Mechanism of Action of KL-50, a Candidate Imidazotetrazine for the Treatment of Drug-Resistant Brain Cancers

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ABSTRACT: Aberrant DNA repair is a hallmark of cancer, and many tumors display reduced DNA repair capacities that sensitize them to genotoxins. Here, we demonstrate that the differential DNA repair capacities of healthy and transformed tissue may be exploited to obtain highly selective chemotherapies. We show that the novel N3-(2-fluoroethyl)imidazotetrazine “KL-50” is a selective toxin toward tumors that lack the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT), which reverses the formation of O6-alkylguanine lesions. We establish that KL-50 generates DNA interstrand cross-links (ICLs) by a multistep process comprising DNA alkylation to generate an O6-(2-fluoroethyl)guanine (O6FEtG) lesion, slow unimolecular displacement of fluoride to form an N1,O6-ethanoguanine (N1,O6EtG) intermediate, and ring-opening by the adjacent cytidine. The slow rate of N1,O6EtG formation allows healthy cells expressing MGMT to reverse the initial O6FEtG lesion before it evolves to N1,O6EtG, thereby suppressing the formation of toxic DNA–MGMT cross-links and reducing the amount of DNA ICLs generated in healthy cells. In contrast, O6-(2-chloroethyl)guanine lesions produced by agents such as lomustine and the N3-(2-chloroethyl)imidazotetrazine mitozolomide rapidly evolve to N1,O6EtG, resulting in the formation of DNA–MGMT cross-links and DNA ICLs in healthy tissue. These studies suggest that careful consideration of the rates of chemical DNA modification and biochemical DNA repair may lead to the identification of other tumor-specific genotoxic agents.

INTRODUCTION

Genotoxins are a mainstay of cancer therapy, but dose-limiting toxicity arising from DNA damage to healthy tissue frequently limits the efficacy of these agents. We now know that aberrant DNA repair is a hallmark of cancer and that many tumor types display reduced DNA repair capacities. We posited that an understanding of the factors underlying the rate of DNA damage and repair in healthy and transformed tissues may lead to the identification of novel anticancer agents with improved efficacy and tolerability. In this study, we disclose the mechanism of action of “KL-50” (1; Figure 1a), a novel imidazotetrazine that is highly selective for brain tumors that lack the DNA repair protein O6-methylguanine-DNA-methyltransferase (MGMT).

BACKGROUND

Approximately 13,000 people in the United States are diagnosed annually with glioblastoma (aka glioblastoma multiforme, GBM), an aggressive brain cancer. The overall survival following diagnosis is ~15 months. The standard of care for GBM consists of surgical debulking followed by adjuvant radiotherapy and chemotherapy using temozolomide (TMZ, 2; Figure 1a). TMZ (2) releases methyl diazonium (3) under physiological conditions, leading to the production of O6-methylguanine (O6MeG, 4a; Figure 1b). This treatment regimen provides a ~6 month survival benefit to patients harboring tumors that lack MGMT (“MGMT−” tumors), a DNA repair protein that is ubiquitously expressed in healthy tissue but whose expression is lost in ~50% of GBMs. MGMT reverses O6-alkylguanine lesions by transfer of the alkyl substituent to an active site cysteine (Cys145). In the absence of MGMT, O6MeG (4a) induces cell death by a DNA...
mismatch repair (MMR)-dependent pathway.\textsuperscript{7,11} Though most patients harboring MGMT− GBM respond initially to TMZ (2), resistance in \textasciitilde30–40\% of recurrent MGMT− GBM is associated with loss of MMR activity.\textsuperscript{12} The resulting MGMT−, MMR-deficient tumors (referred to hereafter as “MGMT−/MMR−” tumors) proliferate with O\textsuperscript{6}-ethylguanine (O\textsuperscript{6}EtG, 6a) in their genome and accumulate nucleotide transitions.\textsuperscript{13}

