Inhibition of protein disulfide isomerase in glioblastoma causes marked downregulation of DNA repair and DNA damage response genes

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Abstract

Aberrant overexpression of endoplasmic reticulum (ER)-resident oxidoreductase protein disulfide isomerase (PDI) plays an important role in cancer progression. In this study we demonstrate that PDI promotes glioblastoma (GBM) cell growth and describe a class of allosteric PDI inhibitors that are selective for PDI over other PDI family members.

Methods: We performed a phenotypic screening triage campaign of over 20,000 diverse compounds to identify PDI inhibitors cytotoxic in cancer cells. From this screen, BAP2 emerged as a lead compound, and we assessed BAP2-PDI interactions with gel filtration, thiol-competition assays, and site-directed mutagenesis studies. To assess selectivity, we compared BAP2 activity across several PDI family members in the PDI reductase assay. Finally, we performed in vivo studies with a mouse xenograft model of GBM combining BAP2 and the standard of care (temozolomide and radiation), and identified affected gene pathways with nascent RNA sequencing (Bru-Seq).

Results: BAP2 and related analogs are novel PDI inhibitors that selectively inhibit PDIA1 and PDIp. Though BAP2 contains a weak Michael acceptor, interaction with PDI relies on Histidine 256 in the b’ domain of PDI, suggesting allosteric binding. Furthermore, both in vitro and in vivo, BAP2 reduces cell and tumor growth. BAP2 alters the transcription of genes involved in the unfolded protein response, ER stress, and apoptosis, and PDI inhibition downregulates DNA repair and DNA damage response genes.

Conclusion: These results indicate that BAP2 has anti-tumor activity and the suppressive effect on DNA repair gene expression warrants combination with DNA damaging agents to treat GBM.

Keywords: Protein disulfide isomerase, drug discovery, allosteric inhibition, cancer, DNA repair
Graphical Abstract

BAP2

$IC_{50} = 930 \pm 90 \text{ nM}$
Introduction

Glioblastoma (GBM) is the most common and most aggressive type of primary human brain tumor [1]. Surgery followed by radiotherapy in combination with temozolomide (TMZ) is the standard of care for GBM; however, only 5.5% of patients survive more than 5 years after diagnosis [2]. As an alkylating agent that induces DNA damage, TMZ is toxic and exhibits limited prolongation of survival [3, 4]. No successful drug treatment for GBM has been approved for over three decades. Additionally, GBM tumor resistance may arise from therapy-resistant cancer stem cells (CSC). CSCs are a subpopulation of tumor cells that have self-renewal properties, are self-sustaining, and can differentiate into nontumorigenic cancer cell types [5]. CSCs can arise from neural stem cells and allow repopulation of the tumor mass after treatment. Transcription factors including Sox2 and OLIG2 maintain the stem cell like properties of the cells[6, 7]. Thus, a critical unmet need exists for new, targeted chemotherapeutics with improved efficacy and safety for GBM patients.

Overexpression of protein disulfide isomerase (PDI) promotes cancer growth, suggesting that it represents a potential cancer therapeutic target [8-10]. PDI is a dithiol-disulfide oxidoreductase with molecular chaperone functions [11, 12]. Cysteines (Cys) in the catalytic sites of the homologous $\alpha$- and $\alpha'$-domains are essential for enzymatic function and can transition between oxidized (disulfide) and reduced (dithiol) states [13, 14]. Mainly located in the endoplasmic reticulum (ER), PDI mediates folding of specific polypeptides and is upregulated by the unfolded protein response (UPR) as a cancer survival mechanism [15]. Concordantly, PDI inhibition results in the accumulation of unfolded and misfolded proteins, ER stress, and cell death [16]. Several inhibitors of PDI have been identified, including PACMA31 for the treatment of ovarian
cancer [17], 16F16 and LOC14 for the prevention of neurodegeneration [18, 19], CCF642 for the treatment of multiple myeloma [20], juniferdin for the inhibition of HIV-1 infection [21], bepristats and quercetin-3-rutinoside for the suppression of thrombus formation [22-24], and KSC-34 and analogs as a domain-selective PDI probes [25]. PDI inhibition results in synergistic cell killing in combination with TMZ [26] and sorafenib [27]. However, no PDI inhibitors have been approved for clinical use.

We previously validated PDI as a therapeutic target wherein PACMA31 was demonstrated to have anti-tumor activity [17]. PACMA31 has been demonstrated by our lab in this report and others to be non-specific towards PDI, and can inhibit other PDI family members, such as ERp57.[22] Furthermore, we identified a potent PDI inhibitor, 35G8, that was toxic in a 2D cancer model [28]. However, 35G8, as a known redox-cycling molecule, does not possess drug-like properties. This prompted us to pursue a PDI inhibitor with a novel scaffold and more appropriate drug-likeness. In this study, we investigate chalcone-containing derivatives as PDI inhibitors and demonstrate that PDI promotes GBM cell growth. Chalcones (benzylideneacetophenones) are simple privileged molecules, and, although various chalcones have anti-cancer activities, some of their molecular targets have not been fully validated [29]. Therefore, an improved understanding of their mechanisms of cytotoxicity is critical for further development. Though the discovered chalcone compounds contain a Michael-acceptor moiety, a weak electrophile, our lead chalcone-containing compound BAP2 binds to an allosteric site on PDI, selectively inhibits PDI activity, and suppresses cell growth in a model with GBM patient-derived cells. We further discovered that PDI knockdown and inhibition abrogate the stem-like phenotype of GBM cells. Bromouridine labeling and sequencing (Bru-seq) of nascent RNA demonstrated that PDI inhibition modulates transcriptional pathways associated with ER stress.
and the UPR. More significantly, PDI inhibition caused a global downregulation of DNA damage response (DDR) genes. These findings warrant further development of these compounds as a novel targeted approach for the treatment of GBM and in combination with DNA-damaging chemotherapy.
Experimental Procedures

**Reagents.** Control and PDI siRNAs were purchased from OriGene Technologies (Rockville, MD). Opti-MEM medium, Lipofectamine RNAiMAX transfection reagent, propidium iodide, and AlamarBlue Cell Viability Reagent were purchased from Life Technologies (Grand Island, NY). PDI (1:4000, #3501), E2F1 (1:500, #3742), RAD51 (1:500, #8875S), Sox2 (1:1000, #3579), and phospho-histone H2A.X (1:500, #9718) PARP (1:1000, #5625), and cleaved Caspase 3 (1:1000, #9664) antibodies were purchased from Cell Signaling Technology (Danvers, MA). Actin (1:3000, sc-47778), BRCA2 (1:1000, sc-135731), ATR (1:1000, sc-515173), ATM (1:1000, sc-135663), WRN (1:1000, sc-135807), and HSPA6 (1:1000, sc-376193) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). CD133/1 (AC133)–APC antibody was purchased from Milteny Biotec (Auburn, CA). Secondary antibodies were purchased from Cell Signaling (anti-rabbit, 1:7500, #35568 and anti-mouse, 1:5000, #35518).

