

Review Article

Examining the Role of Specialized DNA Polymerases in the Development of Temozolomide Resistance in Glioblastoma Multiforme

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Abstract

Glioblastoma multiforme (GBM) is an extremely malignant type of primary brain tumor that exhibits a high mortality rate. Current standard therapy involves surgery followed by radiation and treatment with the DNA-alkylating agent, temozolomide (TMZ). While TMZ treatment can extend post-operative survival, most patients develop resistance to TMZ which leads to a significant increase in mortality. At the molecular level, TMZ produces a variety of different DNA lesions including N⁷-methylguanine, N³-methyladenine, and O⁶-methylguanine. Although each DNA lesion possesses a unique molecular structure, they all elicit cytostatic and cytotoxic effects against GBM cells. This review article describes the molecular and cellular mechanisms accounting for the anti-cancer activity of TMZ as well as the mechanisms responsible for both inherent and TMZ-induced drug resistance. Special emphasis is placed on understanding the roles that various DNA polymerases play toward the initiation and progression of GBM in addition to mediating resistance to TMZ. This review concludes with discussions on several new approaches that show promise in combating TMZ-resistance, specifically using small molecules to block the replication of DNA lesions catalyzed by various DNA polymerases.



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Keywords

Glioblastoma multiforme; DNA damage; DNA polymerization; chemotherapy; nucleoside analogs

1. Introduction

Glioblastoma multiforme (GBM) is the most common type of brain cancer and afflicts over 12,000 children and adults each year in the United States [1]. GBM is also the deadliest of all cancers, having 5-year survival rates of less than 10% [2]. The current standard of treatment for GBM is referred to as the Stupp protocol published in 2005 [3]. This landmark study demonstrated a survival advantage for concomitant and adjuvant temozolomide (TMZ) treatment with radiotherapy in patients with GBM [3]. This study comprised a total of 573 patients with a median age of 56 years and included only patients with newly diagnosed and histologically confirmed GBM. In addition, the majority of patients (>80%) had undergone debulking surgery prior to treatment. Patients were randomly assigned to receive a total of 60 Gy alone (fractionated focal irradiation in daily fractions of 2 Gy given 5 days per week for 6 weeks) or radiotherapy combined with daily TMZ (75 mg per square meter of body-surface area per day, 7 days per week from the first to the last day of radiotherapy). This was followed by six cycles of adjuvant TMZ (150 to 200 mg/m² for 5 days during each 28-day cycle). After 28 months, the median overall survival (OS) was 14.6 months in patients receiving a combination of TMZ and radiotherapy which was longer than 12.1 months observed with radiotherapy treatment alone. The two-year OS rate with combining TMZ with radiotherapy was 26.5%, and this was statistically higher than 10.4% obtained using radiotherapy alone.

Collectively, these data showed that combining TMZ and radiotherapy provided significant survival benefits for newly diagnosed GBM patients without produced overt toxicities. Since this study was published, TMZ remains the primary FDA-approved drug used to treat GBM [4]. TMZ is an orally administered DNA alkylating agent that can effectively cross the blood-brain barrier [5]. Once inside a tumor, TMZ produces several distinct forms of DNA damage that produce cytostatic and cytotoxic effects. Unfortunately, even with aggressive treatments using IR and TMZ, the median survival time for most GBM patients is less than 16 months [6]. Much of this poor prognosis lies in the development of drug resistance that is caused in part by the pro-mutagenic replication of DNA lesions produced by TMZ. However, a number of new treatments show promise against GBM. For example, carmustine (1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)) is an anti-cancer agent that also alkylates and cross-links DNA during all phases of the cell cycle [7]. These modifications disrupt DNA replication and transcription to induce cell cycle arrest and/or apoptosis. In addition, carmustine carbamoylates proteins such as DNA repair enzymes, and this modification can impair their activity to increase the cytotoxicity of carmustine. Unlike TMZ, however, carmustine cannot effectively pass through the blood-brain barrier and is thus placed on wafers (Gliadel®) and used as an intracranial implant to achieve localized chemotherapy [8, 9]. Another innovative treatment is aldodoxorubicin, an analog of the anti-cancer drug doxorubicin (DOX) which is effective against many types of hematological and solid cancers [10]. DOX primarily generates double strand DNA breaks (DSBs), a highly cytotoxic form of DNA damage [10]. Unlike TMZ, DOX is unable to effectively penetrate the blood-brain barrier and is thus not used to treat GBM. However, aldodoxorubicin is a prodrug of

DOX that contains a pH sensitive linker that allows for transport across the blood-brain barrier and preferential release of the drug within GBM cells [11]. In fact, results from a recent phase II clinical trial in relapsed GBM patients showed that idoxorubicin penetrates the blood-brain barrier and is associated with objective tumor responses via MRI imaging and prolonged survival [12]. Finally, the anti-angiogenic agent bevacizumab (Avastin®) has received attention as second line therapy against GBM. While bevacizumab does not improve overall survival, it does improve the management of symptoms and overall quality of life for GBM patients [13, 14].

It is beyond the scope of this review to critically evaluate all of these therapeutic approaches against GBM. As such, this review focuses on the molecular and cellular mechanisms accounting for the anti-cancer activity of TMZ, mechanisms accounting for TMZ resistance, and approaches being developed to combat drug resistance. Special emphasis is placed on discussing new approaches to block the replication of DNA lesions that persist after exposure to TMZ.