Lomustine (aka CCNU, 7; Figure 1a) is used as salvage therapy in recurrent MGMT−/MMR− GBM.\textsuperscript{13} Lomustine (7) transfers a 2-chloroethyl substituent to O\textsuperscript{6}-guanine to form O\textsuperscript{6}-(2-chloroethyl)guanine (O\textsuperscript{6}ClEtG, 4b). O\textsuperscript{6}ClEtG (4b) evolves to the \([\text{G}(\text{N}1)\text{C}(\text{N}3)]\text{Et}\) DNA interstrand cross-link (ICL) 10a by unimolecular displacement of chloride to form the \(\text{N}1,\text{O}\text{EtG}\) intermediate 8a followed by ring-opening (with C=O bond cleavage) by the adjacent cytidine residue (dG, 9a) to give the ICL 10a.\textsuperscript{14} As lomustine (7) is toxic to MGMT−/MMR− cells, this indicates that ICL 10a-induced toxicity does not depend on mismatch repair.\textsuperscript{15} Consistent with this, we previously showed that lomustine (7) was toxic to MGMT−/MMR− LN229 GBM cells.\textsuperscript{16} However, the same assays also showed that MGMT provides a smaller degree of protection from lomustine (7) than it does from TMZ (2). This is consistent with clinical data wherein off-target toxicity limits the dosing of lomustine (7).\textsuperscript{17} Mitozolomide (MTZ, 11; Figure 1a) is an N3-(2-chloroethyl)imidazotetrazine that advanced to clinical trials but was abandoned due to toxicity.\textsuperscript{18}

Though its mechanism of action has not been studied in the same level of detail, MTZ (11) transfers a 2-chloroethyl substituent to DNA and likely cross-links DNA by a pathway analogous to lomustine (7; Figure 1c).\textsuperscript{19}

We envisioned that an alkylating agent that retained the ability of MGMT to mitigate toxicity to healthy MGMT+ tissues might prove effective against recurrent, TMZ (2)-resistant MGMT−/MMR− GBM. We initially hypothesized that rapid \((t_{1/2} \sim 18 \text{ min}; \text{see also Figure S1})\) conversion of the initial O\textsuperscript{6}ClEtG lesion (4b) to the electrophilic \(\text{N}1,\text{O}\text{EtG}\) intermediate 8a occurred before MGMT could repair O\textsuperscript{6}ClEtG (4b),\textsuperscript{21} leading to DNA ICLs.

![Figure 1. Overview of this study. (a) Structures of DNA alkylating agents examined in this study. (b) The cytotoxicity of TMZ (2) requires an intact DNA mismatch repair (MMR) pathway. (c) Alkylation at O\textsuperscript{6}-guanine by lomustine (CCNU, 7) and MTZ (11) affords O\textsuperscript{6}-(2-chloroethyl)guanine (O\textsuperscript{6}ClEtG, 4b) while alkylation at O\textsuperscript{6}-guanine by KL-50 (1) affords O\textsuperscript{6}-(2-fluoroethyl)guanine (O\textsuperscript{6}FEtG, 4e). In the absence of MGMT, these lesions evolve to the ICL. 10a by intramolecular halide displacement to form N1,\text{O}\text{EtG} (N1,\text{O}\text{EtG}, 8a) followed by ring-opening by the adjacent cytidine base (9a). Based on literature rate data, we hypothesized that N1,\textsuperscript{O}\text{EtG} (8a) accumulates in MGMT+ cells treated with KL-50 (1), thereby preventing accumulation of N1,\text{O}\text{EtG} (8a). Ring-opening of N1,\textsuperscript{O}\text{EtG} (8a) by MGMT generates the DNA–MGMT cross-link 13. Alternatively, MGMT reversal of O\textsuperscript{6}ClEtG (4b) and O\textsuperscript{6}FEtG (4e) forms the 2-haloethylsulfides 6b and 6c, respectively, that may cross-link to DNA via the episulfonium ion intermediate 12.]}
in MGMT+ cell lines. However, prior studies demonstrated that treatment of the MGMT+ human embryo cell line IMR-90 with lomustine (7) and MTZ (11) led to negligible DNA ICL formation.\textsuperscript{19d,22} Simultaneously, we noted reports showing that the addition of MGMT to DNA treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) led to the formation of covalent DNA–MGMT cross-links between the N1 position of guanine and an MGMT cysteine sulfur, suggesting that these DNA–protein cross-links (DPCs) arise from ring-opening of N1,0\textsuperscript{EtG} (8a) by MGMT (Figure 1c).\textsuperscript{23} Additionally, MGMT–DNA DPCs can form via an episulfonium ion pathway (see 12).\textsuperscript{24} While the clinical significance of these DPCs is currently unknown, evidence suggests that DNA–MGMT cross-links are toxic,\textsuperscript{24a,25} leading us to hypothesize that they contribute to the toxicity of lomustine (7) and MTZ (11) in MGMT+ cells (for further discussion, see the Supporting Information).