**Cell culture.** GBM cell lines U87MG, NU04, and U118MG were kindly provided by Dr. Alan L. Epstein (University of Southern California, Los Angeles, CA) and were maintained in RPMI-1640 (Life Technologies) supplemented with 10% FBS (Fisher Scientific, Pittsburgh, PA). A172 cells were obtained from the American Type Culture Collection (ATCC). All cell lines were authenticated with STR DNA profiling (University of Michigan) and matched to reference profiles from the ATCC database. Cells were grown as monolayers at 37 °C in a humidified atmosphere of 5% CO2. Four patient-derived primary cell lines (HF2303, HF2587, HF2927, and HF3016 cells) were provided by Dr. Tom Mikkelsen and Dr. Ana C. deCarvalho (Henry Ford Hospital, Detroit, MI). Establishment of primary tumor cell culture was described previously [30]. Primary GBM cell lines were maintained in neurosphere medium composed of DMEM/F-12 supplemented with N-2 (Gibco), 0.5 mg/ml BSA (Sigma), 25 μg/ml gentamicin (Gibco),
0.5% antibiotic/antimycotic (Invitrogen), 20 ng/ml bFGF, and 20 ng/ml EGF (Peprotech). Cells were maintained in culture up to 20 passages. Cells were checked for *Mycoplasma* contamination with PlasmoTest kit (InvivoGen).

**Bioinformatics analysis.** Rembrandt gene expression data was sourced from Array Express and processed using the robust multiarray analysis algorithm. Rembrandt patient metadata was sourced from the Georgetown Database of Cancer [31]. Gravendeel normalized gene expression data was sourced from GEO and original paper supplemental data [32]. GBM and LGG TCGA data were downloaded from the Genome Data Analysis Center (GDAC) Firehose [33]. Kruskal-Wallis and survival analysis statistics were calculated using the R statistical programming language [34]. GSEA v2.2.3 was used with v6 gene sets sourced from MSigDB. To quantify enrichment significance, 10,000 gene set permutations were performed using weighted mode scoring and Pearson metric.63

**PDI reductase assay.** PDI activity was measured as described previously [35]. Briefly, purified recombinant PDI (0.5 μM PDI, 1.6 μM of the a or a’c domain, 1.6 μM PDIp, or 0.4 μM ERp57) was incubated with indicated compounds at 37 °C for 1 h in buffer (100 mM sodium phosphate, 2 mM EDTA, 8 μM DTT, pH 7.0), followed by addition of DTT (100 μM) and bovine insulin (130 μM). The a and a’c domains in the PDI assay were incubated for 45 minutes at room temperature before addition of insulin. Resulting aggregation of reduced insulin B chain was measured at 650 nm.

**Denatured and reduced RNase A (drRNase A) assay.** drRNase assay was performed as described [36]. Briefly, oxidative renaturation of denatured and reduced RNase A was measured spectrophotometrically in the presence of cCMP as an RNase A substrate. Tris-acetate buffer
(pH 8.0), 4.5 mM cCMP, GSH and GSSG (at concentrations to yield a redox buffer of chosen composition), and 1.4 µM PDI were equilibrated at 25 °C. The assay was initiated by addition of reduced, denatured RNase A. The hydrolysis of cCMP resulting from the gain in ribonuclease activity was recorded as an increase in absorbance at 296 nm.

**PDI knockdown.** PDI knockdown was modified from a previous approach [35]. Control or PDI siRNA (100 nM) with Lipofectamine RNAiMAX transfection reagent was added to cells in Opi-MEM medium. After 10 h, 1 mL of RPMI-1640 with 20% FBS was added to each well. 24 h after siRNA addition, cell culture medium was replaced with RPMI-1640 with 10% FBS.  

**MTT assay.** MTT growth inhibition assay was conducted as described [37]. Cells were seeded and allowed to attach overnight. Cells were treated with compounds and, after 72 h, were incubated with 0.3 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco, Solon, OH) for an additional 3 h at 37 °C. After removal of the supernatant, DMSO was added, and absorbance was read at 570 nm. Percent cell growth inhibition was expressed as (1 - A/C) × 100% (A and C are absorbance values from experimental and control cells, respectively). Standard error of the mean (SEM) was calculated based on IC_{50} values obtained from at least three independent experiments.  

**Alamar Blue viability assay.** Neurospheres were dissociated and seeded at 5,000 cells/well in 96-well plates. Cells were treated after 24 h. Alamar Blue reagent was added to cells per manufacturer protocol after 72 h. Fluorescence signals were determined by EnVision Xcite Multilabel Reader (PerkinElmer).  

**Colony formation assay.** Briefly, 600 (NU04 and U87MG) or 300 (HCT116) cells were seeded per well in 24-well (NU04 and U87MG) or 96-well (HCT116) plates and allowed to attach. After
24 h, serial dilutions of compounds were added to the culture medium and incubated for 24 h. Cells were cultured until colonies formed (12 days (NU04 and U87MG) or 7 days (HCT116)), stained with crystal violet solution (0.25% crystal violet, 10% formaldehyde (37% v/v), 80% methanol) for 1 h, and thoroughly washed with water.

**Sphere formation assay.** Sphere formation assay was adapted from previous studies [38]. Spheres were dissociated and 50 cells/μl were seeded in 96-well plates. After 24 h, cells were treated with indicated compounds and incubated for 7 days in neurosphere medium. The number of floating spheres in nine individual fields per sample were counted.

**Thermal shift assay.** The thermal shift of PDI (0.3 mg/ml in 100 mM Na₂PO₄, pH 7.0) in the presence or absence of compound was determined with the Protein Thermal Shift kit (Applied Biosystems), following the kit protocol. PDI was mixed with buffer, compound at indicated concentrations, and 1× ROX dye to a total volume of 20 μL in a 384-well plate. The plate was heated from 25 to 99 °C at a rate of 1 °C/min in the ViiA7 Real-Time PCR instrument (Thermo Fisher Scientific). Melting curves and temperatures were calculated using the Boltzmann method in the Protein Thermal Shift software (Thermo Fisher Scientific).