2. Results

2.1 Molecular and Cellular Mechanisms of Temozolomide

The chemical mechanism responsible for the ability of TMZ to produce different forms of DNA damage is illustrated in Figure 1. The key step in this reaction is the ability of TMZ to undergo spontaneous hydrolysis at physiological pH (~7.4) to form a methyldiazonium ion. The formation of this strong electrophile enables an S_N1-type alkylation reaction in which the methyldiazonium ion effectively reacts with nucleophilic functional groups (oxygen and nitrogen) present on single-strand and duplex DNA [15]. Reactions on duplex DNA predominantly occur primarily at the N7 position of guanine (70%), the N3 position of adenine (10%) and the O6 position guanine (7%) [16]. Additional sites for alkylation include the N1 of adenine and the N3 of cytosine. However, these reactions occur more frequently in single-stranded DNA that typically forms at DNA replication forks during DNA synthesis and at sites of active gene transcription [17].

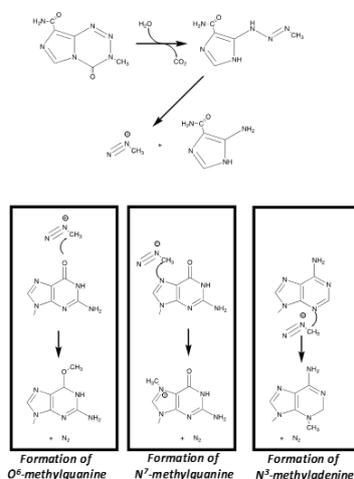


Figure 1 Chemical mechanism for the activation of temozolomide (TMZ) to produce different DNA lesions. TMZ undergoes spontaneous hydrolysis to form a methyldiazonium ion which reacts with nucleophilic functional groups present on single- and double-stranded DNA. Primary sites for modification are the N7 position of guanine, the N3 position of adenine, and the O6 position of guanine.

The most commonly formed DNA lesions, N⁷-methylguanine, N³-methyladenine, and O⁶-methylguanine, all produce cytostatic and cytotoxic effects. However, since each DNA lesion is structurally unique, the mechanisms accounting for their anti-cancer effects differ at the cellular level. As a consequence, the underlying mechanisms for resistance are different, and these differences have significant ramifications on therapeutic response. DNA damage caused by TMZ induces cellular senescence which is characterized by cell-cycle arrest at the G₂/M phase [18]. Recently, Aasland *et al.* showed that TMZ-induced senescence is initiated by damage recognition through the MRN complex followed by activation of ATR/CHK1 kinases and degradation of CDC25c [19]. However, TMZ-induced senescence also depends upon functional p53 in addition to sustained p21 induction. While the NF-κB pathway is also required for TMZ-induced senescence, neither p14 nor p16 (which are targets of p53) do not appear to play essential roles. In addition, TMZ treatment represses expression of several MMR pathway proteins including MSH2, MSH6, and EXO1. Surprisingly, the homologous recombination protein RAD51 is also downregulated. Both effects appear to be dependent upon p53 activity since their repression is not observed in p53-deficient cells.

Although the focus of this review is on drug resistance caused by the replication of lesions produced by TMZ, it is necessary to first understand how these DNA adducts are repaired. Provided below is a brief description of the major DNA repair pathways involved in correcting the three most common lesions formed by TMZ. The cytotoxic effects of a methylated nucleobase are typically caused by the ability of the formed adduct to transiently disrupt the continuity of DNA replication or to block this process completely [10]. For example, methyl adducts such as N³ adenine occur at positions on a nucleobase that directly inhibit DNA synthesis catalyzed by a DNA polymerase. In these cases, methylation at this and other key nucleobase positions disrupt essential contacts between the nucleobase and critical active site amino acid residues to block DNA synthesis [20, 21]. This inhibition causes the formation of blocked replication forks that are unstable and ultimately collapse to produce double-strand breaks (DSBs) which are highly cytotoxic. In contrast, methylation at other nucleobase positions can perturb interactions associated with proper Watson–Crick base-pairing interactions. For example, methylation at the O⁶ position of guanine produces O⁶-methylguanine that does not permanently block replication fork progression but rather allows for the incorporation of either cytosine or thymine. Neither nucleotide can correctly base-pair with the adduct. As such, the resulting mis-pair is excised and repaired by the mismatch repair (MMR) pathway [22]. As described in more detail later, the MMR pathway works to first excise newly synthesized DNA to produce a long single-strand gap. The gap is then filled in by repair DNA synthesis. However, since the DNA lesion originally present in the template strand is not excised, gap-filling DNA synthesis simply generates another mis-pair. This produces a futile cycle caused by repetition in mis-pair formation, excision, and reformation of a mis-pair. Eventually, the resulting single-strand gap that forms during this futile cycle generates a DSB during the next S-phase to induce cell death [23].

The most frequently formed DNA lesion is N⁷-methylguanine [24]. At face value, this lesion would appear to be harmless since the modification occurs at a position in the major groove of DNA which should not directly influence DNA polymerase activity. However, methylation at this and the N³ position of guanine as well as the N⁷ position of adenine accelerates the rate of non-enzymatic hydrolysis of the glycosidic bond between the nucleobase and deoxyribose [25, 26]. This

depurination reaction forms a new distinct lesion termed an abasic site which functions as a strong block to DNA synthesis catalyzed by most DNA polymerases [27-29].

2.2 Repair Pathways for Alkylated DNA

There are several distinct DNA repair pathways responsible for correcting DNA lesions produced by mono-functional alkylating agents such as TMZ. These include direct repair of O⁶-methylguanine by the enzyme O⁶-methylguanine methyltransferase (MGMT), the mismatch DNA repair (MMR) pathway, and the base excision repair (BER) pathway. In normal cells, these pathways are essential for protecting the nuclear genome as they correct methylated and other types of DNA lesions that arise from endogenous and exogenous sources. However, their activity has long been demonstrated to have deleterious effects on patient responses to TMZ by promoting drug resistance [30-32]. In addition, defects in the MMR and BER pathway can produce equally devastating effects by allowing DNA lesions caused by TMZ to persist. This allows the unrepaired DNA lesions to be misreplicated, and this process can drive drug resistance and/or mutagenesis. The MGMT, MMR, and BER pathways have been extensively reviewed elsewhere [33-38]. However, provided below are brief descriptions of each pathway to emphasize their roles in generating TMZ-resistance, particularly with the inappropriate replication of unrepaired DNA lesions.