We reasoned that slowing the rate of formation of N1,0\textsuperscript{EtG} (8a) without attenuating the rate of MGMT reversal of the initial O\textsuperscript{5}-guanine alkylation product might decrease the amount of DNA–MGMT DPCs in healthy tissue and lead to an agent effective against recurrent, MGMT−/MMR− GBM. Inspired in part by a report that O\textsuperscript{5}-(2-fluoroethyl)guanosine (4d) transforms to the N1,0\textsuperscript{EtG} 8b with a half-life of \approx 18.5 h (pH 7.4, 37 °C; Figure S1),\textsuperscript{26} we identified the N3-(2-fluoroethyl)imidazotetrazine KL-50 (1) as the first reported compound to overcome acquired TMZ (2) resistance while maintaining high selectivity for MGMT− cells (Figure 1a).\textsuperscript{26} Herein, we show that the superior efficacy and tolerability of KL-50 (1) derives from slow rates of DNA ICL formation and a near complete elimination of DNA–MGMT cross-links, which allows one to achieve efficacious concentrations of drug in tumor tissue without toxicity to healthy (MGMT+) cells.

\section*{RESULTS}

The synthesis of KL-50 (1) proceeds in two steps and 23% yield from commercial reagents.\textsuperscript{16} We evaluated the activity of TMZ (2), KL-50 (1), and MTZ (11) in clonogenic survival assays, which measure the long-term replication potential of cells following drug exposure and are regarded as more rigorous than short-term growth delay assays, which measure the metabolic activity of cells following drug exposure and are regarded as more rigorous than short-term growth delay assays.\textsuperscript{27} We previously reported the activities of TMZ (2) and KL-50 (1) in this assay\textsuperscript{16} and have recapitulated them here for comparison to MTZ (11). As anticipated, TMZ (2) was efficacious against MGMT−/MMR+ cell lines but not MGMT+/MMR− or MGMT−/MMR− lines (Figure 2a). We observed surviving fractions of 6.3 × 10\textsuperscript{-4} and 4.0 × 10\textsuperscript{-5} in MGMT−/MMR+ and MGMT−/MMR− cell lines, respectively, treated with 30 μM KL-50 (1, Figure 2b); in contrast, MGMT+ cells proved highly resistant to KL-50 (1) as we only observed \approx 60–70% cell kill at 200 μM KL-50 (1). Thus, KL-50 (1) is highly selective for MGMT− cell lines over MGMT+ cell lines. The activity of MTZ (11) in this assay mirrored results we obtained previously with lomustine (7);\textsuperscript{16} thus, MTZ (11) eradicated the growth of MGMT− cell lines irrespective of MMR status but was toxic to MGMT+ cell lines at higher concentrations (Figure 2c). Taken together, these assays establish KL-50 (1) as highly efficacious in MGMT− GBM cell lines irrespective of MMR status, while the profiles of lomustine (7)\textsuperscript{16} and MTZ (11) in this assay are consistent with the lower tolerability of these reagents deriving from toxicity to healthy MGMT+ cell lines.

To further probe the cell line selectivity of KL-50 (1), we evaluated its activity against 902 human cancer cell lines using the high-throughput PRISM (Profiling Relative Inhibition Simultaneously in Mixtures) screen (Figures S2–S3).\textsuperscript{29} Cell viability was correlated to expression of \approx 19,000 protein-coding genes across seven doses (91 nM–200 μM) of KL-50 (1). From this analysis, MGMT expression emerged as the unique determinant of sensitivity. Comparison of toxicity to levels of MGMT expression showed a high Pearson’s correlation coefficient (r = 0.642), indicating that there is a strong positive correlation between MGMT gene expression and cell viability across all cell lines assayed. Significantly, this correlation is stronger than several “targeted therapeutics”, such as the BCL2 inhibitor venetoclax (vs BCL2 expression; r
$z = -0.590$, the EGFR inhibitor erlotinib (vs EGFR expression; $r = -0.358$), and the TOP2α inhibitor etoposide (vs TOP2α expression; $r = -0.185$) based upon AUC data obtained in similar screens from the Wellcome Sanger Institute’s Genomics of Drug Sensitivity in Cancer project (Figure S4). These data corroborate the selectivity of KL-50 (1) for MGMT− cells first observed in LN229 GBM cells across a much larger array of cancer cell lines and suggest that KL-50 (1) may find use in treating other MGMT-deficient tumors.