**Cellular thermal shift assay.** For the cellular thermal shift assay [39, 40], U87MG or A172 cells were seeded at 2 × 10⁶ cells/100 mm dish and allowed to attach overnight. Cells were treated with indicated concentrations of compounds, or DMSO as the negative control, for 2 h at 37 °C, 5 % CO₂. After treatment, cells were trypsinized, washed with DPBS, and suspended in 600 μL DPBS. Cells were split into 100 μL aliquots, heated at indicated temperatures for 3 min in the Veriti Thermal Cycler (Applied Biosystems), and incubated for 3 min at room temperature. The cells were flash-frozen twice and spun at 14 × g for 20 min at 4 °C.
Supernatants were collected and loaded onto a 10 % polyacrylamide gel at a volume of 16 μL, with 4 μL 4× SDS loading dye. Subsequently, Coomassie staining using standard procedure or Western blotting was performed.

**Western blot.** Western blot was modified from previously described protocols [41]. Briefly, cells were washed with PBS and lysed in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, pH 7.5 with 1× protease inhibitor (Sigma-Aldrich, St. Louis, MO) and phosphatase inhibitor (Alfa Aesar, Ward Hill, MA) cocktails. Protein concentration was determined by the BCA assay (Thermo Scientific, Rockford, IL). Proteins were resolved on 10% acrylamide gels via SDS-PAGE and electrotransferred to immobilon-FL PVDF membrane (Millipore, Billerica, MA). After incubation with StartingBlock (TBS) blocking buffer (Thermo Scientific), membranes were probed with primary antibodies in TBST with 5% FBS, subsequently with DyLight 800 conjugated anti-rabbit IgG (Thermo Scientific) in TBST with 5% milk and 0.01% SDS and scanned using the ODYSSEY CLx infrared imaging system (LI-COR, Lincoln, NE).

**Competition assay.** Full-length recombinant PDI (15 μM) was incubated with BAP2, BAP30, or PACMA31 at indicated concentrations in sodium phosphate buffer for 1 h at 37 °C followed by 1 h incubation with 20 μM PACMA57. Solutions were then mixed with 5× SDS sample buffer for SDS/PAGE. Gel fluorescence was analyzed using FluorChem M system (ProteinSimple, San Jose, CA).

**Gel filtration.** Full-length PDI-BAP2 complex was prepared by overnight incubation of 5 μM PDI at room temperature with 200 μM BAP2, before being subjected to a Superdex 200 HR 10/60 column (GE Healthcare) and chromatographed at 1 ml/min using 50 mM Tris/HCl buffer.
(pH 7.6) containing 150 mM NaCl and 2 mM EDTA. The main peak was collected, and aliquots were stored at -80 °C for the PDI reductase assay.

**AMS assay.** 20 μM isolated a or a’c domains from PDI were incubated with or without 40 μM BAP2 or PACMA31 for 1 h in PBS at 25 °C. Free thiols were blocked with 2 mM AMS (Invitrogen). Samples were analyzed by SDS-PAGE and stained with Coomassie blue.

**Site-directed mutagenesis.** Forward and reverse primers for H256A mutant of PDI were ordered from Integrated DNA Technologies (Forward Primer: GGTGAAATCAAGACTGCCATCCTGCTGTTC; Reverse Primer: GAACAGCAGGATGGCAGTCTTGATTTCACC). Site-directed mutagenesis was performed with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) following manufacturer protocol with some modification. Briefly, reactions containing 10 ng DNA template, 100 ng primers, dNTP mix, reaction buffer, QuikSolution reagent, and 2.5 U of PfuUltra HF DNA polymerase were cycled at 95 °C for 1 min, 18 cycles of 95 °C for 50 s, 56 °C for 50 s, 68 °C for 7 min, and 68 °C for 6 min. Individual colonies were grown in 5 ml LB containing 200 μg/ml ampicillin for 8-16 h at 37 °C. DNA was purified (QIAprep Spin Miniprep Kit, Qiagen) and mutations were validated with Sanger sequencing conducted by the University of Michigan DNA Sequencing Core (Figure S1). Plasmids were transformed into BL21(DE3) competent cells (Promega) following the QuikChange II XL Site-Directed Mutagenesis protocol.

**Protein purification.** Wild-type and mutant PDI expression and purification were performed as previously described[17] with slight modifications. The a and a’c domains of PDI in PQE30 vectors were transformed into Express Fl Competent E. coli (New England BioLabs, Inc.). PDIp in a pET23b vector and ERp57 in a PQE30 vector were transformed into Rosetta BL21(DE3)
pLysS (Novagen) and Express $\Phi$ Competent E. coli (New England BioLabs, Inc.), respectively. Bacteria was grown in LB medium with 200 μg/ml ampicillin (EMD Biosciences, La Jolla, CA) at 37 °C and incubated at an OD$_{600}$ of 0.5 for 4 h with 1 mM isopropyl β-D-1-thiogalactopyranoside (GoldBio) or OD$_{600}$ of 0.8 for 16 hours at 16 °C for PDIp and ERp57. Cells were harvested by centrifugation and re-suspended in Buffer A (20 mM sodium phosphate, pH 7.3). Cells were lysed by sonication and the cell debris was removed by centrifugation. The supernatant was applied to a Ni-nitrilotriacetic acid beads (Qiagen, Hilden, Germany), equilibrated with 10 ml of Buffer A and incubated at 4 °C, overnight. After incubation, the nickel beads were added to a column and washed in Buffer A and then in Buffer B (20 mM sodium phosphate, 0.5 M sodium chloride and 50 mM imidazole, pH 7.3). The his-tagged proteins were eluted using Buffer C (20 mM sodium phosphate and 250 mM EDTA, pH 7.3) and eluent was dialyzed in 100 mM sodium phosphate buffer (pH 7.0) with 2 mM EDTA.

**Stemness marker analysis.** $1 \times 10^6$ cells/25 cm$^2$ flask were seeded and treated with indicated compounds at 10 μM after 24 h. Cells were incubated for 72 h before collection and dissociation. CD133 staining was carried out per manufacturer protocol. Propidium iodide (PI) was included as a cell viability marker. Percentage of CD133-positive/PI negative cells was determined and recorded with the BD FACS Aria (BD Biosciences).

**Bromouridine (Bru)-RNA sequencing.** Bru-RNA sequencing was performed as previously described, with some modification [42]. U87MG cells were treated with BAP2 at 3 μM for 4 h, and 2 mM Bru was added for the last 30 min of treatment. Cells were collected, and total RNA was isolated with TRIzol reagent. Bru-labeled RNA was captured from total RNA by incubation with anti-BrdU antibodies (BD Biosciences) conjugated to magnetic beads (Dynabeads, Goat anti-Mouse IgG; Invitrogen) under gentle agitation at room temperature for 1 h. Bru-containing
RNA population was isolated and sequenced. Sequencing reads were mapped to the HG19 reference genome. Pre-ranked gene lists were generated for each treatment ranking genes by fold change in transcription compared to control. Sequencing results were filtered using cutoff value of gene size > 300 base pairs and mean reads per kilobase per million > 0.5.

