Cell Signaling); Chk1, Chk2 (Millipore); phospho-S824-KAP1, PARP2 (Abcam); PAR (Trevigen) and KAP1 (Santa Cruz) as described in supplementary methods. Densitometry on original blots was quantified by ImageJ software and the signal was normalized to the corresponding control lane. Chromatin trapping of PARP1 and PARP2 was assessed by incubating U251 cells for three hours with graded concentrations of TAL in the presence of 100 µmol/L of TMZ. Chromatin-bound proteins isolated by acid extraction were analyzed by western blotting. To assess Ki67, glass slides carrying tissue sections were subjected to antigen retrieval in boiling Tris-sodium chloride buffer (pH 9.0) followed by immunostaining with Ki-67 specific rat monoclonal antibody (eBioscience cat# SolA15) and processed for immunofluorescence detection and analysis as described in supplementary methods. For detection of γH2AX and RPA foci, cells cultured on glass coverslips were treated with TMZ and/or TAL for 24 or 72 hours and processed as previously described (15).

Flow cytometry

Cells treated with TMZ and/or TAL were fixed in ice cold 70% ethanol, incubated with RNase A and propidium iodide and subsequently analyzed by flow cytometry as previously described (27). Modeling of cell cycle events was performed using Modfit-LT version 4.

In vivo efficacy studies

All animal experiments were preapproved by the Institutional Animal Care and Use Committee (IACUC), and conducted in accordance with IACUC guidelines. Xenografts were established in female athymic nude mice (Hsd:athymic Nude-Foxn1^{nu}, ages 6–7 weeks; Envigo, Indianapolis, IN) as previously described (15, 16). Mice with established tumors were randomized into treatment groups. Treatment arms included placebo, a range of TMZ dosing schedules (5–50 mg/kg, days 1 to 5, repeated every 14 or 28 days), a range of TAL (0.025 or 0.15 mg/kg, days 1 to 5, repeated every 14 or 28 days) or a TAL/TMZ combination. Heterotopic flank tumors were measured thrice weekly, and mice were euthanized when tumor volume exceeded 2000 mm³. Mice with intracranial xenografts were observed daily and euthanized upon reaching a moribund state.

Evaluation of pharmacodynamic effects

Pharmacodynamic (PD) effects were evaluated in mice with established flank tumors treated with vehicle, TMZ (5 mg/kg/d), TAL (0.15 mg/kg, twice daily) or the combination of TMZ and TAL for 5 days and then euthanized 72 hours after the last TMZ dose. Tumors were harvested and processed for protein extraction and immunoblotting as previously described (15).

Central nervous system (CNS) drug distribution

Non-tumor bearing FVB wild-type (WT), Mdr1a/b^{-/-} (MKO), Bcrp^{-/-} (BKO) and Mdr1a/b^{-/-}Bcrp^{-/-} knockout (TKO) mice were treated with a single dose of TAL (0.15 mg/kg) and euthanized 2 hours after drug administration; whole blood and brain were collected for analysis. Plasma isolated by centrifugation and the brain tissues were flash frozen on dry ice and homogenized in three volumes of 5% bovine serum albumin per plasma volume (or brain weight; TAL concentrations were determined by liquid chromatography – tandem mass spectrometry (LC/MS-MS).

In vitro analyses of intracellular TAL accumulation

In vitro intracellular TAL accumulation was assessed by using vector-controlled MDCKII cells that overexpressed either human MDR1 or murine Bcrp1 as previously described(28). Cells were cultured to 80% confluence, rinsed with serum-free assay buffer, and pre-treated with (or without) 1 μmol/L zosuquidar or 0.2 μmol/L Ko143 for 30 minutes, and subsequently supplemented with TAL, ³H-vinblastine or ³H-prazosin. After incubation at 37°C for 1 hour, cells were rinsed with ice cold PBS and lysed with 1% Triton X-100 (Thermo Scientific) and TAL concentrations determined by LC/MS-MS.

Analysis of TAL by LC/MS-MS

TAL was extracted from cell lysate, brain homogenate or plasma with cold ethyl acetate. The organic supernatant was dried with nitrogen, and the sample was reconstituted in 50 μL of 5 mmol/L ammonium acetate in acetonitrile (mobile phase). Five μL samples were injected onto the column (Agilent Zorbax SB C18 5 μm ; 150 X 0.5 mm), and LC/MS-MS was performed on an LTQ Orbitrap Velos instrument (Thermo Scientific, Waltham, MA) interfaced with a Nano2D–LC HPLC (Eksigent, Dublin, CA) using positive electrospray ionization. Separation on the column was performed using a linear gradient (relative acetonitrile to 5 mM ammonium acetate concentrations varied between 20–90%) at a mobile phase flow rate of 15 $\mu\text{L}/\text{min}$. Analysis was performed using accurate mass extracted ion chromatograms of m/z 285.08997+ and 298.07922+ (parent ion m/z 381.1) for TAL with a mass tolerance of 2 ppm.

Statistical analyses

Unless stated otherwise, all *in vitro* data presented are mean $\pm$ standard error of the mean (SEM) from three or more experiments. Two-sample t-tests were used to compare measures across treatment groups. The IC_{50} values were calculated by fitting the experimental data to a sigmoidal curve using GraphPad Prism. Combination effects of TAL and TMZ on cell growth were assessed by the Bliss independence log synergy volumes ($\mu\text{M}^2\%$), which were calculated using MacSynergy II and reported in the form of 3-dimensional graphs with TMZ and TAL concentrations plotted on the X and Y dimensions, and Bliss independence log synergy volume on Z dimension(29). Median survival and tumor progression beyond 1,500 mm^3 (time to endpoint) were estimated by the Kaplan-Meier method and compared using the log rank test. In all cases, p-values <0.05 were considered statistically significant.

