



REVIEW ARTICLE

# Novel Strategies for Improved Treatment of O6-Methylguanine-DNA Methyltransferase Promoter-Methylated Glioma

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## ABSTRACT

Adult diffuse gliomas are primary brain tumors notorious for leading to devastating neurologic consequences from both tumor progression and therapeutic interventions. The arsenal of current established treatments primarily includes surgery, radiotherapy, and DNA alkylating chemotherapy agents. Unfortunately, even with aggressive treatments, long-term cure is typically not attainable, except in certain cases of low-grade gliomas amenable to complete surgical resection. Grade 4 glioblastoma (GBM) represents the most aggressive and most common type of glioma in adults, is often resistant to current therapies, and is associated with a median survival of approximately 15 months. While biomarker-based therapies for gliomas are limited, O6-methylguanine-DNA methyltransferase (MGMT) is one well-established prognostic marker in GBM and is associated with improved response to the alkylating agent temozolomide (TMZ). Methylation of the MGMT promoter leading to loss of MGMT expression occurs in approximately half of GBMs and 70-80% of anaplastic and low-grade gliomas. While MGMT promoter-methylated gliomas are responsive to TMZ, a characteristic resistance mechanism of mismatch repair loss often emerges, resulting in recurrent drug-resistant disease. In prior work, we identified a new TMZ derivative “KL-50” which overcomes resistance to TMZ driven by loss of mismatch repair in preclinical glioma models. KL-50 functions via a novel DNA-modifying mechanism involving evolution of a primary alkyl lesion to a DNA interstrand crosslink specifically in the absence of MGMT. Research is ongoing to establish this new class of agents as a potential improved therapy in human gliomas. In this review, we provide an overview of the history and evolution of alkylator use in GBM, discuss the mechanisms and pitfalls of current therapies including toxicity or susceptibility to resistance mechanisms, and present the potential of a new wave of DNA modifiers to improve outcomes in gliomas.

## I. Introduction

Glioblastoma (GBM), IDH-wild-type, is the most common and aggressive form of diffuse glioma and accounts for the majority of malignant primary brain tumors in adults. It is defined by its highly infiltrative growth, rapid progression, and poor prognosis, with a median survival of approximately 15 months and a five-year survival rate of less than 10% despite maximal therapy<sup>1,2</sup>. The current standard-of-care, which includes surgical resection followed by radiation and concomitant and adjuvant temozolomide (TMZ), offers only modest, transient benefit<sup>3,4</sup>. Other diffuse gliomas, including IDH-mutant astrocytomas and oligodendrogliomas, follow a more protracted clinical course but remain incurable and ultimately progress to treatment-refractory disease. Even among patients with WHO grade 2–3 diffuse gliomas, median overall survival ranges from 5 to 10 years, with outcomes highly dependent on age, extent of resection, and molecular subtype<sup>5</sup>. Across this spectrum, recurrence is common and effective salvage therapies are lacking<sup>6</sup>.

The primary alkylating agent used in the treatment of diffuse gliomas, TMZ, is limited by predictable resistance mechanisms. In tumors with intact O6-methylguanine DNA methyltransferase (MGMT) expression, TMZ-induced O6-methylguanine lesions are rapidly repaired, preventing cytotoxicity<sup>7–9</sup>. In MGMT-silenced tumors, which are typically more responsive to TMZ, selective treatment pressure frequently leads to acquired mismatch repair (MMR) deficiency, most often through mutations in MSH6<sup>9–13</sup>. These recurrences can exhibit a hypermutator phenotype yet remain unresponsive to alkylator re-challenge and immune checkpoint blockade<sup>13–15</sup>. Nitrosoureas, such as BCNU (carmustine) and CCNU (lomustine) provide only incremental benefit and are constrained by significant hematologic toxicity and lack of tumor specificity<sup>16</sup>. The need for therapies that can overcome TMZ resistance while maintaining a favorable therapeutic index remains unmet in both GBM and other molecularly defined diffuse gliomas.

In this review, we trace the evolution of alkylating agents in diffuse glioma, examine the molecular determinants of resistance to current treatments, and explore the therapeutic potential of novel agents engineered to address these shortcomings. We focus on KL-50, a next-generation imidazotetrazine designed to induce MGMT-dependent, MMR-independent DNA crosslinks, representing a rational approach to selectively target MGMT-silenced, treatment-refractory gliomas.