We previously obtained evidence for DNA ICL formation by KL-50 (1) using a modification of the alkaline comet assay in LN229 MGMT+/−/MMR+ and MGMT−/−/MMR− cells. To determine if MGMT can rescue cells from DNA ICL formation by KL-50 (1), we repeated this assay across the full panel of isogenic LN229 MGMT±/MMR± cell lines (Figure 3a). We found that KL-50 (1) only reduced %DNA in the tail in the MGMT−/−/MMR± cell lines, indicating that KL-50 (1) does not form high levels of DNA ICLs in the presence of MGMT. This mirrors the lack of appreciable ICL formation observed for the 2-chloroethylyating agents lomustine (7) and MTZ (11) in MGMT+ IMR-90 human embryo cells (vida supra).19d,22

To more closely evaluate the structure of the DNA lesions derived from KL-50 (1) treatment, we carried out in vitro alkylations studies. By analogy to lomustine (7) and MTZ (11), we anticipated that treatment of DNA with KL-50 (1) would result in the initial formation of O\textsuperscript{\text{\textprime}}\text{EtG} (4e) lesions that would evolve to the $[\text{G(N1)=C(N3)}]_{\text{Et ICL}}$ (10a; Figure 1c). As such, in vitro DNA alkylation followed by enzymatic digestion should yield a pool of nucleosides containing O\textsuperscript{\text{\textprime}}\text{EtG} (4e) and the [dG(N1)-dC(N3)]Et ICL (10b; Figure 4a). We initiated these studies by synthesizing the requisite deoxynucleosides 4f and 10b (Figure S5). Though both 4f and 10b have been previously described, to the best of our knowledge, detailed spectroscopic data have not been reported for either compound. The Supporting Information of this manuscript contains detailed experimental procedures for the preparation of 4f and 10b and complete NMR and high-resolution mass spectrometry (HRMS) characterization data for each compound (Figures S6–S11). Comparison of experimental carbon-13 NMR shifts of synthetic 10b to theoretical carbon-13 NMR shifts of 10b as well as three additional [dG-dC]Et ICL isomers (S1–S3) in combination with 2-D NMR data (Figures S8–S10) provide further support for the UV spectroscopy-based structural assignment of the [dG(N1)-dC(N3)]Et ICL (10b) by Ludlum and co-workers in 1982.14a

With the required synthetic standards in hand, calf thymus DNA (4 mg/mL) was incubated with KL-50 (1, 1 mM) for 5 or 72 h at 37 °C (Figure 4a). Following removal of small molecules by size exclusion spin filtration, the alkylated DNA was digested with exonuclease III, exonuclease T5, and QuickCIP phosphatase. Analysis of the resulting nucleoside mixtures using liquid chromatography (LC) combined with HRMS showed the presence of O\textsuperscript{\text{\textprime}}\text{EtGd}(4f) after 5 and 72 h, while the [dG(N1)-dC(N3)]Et ICL (10b) was only observed after 72 h (Figure 4b–d; see also Figures S12–S15). The structures of these products were verified by targeted LC/MS and comparison with the synthetic standards (Figures S16–S17). Neither O\textsuperscript{\text{\textprime}}\text{EtGd}(4f) nor the [dG(N1)-dC(N3)]Et ICL (10b) was observed in control experiments in which calf thymus DNA was treated with vehicle for 5 or 72 h at 37 °C. These results are consistent with initial formation of an O\textsuperscript{\text{\textprime}}\text{EtG} lesion (4e) in DNA that evolves to the $[\text{G(N1)=C(N3)}]_{\text{Et ICL}}$ (10a) in a manner analogous to the O\textsuperscript{\text{\textprime}}\text{ClEtG} DNA lesion (4b) formed by lomustine (7) and MTZ (11; Figure 1c).

Having validated that KL-50 (1) forms O\textsuperscript{\text{\textprime}}\text{EtG} (4e) and the $[\text{G(N1)=C(N3)}]_{\text{Et ICL}}$ (10a) in DNA, we proceeded to compare the rates of DNA ICL formation for O\textsuperscript{\text{\textprime}}\text{EtG} (4b) and O\textsuperscript{\text{\textprime}}\text{EtG} (4e). In a modified alkaline comet assay time-course ($t = 2, 8, 24$ h), we previously demonstrated that MTZ (11) treatment (200 μM) induces ICL formation within 2 h and maximum cross-linking within 8 h in LN229 MGMT−/−/MMR− cells.16 In contrast, DNA cross-linking is only observed after 8 h in cells treated with KL-50 (1, 200 μM). To study the kinetics of cross-linking in more detail and set the stage for probing DNA–MGMT cross-linking, we targeted the synthesis of a well-defined deoxyoligonucleotide duplex containing a single O\textsuperscript{\text{\textprime}}\text{ClEtG} (4b) or O\textsuperscript{\text{\textprime}}\text{EtG} (4e) residue. While formation of these O\textsuperscript{\text{\textprime}}\text{ClEtG} and O\textsuperscript{\text{\textprime}}\text{EtG} containing duplexes from a common, activated single-strand oligonucleotide precursor was attractive,34 the instability of O\textsuperscript{\text{\textprime}}\text{ClEtG} (4b) toward N1,O\textsuperscript{\text{\textprime}}-EtG (8a) formation30 (see Figure 1c) necessitated the synthesis of each duplex from the corresponding phosphoramidite building block in a protected form. Luedtke and co-workers previously disclosed the use of the photolabile N2-(2-nitrobenzoyloxy carbonyl) (NBOC) protecting group to cage O\textsuperscript{\text{\textprime}}\text{ClEtG} (4b) and studied its DNA cross-linking after photodeprotection (however, this study did not assess the rate of cross-linking).35 We envisioned that this photodeprotection approach would provide a defined $t_d$ for our kinetic studies.

