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Review

DNA repair mechanisms and their clinical impact in glioblastoma

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ABSTRACT

Despite surgical resection and genotoxic treatment with ionizing radiation and the DNA alkylating agent temozolomide, glioblastoma remains one of the most lethal cancers, due in great part to the action of DNA repair mechanisms that drive resistance and tumor relapse. Understanding the molecular details of these mechanisms and identifying potential pharmacological targets have emerged as vital tasks to improve treatment. In this review, we introduce the various cellular systems and animal models that are used in studies of DNA repair in glioblastoma. We summarize recent progress in our knowledge of the pathways and factors involved in the removal of DNA lesions induced by ionizing radiation and temozolomide. We introduce the therapeutic strategies relying on DNA repair inhibitors that are currently being tested in vitro or in clinical trials, and present the challenges raised by drug delivery across the blood brain barrier as well as new opportunities in this field. Finally, we review the genetic and epigenetic alterations that help shape the DNA repair makeup of glioblastoma cells, and discuss their potential therapeutic impact and implications for personalized therapy.

Contents

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1. Introduction

Glioblastoma (GBM, grade IV glioma)[2] represent the most frequent and aggressive malignant primary brain tumors in humans. GBMs encompass secondary GBMs (about 5% of the cases) which develop from lower-grade diffuse astrocytoma (WHO grade II) or anaplastic astrocytoma (WHO grade III), and primary GBMs (about 95% of the cases) which arise rapidly de novo without clinical or histological evidence of a less malignant precursor. GBMs display very poor prognosis and lack therapeutic options, further complicated by the presence of the blood brain barrier (BBB). Current management of GBMs usually consists of surgical resection, followed by radiotherapy (RT) with concomitant and adjuvant chemotherapy with the DNA alkylating agent temozolomide (TMZ) [1], both treatments inducing DNA damage. Although TMZ displays antitumor activity and limited toxicity, its survival benefit remains unsatisfactory – a mere 2.5 months [1], due to rapid occurrence of resistance and tumor relapse.

GBMs are characterized by an important intertumor and intratumor heterogeneity both at the cellular and genomic levels [2,3]. Significant progress has been made in our characterization of the molecular alterations found in GBM biopsies, leading to a comprehensive landscape of somatic genomic alterations in glioblastoma and refining the list of putative GBM driver genes [4]. Adult and pediatric GBM subtypes with different presentation and/or progression courses as well as therapeutic responses have been proposed based on molecular analyses [5]. Likewise, advances in surgical sample collection, integrated genomic analyses and single-cell technology have shed new light on the (regional) intratumor heterogeneity within GBM patients, revealing patterns of cancer evolution at the single-patient level [6,7]. Although what drives such dynamic heterogeneity at the cellular level remains unclear ([8], and references therein), it may hold important keys to understanding the response of GBM cells to genotoxics and tumor recurrences [9,10].

Sophisticated cellular mechanisms have evolved to detect, signal and repair the various DNA lesions inflicted in our chromosomes by endogenous or exogenous genotoxics. The robustness of the DNA repair mechanisms composing this DNA damage response (DDR) is ensured in part by the inherent redundancy of the many pathways that can remove a specific lesion and also by the fact that DNA repair pathways function in complex networks, with DNA-repair intermediates produced by a given pathway oftentimes forming substrates for another pathway [11]. In non-neoplastic cells, accurate DNA repair is essential to prevent genetic instability, a driving force in tumorigenesis. Thus, cancer cells often display genetic or epigenetic alterations that affect DNA repair factors [12]. Paradoxically, DNA repair mechanisms are also paramount to the removal of lesions induced by genotoxic anti-cancer agents, and a crucial factor contributing to the development of resistance and tumor relapse. Furthermore, tumorigenic cells experience greater dependence on residual DNA repair mechanisms that help them cope with exacerbated DNA damage resulting from increased cellular metabolism (i.e., oxidative DNA damage) as well as replication and/or mitotic stress [13], whereas conditions imposed by the tumor microenvironment (e.g., hypoxia) can profoundly alter the expression and function of DNA repair genes. Understanding the molecular details underlying the response of tumor cells to genotoxics and deciphering the exploitable cancer-specific genetic alterations in the DNA damage response are recognized as crucial steps in the development of strategies that will improve cancer management. In recent years, several therapeutic strategies and DNA repair inhibitors have been elaborated to take advantage of defective DNA repair or saturate altogether the DNA repair capacities in cancer cells [12,14].

