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Targeting DNA Repair Mechanisms to Treat Glioblastoma

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1. Introduction

The current standard of care for the treatment of glioblastoma multiforme (GBM) includes the surgical resection of the tumor in combination with ionizing radiation and the DNA alkylating agent Temozolomide (TMZ). The introduction of TMZ into clinical use has improved patient outcomes [1, 2]. Stupp et al. showed that the addition of TMZ to radiotherapy lengthened the median survival in patients with GBM from 12.1 months to 14.6 months [2]. TMZ exerts an effect upon GBM cells by preferentially damaging the DNA of the rapidly growing tumor cells, ultimately resulting in their death. While the combined use of TMZ and ionizing radiation can increase overall survival, the long-term survival for GBM patients is still poor [3]. What has recently become apparent is that GBM tumors can develop several forms of resistance to the DNA damage-induced cell death caused by radiotherapy and TMZ treatments. In this way, GBM tumors can survive and generate new tumors when they should otherwise not survive. This review will discuss mechanisms of resistance to DNA damage-induced cell death in GBM tumors and will outline some DNA repair functions that can be targeted to potentially improve treatment outcomes.

Maintaining the integrity of the genome is essential for the health and survival of multicellular organisms. The continuous exposure of cellular DNA to potentially harmful environmental and internal insults necessitates redundant and overlapping DNA repair mechanisms. Several excellent reviews have extensively described the wide variety of DNA repair mechanisms used by cells in response to DNA damage [4-6]. Damage to DNA can result in cell cycle arrest to allow for DNA repair mechanisms to occur, or can stall replication forks during DNA repli-

cation causing senescence. Proliferating cells, like those in GBM tumors, are affected to a greater extent than quiescent cells following DNA damage, causing the cells to arrest at particular points within the cell cycle [7]. However, cancers such as GBMs are quite adept at repairing the DNA damage or over-riding the cell cycle checkpoints to allow cell proliferation to continue despite the damage.

GBM tumors respond to DNA damage induced by ionizing radiation and TMZ treatment through increased expression of DNA repair enzymes, including the proteins O-6-methyl-guanine-DNA methyltransferase (MGMT) and Poly (ADP-ribose) Polymerase 1 (PARP-1) [8]. Furthermore, tumors are able to eliminate chemotherapeutic compounds from cells through the increased expression and activity of ABC transporters, specifically ABC-1 [9]. Compounding this issue is the growing body of evidence indicating that a small population of slow-growing cancer stem cells reside within the GBM tumor (also called glioma-initiating cells) and are responsible for the subsequent recurrence of GBM tumors [10, 11]. Glioma initiating cells are particularly resistant to standard treatments, in part through the elevated expression of enzymes responsible for repair of DNA damage [12, 13]. Therefore, successful destruction of GBM tumors may require a combined approach utilizing standard treatments in combination with inhibition of DNA repair pathways. This approach to cancer treatment, called synthetic lethality, preferentially affects cancer cells by inhibiting several molecular processes necessary for tumor survival without significantly affecting normal tissues [14, 15]. This treatment approach utilizing the DNA repair enzymes MGMT and PARP-1 have been a focus of the research conducted at the Upper Michigan Brain Tumor Center, and this review will be supplemented with findings from our laboratory.

Temozolomide. TMZ is easily absorbed after oral administration, readily crosses the blood-brain barrier and is better tolerated than its parent compound, mitozolomide. TMZ is an imidazo-tetrazine prodrug that is converted to a compound, 5-(3-methyltriazen-1-yl)imidazole-4-carboximide (MTIC), capable of alkylating DNA and displays antitumor activity in a variety of cancer types [16]. Spontaneous conversion of TMZ to MTIC is pH dependent and is a chemically controlled reaction [1]. MTIC's most common sites of methylation are at the N7 position of guanine followed by the N3 position of adenine. The N7-methylguanine is stable and makes up 80-85% of all alkyl adducts, whereas, N3-methyladenine is readily hydrolyzed and comprises only 8-18% of adducts [8, 17]. The O6 position of guanine only makes up 5% of lesions but is the most stable of the three and persists in the DNA in the absence of MGMT enzyme activity [8, 17]. O6-methylguanine is considered to be the most lethal of the alkyl adducts. For a visual representation of these common adducts, see Figure 1.

O-6-Methylguanine-DNA Methyltransferase. The MGMT enzyme reduces the cytotoxicity of O6-alkylating agents like TMZ by catalyzing the transfer of methyl groups from the O6 position of guanine to a cysteine residue within the active site of the MGMT enzyme [1]. The transfer of ethyl or alkyl groups to MGMT renders the MGMT enzyme inactive, leading to its degradation via the proteosomal pathway [18]. Therefore, continued MGMT function requires the de novo expression of MGMT protein in order to provide resistance to chemotherapeutic agents. Unfortunately, many GBM tumors exhibit increased MGMT expression, which reduces the effectiveness of alkylating agents such as TMZ. GBM tumors with hypermethylated MGMT