2.3 Direct Repair by O⁶-Methylguanine-DNA Methyltransferase

O⁶-methylguanine DNA methyltransferase (MGMT) directly corrects damaged guanine nucleobases by transferring the methyl group from the O⁶ position of guanine to an active site cysteine residue [39]. MGMT is considered a “suicide” DNA repair enzyme since methylation of the active site cysteine inactivates the enzyme. This limits MGMT to a single-round of DNA repair as opposed to catalyzing multiple turnovers of nucleobase correction [40]. Despite this limitation, MGMT activity is important for maintaining genomic integrity by preventing mutagenesis and possible tumorigenesis caused by DNA alkylation. While the importance of MGMT activity in a normal cell is intuitively obvious, the loss of MGMT activity can have surprisingly beneficial outcomes for cancer patients being treated with TMZ. For example, molecules such as O⁶-benzylguanine have been used to inhibit MGMT activity, and this inhibition can sensitize cancer cells to the cell killing effects of DNA alkylating agents [41-43]. In addition, MGMT expression is an important feature in therapeutic responses to TMZ. Several studies have shown that the loss of MGMT expression is not due to gene deletion, mutation, rearrangement or unstable RNA, but due to methylation of CpG island of MGMT promoter [44-46]. In fact, epigenetic silencing of the MGMT promoter methylation is associated with longer survival in GBM patients receiving TMZ as part of their treatment [47]. Finally, the methylation status of the MGMT promoter is proposed to be an accurate biomarker in predicting the prognosis of GBM patients [48-50].

2.4 DNA Mismatch Repair

The mismatch repair (MMR) pathway is primarily involved in correcting replication errors that have escaped the proofreading activity of high-fidelity DNA polymerases during chromosomal replication [51]. Mismatches fall into two categories, the first being base–base mis-pairs such as T paired opposite G while the second are insertion and deletion mismatches caused by strand slippage

at repetitive DNA sequences. As indicated earlier, MMR plays an important role in processing mispairs such as C and T paired opposite O⁶-methylguanine caused by TMZ exposure. In humans, the MMR pathway features two families of MMR proteins that are heterodimeric homologs of bacterial MutS (MSH) or MutL (MLH). Both proteins have been extensively studied at the biochemical and cellular levels [52-54]. The MMR pathway is generally divided into three distinct phases that include mismatch recognition, excision, and re-synthesis of excised DNA. Each phase is catalyzed by a confederation of proteins, and their activities are tightly regulated to ensure complete and accurate repair of damaged DNA.

The first stage of MMR is mismatch recognition by MutS α or MutS β . The choice of initiator is dictated by the type of DNA lesion. MutS α initiates the repair of base–base mispairs and small deletions of 1 to 2 nucleotides. In contrast, MutS β is primarily responsible for initiating the repair of DNA segments containing larger deletions (1 to 15 nucleotides). Binding to a mismatch induces an exchange of ADP for ATP in both MutS α and MutS β , and this exchange converts both proteins into sliding clamps that allows the proteins to perform a two-dimensional search along DNA to identify other mismatches and to interact with MutL α .

The second phase is DNA excision in which the newly synthesized DNA strand containing the error is excised while leaving the templating strand unaltered. Although the mechanism for strand recognition is not completely understood in eukaryotes, recent results suggest that PCNA plays an important role in regulating this process [55]. *In vitro* and *in vivo* evidence demonstrate that PCNA can interact with MutS and MutL in addition to other proteins such as EXO1 as well as Pol δ , and Pol ϵ which are involved in re-synthesizing excised DNA [56-58]. *In vitro* MMR reactions require a preexisting nick or single-strand gap in the heteroduplex DNA substrate that can reside on the 5′- or 3′- side of the mismatch. In 5′-MMR reactions, excision is catalyzed by EXO1, a 5′ to 3′ exonuclease that is activated by MutS α in a mismatch-dependent manner. Excision in the case of 3′-MMR (a break or gap located on the 3′ side of the mismatch) is less well understood since EXO1 is currently the only exonuclease that appears to be involved in MMR.

The third and final phase of MMR involves DNA gap filling by a high-fidelity polymerase followed by ligation catalyzed by DNA ligase I. These activities collectively generate a corrected and intact DNA duplex. During this process, PCNA is loaded at a 3′ terminus by RFC, and replicative DNA polymerases, pols δ or pol ϵ , replicate the RPA-coated gapped DNA. DNA ligase I generates the final phosphodiester bond to fully repair the DNA.

As expected, proper MMR function can be compromised by mutation or epigenetic silencing to generate a hypermutator phenotype, and this can produce devastating pathological effects by altering genomic fidelity and integrity. For example, dysfunctional MMR activity is responsible for increased susceptibility for inherited cancers such as Lynch syndrome [59].

2.5 Base Excision Repair Pathway

The most abundant TMZ-induced adducts, 7-meG, 3-meA, and abasic sites, are repaired by the short patch base excision repair (BER) pathway. The BER pathway primarily functions to repair endogenous forms of DNA damage caused by reactive oxygen species generated during oxidative metabolism [60]. Spontaneously generated forms of damaged DNA include oxidized bases (8-oxoguanine), alkylated bases (N³-methyladenine), abasic sites, and single-strand breaks (SSBs). In a normal cell, it is estimated that as many as 50,000 BER-repaired lesions form per cell during the

course of a single day [61]. However, treatment with chemotherapeutic agents such as TMZ put a remarkable strain on BER since the number of these lesions increases by several orders of magnitude.