The dataset was interrogated with the Database for Annotation, Visualization and Integrated Discovery (DAVID), IPA, and GSEA. Genes with over 8-fold change in transcription (142 upregulated and 95 downregulated) after BAP2 treatment were submitted to DAVID (david.abcc.ncifcrf.gov) for functional annotation [43, 44]. Upregulated and downregulated genes were categorized into 235 and 81 functional gene clusters, respectively. For IPA, a dataset of ~2,500 genes (cutoff value: 2-fold change) was used. The identified pathways were ordered by P-value of significance and maximum number of genes in the pathway. A pre-ranked gene list of 448 genes with a log2(fold change) ≥ 3 was also analyzed for gene enrichment using GSEA gene sets based on the Kolmogorov-Smirnov statistic. For each gene set, an enrichment score (ES) was normalized to account for the difference in gene set size, and a false discovery rate (FDR) was calculated based on the normalized enrichment score (NES) values.

**Xenograft implantation and treatment.** 250,000 U87MG or D54 cells were implanted subcutaneously into each flank of four to six-week old Athymic Nude- Foxn1nu mice in 100 µL RPMI:BD Matrigel Basement Membrane Matrix (1:1) suspension. Caliper measurement was conducted twice weekly and tumor volume was determined as $V = (\text{length}/2) \times (\text{width}^2)$. Mice were randomized into four groups when tumors reached 100 mm$^3$ and treatments: vehicle control, TMZ alone (5mg/kg in Ora-Plus (Perrigo) via oral gavage five days/week) (LKT Laboratories), BAP2 alone (20 mg/kg via intraperitoneal injection in 5% DMSO / 65% 1,2-propanediol / 30% saline five days/week), or local radiation (Kimtron Medical, IC-320) alone (2
Gy × 5 doses/week) were given for two weeks. Western blot was performed on mouse tumor tissue. Briefly, cells and snap-frozen tissues were washed with PBS and lysed in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, pH 7.5 with 1x protease inhibitor (Sigma-Aldrich) and phosphatase inhibitor (Alfa Aesar) cocktails. Protein concentration was determined by the BCA assay (Thermo Scientific). Proteins were resolved on 10% acrylamide gels via SDS-PAGE and electrotransferred to immobilon-FL PVDF membrane (Millipore). After incubation with StartingBlock (TBS) blocking buffer (Thermo Scientific), membranes were probed with primary antibodies in TBST with 5% FBS, subsequently with DyLight 800 conjugated anti-rabbit IgG (Thermo Scientific) in TBST with 5% milk and 0.01% SDS and scanned using the ODYSSEY CLx infrared imaging system (LI-COR). Alternatively, Horseradish peroxidase-conjugated anti-rabbit or mouse IgG (H+L) (Jackson ImmunoResearch) were used as secondary antibodies and Pierce™ ECL (Thermal Scientific) or ECL™ prime (GE Healthcare) were used as substrate.

Statistics analysis. Significance was evaluated with one-way ANOVA or student t-test using Prism (version 6.01, La Jolla, CA). Differences were statistically significant at \( p < 0.05 \).

Results

\textit{P4HB expression correlates with tumor grade and is associated with reduced overall survival.} Using data published by Rembrandt (n = 287) and Gravendeel (n = 284), we discovered \textit{P4HB} (gene name for PDIA1) was significantly overexpressed in brain tumors compared to normal brain tissues (Figure 1). Differences in expression were quantified using a Kruskal-Wallis test across glioma grade and normal tissues (Rembrandt: \( p = 2.84\times10^{-21} \) (Figure 1A) and Gravendeel: \( p = 1.72\times10^{-15} \) (Figure 1B)). In both datasets, \textit{P4HB} expression was associated with progression. In TCGA dataset (n = 667), \textit{P4HB} expression was higher in patients
with GBM compared to patients with LGG (lower grade glioma) \( (p = 8.83E-55, \text{ Figure 1C}) \). Patients with high \textit{P4HB} expression (regardless of grade) exhibit reduced overall survival in Rembrandt \( (p = 5.28E-10, \text{ Figure 1D}) \) [31], Gravendeel \( (p = 1.77E-9, \text{ Figure 1E}) \) [32], and TCGA \( (p = 2.2E-15, \text{ Figure 1F}) \) datasets. Compared to other PDI family members, mRNA expression of \textit{P4HB} is significantly upregulated in GBM patient samples, which further supports \textit{P4HB} as a critical GBM tumor growth gene (Figure S2). These findings suggest that overexpression of PDI may be linked to brain tumor progression and aggressiveness.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{\textit{P4HB} expression increases with cancer grade and is associated with reduced survival. Significant differences in \textit{P4HB} expression were observed using Kruskal-Wallis (KW) statistics across glioma grade and normal tissue. \textit{P4HB} had significantly increased expression in patients with GBM compared to patients with LGG in the Rembrandt (a), Gravendeel (b), and TCGA (c) datasets. Patients with increased \textit{P4HB} expression (regardless of grade) exhibited reduced survivability in three glioma datasets (d-f). Populations were stratified using median \textit{P4HB} expression per dataset.}
\end{figure}
To investigate the role of PDI in cell proliferation, we silenced PDI expression in two GBM cell lines (Figure S3A). PDI knockdown significantly decreased cell proliferation (Figure S3B) and viability (Figure S3C), suggesting that PDI promotes GBM cell survival. Furthermore, the shRNA data in Project Achilles (http://portals.broadinstitute.org/achilles) show that PDI knockdown results in negative shRNA scores (Figure S3D). shRNA score is the log2-based change in shRNA compared with pooled shRNA in several cancer cell lines and a negative shRNA score is indicative of decreased cell proliferation. This suggests that PDI knockdown reduced cell viability among over 95% of cancer cell lines transfected, further supporting PDI as a drug target in cancer.

Chalcone-containing compounds inhibit PDI. We performed a two-step screening of a subset of our in-house drug-like small molecule libraries (Figure 2A) [28]. From an initial screen for cytotoxicity, 443 compounds with high potency were identified and tested for PDI inhibition. The screening campaign provided us with two lead compounds that contained the chalcone or benzylideneacetophenone scaffold (BAPs): BAP1 (IC$_{50}$ = 0.83 ± 0.20 µM) and BAP2 (IC$_{50}$ = 0.93 ± 0.09 µM) (Figure 2B, Table S1).