Following Luedtke’s synthetic strategy with minor modifications,35,36 we synthesized the requisite phosphoramidite building blocks 14a and 14b in seven steps from commercially available 2′-deoxyguanosine (5b; Figure 5a; see Figure S18 for the synthesis of 14a and 14b). Solid phase oligonucleotide synthesis (SPOS) using the photocaged phosphoramidites 14a and 14b as well as commercially available N2-phenoxyacetyl-6′-deoxyadenosine, 2′-deoxythymidine, and unmodified N2-isopropyl-phenoxyacetyl-2′-deoxyguanosine phosphoramidites52 provided the photocaged oligonucleotides 15a and 15b, respectively.32 The desired 5′-End labeling of 15a and 15b followed by hybridization to a complementary oligonucleotide generated the deoxyoligonucleotide duplexes 16a and 16b, respectively (Figure 5b). Notably, the use of a hydroquinone-0,0′-diacetic acid linker (“Q-linker”)38 that enabled deprotection using diisopropylamine in methanol proved
critical as attempts to free the oligonucleotides 15a and 15b from resins bearing the more common succinyl linker using this mixture resulted in low conversion, 37 while use of potassium carbonate in methanol with the succinyl linker provided mixtures of oligonucleotides containing varying amounts of side products arising from cleavage of the NBOC protecting group and/or intramolecular halide displacement (see Figures S19–S22).

To carry out the cross-linking experiments, deoxyoligonucleotide duplexes 16a and 16b were separately irradiated (368 nm LED, 5 min; see Figure S23 for optimization of irradiation time and Figure S24 for a visual depiction of the photo-irradiation setup) and then incubated at pH 7.2 (20 mM sodium phosphate, 250 mM sodium chloride) at 37 °C (Figure 5b). Aliquots were removed immediately after irradiation (t₀) and diluted with denaturing formamide buffer to arrest cross-linking. Upon completion of the study, the samples were resolved by denaturing polyacrylamide gel electrophoresis (dPAGE, 18%; Figure 5c and 5e, left-hand lanes). For both duplexes, we observed the time-dependent appearance of cross-linked DNA (10c) as a band with lower mobility relative to the single-stranded oligonucleotide. 35 As expected, the OCEtG containing deoxyoligonucleotide duplex 16a formed DNA ICLs more rapidly than the OPEtG containing duplex 16b. Quantification of the bands by autoradiography established that the OCEtG containing duplex 16a transformed to the [G(N1)−C(N3)]Et ICL 10c with a half-life (relative to the maximum amount of cross-linking) of 6.30 ±0.04 h (Figure 5d), while cross-linking by the OPEtG containing duplex 16b was >10-fold slower (half-life = 80 ±4 h; Figure 5f). To verify the identity of the ICL (10c), we performed a preparative-scale (101 nmol) cross-linking experiment using unlabeled OCEtG containing duplex 16a' (Figure S25). Irradiation, incubation (70 h), purification of the ICL by dPAGE, and enzymatic digestion yielded a mixture of unmodified nucleosides and the [dG(N1)−dC(N3)]Et ICL 10b. HPLC co-injection with the synthetic standard as well as tandem mass spectrometry confirmed the formation of the [dG(N1)−dC(N3)]Et ICL 10b. We carried out density functional theory (DFT) calculations to determine the activation energy for cyclization of O'-{(2-chloroethyl)-N9-methylguanine (4g)} to N1,O'-ethano-N9-methylguanine (8c) and O'-{(2-fluoroethyl)-N9-methylguanine (4h)} to N1,O'-ethano-N9-methylguanine (8c) in water (Figure S26). Consistent with intuition and our experimental findings, we found that the activation energy for cyclization of O'-{(2-fluoroethyl)-N9-methylguanine (4h)} is significantly higher than O'-{(2-chloroethyl)-N9-methylguanine (4g); 23.3 and 17.8 kcal/mol, respectively.}

As is evident in Figures 5c and 5e, a large number of bands with decreased mobility compared to the cross-linked product 10c were observed by autoradiography. As these bands were observed at t₀ with an intensity that did not increase with time, we speculated that they may be the result of nonselective,
irradiation-induced cross-linking. However, irradiation of the corresponding deoxyoligonucleotide duplex containing an unmodified guanine residue (16c; Figure S27) did not give rise to these bands, indicating they did not arise from UV-mediated damage to the duplex. As such, we hypothesized that these bands derived from nonspecific side reactions of the duplex with the reactive nitrosoarene formed on photodeprotection.