promoter regions do not develop resistance to the drug indicating that reduced MGMT expression in tumors is a clinically relevant and potentially important cellular phenotype to consider during treatment.
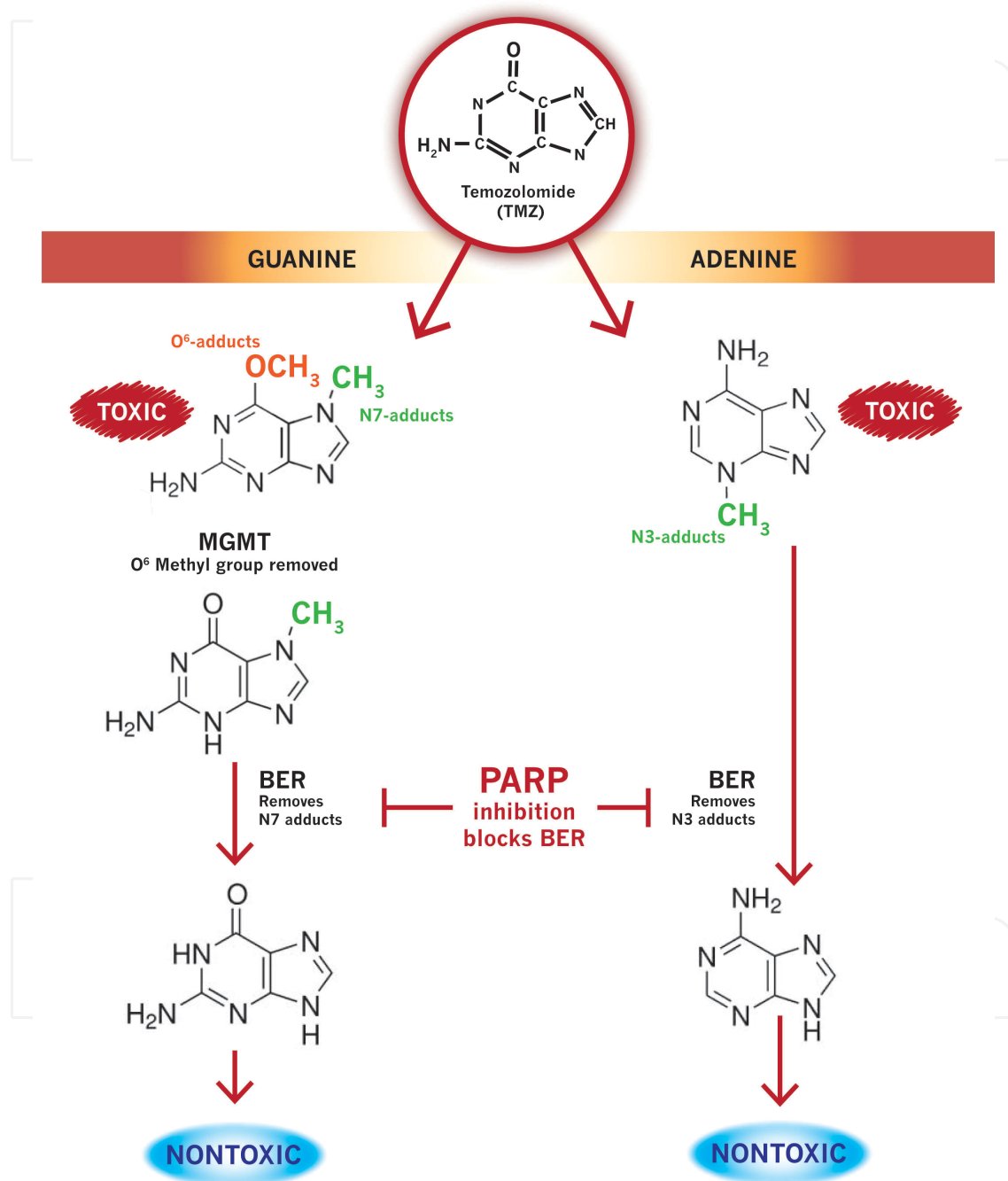

Figure 1. Common temozolomide-induced DNA lesions appear on guanine and adenine. N7-methylguanine, N3-methyladenine and O6-methylguanine DNA adducts account for roughly 70%, 10% and 5% of these lesions respectively. O6-methylguanine adducts are the most cytotoxic, yet are readily repaired by MGMT, resulting in TMZ resistance. The less toxic N7-methylguanine and N3-methyladenine adducts are readily repaired by the base excision repair (BER) system. The inhibition of PARP blocks BER and increases toxicity.

Poly (ADP-ribose) Polymerase 1. PARP-1 is a cell-survival factor that functions in single-stranded break repair (SSBR) to maintain genomic integrity [19]. The zinc-finger domain of PARP-1 binds to DNA nicks and adds the polyanion ADP-ribose (PAR) to histone proteins H1 and H2B [20]. PAR addition to histones relaxes the 30nm chromatin allowing access of DNA repair enzymes to the DNA. Inhibition of PARP-1 in proliferating cells sensitizes cells to DNA damage resulting in cell cycle arrest [21].