Results

TAL sensitizes GBM cells to TMZ

The inhibitory dose-response of PARP activity by TAL was initially evaluated in U251 and T98G cell lines exposed to 100 $\mu\text{mol}/\text{L}$ of hydrogen peroxide to maximally stimulate PARP activity. A near complete suppression of PARylation occurred at 3 nmol/L and 10 nmol/L of TAL in U251 and T98G cells, respectively (Figure 1A). In the presence of 100 $\mu\text{mol}/\text{L}$ of TMZ, TAL also induced robust trapping of PARP1 and PARP2 at concentrations of 3 nmol/L and higher in U251 cells (Figure 1B). These results affirm the potent PARP inhibitory and trapping effects of TAL in GBM cells.

Higher concentrations of TAL (10 nmol/L in U251; 30 nmol/L in T98G cells) were needed for enhanced cytotoxicity as compared to controls (Figure 1C–D). Although combining TAL with TMZ significantly increased cytotoxicity in U251 cells (Figure 1C, Figure S1A), cumulative synergy was minimal (12.4 $\mu\text{M}^2\%$) (Figure 1C, E). In contrast, low concentrations of TAL (1–3 nmol/L) robustly enhanced TMZ (3–100 $\mu\text{mol}/\text{L}$) cytotoxicity in T98G cells (Figure 1D, Figure S1B) and demonstrated marked synergy (84.0 $\mu\text{M}^2\%$) (Figure 1D, E). The increased sensitivity to TAL in the MGMT expressing T98G cell line is predicted because of the lack of lethal O^6MeG . The presence of O^6MeG in TMZ treated U251 cells (MGMT non-expressing) minimizes the impact of TAL induced conversion of N^7

and N³ adducts to cytotoxic lesions. Similar sensitizing effects of TAL were also observed in clonogenic assays with an additive effect of TAL combined with TMZ (3.4 $\mu\text{M}^2\%$) in U251 cells (Figure S2A, C), and a more significant synergy (34.3 $\mu\text{M}^2\%$) with low dose TAL combined with TMZ in T98G cells (Figure S2B, C). These results suggest that TAL potentiates TMZ efficacy in both TMZ sensitive and resistant GBM cell lines.

TAL potentiates DNA damage signaling and G2/M cell cycle arrest

Incubation with TAL (1–10 nmol/L) had no significant impact on the phosphorylation of Chk1, Chk2 or KAP1 in either of the glioma cell lines while exposure to clinically relevant TMZ concentrations expectedly induced DNA damage signaling in U251 cells but not in T98G cells (Figure 2A). However, co-treatment with TAL (3 or 10 nmol/L) and TMZ (10 or 30 $\mu\text{mol/L}$) robustly increased phosphorylation of Chk1, Chk2 and KAP1 in U251 cells. Similarly, TAL (1–10 nmol/L) combined with supra-therapeutic concentrations of TMZ (300 $\mu\text{mol/L}$) increased the phosphorylation of all three DNA damage signaling proteins in T98G cells, while combining TAL (3 or 10 nmol/L) with 30 $\mu\text{mol/L}$ TMZ increased the phosphorylation of KAP1 and Chk2 but had no impact on phospho-Chk1 (Figure 2A, Figure S3A). Similarly, TAL (3 nmol/L) had no effect on γH2AX or RPA foci compared to DMSO treatment in either U251 or T98G cells, while treatment with TMZ (100 $\mu\text{mol/L}$) increased γH2AX and RPA foci in U251 (but not in T98G) cells. Co-treatment with TAL and TMZ increased foci formation in both glioma cell lines (Figure 2B–C). Cell cycle analyses by flow cytometry demonstrated increased G2/M arrest with TAL/TMZ co-treatment as compared to treatment with either drug alone (figure S4). These results suggested that TAL promotes TMZ induced DNA damage and signaling response in GBM cells.

TMZ sensitizing effects of TAL on primary neurosphere growth in vitro

The *in vitro* efficacy of TAL was also assessed in the MGMT promoter hypermethylated GBM12 PDX line. GBM12 is sensitive to TMZ alone with an IC₅₀ of 5.1 $\mu\text{mol/L}$, and treatment with TAL (3 or 10 nmol/L) alone also led to reduced neurosphere formation (Figure 3A, Figure S1C). Co-treatment of TAL with TMZ led to a further 55–70% reduction in neurosphere formation with a consequent reduction in TMZ IC₅₀ to 3.1 $\mu\text{mol/L}$ and 81 nmol/L with TAL concentrations of 3 or 10 nmol/L, respectively. However, this drug interaction was not associated with any significant synergy (Figure 3B–C). These results are similar to the limited synergy observed in U251 cells.

The effect of TAL, TMZ and the combination on DNA damage signaling was also examined in neurosphere cultures. Low dose TAL (1 or 3 nmol/L) had a modest effect on PARP activity, as measured by the auto-PARYlation of PARP1, while 10 nmol/L TAL effectively suppressed PARP activity (Figure 3D, Figure S3B). TMZ (10 or 30 $\mu\text{mol/L}$) had no significant effect on PARP activity. TAL (1–10 nmol/L) alone had no significant impact on the phosphorylation of Chk1, Chk2 or KAP1 in GBM12. However, as compared to DMSO control or TAL (1–10 nmol/L) alone, treatment with TMZ (10 or 30 $\mu\text{mol/L}$) led to increased KAP1 phosphorylation, and a modest but dose-dependent increase in phosphorylation of Chk1 and Chk2. The combination of TAL (10 nmol/L) and TMZ (10 or 30 $\mu\text{mol/L}$) robustly increased phosphorylation of all three DNA damage signaling proteins (Figure 3D). The effect of combining lower dose TAL (1 or 3 nmol/L) with TMZ on DNA damage signaling

was modest and resulted in a minor increase in pKAP1, and no significant change in pChk1 or pChk2. These results are consistent with the idea that inhibition of PARP activity is necessary to potentiate the effect of TMZ on DNA damage signaling in neurosphere cultures of GBM12. These *in vitro* analyses suggest that the combination of TAL and TMZ may be an effective strategy to suppress GBM growth.