To test this hypothesis, we synthesized the deoxyoligonucleotide duplexes 16d and 16e containing an O6MeG residue or an N2-NBOC-O6MeG residue, respectively, in place of the N2-NBOC-O6-(2-haloethyl)guanine residues in 16a and 16b. In support of our hypothesis, a pattern of lower-mobility bands similar to those shown in Figures 5c and 5e were observed only when the NBOC-protected deoxyoligonucleotide duplex 16e, but not 16d, was irradiated and analyzed by denaturing gel electrophoresis.

The DNA cross-linking studies presented above establish that the O6EtG lesion (4e) derived from KL-50 (1) generates DNA ICLs more slowly than the O6ClEtG lesion (4b) derived from MTZ (11) or lomustine (7). Because this cross-linking mechanism proceeds through the common intermediate N1,O6EtG (8a), the difference in observed rates of DNA ICL formation must derive from the slower rate of cyclization of O6EtG (4e) to N1,O6EtG (8a), relative to O6ClEtG (4b). A central element of our mechanistic analysis (see Figure 1c and associated text) is that the slower rate of cyclization of O6EtG (4e) to N1,O6EtG (8a) will provide more time for MGMT to reverse O6EtG (4e) in healthy tissue, thereby

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**Figure 5.** Kinetic study of DNA ICL 10c and DNA–MGMT cross-link formation using the O6XEtG containing deoxyoligonucleotide duplexes 16a (X = Cl) and 16b (X = F) in the absence and presence of MGMT. (a) O6XEtG containing deoxyoligonucleotides 15a (X = Cl) and 15b (X = F) were synthesized from phosphoramidites 14a and 14b, respectively (see Figure S18 for the synthesis of 14a and 14b). (b) Experimental design. (c) Representative denaturing gel for the kinetic study of the O6ClEtG containing duplex 16a ± 5 equiv of human MGMT. (d) Plot of %DNA cross-linked versus time as quantified by autoradiography for the kinetic study of the O6ClEtG containing duplex 16a ± 5 equiv MGMT. (e) Representative denaturing gel for the kinetic study of the O6FEtG containing duplex 16b ± 5 equiv of human MGMT. (f) Plot of %DNA cross-linked versus time as quantified by autoradiography for the kinetic study of the O6FEtG containing duplex 16b ± 5 equiv MGMT. For (b), only the 5′-32P-labeled products of the reaction are drawn. For (d) and (f), points indicate mean; error bars indicate standard deviation; n = 3 biological replicates.
reducing the amount of potentially toxic DNA–MGMT cross-links derived from the reaction of N1,0 EtG (8a) with MGMT. We reasoned that addition of MGMT to solutions of the O EtG containing deoxyoligonucleotide duplexes 16a (X Φ Cl) and 16b (X Φ F) immediately after photodeprotection would allow us to test this hypothesis. Additionally, this assay would also enable us to directly assess the degree to which MGMT can prevent DNA ICL formation from 16a and 16b.

We found that DNA ICL production was reduced but not completely ablated when the O EtG containing duplex 16a was exposed to MGMT (5.0 equiv) immediately following photodeprotection (Figure 5c, right-hand lanes). A plot of %DNA cross-linked as a function of time revealed a ~2-fold reduction in the formation of DNA ICL 10c after 2 h (Figure 5d). A band of low mobility, consistent with a DNA–MGMT cross-link, was observed at the top of the gel immediately after addition of MGMT. This nearly instantaneous formation of DNA–MGMT cross-links is consistent with rapid formation of N1,0 EtG (8a) following photodeprotection of the O EtG containing duplex 16a (Figure 5d; see also Figure S29c, vide infra). By comparison, we found that production of the DNA ICL 10c was almost completely ablated when the O EtG containing duplex 16b was exposed to MGMT (5.0 equiv) immediately following photodeprotection (Figure 5e, right-hand lanes). A plot of %DNA cross-linked as a function of time reveals that the addition of MGMT reduced DNA-cross-linking by ~8 fold at 70 h (Figure 5f). We detected trace amounts of DNA–MGMT cross-links at the final timepoint in this assay (70 h).