2. O-6-methylguanine-DNA methyltransferase

MGMT Mechanism of Action. The MGMT gene is located on chromosome band 10q26.3 spanning 300kb, with 5 exons and 4 introns. It encodes the 207 amino acid MGMT repair enzyme that is highly conserved among species and plays a critical role in maintaining the integrity of genomic DNA. The MGMT 1.2kb promoter region is TATA-and CAAT-box free with numerous CpG islands. Expression levels of MGMT vary significantly between tissues and can be regulated by glucocorticoids, cAMP, protein kinase C, and DNA damage [22].

The transcription factor Sp1 functions in transcriptional regulation of the MGMT gene, and CpG methylation within the promoter sequence affects chromatin structure to affect Sp1 access to the promoter site [23]. Methylation of specific CpG clusters in the promoter is correlated with MGMT gene silencing [24]. However, the overall amount, location, and homogeneity of MGMT promoter methylation is variable in GBM [25]. Dunn et al. investigated 109 newly diagnosed GBMs, and found that 58 tumors had an elevated methylation status compared to non-neoplastic brain tissue ($\geq$ 9% methylated). Furthermore, 19 of the tumors examined with a methylation status greater than 35% correlated with the highest 2-year survival rates [26]. It is not fully understood what determines MGMT promoter methylation levels, but recent evidence indicates that p53 may play a role. Using human lung cancer cells, Lai et al. showed that the knockdown of p53 increases MGMT promoter methylation in wild type p53 lung cancer cells [27] while Srivenugopal et al. reported that inducible p53 expression suppresses MGMT levels in a p53-null lung cancer cell line [28]. In contrast, published work by Wang et al. suggests that hypermethylation of CpG islands within the MGMT gene does not strictly correlate with reduced MGMT protein expression [29]. A number of studies have highlighted the variability in MGMT promoter methylation and MGMT gene expression levels indicating both a variability in MGMT activity within and between tumors [30]. More recently, Kanemoto et al. performed deep sequencing analyses of the entire MGMT promoter to develop a diagnostic assay for progression-free survival of GBM patients based upon hypermethylation of CpG islands. Despite the evidence that variability in MGMT promoter methylation does exist, the data confirm the general hypothesis that hypermethylation of the MGMT promoter does correlate with reduced MGMT enzyme activity [31].

The MGMT enzyme functions as both a transferase and acceptor of alkyl-groups. MGMT activity does not require cofactors or other enzymes, rapidly removes DNA adducts from the O6 position of guanine, and transfers them to an internal cysteine residue (Cys145) within the enzyme active site [32]. This reaction is stoichiometric and once the MGMT protein has been alkylated, it is inactivated and undergoes ubiquitin-mediated degradation [33].

Most alkylating agents used to therapeutically induce cell death target the O6-methylguanine adduct. While MGMT primarily repairs O6-methylguanine DNA adducts, it has the ability to repair adducts of greater size (i.e. O6-ethylguanine) as well as the minor alkylation product O4-methylthymine. MGMT-mediated repair pathways correct the damage caused by alkylating chemotherapeutic agents utilized in the treatment of gliomas, melanomas, carcinoid tumors, and lymphomas, such as carmustine, temozolomide, streptozotocin, procarbazine, and dacarbazine [32].

Clinical Implications of MGMT. As noted above, MGMT specifically reverses the DNA damaging effects of TMZ, and hypermethylation of the promoter for the MGMT gene correlates with reduced MGMT production. This finding has clinical implications, as highlighted by Stupp et al. and Hegi et al. in companion 2005 papers [2, 34]. Stupp et al. reported results from a multicenter, randomized trial comparing adjuvant radiation therapy to radiation therapy plus temozolomide for the treatment of glioblastoma. The median survival for the temozolomide group was 14.6, versus 12.1 months in the radiation only group. The two year survival rate was 26.5% in the temozolomide group versus 10.4% in the radiation only group. Perhaps the more interesting findings came from subgroup analysis. Hegi et al. stratified the two treatment groups based on MGMT promoter methylation. Though less than half of the patients enrolled in the clinical trial had usable DNA methylation data, the results, summarized in Table 1, are nonetheless compelling. In nearly a decade since the publications of these papers, the conclusion that MGMT promoter methylation sensitizes malignant glioma to temozolomide has been confirmed in multiple clinical and cohort studies [35-38]. MGMT promoter methylation status is an important prognostic biomarker and it appears that MGMT methylation status should be considered when formulating the treatment plan [39].

3. Inhibition of O-6-methylguanine-DNA methyltransferase

The key mechanism of resistance to alkylating agents in GBM is the presence of MGMT enzyme, and most human tumors exhibit high levels of MGMT expression and activity. As mentioned previously, elevated expression of MGMT is inversely correlated with survival [34, 40, 41]. Thus, suppression of MGMT activity could render cells more sensitive to alkylating agents, augmenting cytotoxicity.