In vivo tolerability of TAL/TMZ in mice

In vivo studies in athymic nude mice were initially performed to define a tolerable combination dosing regimen. Low dose TAL (0.025 mg/kg twice daily) combined with standard TMZ (50 mg/kg/d) on days 1–5 of a 28-day cycle led to substantial weight loss (23%) and 100% mortality within one week (Table 1). Combining TAL 0.15 mg/kg twice daily with a metronomic TMZ regimen (5 mg/kg/d, days 1–5 every 7 days) was better tolerated with a median weight loss of 16% and a 42% mortality rate within 28 days. Reducing the dosing frequency (days 1–5 every 14 days) further improved tolerability (7% weight loss and 9% mortality in the first 28 days); therefore this regimen was selected for further *in vivo* studies.

In vivo survival studies in PDX GBM12 treated with TAL/TMZ

The efficacy of TAL and TMZ was initially evaluated in flank GBM12 tumor models to avoid the potential confounding influence of drug delivery across the BBB. TAL (0.15 mg/kg, twice daily, days 1–5 every 14 days) was ineffective (11 vs. 14 days with control, $p=0.19$) while TMZ (5 mg/kg, days 1–5, every 14 days) significantly prolonged the time to tumor growth endpoint (50 vs. 11 days with placebo, $p<0.001$; Figure 4A, Figure S5). Co-treatment with TAL and TMZ further extended the time to tumor growth endpoint (76 days vs. 50 days with TMZ alone, $p=0.005$; and 76 days vs. 11 days with placebo, $p<0.001$; Figure 4A). Terminal tumors acquired from animals treated with TMZ or TAL/TMZ showed no difference in Ki-67 staining compared to those treated with placebo or TAL alone (Figure S6). To assess pharmacodynamic effects of the treatment, mice with established flank GBM12 tumors were treated for 5 days and tumors were harvested 72 hours after treatment completion to analyze DNA damage signaling. Treatment with TAL had no effect on the phosphorylation of signaling proteins compared to control, while phosphorylation of KAP1, Chk1 and Chk2 signaling proteins clearly increased with TMZ (Figure 4B–C). The combination of TAL with TMZ significantly increased pKAP1 and marginally increased pChk2, but did not influence Chk1 phosphorylation (Figure 4B–C). These results indicate that TAL/TMZ co-treatment is an effective strategy in heterotopic GBM12 xenografts.

The efficacy of TAL/TMZ regimen was then tested in an orthotopic GBM12 tumor model. TAL (0.15 mg/kg, twice daily, days 1–5, every 14 days) was ineffective (14 vs. 14 days with placebo, $p=0.46$); while TMZ (5 mg/kg, days 1–5, every 14 days) doubled the median survival (30 days vs. 14 days with placebo, $p<0.001$, Figure 4D). Notably, this survival benefit with low dose TMZ was inferior to the survival extension previously reported for a more standard TMZ regimen (50 mg/kg, days 1–5, every 28 days) in orthotopic GBM12 models (59 days) (16). In contrast to the flank study, TAL/TMZ co-treatment did not significantly increase median survival compared to TMZ alone (37 days vs. 30 days with TMZ alone, $p=0.93$; 37 days vs. 14 days with placebo, $p<0.001$, Figure 4D). These results

indicate that TMZ was effective in both heterotopic and orthotopic GBM12 PDX tumors despite dose reduction, but the combination of TMZ with TAL was only effective in heterotopic tumors.

TAL brain/plasma distribution and efflux liability

The lack of TAL efficacy in the orthotopic tumor model may be explained by limitations in drug delivery across the BBB. Brain and plasma concentrations of TAL at two hours after a single dose (0.15 mg/kg) were 0.49 ± 0.07 ng/g (1.3 nmol/L) and 25.5 ± 4.1 ng/ml (67.1 nmol/L), respectively (brain-to-plasma ratio 0.02). Efflux activity of MDR1 and/or BCRP1 is known to influence the delivery of numerous chemotherapeutic drugs across the BBB, so the brain-to-plasma ratio of TAL was additionally assessed in MKO, BKO and TKO FVB mice. TAL concentrations in the brain of BKO mice was no different than that of WT mice, while the concentrations of TAL were significantly higher in brain tissues of MKO and TKO mice, (17.2 ± 2.1 and 17.3 ± 3.8 ng/g; brain-to-plasma ratio 0.23 and 0.24, respectively; Figure 5A). These results suggest that MDR1-mediated efflux at the BBB may significantly contribute to the limited distribution of TAL in the brain.