To verify that MGMT decreased the formation of DNA ICL 10c and caused the formation of DNA–MGMT cross-links via the active site cysteine residue of the protein (Cys145), we performed two assays. In the first, we used a restriction enzyme-based MGMT activity assay to demonstrate that pre-exposure of MGMT to irradiated, unlabeled duplexes 16a and 16b′ inhibits the ability of the protein to repair a duplex containing a PstI restriction site blocked by an O MeG residue (S4; Figure 6a; see Figure S28 for a schematic describing the assay). In this assay, unlabeled duplexes 16a and 16b′ were separately irradiated (368 nm) then incubated with human MGMT (3 h, 37 °C), allowing for inactivation of MGMT by transfer of the O EtG lesion to the active site cysteine of the protein. As such, upon addition of the 32P-labeled, O MeG containing duplex S4 (Figure S28), the O MeG lesion is not repaired and subsequent addition of PstI does not produce the cleavage product 18, leading to the observation of uncleaved O MeG containing oligo 17 by dPAGE analysis (as in Figure S28, left). In contrast, repetition of the experiment with nonirradiated duplexes 16a and 16b′ produces the PstI cleavage product 18 upon dPAGE analysis, indicating that MGMT was not able to repair the O EtG lesion in the presence of the N2-NBOC protecting group (as in Figure S28, right). Control experiments show that the cleavage product 18 is only observed when the O MeG containing duplex S4 is treated with MGMT prior to the introduction of PstI and that inactivation of MGMT via pretreatment with the known MGMT substrate O benzylguanine (O BnG, 20 equiv, 1 h)12 prevents repair of the O MeG lesion upon addition of the labeled O MeG containing duplex S4, leading to an absence of the cleavage product upon introduction of PstI. Notably, this assay also showed that the small amount of DNA–MGMT cross-links observed for 16b does not result from insufficient interaction between MGMT and the duplex. In a second assay, we verified that the formation of DNA–MGMT cross-links and decrease in ICL formation observed upon addition of MGMT to the O EtG-containing duplex 16a following photodeprotection specifically involves the active site cysteine of the protein (Cys145). We inactivated MGMT by treatment for 1 h with O BnG (20 equiv)42 then added this deactivated MGMT to photodeprotected duplex 16a. We found that addition of O BnG treated MGMT resulted in no DNA–MGMT cross-link formation and increased the amount of DNA ICLs 10c to nearly the same level as observed in the absence of MGMT (Figure 6b).

It is important to note that, due to the short half-life for cyclization of O EtG (4b) to N1,0 EtG (8a; t 1/2 ~ 18 min for the isolated nuclease 4c;20 Figure S1), the 5 min duration of the photodeprotection prior to the introduction of MGMT may allow O EtG (4b) lesions that would have been repaired by MGMT to instead evolve to N1,0 EtG (8a) in the absence of the protein. This would artificially increase the amount of DNA–MGMT cross-links observed as, under physiological conditions, ring-opening of N1,0 EtG (8a) to the ICL 10a is comparatively slow (t 1/2 ~ 4–8 h).51,52 Figure 1c), and MGMT is present before introduction of the DNA alkylating agent. To address this, we carried out an experiment wherein MGMT was added immediately before irradiation (Figure S29). While it is theoretically possible that MGMT may reverse the O EtG in 16a before photodeprotection, we

![Figure 6](https://doi.org/10.1021/jacs.3c06483)
demonstrated above that nonirradiated duplex 16a did not deactivate MGMT (Figure 6a). To control for potential light-mediated degradation of MGMT, we conducted a control experiment wherein the O6ClEtG containing duplex 16a and MGMT were irradiated separately and then combined prior to incubation. We found that the levels of ICL 16c formation (Figure S29a) and DNA–MGMT cross-linking (Figure S29b) were not measurably altered by the addition of MGMT to the O6ClEtG containing duplex 16a before or after irradiation. Furthermore, we found little difference between the results from postirradiation addition of nonirradiated and irradiated MGMT, indicating that the irradiation does not affect the activity of MGMT. As such, the 5 min delay between the start of photodeprotection and the addition of MGMT does not artificially increase the amount of DNA–MGMT cross-links. This indicates that the experiments described above with the O6ClEtG containing duplex 16a in the absence and presence of MGMT accurately model the physiological treatment of MGMT− and MGMT+ cells, respectively, with 2-chloroethylating agents. We regard the experiments described above with the O6FeTG containing duplex 16b as even more robust owing to the slower rate of conversion of O6FeTG (4e) to N1,O6EtG (8a) (t1/2 ~ 18.5 h for the isolated nucleoside 4d) (Figure S1).