Repair of N³-methyladenine and N⁷-methylguanine is initiated by recognition of the lesions by alkylpurine-DNA-*N*-glycosylase (AAG). AAG cleaves the glycosylic bond between the damaged base and deoxyribose to produce an abasic site as the key intermediate in the repair process. After the hydrolysis reaction, AAG remains bound to the abasic site and recruits the endonuclease, Ape1, to the site of damage. Ape1 cleaves the DNA phosphodiester backbone to produce a SSB containing a 3'-OH and a 5' deoxyribose phosphate (5'-dRP) termini [62]. Ape1 is then replaced by DNA polymerase β , a repair DNA polymerase that possesses 5' lyase activity that can excise the 5'-dRP to produce a single nucleotide gap. The formed gap is subsequently filled in by DNA polymerase β , and DNA ligase seals the nick to finalize the repair process.

2.6 Consequences of Ineffectual DNA Repair

The most common mechanisms associated with TMZ resistance involve alterations in DNA repair pathways such as MGMT [63] and MMR [64]. As expected, the lack of efficient DNA repair makes cancer cells much more susceptible to mutagenesis which can produce more malignant cancers which possess additional mechanisms of drug-resistance. Indeed, a landmark study by Johnson *et al.* showed that genomic DNA isolated from recurrent GBM tumors initially treated with TMZ were highly mutated [65]. Their results showed that TMZ-treated tumors contained ~30 to 90 mutations per megabase compared to initial tumors which had significantly lower mutation frequencies (<4 mutations per Mb). Furthermore, recurrent GBM tumors were drug resistant, and this coincided with the accumulation of acquired somatic mutations in genes associated with MMR, retinoblastoma, and mammalian target of rapamycin (Akt-mTOR) pathways [65]. These results strongly implicate a mechanism in which DNA lesions formed by TMZ are inappropriately replicated due to ineffectual DNA repair.

Unrepaired DNA lesions can be misreplicated by various DNA polymerases in a biological process known as translesion DNA synthesis (TLS). First identified in bacterial cells, the ability to replicate damaged DNA via TLS activity was considered a "necessary evil" by allowing cells to tolerate unrepaired DNA lesions in order to survive [66]. It is now established that TLS is a highly conserved mechanism that occurs in both prokaryotes and eukaryotes [67]. In humans, however, TLS activity is often considered a "double-edged sword" since it can produce both beneficial and adverse effects on genomic fidelity and cellular homeostasis. One example of the beneficial effects of TLS activity comes from the disease known as xeroderma pigmentosum variant (XP-V) which occurs in individuals possessing mutations in a specialized DNA polymerase denoted as polymerase eta (pol η) [68]. This polymerase is unique as it can bypass unrepaired thymine dimers with high efficiency while maintaining genomic fidelity [69]. Individuals lacking pol η activity display an increased incidence of squamous cell skin carcinomas in response of UV-exposure and this highlights an important role for replicating damaged DNA [70]. However, unregulated TLS activity can have equally devastating effects as this activity is associated with generating somatic mutations that drive drug resistance to chemotherapeutic agents. As discussed in more detail below, there are several unique challenges toward a complete understanding of how TLS activity drives drug resistance. For example, there are a number of different DNA polymerases that can replicate lesions generated by

anti-cancer agents such as TMZ. Thus, it is difficult to unambiguously determine which specific DNA polymerase(s) is(are) responsible for generating resistance. In this case, the inherent redundancy in DNA polymerase utilization during TLS represents another set of challenges to develop prognostic biomarkers that accurately correlate TLS activity with TMZ-resistance. In addition, the redundancy may pose challenges toward developing therapeutic agents that block TLS activity in response to chemotherapy.

In humans, TLS activity is believed to be primarily catalyzed by a family of DNA polymerases that include polymerase eta (pol η), polymerase iota (pol ι), polymerase theta (pol θ), polymerase zeta (pol ζ), polymerase kappa (pol κ), and the Rev1 protein. These unique polymerases are coined "specialized" due to their ability to replicate a wide variety of structurally distinct DNA lesions with high efficiency and remarkable fidelity. For example, pol η displays an usually high error rate of making 1 mistake every 100 opportunities when replicating undamaged DNA [71]. However, pol η is highly proficient at replicating naturally occurring DNA lesions such as thymine dimers and abasic sites as well as lesions including O⁶-methylguanine and cisplatinated DNA that are produced during treatment with anti-cancer drugs [72-74].

The unique activity displayed by specialized DNA polymerases directly contrasts that inherent to "high-fidelity" DNA polymerases such as polymerase alpha (pol α), polymerase delta (pol δ), polymerase epsilon (pol ϵ), telomerase, and polymerase gamma (pol γ). Since these polymerases are intimately involved in chromosomal and mitochondrial DNA synthesis, they display optimal activity on undamaged DNA and low activity with damaged DNA. However, there are reported instances in which high-fidelity polymerases can efficiently replicate small, miscoding DNA lesions such as O⁶-methylguanine and 8-oxo-guanine [75-77].

Finally, there is a third class of DNA polymerases that primarily function in DNA repair pathways. These include polymerase beta (pol β) which is responsible for processing damaged DNA repaired by BER. In addition, polymerase lambda (pol λ) and polymerase mu (pol μ) play important roles in properly repairing DSBs formed during homologous recombination and non-homologous end-joining [78]. Finally, the fascinating polymerase known as terminal deoxynucleotidyl transferase (TdT) is important for generating immunological diversity by randomly incorporating nucleotides into single strand DNA during V(D)J recombination [79]. Each of these polymerases display variable fidelity depending upon whether they replicate undamaged or damaged DNA.