The efflux liability for TAL was further examined by assessing *in vitro* intracellular TAL accumulation in MDCKII cells modified to overexpress either human MDR1 or murine Bcrp1. Similar to the limited uptake of ^3H -vinblastine (a known MDR1 substrate), TAL accumulation was restricted in MDCKII-MDR1 cells to $18.6 \pm 1.2\%$ of the TAL concentrations in control cells ($p < 0.001$, Figure 5B). Furthermore, the impact of MDR1 on TAL accumulation was completely reversed in the presence of $1 \mu\text{mol/L}$ zosuquidar (Figure 5B). MDR1 inhibition also led to a smaller increase in TAL accumulation in control cells, which is consistent with known basal expression of MDR1 in MDCKII-WT cells. TAL accumulation in MDCKII-BCRP1 cells was not altered ($83.1 \pm 4.4\%$ as compared to control cells, $p = 0.06$; Figure 5C), and inhibition of BCRP by 200 nmol/L Ko143 slightly increased TAL accumulation in MDCKII-BCRP cells but remained statistically insignificant relative to control cells ($242 \pm 46.9\%$ vs. $99.1 \pm 15.5\%$, $p = 0.13$). These results confirm a selective efflux liability of TAL towards MDR1.

Discussion

Combination therapy with PARP inhibitors and TMZ is a promising strategy and is currently being clinically evaluated in several malignancies including breast cancer (NCT01506609, NCT01009788), small cell lung cancer (NCT02152982, NCT01390571) and GBM (NCT02152982, NCT01390571). The potency of TAL at nanomolar concentrations (18) and the specific synergy with TMZ(18, 30) has generated interest for this combination. After showing promise in heterotopic xenografts of GBM and Ewing sarcoma(22), TAL/TMZ has demonstrated preliminary efficacy in a phase I clinical trial in multiple tumor types(31). However efficacy in heterotopic GBM xenografts does not necessarily translate into orthotopic efficacy (32), so further investigation was critical prior to GBM clinical trial development. This study demonstrates a lack of efficacy of TAL/TMZ in orthotopic GBM models in association with limited brain distribution of TAL, therefore suggesting that TAL/TMZ may not be appropriate for clinical translation in GBM.

The mechanism of TMZ sensitization by PARP inhibitors at clinically achievable concentrations remains unclear (15). PARP1 and 2 have a pleiotropic role in DNA repair, including the processing of TMZ induced N³MeA and N⁷MeG DNA lesions (11, 33). PARP inhibitors are thought to potentiate TMZ by suppressing the BER mediated repair of N³MeA and N⁷MeG DNA lesions. Additionally, PARP-trapping initiates cytotoxicity by generating protein-DNA complexes at BER intermediates, a mechanism that is distinct from the O⁶MeG mediated cytotoxicity of TMZ alone (22). Supporting this notion, TAL (like veliparib) synergized the *in vitro* cytotoxic effects of TMZ to a greater extent in TMZ-resistant GBM cells (Figure 1C). However, TMZ resistance prevents *in vivo* sensitization by veliparib (14–16) suggesting that PARP inhibitor concentrations achievable *in vivo* may have little impact on BER. PARP additionally regulates MRN to promote the restart of stalled replication forks (34). TMZ-induced O⁶MeG lesions mispair with thymidine in cells lacking MGMT, triggering futile cycles of mismatch repair, stalled replication and double stranded DNA breaks(3). Thus, PARP inhibition and trapping may potentiate TMZ efficacy by compromising fork stability and/or delayed recovery from replication stress. This paradigm is supported by the increased DNA damage signaling observed after *in vivo* treatment of MGMT promoter hypermethylated GBM xenografts with veliparib/TMZ compared to TMZ alone(16). The effect of TAL combined with low dose TMZ in the *in vivo* setting may be below the threshold required to induce a detectable impact on fork stability, despite a delayed recovery from replication stress as demonstrated by the prolongation of tumor stasis in heterotopic tumor xenografts (Figure 4A–C). A dose-dependent influence on recovery from replication stress may be an alternative mechanism for TAL-mediated potentiation of TMZ efficacy.

Dose reductions of TMZ are necessary to promote the tolerability of some PARP inhibitors. In clinical trials of TMZ combined with veliparib and rucaparib, 25–30% dose reductions were essential to prevent significant myelotoxicity (35, 36). In contrast, the addition of TAL to TMZ required an 80% reduction in TMZ dose. This higher toxicity of TAL/TMZ may be related to superior PARP trapping by TAL. Mouse xenograft studies have revealed an inverse correlation between PARP trapping capacity and the tolerability of PARP inhibitors when combined with TMZ (20). Although there is clinical precedent for the use of lower doses of TMZ per day for the treatment of GBM (RTOG 0525, RESCUE), the evaluated TMZ doses (50–75 mg/m²) exceeded the anticipated dose reduction for TAL/TMZ tolerance, and these regimens were designed to deplete MGMT by increasing the overall treatment dose density (37, 38). With the scarcity of efficacy data for low dose TMZ in patients with newly diagnosed GBM, significant dose reductions of the only chemotherapy agent with known survival benefit to permit tolerability of a novel experimental agent with unknown clinical value may not be feasible. Based on synergy analysis in GBM cell lines low dose TMZ regimens are more amenable for initial study in patients with recurrent malignancy (NCT02116777). However, TAL/TMZ synergy in TMZ resistant models (T98G) was only possible at clinically unachievable TMZ concentrations, rather than the lower doses needed for tolerability. These potential issues would need to be addressed before attempting further development of TAL/TMZ in patients with GBM.