Having demonstrated in a chemically controlled system that switching from an O6ClEtG to an O6FeTG lesion decreases the amount of DNA–MGMT cross-linking, we next wanted to validate this finding in a cellular context. To this end, we isolated genomic DNA from MGMT+ DLD1 cells treated with TMZ (2), KL-50 (1), lomustine (7), or MTZ (11) for 8 h (Figure 7). Immunoblotting using an anti-MGMT antibody showed a lack of DNA–MGMT cross-links for vehicle- and TMZ (2)-treated samples, as expected. In contrast, cells treated with the 2-chloroethylating agents lomustine (CCNU, 7) and MTZ (11) showed robust formation of DNA–MGMT cross-links. To our knowledge, this is the first time that the specific formation of DNA–MGMT cross-links has been observed in DNA isolated from cells treated with 2-chloroethylating agents.45 Finally, in agreement with our studies of the O6FeTG containing duplex 16b, DNA–MGMT cross-links were not observed after treatment of DLD1 cells with the 2-fluoroethylation agent KL-50 (1) for 8 h.

**DISCUSSION**

In this study, we have rigorously characterized the mechanism of action of KL-50 (1), a novel lead for the treatment of MGMT-silenced, drug-resistant glioblastoma. Our studies support a model wherein the selectivity of KL-50 (1) derives from the slow rate of formation of the key ethanoguanine intermediate N1,O6EtG (8a), which provides time for MGMT to reverse the initial alkylation product O6FeTG (4e) in healthy tissue. This rate differential prevents the accumulation of N1,O6EtG (8a) in tissues that express MGMT, nearly eliminating the formation of DNA ICLs and DPCs in the presence of MGMT. In contrast, the more rapid conversion of O6ClEtG (4b) (derived from 2-chloroethylating agents) to N1,O6EtG (8a) results in the formation of DNA–MGMT cross-links and DNA ICLs in the presence of MGMT.

It is important to note that MGMT activity has also been characterized as a mechanism of resistance to DNA alkylating agents, including TMZ (2),44 and efforts to develop “MGMT− independent” therapeutics have been recorded. For example, VAL-083 (aka dihydrodigalactitol) is a diepoxide-based DNA cross-linking agent that is being evaluated in clinical trials of MGMT+ GBM, with the goal of overcoming MGMT-based resistance to TMZ (2).45 Additionally, TMZ derivatives that exert toxicity independent of MGMT status have been described.46 The notion that MGMT-silencing might provide a therapeutic index when alkylating agents are administered systemically to patients harboring MGMT− tumors was first codified in 1980.47 Our own opinion is that it will be difficult to achieve efficacious level of exposure to systematically administered DNA alkylating agents without a mechanism, such as MGMT silencing, to obtain selective tumor toxicity. While our work is unlikely to benefit patients with MGMT+ tumors, it may provide a treatment option for recurrent, TMZ-resistant, MGMT− GBM. Additionally, the comparable activity of KL-50 (1) to TMZ (2) in MGMT−/MMR− models of GBM16 suggests its potential use in the up-front setting.

Historically, genotoxins have been prescribed based on tumor histology and site of origin, without regard to the underlying DNA repair capacity of the tumor. The work here demonstrates that a detailed analysis of the chemical kinetics of DNA modification, coupled with an understanding of the rates of biochemical DNA repair, could lead to highly selective, systemically administered therapies. Though further study is required to evaluate the safety of KL-50 (1) in humans, our data suggest it is much more selective than lomustine (7) or MTZ (11). Further study of the rates of chemical DNA modification and biochemical DNA repair may lead to the identification of additional targeted therapeutics that exploit specific, tumor-associated DNA repair defects.

**ASSOCIATED CONTENT**

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.3c06483.

Supplementary figures and tables, detailed experimental procedures, and characterization data for all new compounds (PDF)
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Notes

The authors declare the following competing financial interest(s): S.B.H., R.S.B., and K.L. are cofounders and hold equity in Modifi Biosciences, which has exclusive rights to the findings herein. S.B.H. and R.S.B. are officers of Modifi Biosciences. E.D.H. was a consultant for Modifi Biosciences. K.L., S.E.G., R.K.S., E.D.H., and S.B.H. are inventors on US Provisional Application 63/290,572 submitted by Yale University, which covers the mechanism of action and use of KL-50 (1) for the treatment of MGMT−/−MR− GBM. K.L., R.S.B., and S.B.H. are inventors on US Provisional Applications 63/290,763, 63/212,410, 63/247,645, 63/290,622, 63/290,627, and 63/290,630, which cover KL-50 (1) and related derivatives for the treatment of MGMT-deficient tumors.

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