We next identified 41 analogs of BAP1 and BAP2 with drug-like properties and characterized their PDI inhibitory potential (Figure S4, Table S1). Four of 41 additional BAP2 derivatives inhibited PDI with an IC$_{50}$ value < 5 µM: BAP3 - 6 (Figure 2C, Table S1). Thus, modest structural changes around the chalcone scaffold had marked effects on PDI inhibition. Gel filtration analysis demonstrated that BAP2 forms a stable complex with PDI, and binding induces a conformational change (Figure 2D). PDI incubated with BAP2 was eluted with a smaller volume. After removing the excess BAP2 via gel filtration, the 76 mL eluted sample lost the isomerase and reductase activity, which may indicate that PDI and BAP2 form a stable
However, it is unclear whether BAP2 and PDI form intermolecular covalent bond(s) and whether the binding of BAP2 to PDI is irreversible.

**Figure 2.** BAP2 derivatives are potent PDI inhibitors. (a) 20,000 drug-like compounds were screened in the MTT assay. Compounds that inhibited 40% of cancer cell growth in the MTT assay were screened in the PDI reductase assay. Numbers indicate compounds identified after each screening filter. (b) Inhibitory effects of BAP1 and BAP2 in the PDI reductase assay. (c) Inhibition profile of BAP analogs 3-6 in the PDI reductase assay. (d) PDI-BAP2 complex preparation by gel filtration. (e) The reductase and isomerase activity of PDI-BAP2 complex.
To further validate that BAP2 targets PDI, we tested the ability of BAP2 to stabilize PDI using the thermal shift assay. BAP2 stabilized PDI to thermal unfolding, similar to endogenous ligand estradiol [45], in both the thermal shift assay and cellular thermal shift assay (CETSA) [39] (Figure S5). The CETSA measures the ability of intracellular proteins to withstand thermal degradation in the presence of inhibitors. In this study, BAP2 stabilizes PDI to thermal degradation about +1-2 °C (Figure S5C). These results demonstrate intracellular target engagement of PDI by BAP2. To further determine the selectivity of BAP2 on the proteome, we performed CETSA and examined the total lysate on a Coomassie-stained gel (Figure S5D). We did not see any visible stabilization of any other proteins in the lysate by BAP2; we did, however, observe stabilization of a protein around 70 kDa by estradiol, likely estrogen receptor alpha, the main target of estradiol. We did not observe stabilization of PDI with the total cell lysate likely because many proteins share a similar molecular weight (~57 kDa).

BAP2 is a selective allosteric PDI inhibitor. To determine how BAP2 interacts with PDI to inhibit its activity, we first tested two PDI mutants with one active catalytic site in each mutant: PDI-C1 (SGHS in a domain, CGHC in a’ domain) and PDI-C2 (CGHC in a domain, SGHS in a’ domain) [46]. BAP2 inhibited PDI-C1 and PDI-C2 more potently than PDI (Figure 3A), but there was no difference in the potency of BAP2 between the two mutants. To examine active site cysteine accessibility, we used 4-acetamido-4’-maleimidylstilbene-2,2’-disulfonic acid (AMS), which reacts with cysteine thiols and adds ~ 500 Da for each modification, to modify isolated BAP2-treated a and a’c domains (Figure 3B). Incubation with BAP2 blocked AMS binding to the a’c domain, but not the a domain (Figure 3C). In contrast, PACMA31 blocked AMS binding to cysteines in both a and a’c domains. In addition, BAP2 prevented covalent binding of PACMA57 (fluorescent analog of PACMA31) to PDI active-site cysteines, whereas BAP30 (an
inactive derivative) had no significant effect (Figure 3D). To further test \textbf{BAP2} binding to the active site cysteines, we purified the \textit{a} and \textit{a’c} domains of PDI, which have isolated reductase activity.[47] \textbf{PACMA31} (100 \(\mu\text{M}\)) and \textbf{35G8} (1 \(\mu\text{M}\)) inhibit reductase activity of the isolated domains, whereas inhibitors estradiol, bepristat 1a, and \textbf{BAP2} did not inhibit the activity of the isolated domains (Figure S6). These results indicated \textbf{BAP2} may not bind directly on the \textit{a} or \textit{a’c} domains. To explore the possibility that \textbf{BAP2} did not bind in the active site, we tested the activity of a saturated analog of \textbf{BAP2} (Figure S6B). Without the Michael acceptor, the analog retained modest potency, which suggests that inhibition of PDI is not solely dependent on the alpha-beta unsaturated ketone scaffold.
Figure 3. BAP2 targets an allosteric site in PDI selectively. (a) Comparison of inhibitory effects of BAP2 on active sites in the a and a’ domains. PDI (CGHC in a, CGHC in a’), PDI-C1 (SGHS in a, CGHC in a’) and PDI-C2 (CGHC in a, SGHS in a’) at 0.5 μM were incubated with or without 10 μM BAP2 at 37 °C for 1 h. Statistical significance determined via unpaired t test. ** p < 0.01; **** p < 0.0001. ns: not significant. (b) Schematic of AMS reactivity with active-site thiols on PDI. (c) Thiol reactivity of the active site of a and a’c domains of PDI with AMS before and after BAP2 treatment. (d) BAP2 inhibited binding of PACMA57, a fluorescent analog of PACMA31, to PDI. (e) Activity of wild-type PDI and H256A PDI in the PDI reductase assay. (f) BAP2 treatment in the PDI reductase assay with wild-type and H256A PDI. (g) Domain architecture of PDI. Active site CGHC residues and b’ domain His256 are shown. (h) Domain architecture of PDI family members with the CXXC active site tested. (i) Inhibition of PDI family members (1 μM) by BAP2 (40 μM) and PACMA31 (40 μM) in the PDI reductase assay. (j) Reductase activity calculated by the maximal slope of the curve relative to lag time. Enzyme activity of each protein incubated with DMSO was used as 100%. Data expressed as mean ± S.D. (n ≥ 3).

His256 is an important residue for substrate recognition in the b’ domain of PDI family members [48, 49]. Several PDI inhibitors, including quercetin 3-rutinoside[47] and bepristat 1a[22], bind in the b’ domain; thus, we sought to determine whether BAP2 may also bind in the hydrophobic substrate-binding pocket. BAP2 and analogs contain a key hydroxyl group required for activity, similar to estradiol, an endogenous PDI ligand that binds in the b’ domain of PDIp [49].

Reductase capabilities of PDI were not hindered by the histidine-to-alanine mutation H256A (Figure 3E); however, BAP2 was much less active against the H256A mutant than the wild-type protein (Figure 3F). These results indicate that BAP2 interaction with H256 is important for activity. BAP2 binding in the b’ domain may induce a conformational change in PDI that blocks the a’ active site (Figure 3G).