Drug exclusion from the brain by the BBB is a critical variable that limits the efficacy of many CNS-directed pharmaceutical agents. The BBB is a complex neurovascular unit which

includes tight junctions between brain capillary endothelial cells and ATP-binding cassette transporters expressed on their luminal surfaces. The historical narrative has been that drug exposure in high grade gliomas should be adequate because the contrast enhancement on magnetic resonance imaging should correlate with BBB disruption. However infiltrating glioma cells extend well beyond the margins of contrast enhancement (39, 40). Additionally, blood-glioma barrier breakdown is regional and heterogenous (28). The concentration of many drugs is significantly lower at the infiltrating GBM rim as compared to the necrotic tumor core(28). This impaired drug delivery to infiltrating glioma may be associated with diminished efficacy of these drugs in orthotopic glioma models (32, 41–43). TAL mirrors these experiences by demonstrating a lack of orthotopic efficacy despite activity in heterotopic models. Furthermore, the concentration of TAL in the mouse brain (0.5 ng/g, or 1.3 nmol/L) was lower than required for effective PARP inhibition *in vitro*, hence explaining the observed lack of intracranial efficacy. Moreover, this concentration increased 35-fold (17.2 ng/g) in MKO, providing clear evidence of the prominent liability of TAL to MDR1-mediated efflux at the BBB. These findings reinforce our hypothesis that the BBB restricts TAL delivery into the brain and prevents sufficient drug delivery for efficacy in brain tumors.

The repeated failures of numerous promising drugs in clinical trials for patients with GBM testifies for the necessity of careful preclinical evaluation of critical clinically relevant parameters prior to human study. The *in vivo* TAL dosing strategy and CNS distribution evaluation in this study were pertinent because the plasma concentration of TAL achieved two hours after drug administration was similar to that measured in the phase I clinical trial (67.1 nmol/L in mice vs. 50 nmol/L in human)(44). Comparing the pharmacokinetics of TAL to other PARP inhibitors, the brain-to-plasma concentration ratio for TAL (0.02) is lower than that of rucaparib (0.11), which also lacked efficacy in orthotopic glioma models(42). Alternatively, veliparib has a much higher brain-to-plasma concentration ratio (0.47) than either TAL or rucaparib despite the efflux liability of veliparib to MDR1 and BCRP (16, 45). Furthermore, veliparib has clear efficacy in TMZ-sensitive orthotopic glioma models despite being significantly less potent than TAL (inhibition of hydrogen peroxide induced PARylation at 1000 nmol/L vs. 3 nmol/L with TAL)(16). Based on these data, the phase II/III clinical trial A071102 was launched to evaluate the impact of veliparib added to standard adjuvant TMZ therapy in patients with newly diagnosed, MGMT promoter hypermethylated GBM (tolerable doses of TMZ/veliparib lack synergy in TMZ refractory GBM, as demonstrated in preclinical models(14, 16) and RTOG-0929 clinical trial(46)). A comparison of the properties of drugs from a single class provides insight on the relative significance of variables such as drug potency, BBB penetrability and efflux liability for efficacy in orthotopic glioma models. These considerations emphasize the necessity to give special attention to brain pharmacokinetics and drug tolerability at clinically relevant concentrations for the successful design of GBM-directed novel therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hegi ME, Diserens AC, Gorlia T, Hamou MF, de Tribolet N, Weller M, et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med*. 2005; 352(10):997–1003. [PubMed: 15758010]
2. Friedman HS, Kerby T, Calvert H. Temozolomide and treatment of malignant glioma. *Clin Cancer Res*. 2000; 6(7):2585–97. [PubMed: 10914698]
3. Sarkaria JN, Kitange GJ, James CD, Plummer R, Calvert H, Weller M, et al. Mechanisms of chemoresistance to alkylating agents in malignant glioma. *Clin Cancer Res*. 2008; 14(10):2900–8. [PubMed: 18483356]
4. Kitange GJ, Carlson BL, Mladek AC, Decker PA, Schroeder MA, Wu W, et al. Evaluation of MGMT promoter methylation status and correlation with temozolomide response in orthotopic glioblastoma xenograft model. *J Neurooncol*. 2009; 92(1):23–31. [PubMed: 19011762]
5. Agnihotri S, Gajadhar AS, Ternamian C, Gorlia T, Diefes KL, Mischel PS, et al. Alkylpurine-DNA-N-glycosylase confers resistance to temozolomide in xenograft models of glioblastoma multiforme and is associated with poor survival in patients. *J Clin Invest*. 2012; 122(1):253–66. [PubMed: 22156195]
6. Agnihotri S, Burrell K, Buczkowicz P, Remke M, Golbourn B, Chornenkyy Y, et al. ATM regulates 3-methylpurine-DNA glycosylase and promotes therapeutic resistance to alkylating agents. *Cancer Discov*. 2014; 4(10):1198–213. [PubMed: 25100205]
7. Cahill DP, Levine KK, Betensky RA, Codd PJ, Romany CA, Reavie LB, et al. Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. *Clin Cancer Res*. 2007; 13(7):2038–45. [PubMed: 17404084]
8. Yip S, Miao J, Cahill DP, Iafrate AJ, Aldape K, Nutt CL, et al. MSH6 mutations arise in glioblastomas during temozolomide therapy and mediate temozolomide resistance. *Clin Cancer Res*. 2009; 15(14):4622–9. [PubMed: 19584161]
9. Fortini P, Pascucci B, Parlanti E, D'Errico M, Simonelli V, Dogliotti E. The base excision repair: mechanisms and its relevance for cancer susceptibility. *Biochimie*. 2003; 85(11):1053–71. [PubMed: 14726013]
10. Nagel ZD, Kitange GJ, Gupta SK, Joughin BA, Chaim IA, Mazzucato P, et al. DNA Repair Capacity in Multiple Pathways Predicts Chemoresistance in Glioblastoma Multiforme. *Cancer Res*. 2016
11. Helleday T, Petermann E, Lundin C, Hodgson B, Sharma RA. DNA repair pathways as targets for cancer therapy. *Nat Rev Cancer*. 2008; 8(3):193–204. [PubMed: 18256616]
12. Liu X, Shi Y, Guan R, Donawho C, Luo Y, Palma J, et al. Potentiation of temozolomide cytotoxicity by poly(ADP)ribose polymerase inhibitor ABT-888 requires a conversion of single-stranded DNA damages to double-stranded DNA breaks. *Mol Cancer Res*. 2008; 6(10):1621–9. [PubMed: 18922977]
13. Calabrese CR, Almassy R, Barton S, Batey MA, Calvert AH, Canan-Koch S, et al. Anticancer chemosensitization and radiosensitization by the novel poly(ADP-ribose) polymerase-1 inhibitor AG14361. *J Natl Cancer Inst*. 2004; 96(1):56–67. [PubMed: 14709739]
14. Clarke MJ, Mulligan EA, Grogan PT, Mladek AC, Carlson BL, Schroeder MA, et al. Effective sensitization of temozolomide by ABT-888 is lost with development of temozolomide resistance in glioblastoma xenograft lines. *Mol Cancer Ther*. 2009; 8(2):407–14. [PubMed: 19174557]