O6-benzylguanine. Of the agents to target MGMT suppression, O6-benzylguanine (O6BG) was the first developed and was thought to have the greatest potential. O6BG is an MGMT substrate that inactivates MGMT in a suicide manner by binding to the protein. In the early/mid 1990's, studies using O6BG to suppress MGMT in GBM, both *in vitro* [42, 43] and *in vivo* [44], showed increased sensitivity and cytotoxicity to alkylating agents. These data initiated several clinical trials [45-49]. Unfortunately, patients in these early phase trials also exhibited significant hematological toxicity to O6BG and late phase trials were not pursued. Although O6BG may someday find a very useful place in neuro-oncology, the data from these clinical trials suggested that safer and more effective therapeutic approaches were needed to target MGMT.

Methylation Status	Radiation Alone	Radiation and Temozolomide
MGMT Methylated		
Patients	46	46
Median progression free survival (months)	5.9	10.3
Median overall survival (months)	15.3	21.7
2 year survival (%)	22.7	46
MGMT Unmethylated		
Patients	54	60
Median progression free survival (months)	4.4	5.3
Median overall survival (months)	11.8	12.7
2 year survival (%)	0	13.8

Table 1. Data summarized from Hegi et al. 2005.

Gene Therapy. While O6BG was proving to be too toxic for systemic delivery, a promising method for treating malignant tumors was being developed, gene therapy. Several studies have targeted oncogenes both *in vitro* and *in vivo* using antisense oligonucleotides with varying success, as described in the review by Caffo et al. [50]. Further, multiple studies have applied RNA interference strategies to treatment of gliomas - a Pubmed search in September of 2014 using the key words "RNA interference and glioma" generated 707 citations. The disruption of genes via synthetic nucleotide sequences may provide a specific inhibition of tumor growth with minimal off-target effects.

In 2008, our lab used RNA interference *in vitro* to silence the MGMT gene in GBM cell lines expressing high (U138MG), low (U87MG) and non-detectable levels (LN229) of MGMT [51]. Baseline TMZ dose response curves indicated that GBM cells with high levels of MGMT expression exhibit the greatest resistance. The half maximum effective concentration (EC50) of TMZ was consistently higher in cells with higher MGMT expression, this finding was similar to previous reports [52]. After MGMT mRNA knockdown, U138 cells became more sensitive to TMZ as expected (Figure 2). Interestingly, we were unable to detect a gene expression change in the U87MG cells, even though siRNA treatment rendered these cells more sensitive to TMZ treatment. No gene knockdown occurred in the LN229 cells (MGMT was not detectable at baseline) and the EC50 in these cells did not change after attempting RNA interference. These data are consistent with the work of others [13, 53] suggesting that siRNA could be an effective therapeutic agent.

Although it is possible for siRNA to function efficiently using cells grown in culture, siRNA application within organisms is difficult. These siRNA macromolecules do not cross cell membranes easily and would make crossing the blood brain barrier problematic. Thus, novel delivery systems for siRNA are being investigated. One successful nanoparticle system being developed is the LipoTrust EX Oligo liposome delivery system [13]. Kato et al. reported that liposome delivery of siRNA to downregulate MGMT was effective in sensitizing GBM to TMZ

in both *in vitro* and *in vivo* models [13]. Nanoparticles, whole cells and viruses, alone and in combination, are options being explored for delivery of synthetic nucleotides [54, 55]. It appears that gene therapy is a realistic possibility and may provide patients with "personalized medicine" in the near future. Yet, there are major concerns with the use of the technology, including off-target gene effects. Further, there are concerns about the use of viruses for gene therapy because of their potential to induce mutations. Oncolytic viruses, which preferentially lyse tumor cells, can become more selective and effective when engineered to possess tumor specific transgenes. The conditionally replicating adenovirus is one such oncolytic virus that not only targets the tumor cell but also possesses a gene that blocks the recruitment of the transcriptional coactivator p300 to the MGMT promoter [56].

p53. DNA damage from alkylating agents, ionizing radiation and ultraviolet light induces p53 activity. The role p53 plays in MGMT expression is not fully understood. Knockdown of p53 increases MGMT promoter methylation [27], yet overexpression of wild-type p53 suppresses MGMT and renders human cancer cells more sensitive to TMZ [28]. A previous study also reported MGMT downregulation in p53-null osteosarcoma cells with the introduction of wild-type p53 [57]. Grombacher et al. demonstrated that the overexpression of p53 reduced basal MGMT promoter activity in rodent cells [58]. The mechanism of p53-mediated MGMT knockdown may be through physical binding of the MGMT promoter without altering the methylation status [59]. It has also been shown that p53 prevents Sp1 from binding to the promoter [60]. Because overexpression of p53 may suppress MGMT, targeting p53 may render GBM more sensitive to TMZ. Levetiracetam is often used in GBM patients to manage seizures, but its use may further benefit patients by augmenting p53-mediated MGMT suppression [61]. Interferon beta (IFN-β) was shown to mediate cytotoxicity in human GBM cell lines [62], most likely because IFN-β induces p53 and improves the response of GBM cells to TMZ treatment [63, 64]. More recently, the use of a mitogen-activated protein/extracellular signal-regulated kinase (MEK) inhibitor has shown promise as a TMZ adjuvant by activating p53 [65]. Molecules targeting miRNAs may also be effective at p53-mediated suppression of MGMT. For example, ways to suppress miR-21 may also reduce MGMT levels via p53 activation [66]. The utility of targeting p53 may be limited [64] due to the high incidence of p53 mutation in GBM [63].