We further evaluated BAP2 activity against a panel of PDI family members containing the CXXC active site (PDI, PDIp, ERp57, ERp46, ERp72, P5, and ERp18) (Figure 3H), and a single catalytic domain a of PDI. We found that BAP2 selectively inhibits PDI and PDIp, whereas
**PACMA31** inhibits all tested PDI family enzymes (Figure 3I, S7, S8). PDIp shares the highest degree of similarity with PDI among PDI family proteins in evolutionary divergence [50]. A conserved histidine residue responsible for substrate recognition is found in both PDI and PDIp, providing a potential explanation for activity against both family members. The $b'xa'$ domain found in PDI and PDIp is not present in ERp46 or P5, which could explain why BAP2 is not active against those PDI family members. Furthermore, ERp57 and ERp72 lack the conserved histidine residue that BAP2 requires for activity. ERp18 displayed low disulfide reductase activity making it difficult to detect a significant effect of BAP2. Additionally, BAP2 did not inhibit the isolated PDI $a$ domain, confirming that BAP2 inhibits catalytic activity by binding to an allosteric site located in the $b'xa'c$ domain (Figure 3J).

**BAP compounds reduce GBM cell growth and synergize with TMZ.** Among the BAP derivatives generated, BAP2 was the most potent (IC$_{50}$ value: 5.3 ± 1.8 µM in NU04) (Table S2). Derivatives inhibited colony formation in a 2D cell model of GBM (Figure 4A), and cytotoxicity correlated with PDI inhibitory activity (Figure 4B). Furthermore, BAP2 was active in patient-derived primary GBM cells (HF2303, HF2598, HF2927, and HF3016), whereas inactive analog BAP30 did not show significant cytotoxicity (Figure 4C). BAP2 also sensitized cancer cells to TMZ treatment in the colony formation assay (Figure S9).

**PDI supports maintenance of stemness properties in GBM.** PDI knockdown impaired sphere formation (Figure 4D), a phenotype of CSCs [51], indicating that PDI may be necessary for CSC maintenance. Consistently, BAP2 analogs at non-toxic doses inhibited sphere formation, whereas BAP30 showed no significant inhibition (Figure 4E, S10A). Additionally, BAP2 dose-dependently inhibited sphere formation in patient-derived GBM cells, whereas BAP30 had no effect (Figure 4F, S10B). Furthermore, BAP2 treatment at a non-toxic concentration reduced the
CD133-positive cell population in patient-derived GBM cells, whereas BAP30 did not (Figure 4G). In addition, Sox2 expression, as an orthogonal stemness marker, was dose-dependently abrogated by BAP2 and PACMA31, but not by BAP30 and confirms the reduction in GBM cell stemness induced by BAP2 (Figure 4H). Our findings of the role of PDI in supporting stemness properties in GBM is consistent with a recent report showing that inhibition of PDI induced differentiation of acute myeloid leukemia cells through the activation of SK053 and CCAAT enhancer-binding protein α levels.[52] Taken together, these results suggest that our PDI inhibitors may target the GBM CSC population. Follow-up studies will further investigate the mechanisms of PDI inhibition in suppressing the stemness properties in GBMs.
Figure 4. BAP2 analogs inhibit GBM cell growth and prevent maintenance of stemness properties.
(a) Treatment with BAP1–6 inhibited colony formation. (b) A significant correlation exists between PDI inhibition and compound cytotoxicity. (c) BAP2 induced cell death in patient-derived GBM cell lines. (d) PDI knockdown inhibited U87MG sphere formation. Five representative images were taken for each condition. (e) BAP analogs inhibited U87MG sphere formation. Five representative images are shown for each condition. (f) BAP2 impaired sphere formation in four patient-derived GBM cell lines, whereas BAP30 showed no significant inhibition. (g) BAP2 (10 μM), but not BAP30 (10 μM), reduced CD133 population in human GBM cells. Cells were treated with BAP2 or BAP30 for 72 h. (h) BAP2 downregulated Sox2 expression, as assessed by Western blot analysis. Means ± SEM were calculated from three experiments. Scale bars represent 200 μm. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