15. Gupta SK, Mladek AC, Carlson BL, Boakye-Agyeman F, Bakken KK, Kizilbash SH, et al. Discordant in vitro and in vivo chemopotentiating effects of the PARP inhibitor veliparib in temozolomide-sensitive versus-resistant glioblastoma multiforme xenografts. *Clin Cancer Res*. 2014; 20(14):3730–41. [PubMed: 24838527]
16. Gupta SK, Kizilbash SH, Carlson BL, Mladek AC, Boakye-Agyeman F, Bakken KK, et al. Delineation of MGMT Hypermethylation as a Biomarker for Veliparib-Mediated Temozolomide-Sensitizing Therapy of Glioblastoma. *J Natl Cancer Inst*. 2016; 108(5)
17. Murai J, Huang SY, Das BB, Renaud A, Zhang Y, Doroshow JH, et al. Trapping of PARP1 and PARP2 by Clinical PARP Inhibitors. *Cancer Res*. 2012; 72(21):5588–99. [PubMed: 23118055]
18. Shen Y, Rehman FL, Feng Y, Boshuizen J, Bajrami I, Elliott R, et al. BMN 673, a novel and highly potent PARP1/2 inhibitor for the treatment of human cancers with DNA repair deficiency. *Clin Cancer Res*. 2013; 19(18):5003–15. [PubMed: 23881923]
19. Murai J, Huang SY, Renaud A, Zhang Y, Ji J, Takeda S, et al. Stereospecific PARP trapping by BMN 673 and comparison with olaparib and rucaparib. *Mol Cancer Ther*. 2014; 13(2):433–43. [PubMed: 24356813]
20. Hopkins TA, Shi Y, Rodriguez LE, Solomon LR, Donawho CK, DiGiammarino EL, et al. Mechanistic Dissection of PARP1 Trapping and the Impact on In Vivo Tolerability and Efficacy of PARP Inhibitors. *Mol Cancer Res*. 2015; 13(11):1465–77. [PubMed: 26217019]
21. Aoyagi-Scharber M, Gardberg AS, Yip BK, Wang B, Shen Y, Fitzpatrick PA. Structural basis for the inhibition of poly(ADP-ribose) polymerases 1 and 2 by BMN 673, a potent inhibitor derived from dihydropyridophthalazinone. *Acta Crystallogr F Struct Biol Commun*. 2014; 70(Pt 9):1143–9. [PubMed: 25195882]
22. Smith MA, Reynolds CP, Kang MH, Kolb EA, Gorlick R, Carol H, et al. Synergistic activity of PARP inhibition by talazoparib (BMN 673) with temozolomide in pediatric cancer models in the pediatric preclinical testing program. *Clin Cancer Res*. 2015; 21(4):819–32. [PubMed: 25500058]
23. Capes-Davis A, Reid YA, Kline MC, Storts DR, Strauss E, Dirks WG, et al. Match criteria for human cell line authentication: where do we draw the line? *Int J Cancer*. 2013; 132(11):2510–9. [PubMed: 23136038]
24. Masters JR, Thomson JA, Daly-Burns B, Reid YA, Dirks WG, Packer P, et al. Short tandem repeat profiling provides an international reference standard for human cell lines. *Proc Natl Acad Sci U S A*. 2001; 98(14):8012–7. [PubMed: 11416159]
25. Starling JJ, Shepard RL, Cao J, Law KL, Norman BH, Kroin JS, et al. Pharmacological characterization of LY335979: a potent cyclopropyldibenzosuberane modulator of P-glycoprotein. *Adv Enzyme Regul*. 1997; 37:335–47. [PubMed: 9381979]
26. Allen JD, van Loevezijn A, Lakhai JM, van der Valk M, van Tellingen O, Reid G, et al. Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther*. 2002; 1(6):417–25. [PubMed: 12477054]
27. Sarkaria JN, Tibbetts RS, Busby EC, Kennedy AP, Hill DE, Abraham RT. Inhibition of phosphoinositide 3-kinase related kinases by the radiosensitizing agent wortmannin. *Cancer Res*. 1998; 58(19):4375–82. [PubMed: 9766667]
28. Agarwal S, Mittapalli RK, Zellmer DM, Gallardo JL, Donelson R, Seiler C, et al. Active efflux of Dasatinib from the brain limits efficacy against murine glioblastoma: broad implications for the clinical use of molecularly targeted agents. *Mol Cancer Ther*. 2012; 11(10):2183–92. [PubMed: 22891038]
29. Prichard MN, Shipman C Jr. A three-dimensional model to analyze drug-drug interactions. *Antiviral Res*. 1990; 14(4–5):181–205. [PubMed: 2088205]
30. Engert F, Schneider C, Weibeta LM, Probst M, Fulda S. PARP Inhibitors Sensitize Ewing Sarcoma Cells to Temozolomide-Induced Apoptosis via the Mitochondrial Pathway. *Mol Cancer Ther*. 2015; 14(12):2818–30. [PubMed: 26438158]
31. Wainberg ZA, Hecht JR, Konecny GE, Goldman JW, Sadeghi S, Chmielowski B, et al. Safety and efficacy results from a phase I dose-escalation trial of the PARP inhibitor talazoparib in combination with either temozolomide or irinotecan in patients with advanced malignancies. *Cancer Res*. 2016;76.