Other MGMT Inhibitors. Other targets have been suggested for regulation of MGMT levels in the cell. For example, valproic acid has become an established anti-cancer drug because of it's role in histone deacetylation, but it has also been shown to downregulate MGMT in GBM cells [67]. The proteasome inhibitors Bortezomib and MG132 also suppress MGMT transcription [68, 69]. Altering the expression of tumor suppressor genes may also be an effective treatment strategy. For example, miR-181d has been shown to down regulate MGMT possibly via the K-ras-related Pi3K/AKT and MapK/ERK pathways [70]. Lastly, the cytokine interleukin-24 downregulates MGMT expression in human melanoma cells and the DNA crosslinking agent cisplatin suppresses MGMT in leukemia cells [71, 72].

It is worth noting that individualized therapy based solely on MGMT promoter methylation alone may not always be advantageous. MGMT promoter methylation may not correlate with TMZ sensitivity and survival in some populations [73]. Although it has been reported that the level of MGMT mRNA and protein expression is correlated with promoter methylation status
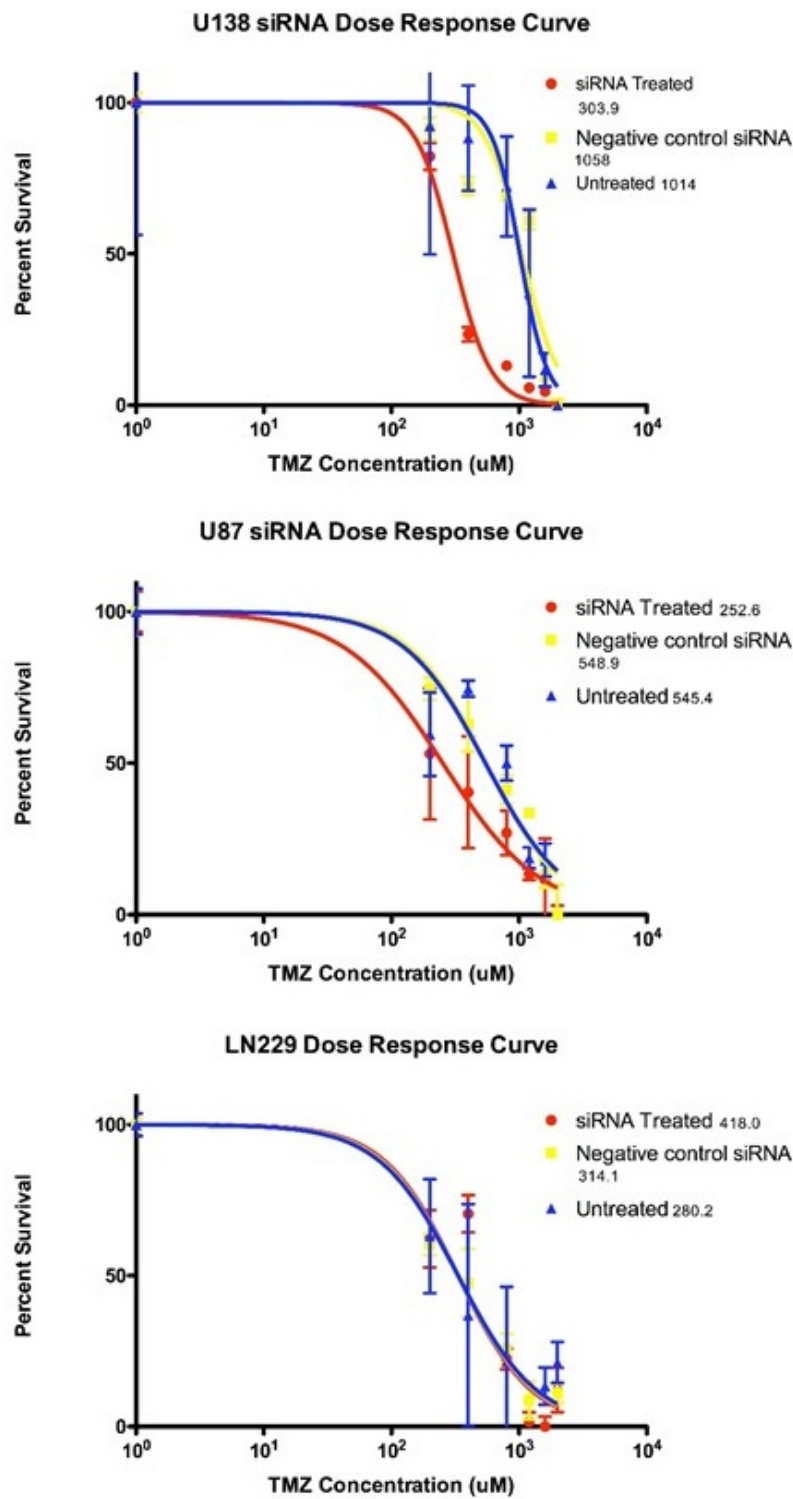
Figure 2. U138MG (high MGMT expression), U87MG (low MGMT expression) and LN229 (no MGMT detected) dose response curves from siRNA experiments (March 2008). The difference in EC50 between the siRNA treated group and the negative and untreated control group was statistically different for U138MG (P<0.0001) and U87MG (P<0.01), with no difference in the LN229 cell line. The figure legend represents the EC50 values for each treatment group in µM of TMZ.