The main DNA lesions induced by ionizing radiation (IR) and TMZ, together with the major factors involved in their repair are recapitulated in Fig. 1. The most severe DNA lesion inflicted by IR are double-strand breaks (DSBs), which are repaired by two mechanisms each composed of several pathways – homologous recombination (HR) and non-homologous end joining (NHEJ) [15,22]. Other forms of DNA damage induced by IR include base damage and single-strand breaks (SSBs), which are repaired, respectively by base excision repair (BER) and SSB repair. The major lesions induced by TMZ are N^7-methylguanine (N^7-meG) and N^3-methyladenine (N^3-meA), which are primarily repaired by BER, and O^6-methylguanine (O^6-meG), a highly cytotoxic lesion which is removed by the O^6-methylguanine-DNA methyltransferase (MGMT) product of the MGMT gene [16]. MGMT is a notable exception to the multi-protein DNA repair pathways, as it works single-handedly in a suicidal reaction that transfers the alkyl group from guanine to an internal cysteine residue. Loss of MGMT by promoter hypermethylation is commonly observed in colorectal carcinomas, gliomas, non-small cell lung carcinomas, lymphomas and head and neck carcinomas [29]: in colorectal cancer, such inactivation is an early event associated with mutagenic consequences [30]. MGMT promoter CpG methylation is observed in about 45% of patients presenting with GBM and is associated with increased sensitivity to TMZ and prolonged survival [31]. In the absence of MGMT, unrepaired O^6-meG can pair with cytosine or thymine leading to O^6-meG/T mispairs that are recognized by the mismatch repair (MMR) machinery. However, MMR action leaves the O^6-meG intact, introducing instead SSBs that are converted to potentially lethal DSBs at replication forks. TMZ-induced mutational inactivation of MMR genes has been observed in recurrent GBM tumors [9,32], consistent with the notion that loss of MMR contributes to resistance to TMZ. Repair of DSBs generated as a result of MMR activity involves the DSB repair machineries as well as proteins belonging the Fanconi Anemia (FA) pathway involved in the recombinational repair of perturbed or broken replication forks [33,34].

2. Scopes of this review

Various reviews have addressed genetic, biochemical and molecular aspects of DNA repair and therapy in glioblastoma [35–37]. Here, we focus on recent developments in the following fields of primary GBM research: i) the experimental and preclinical models used in studies of DNA repair in GBM, ii) the regulation/inhibition of MGMT expression and activity, iii) novel insights into the DNA damage response and DNA repair inhibitors for the treatment of GBM, and iv) DDR-relevant genetic and epigenetic alterations identified in primary GBMs.

Throughout this review, we will also emphasize how strategies driven by RNA interference have contributed to the identification

[2] The abbreviations used in this review are defined in Table 1.