## II. Early history and evolution of alkylator use in glioblastoma

### NITROSOUREAS: BIFUNCTIONAL ALKYLATORS

Alkylating agents have played a central role in the treatment of GBM for over five decades. The introduction of nitrosoureas in the 1970s marked the first class of chemotherapeutics with demonstrable activity against malignant gliomas. Carmustine and lomustine (also referred to as BCNU and CCNU, respectively) were among the first chemotherapeutics to penetrate the blood–brain barrier and produce responses in patients with malignant gliomas<sup>17,18</sup>. These agents act as

bifunctional alkylators, inducing DNA damage by covalently modifying the O6 position of guanine and generating interstrand crosslinks, which block DNA replication and transcription and ultimately lead to cell death<sup>7,8</sup>.

A landmark randomized trial conducted by Walker et al. demonstrated that adding carmustine to postoperative radiotherapy showed modest prolongation of survival, establishing chemotherapy as a viable adjunct to radiation in high-grade glioma<sup>17</sup>. Despite this modest benefit, nitrosoureas were associated with significant hematologic toxicity. Subsequent studies from cooperative groups evaluated various combinations and schedules of nitrosoureas, such as PCV (procarbazine, lomustine, and vincristine), with survival outcomes ranging from 9 to 12 months and 1-year survival rates rarely exceeding 30%<sup>19,20</sup>. Toxicity remained a persistent issue, often limiting dose intensity and duration of therapy.

Despite these drawbacks, nitrosoureas continue to play a role in the recurrent GBM setting, particularly lomustine due to its oral formulation. For example, the BELOB trial, a randomized phase II study, compared lomustine alone, bevacizumab alone, or the combination in recurrent GBM. The combination arm showed improved 9-month overall survival (63% vs. 38% for lomustine alone), although the trial was not powered for definitive comparisons<sup>21</sup>. This study laid the groundwork for the EORTC 26101 phase III trial, which confirmed a modest improvement in progression-free survival with the addition of bevacizumab to lomustine (4.2 vs. 1.5 months) but no difference in overall survival (9.1 vs. 8.6 months). Notably, roughly 50% of patients experienced grade ≥3 hematologic toxicity<sup>22</sup>. These findings underscore both the historical importance and therapeutic limitations of nitrosoureas in GBM.

### TEMOZOLOMIDE: A MONOFUNCTIONAL ALKYLATOR

Seeking improved efficacy and tolerability, researchers developed temozolomide (8-carbamoyl-3-methylimidazo[5,1-d]-1,2,3,5-tetrazin-4(3H)-one), a second-generation imidazotetrazine compound originally described in 1987 and currently the most widely used alkylating agent for treatment of GBM and lower grade diffuse gliomas<sup>7,8,23</sup>. In contrast to the bifunctional alkylators described previously which produce DNA crosslinking, TMZ is a monofunctional methylating agent. TMZ, a prodrug, hydrolyzes under physiological conditions to the linear triazene compound 5-(3-methyltriazene-1-yl)imidazole-4-carboxamide (MTIC), which in turn yields a methyl diazonium cation, the active DNA methylating species<sup>7,23</sup>. DNA methylation preferentially occurs at the N7, N3, and O6 atoms of guanine and N3 atom of adenine, due to the high nucleophilicity of these sites. With the exception of O6-methylguanine, these lesions are substrates for base excision repair enzymes and are readily repaired in most cells. Conversely, O6-methylguanine is a substrate for the direct reversal DNA repair enzyme MGMT, and repair of this lesion depends critically on the cellular activity of MGMT<sup>7,8,24</sup>. Following transfer of the methyl group to a cysteine residue in its active site, MGMT undergoes ubiquitination and subsequent proteasomal degradation.

In the absence or depletion of MGMT, persistent O6-methylguanine lesions mispair with thymidine during replication, leading to recognition and processing by the MutS $\alpha$  and MutS $\beta$  MMR complexes. Excision of thymidine followed by resynthesis of the DNA mispair ultimately generates a process of “futile cycling” which leads to formation of DNA double-strand breaks and eventual cell death<sup>7,25</sup>.

Temozolomide displayed promising efficacy in mouse tumors and was advanced to single agent trials where it demonstrated activity in recurrent GBM<sup>23,26</sup>. Whereas the first generation imidazotetrazine compound mitozolomide and other bifunctional alkylators discussed above were severely hindered by dose-limiting toxicities including myelosuppression in the clinical setting, TMZ was better tolerated<sup>20,26,27</sup>. In addition, TMZ possesses favorable pharmacokinetic properties with oral bioavailability and penetration of the blood-brain barrier<sup>28,29</sup>. In 2005, the seminal randomized clinical trial reported by Stupp et al. demonstrated a survival benefit with the addition of concurrent and adjuvant TMZ to standard radiotherapy for newly diagnosed GBM<sup>3</sup>.