[74-76], this correlation does not hold true in all cases [73, 76-80]. Reports of GBMs with unmethylated MGMT promoter regions and low MGMT mRNA expression as well as GBMs with methylated MGMT promoter regions expressing high MGMT mRNA levels suggest that methylation-independent pathways may alter MGMT mRNA levels [74, 76, 77, 81]. Evidence suggests that mechanisms of post-transcriptional regulation alter MGMT protein expression since protein analysis of MGMT does not correlate with mRNA [76]. Recent data suggest that miRNA regulation of MGMT may explain these discrepancies and miRNAs are currently being investigated as therapeutic targets [70, 82, 83]. Because MGMT expression appears be predictive of progression free and overall survival [74], adequate assessment of tumors may need to include MGMT mRNA and/or protein expression.

MGMT Summary. MGMT plays a critical role in maintaining the integrity of genomic DNA. Unfortunately, elevated MGMT expression is correlated with poorer prognosis in cancer patients. Tumors expressing MGMT will not respond well to alkylating chemotherapy because MGMT corrects O6-methylguanine. Screening the tumor tissue not only for MGMT (expression and methylation status), but also for other genes that may suppress or increase MGMT expression will be important for successful management of GBM. Clinicians should be guarded, however, when determining the treatment strategy because not all GBMs display the same magnitude, locations and homogeneity of methylations [25]. Although many therapeutic targets have been found to suppress MGMT activity, research is still needed to determine which of these molecules will effectively suppress MGMT while being safely administered to the patient.

4. Poly (ADP-ribose) polymerase 1

PARP-1 Mechanism of Action. The human Poly (ADP-ribose) Polymerase (PARP) gene family is evolutionarily conserved and codes for 17 different enzymes. The most important member of the PARP family, PARP-1, is a key DNA repair enzyme located on chromosome 1q42 and is responsible for the majority of PARP activity in the cell. As a component of the base excision repair (BER) pathway, PARP-1 binds to single-strand DNA breaks, catalyzes the formation of ADP-ribose polyanions from its substrate nicotinamide adenine dinucleotide, and recruits additional repair enzymes to the damaged strand. In addition to its central role in DNA repair, the PARP-1 enzyme also regulates other vital biological functions as reviewed in Krishnakumar and Kraus, 2010 [84]. While PARP-1 protein expression is typically low in normal brain tissue, it is highly expressed in GBMs [85]. The transcription of PARP-1 appears to be primarily regulated by the transcription factors SP1 and NFI, but, AP2, YY1 and ETS also bind to promoter sites of the PARP-1 gene [86].

Clinical Implications of PARP-1. In GBM, the effectiveness of radiation and chemotherapy is mitigated by normal cellular DNA repair mechanisms. It follows that interfering with DNA repair will enhance DNA damaging treatments. A number of preclinical studies confirm that PARP inhibition strengthens the efficacy of several DNA damaging anticancer therapies including radiation, DNA methylating agents, and topoisomerase I inhibitors [87]. A particu-

larly exciting application of PARP inhibition is in cancer cells with defects in homologous recombination. The loss of both single strand repair and repair of double strand DNA breaks is fatal [88]. This example of synthetic lethality demonstrates that these two loss-of-function mutations prove fatal, whereas either mutation alone is not. Clinical trials have utilized PARP inhibitors in two ways: in combination with DNA damaging therapies and as a single agent for tumors deficient in homologous repair (e.g., BRCA1/2 mutated breast cancers) [87]. Further, epidermal growth factor receptor (EGFR) status may act as a predictive biomarker for PARP inhibition sensitivity [89]. The EGFR gene is overexpressed in ~50% of GBMs [90]. Nearly 20% of grade III and IV tumors possess the constitutively active class III variant (EGFRvIII) and correlates with elevated expression of the gene [91]. Clark Chen's group recently determined that GBM cells over-expressing EGFRvIII were dependent on the BER system and PARP-1 related function for cell survival [89]. Following pharmacological inhibition or PARP-1 silencing with siRNAs, cytotoxicity was increased in GBM cells expressing elevated levels of EGFRvIII. It was suggested the increased cytoxicity was due to the inability to correct the damage caused by reactive oxygen species and the effect was greater when coupled with radiation [89].

5. Inhibition of poly (ADP-ribose) polymerase 1

Cancer treatments utilizing ionizing radiation and DNA alkylating agents damage DNA, which if not repaired causes cell death. Inhibition of PARP-1 contributes to the sensitization of tumor cells to these treatments and is the basis for multiple preclinical and clinical studies with PARP inhibitors in combination with classical therapies [92].