Aberrant TLS activity has been extensively demonstrated to play a role in tumorigenesis by promoting genetic mutations in addition to driving drug resistance [80-86]. For example, pol ζ has been shown to be overexpressed in several types of cancers including melanoma [81] and esophageal squamous cell carcinomas [82]. Furthermore, higher levels of pol ζ correlate with increased resistance to chemotherapeutic agents such as cisplatin which causes intrastand crosslinks [83, 84]. Likewise, pol η overexpression has been shown to be a common factor associated with producing drug resistance to cisplatin in several types of cancers including breast [85] and head and neck squamous cell carcinomas [86].

TLS activity is also associated with tumorigenesis in gliomas and drug-resistance to TMZ [87-89]. In contrast to cancers such as breast and ovarian cancers which show a reliance on the activities of pol η and pol ζ for tumorigenesis and drug resistance, GBM cells appear to rely on the activities of pol κ and pol ι . For instance, a recent study by Wang *et al.* examined 40 primary glioma samples and 10 normal brain samples to determine if increased expression of various specialized DNA polymerases contributes to cancer initiation and progression [87]. In this study, expression levels of

pol κ , pol ι , and pol η were examined using quantitative RT PCR and Western blot analysis. Results using glioma samples showed no significant expression of pol η . However, overexpression of pol ι was observed in ~28% of samples while pol κ showed an even higher frequency of overexpression (~58%) of samples. The prognostic significance of these higher polymerase levels was confirmed using a population-based tissue microarray derived from a cohort of 104 glioma patients. Immunohistochemical studies showed positive pol ι staining in ~32% of these glioma specimens while 69% were positive for pol κ staining. Furthermore, pol κ - and pol ι -positive staining correlated with reduced survival within this set of glioma patients. Collectively, these findings highlight how deregulated expression of pol κ and pol ι may stimulate tumorigenesis and provide a poor prognosis for glioma patients.

Following these observations, the same group investigated if overexpression of pol κ also plays a role in generating TMZ-resistance [88]. Their results show that pol κ overexpression confers resistance to the drug in GBM cells that display initial sensitivity to TMZ. Likewise, knocking down pol κ expression in GBM cells resulted in TMZ-sensitivity. The basis for these effects is complex as pol κ depletion caused a cascade of cellular events associated with the maintenance of genomic integrity. These include defective homologous recombination-mediated repair and restart of stalled replication forks which led to the inhibition of cell-cycle re-entry and progression.

While specialized DNA polymerases are considered prime suspects in tumorigenesis and drug resistance, they are not the only culprits. In fact, recent evidence from Campbell *et al.* suggests that high-fidelity DNA polymerases, pol ϵ and pol δ that are involved in leading and lagging strand DNA synthesis, respectively, may play significant roles in tumorigenesis in cancers including gliomas [90]. This supposition is based on an assessment of mutation burden obtained through sequencing analysis of >81,000 tumors from pediatric and adult patients [90]. Their findings show that a large number of pediatric cancers possess defects in MMR pathway genes *POLE* and *POLD1*. In addition, ultra-hypermuted pediatric tumors, defined as those possessing greater than 100 mutations per megabase, were all replication repair deficient. These tumors include colorectal cancers, leukemia/lymphomas, and malignant gliomas. In these cases, the authors conclude that while defects in MMR alone can initiate tumorigenesis by increasing mutagenesis, it is the combination of defective MMR coupled with mutations in pol δ and pol ϵ that causes the hyper-mutated state observed in these cancers. Surprisingly, mutations identified in pol δ and pol ϵ do not exclusively occur in the exonuclease-proofreading domain that corrects replication-induced errors. Instead, a wide spectrum of mutations were identified that in regions spanning the polymerization domain, the exonuclease domain, and connection domains. Further studies are needed to correlate specific polymerase mutations with hyper- and ultra-hypermuted genotypes. However, it is clear that mutations in these replicative polymerases alter their intrinsic fidelity and contribute to cancer initiation and drug-resistance.

2.7 Inhibiting TLS Activity as a New Therapeutic Strategy

Cancers such as GBM typically possess defects in one or more DNA repair pathways. These defects give a cancer cell only one option to survive the effects of TMZ – it must replicate damaged DNA inflicted by this agent. This leads to the hypothesis that inhibiting the misreplication of DNA lesions that escape DNA repair could be a new therapeutic strategy to improve patient outcomes, especially with respect to TMZ-resistance [91] (Figure 2).