Importantly, translational studies from this trial identified MGMT promoter methylation as a critical biomarker predicting clinical benefit from TMZ<sup>4</sup>. The MGMT gene promoter contains a 5' CpG island of ~770 base pairs and 97 CpG sites, beginning ~480 base pairs upstream of the transcription start site and traversing through the first non-coding exon<sup>30</sup>. Cytosine methylation in the MGMT promoter leads to chromatin condensation and exclusion of transcription factor binding, resulting in reduced MGMT expression<sup>31,32</sup>. Hegi et al. found that 45% of GBMs harbored MGMT promoter methylation. Among these patients, median survival increased from 15.3 to 21.7 months with the addition of TMZ, whereas in patients without MGMT promoter methylation, overall survival was not significantly affected<sup>4</sup>. Additional studies have since confirmed MGMT silencing in ~40-50% of GBM and have corroborated the predictive value of silencing with respect to TMZ response<sup>33,34</sup>. In current clinical practice, TMZ is utilized for the upfront treatment of newly diagnosed GBM in combination with external beam radiotherapy following maximal safe surgical resection. While studies suggest a greater benefit to TMZ in MGMT-silenced tumors, the lack of other effective treatment options in MGMT unmethylated tumors has led to its recommended use independent of MGMT status<sup>35,36</sup>. For patients over 70 years of age, TMZ treatment alone can also be considered, particularly for those with MGMT promoter methylated tumors or with a poor performance status<sup>36,37</sup>. Unfortunately, GBM nearly inevitably recurs, and no effective standard-of-care has been established for recurrent or progressive cases.

In lower grade gliomas, MGMT promoter silencing is found at even higher rates, approaching 80% of cases. In this setting, MGMT promoter silencing is strongly associated with mutations in isocitrate dehydrogenase 1 and 2 (IDH-1 and -2)<sup>38,39</sup>. Tumor associated IDH1/2 mutations generate neomorphic gain-of-function status leading to overproduction of the oncometabolite 2-hydroxyglutarate, which acts as a competitive inhibitor of

alpha-ketoglutarate-dependent dioxygenases, several of which are implicated in DNA and histone methylation<sup>40-42</sup>. As a result, IDH1/2-mutant gliomas exhibit a CpG-island methylator phenotype (G-CIMP) associated with widespread gene silencing, including MGMT promoter silencing in over 90% of cases<sup>38,39,43,44</sup>.

For WHO grade 3 and high-risk WHO grade 2 astrocytomas, adjuvant TMZ following radiotherapy is typically recommended, stemming from studies demonstrating improved outcomes compared to radiotherapy alone, particularly in IDH-mutant tumors<sup>45,46</sup>. However, while progression-free survival is longer for IDH mutant grade 2-3 gliomas, these tumors still often recur with characteristic signs of TMZ resistance, as discussed further below<sup>15</sup>. The use of TMZ in place of the alternative alkylating regimen PCV (procarbazine, lomustine, vincristine) for oligodendrogliomas defined by 1p/19q codeletion is not yet established, as the phase III CODEL trial is ongoing; however, TMZ remains a commonly used clinical option due to its more favorable tolerability profile<sup>47</sup>. Thus, additional therapies improving upon TMZ for both GBM and lower grade diffuse gliomas are a high clinical priority.

#### IV. Mismatch repair: A key resistance mechanism to temozolomide

As described above, in the absence of MGMT, TMZ induced O6-methylguanine lesions mispair with thymidine during replication, leading to recognition and processing by the MutS $\alpha$  and MutS $\beta$  complexes. This MMR activity leads to excision of thymidine followed by resynthesis of the DNA mispair, ultimately generating a process of “futile cycling” which causes replication fork collapse, DNA double-strand breaks, and cell death via apoptosis or mitotic catastrophe<sup>7,25,48</sup>.