Benzamides. The inhibition of PARP-1 and the role of PARP-1 inhibition has been of interest since the early 1980's when it was demonstrated that PARP could be inhibited using nicoti-namide analogues [93]. The initial interest in PARP inhibition was to determine the role of the enzyme in the cell. The ability to inhibit PARP was responsible for elucidation of the function of PARP and its role in DNA repair. Using 3-aminobenzamide, Sidney Shall's group demon-strated that inhibition of PARP resulted in disruption of the repair of DNA breaks created by treatment with the DNA alkylating agent, dimethyl sulfate. Additionally, they reported that PARP inhibition enhanced the efficacy of dimethyl sulfate as a cytotoxic agent [94]. This study was the first to suggest that combined treatments with DNA alkylating agents and PARP inhibition could be effective in the treatment of cancer. The benzamides were essential in determining the role of PARP and providing "proof of principle" that PARP inhibition could play a role in cancer treatment and increase the efficiency of DNA damaging agents. However, the benzamides are relatively weak PARP inhibitors and have been shown to interfere with cellular pathways not associated with PARP [95]. More recently, specific PARP inhibitors have been produced and several are currently in clinical trials. Second and third generation PARP inhibitors are more potent and require markedly lower effective concentrations to reduce 50% of PARP's activity [96].

New Generation Inhibitors. PARP inhibition results from two different but complementary mechanisms, blocking PARP catalytic activity and the release of the enzyme from the DNA. The enzyme needs to be poly(ADP-ribosyl)ated to dissociate from DNA and if that ability is blocked by the catalytic inhibitor, it remains bound causing a physical obstruction to the repair of the DNA break. This was first proposed by Masahiko Satoh and Tomas Lindahl in 1992, who demonstrated that the repair of nicked plasmid DNA by nuclear extracts was not dependent on PARP, indicating that other repair pathways were still effective [97]. However, if PARP was present then its substrate, NAD+, was necessary for repair to occur. If this PARP-NAD+ repair requirement was blocked by 3-aminobenzamide, which competes for the NAD+ binding site, DNA repair was stopped. This indicated that inactive PARP impeded DNA repair, not only did PARP not recruit repair enzymes to the site of damage but also physically blocked other DNA repair pathways. Recent work expanded our understanding of the role of PARP inhibitors in DNA binding. DNA damage produced by alkylating agents administered concomitantly with a PARP inhibitor resulted in increased PARP–DNA binding compared to PARP–DNA binding with either the alkylating agent or PARP inhibitor alone [98]. In a similar study it was demonstrated that effectiveness of a PARP inhibitor might not only be its ability to inhibit the catalytic ability of the enzyme but also its ability to trap the enzyme on the DNA strand [99]. Using three potent PARP inhibitors (Olaparib (AZD-2281), veliparib (ABT-888), and niraparib (MK-4827)), currently being investigated clinically, it was demonstrated that all are effective in suppression of catalytic activity, having IC50 values that are in the low nanomolar range [100]. If we were to assume that the function of PARP inhibitors is solely explained by catalytic inhibition these three drugs should have similar effect and that effect should not differ from PARP deletion or silencing. However, it was reported that following treatment with the DNA alkylating agent, MMS, that while all three PARP inhibitors were effective in inhibiting catalytic activity, these clinically relevant PARP inhibitors differ markedly in their potency to induce cytotoxic PARP-DNA complexes. The authors indicated that, the potency in trapping PARP differed markedly among inhibitors with niraparib (MK-4827) > olaparib (AZD-2281) >> veliparib (ABT-888), a pattern not correlated with the catalytic inhibitory properties for each drug and suggested that further PARP inhibitor studies should examine both aspects, catalytic inhibition and DNA trapping, in inhibitor evaluation [100].

Inhibitors Versus Deletion or Silencing. It is becoming clear that there are subtle, but important differences between PARP deletion versus PARP inhibition. PARP-1 knockout mice are viable and fertile as are PARP-2 knockout mice. However, the deletion of both enzymes is lethal [101]. This indicates that while either enzyme can fill the DNA repair role, that role is essential. Additionally, it has been shown that PARP-1$^{-/-}$knockout mice are more sensitive to radiation than wildtype mice [102]. In addition to knockout mice, studies examining the effect of PARP silencing using RNA interference have been effective in reduction of PARP and PARP activity and have increased radiosensitivity in a manner equivalent to PARP-1$^{-/-}$knockout mice [103]. In our hands, silencing of PARP-1 in glioblastoma cell lines was generally not as effective as

treatment with a PARP inhibitor [104]. The greater efficacy of PARP inhibitors compared to PARP knockout or PARP silencing likely has to do with the mode of action of the inhibitors. This is an important consideration as it has been shown that inhibitors act on both PARP-1 and PARP-2, usually with similar potency and thus inhibition is more similar to the deletion of both enzymes. Additionally, it is well established that PARP in combination with an inhibitor blocks DNA repair in at least two ways.