BAP2 suppresses tumor growth in a mouse model of GBM. An initial viability assay confirmed that BAP2 treatment significantly lowered viability at concentrations above 1 μM (Figure 5A). Mouse flank xenografts were established, and, when tumors were approximately 100 mm³, treatment was initiated using radiation (Figure 5B, 5E), TMZ (Figure 5F, 5I) or BAP2, each as single agent, or BAP2+TMZ or radiation for five days/week for two weeks. BAP2 treatment caused tumor growth delay (p = 0.003 and 0.0345 compared to vehicle control for D54 and U87MG xenografts, respectively). Radiation and TMZ also caused tumor growth delay (p < 0.0001 (radiation) and p = 0.0012 (TMZ)) (Figure 5C, 5G). TMZ was more efficacious than BAP2 compared to vehicle control (p = 0.0347) (Figure 5G); however, mice treated with TMZ had a 15% decrease in body weight, while mice treated with BAP2 exhibited minimal loss in body weight (Figure 5D, 5H). These results demonstrate that BAP2 may be as effective as TMZ, without the toxicity associated with DNA alkylating chemotherapy.
Figure 5. PDI inhibition is toxic to D54 and U87MG cells and delays tumor progression. (a) D54 and U87MG cells were seeded overnight and BAP2 was added for 72 h. Graph is plotted from three independent experiments and presented as mean ± SEM. D54 or U87MG cells were subcutaneously implanted into Athymic Nude-Foxn1nu mice and vehicle control, TMZ (5mg/kg in Ora-Plus (Perrigo) via oral gavage), BAP2 (20mg/kg via intraperitoneal injection in 5% DMSO / 65% 1,2-Propanediol / 30% saline) or ionizing radiation (2 Gy/day), and TMZ+BAP2 or radiation+BAP2 were given for two weeks (all treatment administered daily for five days per week). Tumor measurement was performed twice weekly to determine volume that was plotted as mean ± SEM in (b) D54 and (f) U87MG xenografts, and tumor volume at Day 18 and Day 12 was used for statistical analysis in (c) D54 (g) and U87MG tumors. Body weight was also recorded twice weekly for mice bearing (d) D54 and (h) U87MG tumors. Tumor doubling time was calculated according to (http://www.chest-x-ray.com/index.php/calculators/doubling-time) for (e) D54 and (i) U87MG xenografts. Study group sizes were: vehicle control – n = 6 (U87MG) or n = 8 (D54), TMZ – n = 6 (U87MG), radiation – n = 5 (D54), BAP2 – n = 9 (U87MG) or n = 6 (D54), TMZ+BAP2 – n = 9 (U87MG), and radiation+BAP2 – n = 6 (D54). * p < 0.05; *** p < 0.001; **** p < 0.0001 compared to control group.
BAP2 affects transcription of genes involved in UPR, ER stress, and apoptosis. Nascent RNA Bru-seq analysis of BAP2-treated U87MG cells identified 237 differentially transcribed genes (≥ 8-fold difference). BAP2 treatment resulted in transcription of genes involved in the UPR, ER stress, and apoptosis (Figure 6A, Table S3). Furthermore, BAP2 decreased transcription of genes responsible for DNA replication and cell cycle function (Figure 6A, Table S4). Ingenuity Pathway Analysis (IPA) identified that 69 genes affected by BAP2 treatment belonged to the protein ubiquitination pathway, which is activated for protein degradation when unfolded and misfolded proteins accumulate during ER stress and UPR (Table S5). Bru-seq nascent RNA sequencing of cells treated with siPDI demonstrated that similar sets of genes decreased, including DNA replication, cell cycle, and nucleus (Figure 6B). REACTOME_CELL_CYCLE (NES = -2.42) and REACTOME_CELL_CYCLE_MITOTIC (NES = -2.27) were the most significant gene sets downregulated following BAP2 treatment with the rigorous cut off of log2(fold change) ≥ 8 (Figure 6C). BAP2 had a pronounced effect on a larger family of HSP proteins, further supporting the role of BAP2 in inducing ER stress (Figure 6D). Moreover, the BAP2 dataset matched gene set enrichment of other ER stress and UPR-inducing compounds (e.g. phospholipids [53], arsenite [54], and epoxomicin [55]) (Table S6-S7).
Figure 6. BAP2 affects ER stress and other signaling pathways. Functional terms represented by genes upregulated and downregulated at least eight-fold (log2(fold change) ≥ 3) by BAP2 (a) or siPDI (log2(fold change) ≥ 1) (b). Pathways highlighted in pink represent gene sets that were identified over both BAP2 and siPDI treatment. (c) BAP2 treatment enriched for downregulation of cell cycle gene sets. NES: normalized enrichment score. FDR q val: false discovery rate q value. Criteria for GSEA was $p < 0.05$ and false discovery rate < 25%. (d) Transcription of 47 of 63 HSPs increased after BAP2 treatment. In particular, transcription of heat shock proteins, HSPA6 (876-fold), HSPH1 (99-fold), and DNAJB4 (28-fold), was upregulated by BAP2 (Figure S11A-C). Highly downregulated genes include mitochondrial methyltransferase-like gene METTL12 (125-fold), GTPase-activating
ARHGAP22 (37-fold), and syntaxin 1A (27-fold), a gene that encodes for a membrane-trafficking protein (Figure S11D-F). Taken together, the nascent RNA transcriptome of BAP2-treated cells indicates PDI is inhibited, causing ER stress and eventual cell death.

PDI inhibition downregulates DNA repair and DDR genes. Multiple genes involved in homologous recombination, Fanconi anemia, non-homologous end-joining, base excision repair, nucleotide excision repair, and mismatch repair pathways were significantly downregulated upon PDI knockdown or BAP2 treatment (Figure 7). From a more complete list of DNA repair and DDR genes, we observed a trend of downregulation not only by BAP2 and PDI-targeting siRNA but also by PACMA31 (Figure S12A, Table S8). PDI knockdown, BAP2, and PACMA31 all downregulated E2F genes (Gene Set: Hallmark E2F Targets), further supporting this hypothesis and demonstrating the similarities between BAP2 treatment and PDI knockdown (Figure S12B, Table S9). In the E2F targets gene set, BAP2 downregulated 65% of the genes, PACMA31 downregulated 72% of the genes, and siPDI downregulated 38% of the genes. These observations link PDI activity to transcription of DNA repair and DDR genes. Additional similarities between BAP2, PACMA31, and PDI siRNA treatment were identified in genes related to hypoxia (Figure S12C). Furthermore, PACMA31 and BAP2 dose-dependently lower E2F1 and RAD51 protein expression in U87MG and D54 cells (Figure S13A). Decrease in protein expression of DDR genes BRCA2, ATR, ATM, WRM, E2F1, and RAD51 upon BAP2 treatment was confirmed with Western blotting (Figure S13B). In vivo downregulation of DDR proteins was confirmed for BRCA2, ATR, RAD51, and E2F1 in response to BAP2+TMZ or radiation (Figure S13C, S13D). Although the mechanistic detail of such a link is beyond the scope of this current study, we hypothesize that PDI inhibition downregulates DNA repair genes such as RAD51 through decreased expression of E2F transcription factors [56].
Figure 7. BAP2 treatment and PDI siRNA knockdown cause global downregulation of DNA repair genes. Four-hour treatment with BAP2 and siRNA-mediated PDI knockdown slows transcription of representative DNA repair genes including RAD51 (a), BRCA1 (b), BRCA2 (c), XRCC2 (d), BLM (e), ATR (f), FANCA (g), MSH2 (h), MRE11A (i), and WRN (j).

DNA repair and E2F gene sets are significantly enriched for genes that correlate with P4HB expression in brain cancer patient samples. We performed GSEA to identify genes that may have functional association with P4HB overexpression, using Pearson statistics in the combined TCGA LGG and GBM patient dataset (N = 676). E2F Targets (NES = 3.09 and FWER <0.0001) and DNA Repair (NES = 2.43 and FWER <0.0001) MSigDB Hallmark gene
sets were significantly enriched for genes that correlate with P4HB mRNA expression (Figure 8A, 8B). E2F Targets gene set co-expression enrichment is consistent with in vitro reduced expression observed in response to PDI inhibition. Taken together, expression of P4HB correlates with expression of E2F and DNA repair genes suggesting a potential mechanism by which targeting PDI in cancer cells may sensitize them to radiation and chemotherapy.

Figure 8. E2F and DNA repair gene sets are significantly enriched for genes that correlate with P4HB expression. GSEA was performed to evaluate the functional enrichment of P4HB co-expressed genes using a Pearson statistic in the combined TCGA Low Grade Glioma and Glioblastoma patient dataset (N = 676). E2F (a) and DNA Repair (b) MSigDB Hallmark gene sets correlate with P4HB expression. ES: enrichment score. NES: normalized enrichment score. FWER: family-wise error rate.
**Discussion**

Glioblastomas are characterized by intratumoral histopathological heterogeneity and molecular heterogeneity [1]. These features contribute to therapeutic resistance and high recurrence rates leading to poor survival [57]. The molecular heterogeneity of GBM has hampered clinical translation of therapies targeting oncogenic pathways, hence inhibition of signaling pathways that are required for maintenance of a transformed phenotype, irrespective of oncogenic driver, is gaining traction.