Loss of MMR function is now recognized as a key mechanism of acquired TMZ resistance in gliomas, particularly in tumors with MGMT promoter methylation. In a seminal study by Hunter et al., targeted sequencing identified somatic inactivating mutations in MSH6 in recurrent gliomas that had been treated with TMZ<sup>49</sup>. Subsequent studies using whole-exome sequencing (WES) and immunohistochemistry (IHC) have corroborated these findings, demonstrating that up to 25–30% of recurrent MGMT-methylated gliomas acquire mutations in core MMR genes, most commonly MSH6, but also MSH2, MLH1, and PMS2<sup>10,13,50-53</sup>. Importantly, MMR loss is shown to be restricted to post-treatment recurrences while absent in untreated primary tumors, strongly implicating acquired MMR deficiency as a major driver of TMZ resistance. This phenomenon is not limited to IDH-wild-type GBM. TMZ-induced hypermutation has been observed in nearly 60% of low-grade, IDH-mutant gliomas that recur with anaplastic transformation following treatment<sup>15,53</sup>.

Mismatch repair deficiency is also relevant in the pediatric setting, where a subset of high-grade gliomas arises in the context of germline mutations in MMR, such as constitutional mismatch repair deficiency (CMMRD)<sup>9,54,55</sup>. These tumors are typically resistant to

TMZ at diagnosis due to a pre-existing lack of functional MMR. Although the co-occurring frequency of MGMT-silencing in the setting of germline MMR deficiency, and the role of MGMT promoter methylation in modulating TMZ sensitivity in pediatric patients is unclear, recent studies suggest that MGMT promoter methylation is common in certain molecular subtypes such as H3.3-G34R and H3K27M wild-type tumors<sup>56-59</sup>.

Functionally, MMR deficiency results in tolerance of O6-meG:T mismatches, allowing tumor cells to escape the cytotoxic effects of TMZ. This enables continued proliferation despite ongoing DNA damage and results in the accumulation of somatic mutations, leading to a hypermutator phenotype characterized by elevated tumor mutation burden (TMB) and extensive genomic instability<sup>13,15,60</sup>. Although microsatellite instability (MSI) is a hallmark of MMR deficiency in other cancers, such as colorectal and those arising in the setting of germline MMR deficiency, it is often absent or minimal in gliomas<sup>13,61-64</sup>. This discrepancy likely reflects the subclonal nature and later emergence of TMZ-induced MMR mutations, which provide limited time for MSI to develop, as well as tissue-specific differences in repair pathway activity<sup>13,65,66</sup>. Thus, WES and IHC remain the most reliable methods for detecting MMR deficiency in glioma.

Notably, while a hypermutator phenotype has been associated with improved responses to immune checkpoint blockade (ICB) in other solid tumors, such as melanoma and colorectal cancer, this has not translated to glioma<sup>13,61,62,67</sup>. Recurrent hypermutant, MMR-deficient gliomas exhibit poor responses to ICB, likely due to the poor fitness and subclonal nature of therapy-induced neoantigens and a highly immunosuppressive tumor microenvironment with impaired antigen presentation<sup>13,66</sup>.

Together, these findings underscore that MMR deficiency is a frequent, treatment-emergent mechanism of resistance to TMZ in MGMT-methylated and IDH-mutant gliomas. This phenomenon contributes to the development of a hypermutant, genomically unstable tumor state that resists further alkylator therapy and typically fails to respond to ICB, reinforcing the need for novel therapeutic approaches.

## V. Beyond temozolomide, testing the next wave of DNA modifiers

### BYPASSING O6-METHYLGUANINE-DNA METHYLTRANSFERASE-MEDIATED RESISTANCE

To improve alkylator therapies for GBM and glioma, some efforts have focused on overcoming the intrinsic resistance to TMZ seen in MGMT unmethylated tumors, namely MGMT expression. One approach is the use of O6-methylguanine mimetics, such as O6-benzylguanine (O6BG) or O6-(4-bromophenyl)guanine (lomeguatrib), as MGMT inhibitors. These compounds inactivate MGMT, lead to its cellular depletion, potentiate the activity of TMZ in preclinical models, and have been shown in human studies to deplete MGMT in peripheral blood mononuclear cells and tumors<sup>68-72</sup>. However, combination

therapy of these inhibitors with TMZ in human clinical trials has necessitated TMZ dose-reductions because of dose-limiting myelosuppression<sup>71,72</sup>. At these reduced doses, O6BG with TMZ showed limited effectiveness in recurrent GBM or anaplastic gliomas, with objective response rates of 3% and 16% respectively<sup>73</sup>. Lomeguatrib with TMZ has primarily been tested in non-glioma solid tumors, including melanoma and colorectal cancer, and has similarly failed to demonstrate significant response rates despite significant hematologic toxicities<sup>74,75</sup>. Several groups have reported drug delivery approaches to reducing systemic toxicity and enhancing CNS exposure to combined O6BG and TMZ, including PLGA nanoparticles, PEGylated liposomes, and exosomes engineered with GBM cell targeted molecules, but these remain in early preclinical stages of investigation<sup>76-78</sup>.