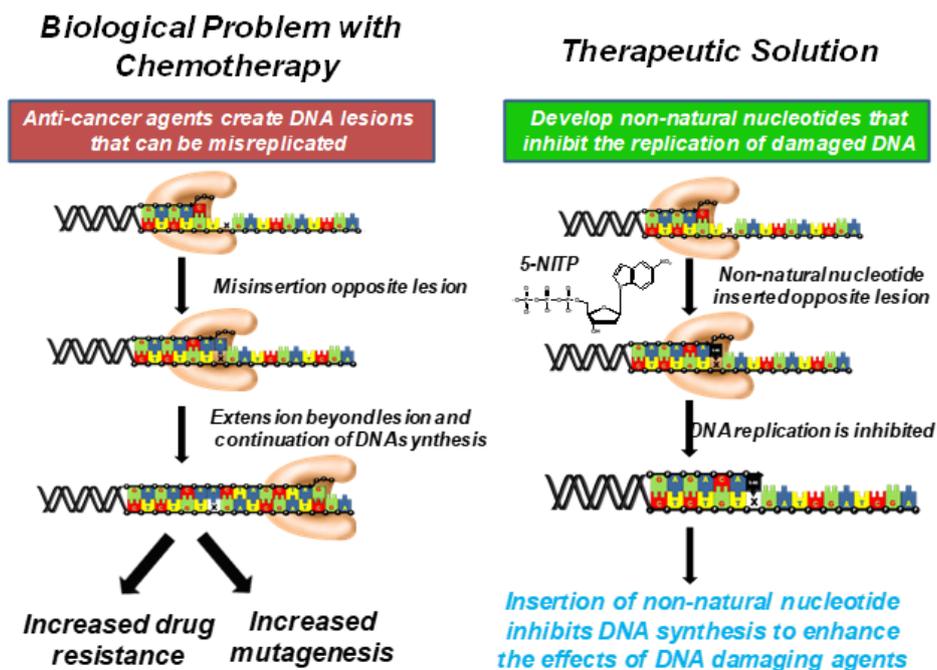


Figure 2 The misreplication of damaged DNA catalyzed by TLS activity can cause mutagenesis in addition to producing drug resistance to anti-cancer agents that produce DNA lesions. One approach to combat these problems is to use artificial nucleotides that can selectively inhibit DNA polymerase involved in replicating DNA lesions generated by therapeutic modalities such as TMZ and ionizing radiation.

This represents a novel approach for several reasons. First, TLS activity can be viewed as the Achilles' heel of GBM cells that are chemoresistant due to defective DNA repair. Secondly, most normal cells are quiescent and possess proficient DNA repair pathways that can effectively correct DNA damage. Since they do not rely on TLS activity for survival unlike their cancerous counterparts, normal cells should be spared from the effects of combining compounds that inhibit TLS activity with low doses of DNA damaging agent. Finally, since TLS activity can be highly pro-mutagenic, it can produce more mutations in a cancer cell to generate more aggressive cancers and/or cause tumor recurrence. In addition, error-prone replication of damaged DNA can transform a normal cell into an oncogenic cell via a phenomenon referred to as "secondary" or "treatment-related" cancers [92]. Thus, inhibiting TLS activity may also be a chemopreventive strategy that prevents these conditions.

To evaluate this approach, the Berdis lab synthesized several artificial nucleotide analogs and characterized their ability to be incorporated opposite an abasic site, the most frequently formed toxic DNA lesion generated by TMZ [93-98]. *In vitro* studies identified one unique analog, designated 5-NITP, that is efficiently and selectively inserted opposite this DNA lesion (Figure 3) [85]. While 5-NITP is efficiently inserted opposite an abasic site, it is refractory to elongation and thus acts as chain terminator against TLS activity. In addition, we demonstrated that 5-NITP is poorly inserted opposite normal DNA (A, C, G, or T). Collectively, these results indicate that the artificial nucleotide retains exquisite selectivity for chain-terminating activity during the replication of an abasic site.

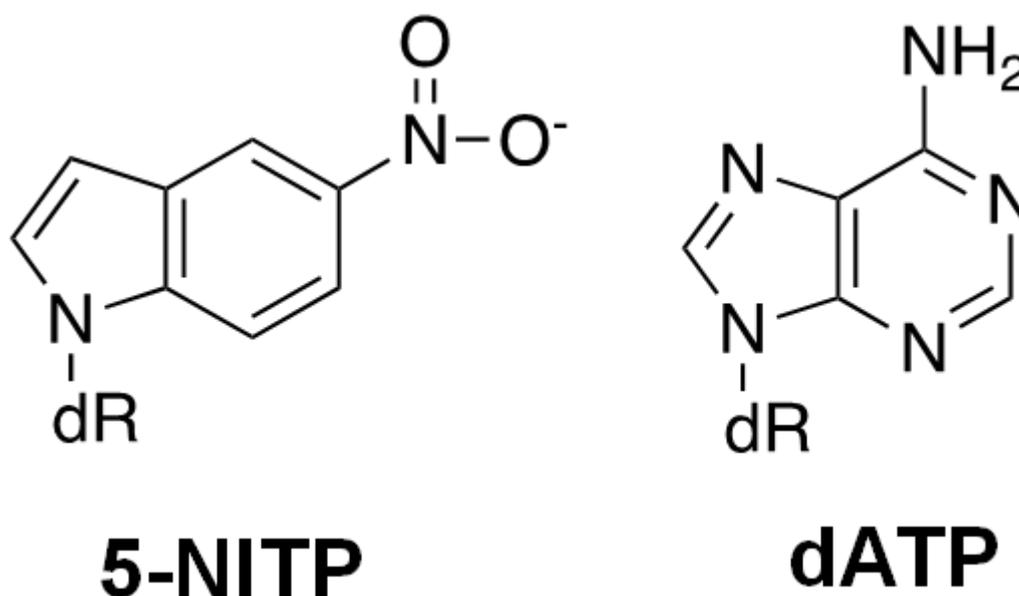


Figure 3 Structural comparison of the artificial nucleotide analog, 5-nitroindolyl-2'-deoxyribose triphosphate (5-NITP) with the natural nucleotide, dATP.

The ability of several human DNA polymerases to incorporate 5-NITP opposite abasic sites was also examined [98]. These studies included two high-fidelity DNA polymerases (pol δ and pol ϵ), three specialized human DNA polymerases (pol η , pol ι , and pol κ), and two DNA polymerases involved in DNA repair (pol λ and pol μ). *In vitro* analyses demonstrated that both high-fidelity DNA polymerases poorly insert the preferred natural substrate, dATP, opposite an abasic site yet efficiently insert 5-NITP opposite the lesion. Likewise, the specialized DNA polymerases, pol η and pol ι , insert 5-NITP opposite an abasic site with 100-fold higher efficiencies compared to dATP. However, pol κ and the repair DNA polymerases, pol λ and pol μ , all replicate an abasic site poorly as none appear to efficiently incorporate dATP and 5-NITP and opposite the lesion.