32. Pokorny JL, Calligaris D, Gupta SK, Iyekegbe DO Jr, Mueller D, Bakken KK, et al. The Efficacy of the Wee1 Inhibitor MK-1775 Combined with Temozolomide Is Limited by Heterogeneous Distribution across the Blood-Brain Barrier in Glioblastoma. *Clin Cancer Res.* 2015; 21(8):1916–24. [PubMed: 25609063]
33. Helleday T. The underlying mechanism for the PARP and BRCA synthetic lethality: clearing up the misunderstandings. *Mol Oncol.* 2011; 5(4):387–93. [PubMed: 21821475]
34. Bryant HE, Petermann E, Schultz N, Jemth AS, Loseva O, Issaeva N, et al. PARP is activated at stalled forks to mediate Mre11-dependent replication restart and recombination. *Embo J.* 2009; 28(17):2601–15. [PubMed: 19629035]
35. Plummer R, Lorigan P, Steven N, Scott L, Middleton MR, Wilson RH, et al. A phase II study of the potent PARP inhibitor, Rucaparib (PF-01367338, AG014699), with temozolomide in patients with metastatic melanoma demonstrating evidence of chemopotential. *Cancer Chemother Pharmacol.* 2013; 71(5):1191–9. [PubMed: 23423489]
36. Su JM, Thompson P, Adesina A, Li XN, Kilburn L, Onar-Thomas A, et al. A phase I trial of veliparib (ABT-888) and temozolomide in children with recurrent CNS tumors: a pediatric brain tumor consortium report. *Neuro Oncol.* 2014; 16(12):1661–8. [PubMed: 24908656]
37. Perry JR, Belanger K, Mason WP, Fulton D, Kavan P, Easaw J, et al. Phase II trial of continuous dose-intense temozolomide in recurrent malignant glioma: RESCUE study. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2010; 28(12):2051–7. [PubMed: 20308655]
38. Gilbert MR, Wang M, Aldape KD, Stupp R, Hegi ME, Jaeckle KA, et al. Dose-dense temozolomide for newly diagnosed glioblastoma: a randomized phase III clinical trial. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology.* 2013; 31(32):4085–91. [PubMed: 24101040]
39. Earnest, Ft, Kelly, PJ., Scheithauer, BW., Kall, BA., Cascino, TL., Ehman, RL., et al. Cerebral astrocytomas: histopathologic correlation of MR and CT contrast enhancement with stereotactic biopsy. *Radiology.* 1988; 166(3):823–7. [PubMed: 2829270]
40. Pafundi DH, Laack NN, Youland RS, Parney IF, Lowe VJ, Giannini C, et al. Biopsy validation of 18F-DOPA PET and biodistribution in gliomas for neurosurgical planning and radiotherapy target delineation: results of a prospective pilot study. *Neuro Oncol.* 2013; 15(8):1058–67. [PubMed: 23460322]
41. Becker CM, Oberoi RK, McFarren SJ, Muldoon DM, Pafundi DH, Pokorny JL, et al. Decreased affinity for efflux transporters increases brain penetrance and molecular targeting of a PI3K/mTOR inhibitor in a mouse model of glioblastoma. *Neuro Oncol.* 2015; 17(9):1210–9. [PubMed: 25972455]
42. Parrish KE, Cen L, Murray J, Calligaris D, Kizilbash S, Mittapalli RK, et al. Efficacy of PARP Inhibitor Rucaparib in Orthotopic Glioblastoma Xenografts Is Limited by Ineffective Drug Penetration into the Central Nervous System. *Mol Cancer Ther.* 2015; 14(12):2735–43. [PubMed: 26438157]
43. Parrish KE, Pokorny J, Mittapalli RK, Bakken K, Sarkaria JN, Elmquist WF. Efflux transporters at the blood-brain barrier limit delivery and efficacy of cyclin-dependent kinase 4/6 inhibitor palbociclib (PD-0332991) in an orthotopic brain tumor model. *J Pharmacol Exp Ther.* 2015; 355(2):264–71. [PubMed: 26354993]
44. De Bono JS, Mina LA, Gonzalez M, Curtin NJ, Wang E, Henshaw JW, et al. First-in-human trial of novel oral PARP inhibitor BMN 673 in patients with solid tumors. *J Clin Oncol.* 2013; 31(15)
45. Li X, Delzer J, Voorman R, de Moraes SM, Lao Y. Disposition and drug-drug interaction potential of veliparib (ABT-888), a novel and potent inhibitor of poly(ADP-ribose) polymerase. *Drug Metab Dispos.* 2011; 39(7):1161–9. [PubMed: 21436403]
46. Robins HI, Zhang P, Gilbert MR, Chakravarti A, de Groot JF, Grimm SA, et al. A randomized phase I/II study of ABT-888 in combination with temozolomide in recurrent temozolomide resistant glioblastoma: an NRG oncology RTOG group study. *J Neurooncol.* 2016; 126(2):309–16. [PubMed: 26508094]