A second approach has been the derivatization of TMZ to effect deposition of O6-guanyl adducts that are irreparable by MGMT. Various substitutions at the N3-methyl position of TMZ have yielded compounds with cytotoxic activity irrespective of MGMT or MMR expression<sup>79-83</sup>. Modifications at the C8 carboxamide position of TMZ have also been shown to reduce MGMT-mediated drug resistance and improve drug metabolism and pharmacokinetic properties<sup>82-85</sup>.

While these various strategies of MGMT inhibitors or MGMT-independent alkylation broaden the range of tumors potentially amenable to treatment to include the MGMT unmethylated cohort, they also abrogate the potential of MGMT methylation to yield tumor-specific sensitization relative to healthy MGMT-expressing cells. Therefore, as noted, many of these strategies have been shown to be limited by off-target normal tissue toxicity.

### A NEW CLASS OF TUMOR-SELECTIVE DNA MODIFIERS

Using a different strategy, our group sought to develop an improved therapy that would maintain the selectivity for MGMT silenced gliomas, but overcome resistance driven by loss of MMR<sup>86</sup>. While this strategy was not designed to target MGMT unmethylated tumors, it was focused on exploiting the genetic vulnerability in MGMT silenced tumors, thereby establishing a therapeutic index in tumors relative to normal tissues. To this end, a panel of TMZ derivatives was synthesized and screened across glioma models with isogenic expression of MGMT and/or MMR, searching for a compound that would selectively kill MGMT negative cells regardless of MMR status. In this manner, KL-50, a novel 2-fluoroethyl derivative of temozolomide, was identified with the desired MGMT-selective, but MMR-independent activity. In isogenic cell line models, KL-50 displayed approximately 24-fold greater potency in MGMT-negative compared to MGMT-positive cells yet showed no decrease in potency upon loss of MMR. In comparison, loss of MMR led to an approximately 100-fold decrease in TMZ activity. The activity of KL-50 was maintained in cell-derived xenograft glioma models in which it displayed effective tumor control in MGMT-silenced tumors with loss of MMR that were impervious to TMZ. KL-50 was well-tolerated in mice based on mouse bodyweights and blood counts at doses effective for tumor control<sup>86</sup>.



As with TMZ, KL-50 is posited to undergo hydrolysis under physiological conditions, forming a 2-fluoroethyldiazonium ion which deposits 2-fluoroethyl lesions on DNA bases. These 2-fluoroethyl DNA lesions are relatively stable given the poor leaving group ability of fluorine (in comparison, for example, to the chloroethyl lesions derived from the bifunctional alkylating agents lomustine or mitozolomide). However, at O6-(2-fluoroethyl)guanine lesions, the fluoride can undergo unimolecular displacement to form an N1,O<sup>6</sup>-ethanoguanine intermediate, which can subsequently be ring-opened by the opposing cytidine residue, generating a DNA interstrand crosslink (ICL). Notably, O6-(2-fluoroethyl)guanosine transforms to N1,O<sup>6</sup>-ethanoguanosine with a half-life of 18.5 hours under physiological conditions<sup>87</sup>, much more slowly than the expected rate of lesion repair, which for O6-ethylguanine is ~3 hours as measured in rat liver DNA<sup>88</sup>. In comparison, the half-life of O<sup>2</sup>-(2-chloroethyl)guanosine, formed by other bifunctional crosslinking agents such as lomustine or mitozolomide, is only 18 minutes, exceeding the rate of MGMT-mediated reversal<sup>89</sup>.

These kinetic considerations suggest a unique ability of KL-50 to form DNA ICLs only in the absence of MGMT, whereas chloroethylating agents form ICLs more indiscriminately. Indeed, in rigorous biochemical studies, O6-(2-fluoroethyl)guanine-containing oligonucleotides slowly produced DNA ICLs (half-life = 80 hours) that were nearly eliminated with the prior addition of purified MGMT<sup>90</sup>. In contrast, O6-(2-chloroethyl)guanine-containing oligonucleotides produced DNA ICLs >10-fold more rapidly (half-life = 6.3 hours) and were only partially reduced with the prior addition of purified MGMT and replaced instead with MGMT-DNA protein crosslinks, which are also a known cause of cell toxicity. In cellulo, KL-50 exposure similarly led to time-dependent formation of ICLs in genomic DNA and delayed induction of markers of DNA damage and replication stress specifically in MGMT-deficient cells<sup>86</sup>. Critically, the formation of DNA ICLs bypasses the need for MMR-induced “futile cycling” to drive toxicity, accounting for the comparable KL-50 activity in both MMR proficient and deficient settings.