GBM tumors may arise from neural stem cells, or hijack pathways used by stem cells for survival, and neural stem cell markers, such as Sox2 and OLIG2 are necessary for glioma growth [7, 58]. Thus, therapy-resistant CSCs within the tumor may contribute to GBM recurrence by repopulating the tumor mass [59-61]. Furthermore, ER stress can change intestinal cells from epithelial stem cells to differentiated cells, suggesting a role for protein folding in regulating stemness [62]. Our report provides preliminary evidence that PDI may be critical for a stem cell phenotype. In addition, we have identified a novel class of PDI inhibitors that inhibit the stem-like phenotype in patient-derived spheroid cultures.

Cell cultures derived from GBM patients preserve the complex genetic profiles of parental tumors and recapitulate aggressive growth characteristics when implanted in immunodeficient mice [63]. Therefore, we used a panel of patient-derived GBM cell lines to mimic tumor heterogeneity. In patient-derived neurosphere lines, BAP2 showed significant efficacy compared to TMZ, suggesting that BAP2 may overcome TMZ resistance in GBMs. Several studies have shown that PDI family members are associated with tumor progression and TMZ resistance in gliomas [64, 65]. PDI, encoded by P4HB, a gene overexpressed in GBM, may therefore be an impactful therapeutic target in GBM.
Posttranslational modifications at PDI active sites, including S-glutathionylation, S-nitrosylation, and succination, suppress its enzymatic activity resulting in accumulation of unfolded proteins and ER stress [66-68]. Most PDI inhibitors, including PACMA31, 16F16, and DTNB, also modify active-site cysteines through covalent binding [8]. Due to this binding characteristic, they lack selectivity among PDI family enzymes, wherein thioredoxin, and other proteins that share the CGHC active site are also inhibited. The Michael acceptor moiety on BAP2 suggests the compound has weak electrophilic activity and binds to the active site cysteines of PDI. However, mutation of H256 inhibits PDI activity up to high concentrations (100 μM). Furthermore, the para hydroxyl group that is critical for BAP2 activity is not forming a hydrogen bond to stabilize the carbonyl group, which is the key interaction for inducing Phase II enzymes such as glutathione transferases.[69, 70] Unlike active-site-targeting PDI inhibitors, BAP2 and analogs bind to an allosteric site located in the b’xa’c domain, resulting in a conformational change in PDI that hinders active-site reactivity. Additionally, BAP2 is active against PDI and PDIp, PDI family members that contain the histidine residue in the b’ domain. Though ERp57 contains active site cysteines, BAP2 does not inhibit it, and ERp57 is one of the isoforms that does not contain the key histidine in the b’ domain. More extensive analysis of the binding position of BAP analogs should be performed to optimize the scaffold further. Ongoing structural determination will provide a clearer visualization and understanding of this unique binding mode.

Resistance to DNA alkylating agent TMZ often develops because GBM cells repair O\(^6\)-methylated DNA with O\(^6\)-methylguanine-DNA methyltransferase (MGMT). Though MGMT depletion has not been an effective therapy for GBM, DNA repair is nonetheless an important mediator in the development of resistance to treatment and is an important aspect of the resistant
nature of CSCs [71]. PDI has been linked to the process of DNA-nuclear matrix anchoring, a critical step to promote DNA repair [72]. In this study, we demonstrate that PDI inhibition by siRNA, PACMA31, and BAP2 decreases global transcription of DNA repair genes (Figure S12). These results suggest that PDI inhibition would be beneficial in combination with DNA alkylating agents such as TMZ to sensitize the tumor and prevent development of resistance. Since BAP2 selectively inhibits PDI and PDIp, these may be the major PDI family members implicated in DNA repair; however, it remains to be determined whether other PDI family members play a role in DNA repair and GBM resistance mechanisms. Furthermore, with evidence that PDI inhibition lowers RAD51 and E2F1 protein expression, a potential mechanism linking PDI to the DNA damage response emerges. E2F1 expression may decrease in response to an ER stress signal triggered by PDI inhibition, which causes RAD51 downregulation. Further investigation into this mechanism is warranted to confirm the role of PDI in DNA repair and the DNA damage response.

The GBM tumor microenvironment is generally hypoxic, selecting for tumor cells and CSCs with an increasingly more invasive and aggressive phenotype. Tumor hypoxia is responsible for decreased drug penetration, increased metastases, and decreased overall survival. Tumor cells respond to hypoxia by activating the UPR and upregulating PDI [73-76]. Our Bru-seq studies show that both PDI knockdown and inhibition of its enzymatic activity highly correlate with downregulation of multiple hypoxic response gene sets, further supporting BAP2 analogs as a potential treatment for hypoxic tumors.

While the effects of BAP2 correlate with siPDI treatment, variations in individual genes may be observed because of the different modes of PDI inhibition. BAP2 is a small-molecule inhibitor of PDI and inhibits PDI in an acute manner, whereas siPDI inhibits PDI in a relatively slower
manner. The cells may respond differently to the two different mechanisms of PDI inhibition; thus, comparing the transcriptomic consequences of the two perturbations should be done carefully. For example, PDI interacts with other proteins under various biological contexts. Such protein-protein interactions are interrupted by siPDI, while they could be maintained in the presence of BAP2.

In conclusion, we have discovered a selective small molecule inhibitor of PDI that binds in a novel allosteric binding site likely spanning the \( b'x \) and \( a' \) domains to block active site thiol activity. Of interest is the novel binding mode of BAP2 and how allosteric binding affects the conformation of the \( a' \) domain. Furthermore, BAP2 is toxic in a panel of primary GBM cell lines, and sensitizes cells to TMZ. Finally, we have demonstrated that PDI inhibition downregulates DNA repair pathways and genes in the E2F pathway. Our findings provide further evidence that PDI inhibition may synergize with DNA alkylating agents and other therapies in which resistance is defined by an upregulation of DNA repair mechanisms. Furthermore, our findings support the exciting possibility of a druggable allosteric site in PDI for future development of selective PDI inhibitors for treating GBM.

**Abbreviations**

GBM: glioblastoma; PDI: protein disulfide isomerase; ER: endoplasmic reticulum; ATCC: American Type Culture Collection; GSEA: gene set enrichment analysis; DDR: DNA damage response; TCGA: The Cancer Genome Atlas; LGG: low grade glioma; TMZ: temozolomide; UPR: unfolded protein response; KW: Kruskal-Wallis; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AMS: 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid;
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Author contributions


Competing interests

The authors have declared that no competing interest exists.
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