Cell-based studies using U87 cells as a model, examined if the corresponding nucleoside, 5-NIdR, potentiates the cytotoxic effects of TMZ by inhibiting TLS activity. This human GBM cell line is resistant to TMZ as the LD₅₀ value for the DNA damaging agent is greater than 100 μ M. Likewise, 5-NIdR displays low potency as the LD₅₀ value is greater than 100 μ g/mL. In this case, the low potency of 5-NIdR is expected since the corresponding nucleoside triphosphate, 5-NITP, is poorly incorporated opposite undamaged DNA. However, combining 5-NIdR with TMZ significantly increases the cell killing effects of the DNA damaging agent as reflected in 4-fold higher levels of early and late stage apoptosis compared to cells treated individually with TMZ or 5-NIdR. Flow cytometry experiments next evaluated the ability of 5-NIdR alone and in combination with TMZ to affect cell cycle progression. U87 cells treated with 100 μ g/mL 5-NIdR had no effect on cell cycle progression as compared to control cells treated with vehicle. This result again indicates that the artificial nucleoside generates a minimal cytotoxic effect in the absence of an added DNA damaging agent. However, treatment with 100 μ M TMZ produces striking effects on cell cycle progression as there is a large increase in cells at G₂/M with a concomitant reduction in cells at G₀/G₁. These changes occur with minimal effects on cells at S-phase or through increases in sub-G₁ DNA levels. The inability of TMZ to affect S-phase coupled with the accumulation of cells at G₂/M suggests that

DNA lesions produced by TMZ do not inhibit chromosomal DNA synthesis and are likely by-passed by specialized DNA polymerases such as pol η and/or pol ι . Consistent with this hypothesis, combining 5-NIdR with TMZ appears to inhibit TLS activity during S-phase as manifest in a ~2-fold increase in cells at S-phase compared to treatment with TMZ or 5-NIdR alone. Furthermore, the increase in sub-G₁ DNA suggests that cell accumulating at S-phase undergo cell death via mitotic catastrophe.

Finally, a heterotopic xenograft mouse model was used to demonstrate that 5-NIdR effectively increases the therapeutic efficacy of TMZ. In these experiments, treatment was initiated with intraperitoneal (IP) injections of TMZ (40 mg/kg) alone or combined with 5-NIdR (100 mg/kg) for 5 consecutive days once GBM tumors reached a volume of ~500 mm³. TMZ treatment alone had a minimal effect on tumor growth whereas combining 5-NIdR and TMZ produced more significant anti-tumor effects. In particular, the median time for death (MTD) for mice treated with TMZ was 45 days whereas the MTD for mice treated with TMZ and 5-NIdR was greater than 250 days. In fact, the majority of mice (>70%) treated with the drug combination showed complete tumor regression with 30 days post-treatment. Finally, toxicology studies show that repeat dosing with 500 mg/kg via intravenous injection produces no adverse effects hematological. In addition, no adverse effects on major organs including brain, heart, liver, and kidney were observed in in male and female mice treated with 500 mg/kg.

Other groups have recapitulated this therapeutic strategy by using different approaches to disrupt TLS activity. For example, Ketkar *et al.* recently synthesized a compound designated IAG-10 ((*E*)-2-((1-(1-naphthoyl)-5-chloro-1*H*-indol-3-yl)-methylene)hydrazine-1-carboximidamide hydrochloride) that inhibits human pol η activity with a low IC₅₀ value of ~7 μ M [99]. In addition, this compound showed high selectivity against pol η as the *in vitro* activity of other specialized and high-fidelity DNA polymerase were unaffected at this concentration of IAG-10. In contrast to the activity of 5-NITP which functions as a selective chain terminating nucleoside analog, IAG-10 inhibits pol η activity by disrupting its ability to bind DNA. Using clonogenic cell-based assays, these authors demonstrated that IAG-10 potentiates the cytostatic effects of TMZ in cells that express pol η . However, IAG-10 did not potentiate TMZ in an isogenic cell line lacking pol η . Finally, combining IAG-10 with TMZ produced an increase in DNA damage as monitored using comet assays to detect SSB formation. It will be of great interest to determine if IAG-10 is effective in an animal model of GBM.

Finally, Wojtaszek *et al.* reported the identification of a 1,4-dihydroquinolin-4-one derivative designated JH-RE-06 that functions as a small-molecule inhibitor of TLS activity [100]. In this case, JH-RE-06 disrupts TLS by targeting pol ζ which plays an important role in extending formed mis-pairs rather than catalyzing their formation. Surprisingly, JH-RE-06 appears to bind to a surface of REV1 that normally interacts with the REV7 subunit of pol ζ . As a consequence, the binding of JH-RE-06 to REV1 induces homodimerization which prevents normal REV1-REV7 interactions. This serves to prevent recruitment of pol ζ to sites of DNA damage to hinder TLS activity. Although not tested against GBM, the authors demonstrated that JH-RE-06 inhibits mutagenic replication catalyzed by TLS activity and increased the cytotoxic effects of cisplatin in human melanoma cells lines. Finally, co-administration of JH-RE-06 with cisplatin lead to significant growth suppression of human melanomas in xenograft mice models. Collectively, these results again reaffirm the validity for using TLS inhibitors as adjunctive agents to improve the therapeutic activity of chemotherapeutic agents that damage DNA.