KL-50 was also recently investigated in patient-derived models of IDH1/2 wild-type GBM<sup>14</sup>. In two different TMZ-naïve intracranial GBM patient-derived xenograft models, one displaying partial MGMT promoter methylation and the other displaying full MGMT promoter methylation, KL-50 displayed robust monotherapy activity compared to vehicle control, approximately doubling mouse median survival. Furthermore, treatment of one of these models with repeated cycles of TMZ led to loss of expression of multiple MMR proteins, including MSH2, MSH6, MLH1, and PMS2. Upon rechallenge of these post-TMZ, MMR-deficient tumors, KL-50 proved more effective than TMZ, extending median survival from 108 to 140 days ( $p = 0.02$ ). Finally, consistent with prior studies in established glioma cell lines, knockout of MSH6 in primary GBM cultures induced resistance to TMZ, but not KL-50. Altogether, these studies support the potential of 2-

fluoroethylating agents both as upfront therapy for MGMT promoter methylated GBM or in the recurrent post-TMZ setting.

## VI. Conclusions

The development of fluoroethylating agents, such as KL-50, represents a rational approach to overcoming key resistance mechanisms in GBM. Preclinical studies have demonstrated that KL-50 induces DNA interstrand crosslinks selectively in MGMT-silenced tumor models and retains activity in the absence of functional MMR, highlighting its potential to fill a critical therapeutic gap in recurrent, treatment-refractory tumors. In addition to its relevance in MGMT-silenced GBM, KL-50 may have therapeutic utility in IDH-mutant gliomas, which frequently exhibit MGMT promoter methylation and are prone to acquiring TMZ-induced MMR deficiency<sup>15,38,91</sup>. Moreover, IDH-mutant gliomas harbor broad defects in DNA repair pathways, including homologous recombination and PARP-dependent base excision repair, due to oncometabolite-induced epigenetic dysregulation, potentially rendering them more sensitive to crosslinking agents like KL-50<sup>92-94</sup>. KL-50 also warrants evaluation in pediatric high-grade gliomas, where MGMT silencing and TMZ responsiveness have historically been less well-defined, but the frequency of underlying germline MMR deficiency syndromes is increasingly recognized<sup>9,54,55</sup>. Together, these observations support KL-50's potential applicability across adult and pediatric gliomas with defined defects in DNA repair.

Future studies should also explore KL-50 in rational combination regimens. Given its unique mechanism of inducing time-dependent DNA interstrand crosslinks in MGMT-silenced cells, KL-50 triggers replication stress and activates downstream DNA repair pathways, including homologous recombination and ATR/CHK1-mediated checkpoint signaling<sup>86</sup>. These responses suggest that there is great potential for combinations with inhibitors of the DNA damage response, such as PARP or ATR inhibitors, to induce synthetic lethality. Furthermore, recent clinical data from the NOA-9 phase III trial also support the rationale for combination alkylator strategies, demonstrating a survival benefit with the addition of lomustine to TMZ in newly diagnosed MGMT-silenced GBM, suggesting that combinations leading to both alkylation damage and DNA crosslinking may enhance antitumor efficacy<sup>95</sup>. The ongoing NRG BN011 trial aims to build on this finding by incorporating more robust stratification and modern trial infrastructure, with the goal of getting FDA-level confirmatory evidence<sup>96</sup>. These findings collectively support the potential for combining TMZ and KL-50, which may enhance the therapeutic index compared to TMZ and lomustine owing to the greater MGMT dependency of KL-50.

Finally, expanding the scope of KL-50 to include extracranial malignancies with MGMT silencing, such as colorectal cancer and other oncometabolite-driven tumors, may further broaden the clinical application of this novel class of agents. Defining predictive biomarkers and resistance mechanisms will be key to optimizing clinical development and realizing the full potential of KL-50.

## Conflict of Interest Statement

R.S.B. is a consultant for Merck. S.E.G. reports royalties from Modifi Biosciences.

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