Figure 1. *In vitro* evaluation of sensitizing effects of TAL in GBM cell lines

A) Western blot images showing dose-dependent inhibition of hydrogen peroxide (H₂O₂) induced PARylation, total PARP1 and β-Actin were used as loading controls. B) Western blots analysis of nuclear lysates to assess chromatin trapping of PARP1 and PARP2 in U251 cells after 3 hours of treatment with each of the TAL concentrations combined with 100 μmol/L of TMZ. C–D) Bar graphs (mean ± SEM from 3 independent experiments) showing effects of TAL ± TMZ on cell growth assessed by CyQuant assay for U251 (C) or T98G (D) cells cultured for 5 days in the absence or presence of indicated concentrations of TMZ ± TAL; * denotes p-value < 0.05 for with TAL treatments versus control, and # denotes p-value < 0.05 for TMZ treatment versus no TMZ. The 3-D plots shown on the right are synergy plots at 95% confidence interval (CI) for the data presented in bar graphs on left. E) Overall drug interaction measured as cumulative synergy or antagonism across the range of TMZ and TAL concentrations.

Figure 2. TAL mediated potentiation of TMZ induced DNA damage

A) Western blot images (representative from 3 independent experiments) showing the impact of a 24 hour pre-treatment with TAL $\pm$ TMZ on DNA damage signaling in U251 (MGMT-low) and T98G (MGMT-high) cells. B) Representative images of γH2AX (red) and RPA (green) foci in U251 and T98G cells treated with or without 3 nmol/L TAL and $\pm$ 100 $\mu\text{mol/L}$ TMZ; magnification bar is 50 μm . C) Quantification of cells with γH2AX or RPA staining (>20 foci/nuclei) from 3 independent experiments; * indicates $p < 0.05$ as compared to control, and # denotes $p < 0.05$ for TMZ vs. TAL+TMZ.

Figure 3. *In vitro* evaluation of sensitizing effects of TAL in GBM12 cells

A) Bar graphs (mean $\pm$ SEM from 3 independent experiments) showing effect of graded concentrations of TAL $\pm$ TMZ on neurosphere formation, * denotes p-value < 0.05 for TAL versus DMSO, and # indicates p-value < 0.05 for TMZ versus control. B) The 3-D plots showing synergy at 95% CI for the TMZ/TAL combinations presented in A. C) Overall drug interaction presented as cumulative synergy and antagonism. D) Western blot images showing the impact of a 24 hour pre-treatment with TAL $\pm$ TMZ on DNA damage signaling in neurosphere cultures, β -Actin used as a loading control.

Figure 4. Evaluation of *in vivo* sensitizing effects of TAL

A) Kaplan-Meier graphs showing time to end point (tumor volume 1500 mm³) in flank xenografts of GBM12 after cyclical treatment with placebo, TAL, TMZ or TAL/TMZ. B) Western blots of PD analysis performed in GBM12 flank xenografts treated as in A for 1 week, and euthanized 72 hours after the last dose of TMZ, equal protein from 3 to 4 tumors per group were analyzed for phosphorylated and total KAP1, Chk2 and Chk1, β-Actin was used as a loading control. C) Bar graphs showing densitometric quantification of damage signalling from western blots depicted in Figure 2B; and D) Kaplan-Meier graphs showing survival over time in mice with established orthotopic GBM12 xenografts treated as in Figure 4A.

Figure 5. TAL distribution in plasma and brain tissues

A) Bar graphs (mean $\pm$ SD) for TAL concentration in brain and plasma of FVB wild-type (WT), *Mdr1a/b*^{-/-} (MKO), *Bcrp1*^{-/-} (BKO), *Mdr1a/b*^{-/-}*Bcrp1*^{-/-} triple knock-out (TKO) mice at 2 hours after a single dose of TAL (0.15 mg/kg), (n=3, * p<0.05 vs. WT controls). B) Bar graphs showing intracellular accumulations of TAL or vinblastine in vector control vs. MDR1-overexpressing MDCKII cells treated with vinblastine (9.3 nmol/L) or TAL (1 μ mol/L) $\pm$ 1 μ mol/L zosuquidar, an MDR1 inhibitor. C) Bar graphs showing intracellular levels of prazosin or TAL in vector or BCRP1-overexpressing MDCKII cells treated with prazosin (0.39 nmol/L) or TAL (1 μ mol/L) $\pm$ BCRP1 inhibitor Ko143 (0.2 μ mol/L), results are mean $\pm$ SD, n=3. * denotes p<0.05 compared to vector controls.

Evaluation of tolerability of treatment regimens combining TAL and TMZ in nude mice implanted with GBM12 cells.

Table 1

#	Tumor location	Dose & schedule	Survival after one week offirst dose	Survival after four weeks of first dose	Maximum weight loss
1	Orthotopic	TMZ: 50 mg/kg, d1-5, q28d + TAL: 0.025 mg/kg, bid, d1-5, q28d	0/5	0/5	23%
2	Orthotopic	TMZ: 30 mg/kg, d1-5, q28d + TAL: 0.05 mg/kg, bid, d1-5, q28d	0/5	0/5	24%
3	Orthotopic	TMZ: 5 mg/kg, d1-5, q28d + TAL: 0.05 mg/kg, bid, d1-5, q28d	5/5	4/5	1%
4	Orthotopic	TMZ: 5 mg/kg, d1-5, q28d + TAL: 0.075 mg/kg, bid, d1-5, q28d	5/5	3/5	6%
5	Flank	TMZ: 5 mg/kg, d1-5, q7d + TAL: 0.15 mg/kg, bid, d1-5, q7d	7/7	4/7	16%
6	Flank	TMZ: 5 mg/kg, d1-5, q14d + TAL: 0.15 mg/kg, bid, d1-5, q14d	11/11	10/11	7%