2.8 Conclusions and Future Directions

While drug resistance to DNA damaging agents is a clinical problem in the treatment of all cancers, it is arguably the most significant issue in the effective treatment of GBM. TMZ is the most widely used anti-cancer agent against GBM, and when combined with radiotherapy, it provides a significant survival benefit for newly diagnosed GBM patients. Unfortunately, TMZ-resistance occurs very rapidly, and this produces a poor prognosis for GBM patients since there are a very limited number of anti-cancer agents that can replace TMZ. Drug resistance is a multi-faceted process and can be caused by increased drug efflux, increased drug metabolism, and increases in levels of glutathione. However, the most common mechanisms associated with TMZ resistance involve alterations in DNA repair pathways including MGMT and MMR (Figure 4).

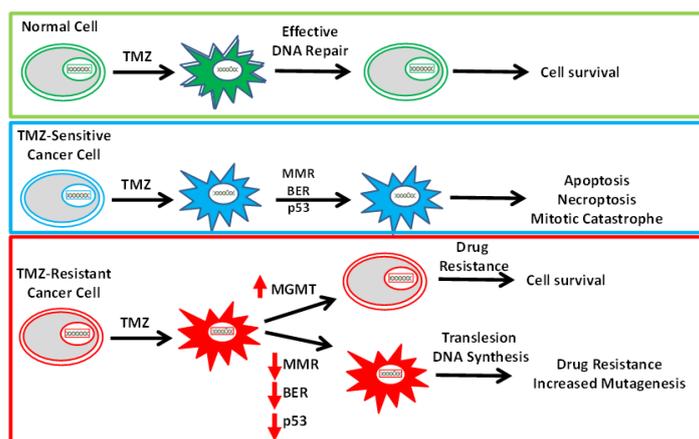


Figure 4 Diagrammatic representation for potential differences in responses and outcomes to DNA damaging agents in a normal cell, a TMZ-sensitive cancer cell, and TMZ-resistant cancer cell. Since most normal cells are quiescent, DNA lesions generated by TMZ can be effectively repaired during the G_0 phase of the cell cycle. Since DNA replication during S-phase is prohibited, there is a low probability that DNA polymerases will misreplicate the damaged DNA prior to DNA repair. In contrast, both TMZ-sensitive and TMZ-resistant cancer cells have re-entered the cell-cycle to become hyperproliferative. In TMZ-sensitive cancer cells, DNA repair pathways such as MMR and BER are still functional, and their activity ultimately drives these cells toward apoptosis. In TMZ-resistant cancer cells, DNA repair proteins may be overexpressed (MGMT) or either not expressed (MMR) and BER) or mutated (MMR and BER) to generate resistance. In many cases, a lack of functional repair allows DNA lesions to persist, and these damaged forms of DNA are inappropriately replicated to further enhance drug resistance.

In some cases, increased MGMT activity can repair the damage inflicted by TMZ, thus making the drug therapeutically inert. As expected, a decrease in MGMT either through epigenetic mechanisms or through the use of pharmacological inhibitors can, in some cases, reverse TMZ-resistance. This beneficial effect appears related to the activity of the MMR pathway. Unfortunately, a lack of MGMT activity coupled with loss of MMR activity also generates TMZ resistance. These combined defects allow lesions generated by the TMZ to persist and subsequently replicated by several different DNA

polymerases. Since this activity can be highly pro-mutagenic, it can increase mutational frequencies in a cancer cell to produce more aggressive cancers and/or cause tumor recurrence. As described here, one newly developed approach to combat this problem is to block the ability of DNA polymerases to replicate lesions produced by TMZ. This can be achieved by several different approaches including the use of artificial nucleoside analogs as well as other small molecules that prevent polymerase binding to damaged DNA or that prevent association with other proteins involved in damage recognition.

Despite significant progress in this area, it is important to acknowledge other new approaches being developed to tackle the challenge of TMZ-resistance in GBM. For example, one interesting study by Tso *et al.* recently used glioblastoma stem cell (GSC) lines expressing MGMT to examine molecular signatures associated with TMZ resistance [101]. Their analyses showed an overall activation of protective stress responses to TMZ treatment which include enzymes associated with biotransformations and detoxification of xenobiotics, blocked endoplasmic reticulum stress-mediated apoptosis, epithelial-to-mesenchymal transition (EMT), and inhibited growth/differentiation. One surprising result was the identification of bone morphogenetic protein 7 (BMP7) as the most down-regulated gene in TMZ-resistant GSCs. To verify the importance of this effect, TMZ-resistant GSCs were treated with BMP7 and showed a marked sensitization to the cytotoxic effects of TMZ. In addition, BMP7 treatment caused a loss in self-renewal and migration capacities as well as inducing senescence and downregulation of genes associated with EMT/migration/invasion, stemness, inflammation/immune response, and cell proliferation/tumorigenesis by changing transcriptional profiles. Pre-clinical animal studies showed that BMP7 treatment prolonged survival time of animals intracranially inoculated with GSC compared to untreated mice or mice treated with TMZ alone. Finally, a more pronounced survival effect was observed by combining BMP7 with TMZ, thus providing a new potential treatment strategy.

Another interesting approach being developed is the use of Tumor Treating Fields (TTFields). Briefly, this method involves applying alternating electric fields using insulated transducer arrays placed directly in the region surrounding the tumor. A recent study by Giladi *et al.* showed that applying TTFields against glioma cells lead to a synergistic enhancement in the cell killing effects of radiotherapy [102]. This enhancement appears to be caused by blocking homologous recombination repair of DNA damage caused by radiotherapy. These preclinical results support the application of TTFields therapy immediately after RT as a viable regimen to increase the overall efficacy of radiotherapy in GBM and other cancer patients.

Author Contributions

Anthony Berdis is the sole author contributing to this work.

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Competing Interests

The author has declared that no competing interests exist.

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