PARP and Glioblastoma. Temozolomide acts by specific methylation of the DNA bases guanine and adenine resulting in inappropriate pairing during DNA replication. O6-methylguanine will trigger the DNA mismatch repair (MMR) pathway and become highly cytotoxic unless it is corrected by MGMT. However, nearly 80% of the total methylation events resulting from TMZ treatment are the N7-methylguanine and N3-methyladenine that trigger the BER system. The disruption of the BER pathway through PARP inhibition renders these lesions cytotoxic and helps overcome the MGMT related TMZ resistance. Another important consideration is the use of PARP inhibitors to increase TMZ sensitivity in tumors that exhibit MMR deficiencies, a relatively common occurrence in sporadic cancers. In these tumors, the lack of MMR, causes the cell to overlook the O6-methylguanine lesion rendering TMZ ineffective. Treatment with a PARP inhibitor renders the N3-methyladenine and N7-methylguanine lesions cytotoxic [105]. However, it has been reported that MMR deficiency does not seem to be responsible for mediating TMZ resistance in adult GBM [106].

Targeting PARP-1 and MGMT. In 2011 we examined the role that PARP-1 inhibition plays in altering GBM cell lines' response to TMZ [104]. We examined any changes in TMZ effectiveness in the GBM cells using either the PARP inhibitor, 3-aminobenzamide (3-AB) and compared its effect to silencing of PARP-1 using RNA interference. In our hands, the use of the PARP inhibitor was generally as, or more, effective than PARP silencing. Additionally, we examined the response to TMZ following PARP-1 silencing combined with MGMT silencing by RNA interference. The response to TMZ following silencing of both PARP-1 and MGMT was compared to the response to TMZ following PARP inhibition and MGMT silencing. In three of four GBM cell lines, the PARP inhibitor, 3-AB, combined with MGMT silencing rendered cells more sensitive to TMZ compared to silencing of both PARP-1 and MGMT or TMZ alone. These data suggest that GBM cells may be more sensitive to PARP inhibition versus PARP silencing, but also suggest that targeting both MGMT and the BER system may result in lasting TMZ-induced lesions leading to cell death.

PARP-1 Summary. PARP-1 and PARP-2 play critical roles which have been demonstrated by the lethality seen when both genes are deleted. One well defined role for these enzymes is DNA repair via the base excision repair pathway. Unfortunately, many cancer treatments including radiation and many types of chemotherapeutic agents act by damaging DNA. Much of this damage can be repaired through the actions of PARP. PARP inhibitors have been shown to bind effectively to both PARP-1 and PARP-2, block the enzymatic activity and may trap the inactivated PARP on the DNA lesion, effectively blocking additional DNA repair mechanisms. These actions have been shown to increase

the sensitivity to radiation and DNA alkylating chemotherapies in preclinical studies. However, while promising, the result of most clinical trials has been somewhat disappointing as the PARP inhibitors as chemopotentiating agents has been limited by an increase in toxicities, necessitating dose reductions of the cytotoxic chemotherapeutic agent and the PARP inhibitor. Ongoing research re-examining the mechanism of action of the PARP inhibitors, including those that bind in sites other than the PARP catalytic site, may eliminate some of the off target binding and reduce inhibition of other members of the PARP family. Also, continued efforts to determine other pathways affected by the PARP inhibitors are necessary to make adequate decisions about clinical usage.

6. Conclusions

Surgical resection, radiation and use of TMZ is currently the standard of care for GBM patients. The alkylating agent, TMZ, induces lesions at the N7 and O6 positions of guanine and N3 position of adenine. However, many of these tumors express MGMT which promptly corrects the most cytotoxic lesion, the O6-methylguanine adduct. Tumors expressing MGMT are, therefore, inherently resistant to TMZ. MGMT inhibition improves the response to TMZ, but MGMT inhibition as standard therapy is still in development. O6BG, although too toxic to give systemically, may find itself useful for future therapy if delivery to the central nervous system can be improved. Gene therapy, with the enhancement of the delivery of synthetic nucleotides like the LipoTrust and oncolytic viruses, may become the standard of care in the future. There are several other molecules targeting pathways that influence MGMT and many more will surely emerge.

The majority (~80%) of these TMZ-induced DNA alkyl adducts, N7-methylguanine and N3-methyladenine, are repaired by the BER system. After the mismatched base has been removed, PARP-1 plays a role in repairing DNA breaks by binding and recruiting other BER proteins. Because of its role in the BER system, PARP inhibitors also improve the response to TMZ. While the results from numerous clinical trials have been disappointing due to systemic toxicities, new inhibitors may improve outcomes.

Modulation of TMZ resistance through the MGMT and BER pathways is clinically viable. New combinations of existing strategies may prove to further compliment TMZ and augment its effectiveness. Although several approaches have been used to modulate PARP and MGMT pathways, molecular screening should be used to identify targets with the greatest therapeutic potential. For example, pre-treatment assessment for MGMT and EGFR expression would provide information regarding the susceptibility to TMZ and PARP inhibitors, respectively. With the growing understanding of the pathways involved with DNA repair, the design of novel strategies or the use of combinations of existing therapies may improve GBM outcome.

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