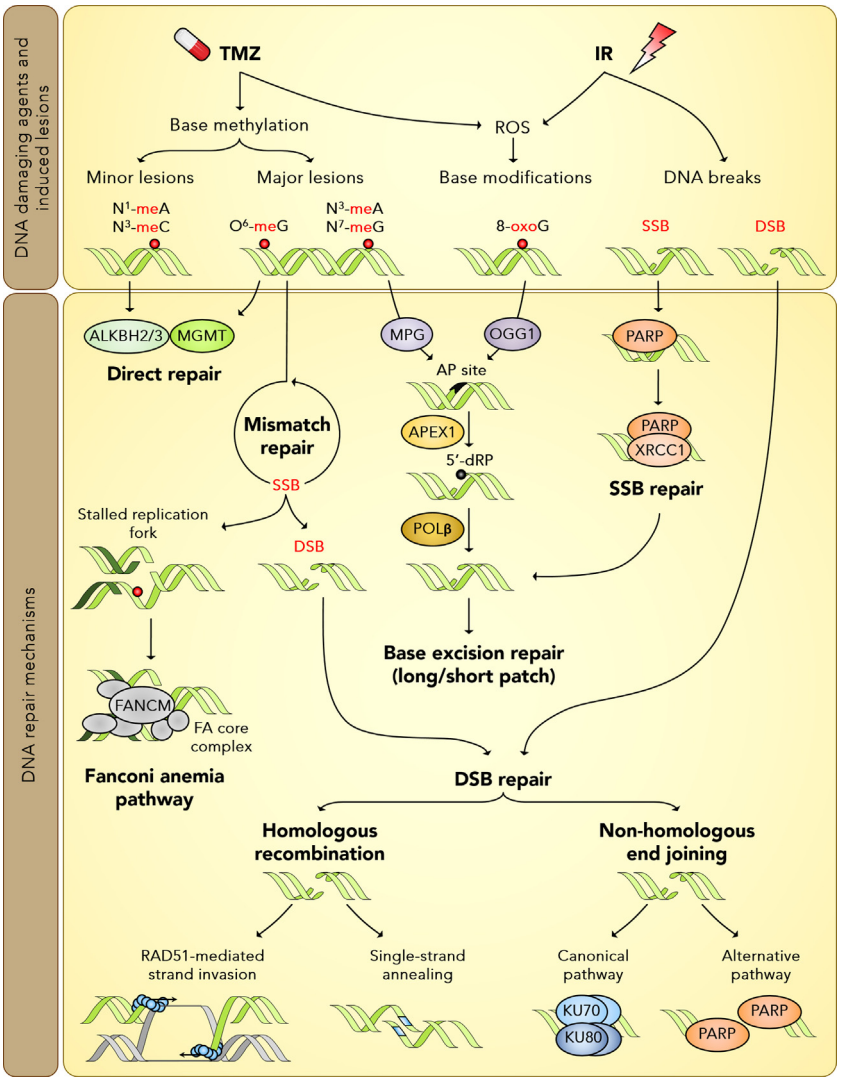

Fig. 1. DNA repair pathways involved in the removal of IR- and TMZ-induced lesions. Schematic overview the lesions induced in DNA by IR and TMZ, as well as the repair mechanisms that have been implicated in their removal in GBM. Only those factors that are discussed in the review have been indicated. The reader is referred to Ciccia [15] for the mechanistic details of each pathway and the molecular composition of the various DNA repair machineries. *Removal of TMZ-induced alkylated bases:* Direct reversal of O^6-meG is mediated by MGMT. In the absence of MGMT, O^6-meG can direct misincorporation of T during DNA synthesis, resulting in mismatch repair (MMR)-driven futile cycles that lead to the generation of DNA single-strand breaks (SSBs), double-strand breaks (DSBs) and replication-associated DSBs. Removal of N^7-meG and N^3-meA, which is mediated by Base Excision Repair (BER), is initiated by the DNA glycosylase MPG. Repair of the resulting apurinic/apyrimidinic (AP) site can involve the replacement of either a single nucleotide (short-patch BER) or several nucleotides (long-patch BER). TMZ can also generate N^1-methyladenine (N^1-meA) and N^3-methylcytosine (N^3-meC) [16,17]. These lesions may be removed by the oxidative demethylases ALKBH2 and ALKBH3 which are capable of directly reversing N^1-meA and N^3-meC in DNA [18,19]. ALKBH2 was found to confer resistance to TMZ in GBM cells [20]. *Repair of oxidative DNA damage:* TMZ- and IR-induced oxidative DNA damage includes oxidized bases (such as 8-Oxo-7,8-dihydroguanine (8-oxoG)) that are recognized and excised by a variety of DNA glycosylases that initiate BER [21]. *SSB repair:* SSBs generated by IR and Reactive Oxygen Species (ROS) or during processing of TMZ-induced DNA lesions are recognized and bound by poly(ADP-ribose) polymerase family members such as PARP1, leading to the recruitment of the scaffold protein XRCC1. Repair is then achieved through short- or long-patch BER repair. *DSB repair:* Repair of DSBs can be achieved by two major pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) DNA repair, each composed of several sub-pathways [15,22]. HR pathways act upon 3′-extended single-stranded DNAs produced by DSB resection. Homology-directed strand invasion, mediated by RAD51 filaments, provides a major mechanism for the recombinational repair of DSBs in GBM cells. When resection exposes complementary sequences (blue segments), repair can occur via RAD52-mediated single-strand annealing. Although this mechanism is involved in the repair of IR-induced DSBs, its relevance in the context of TMZ-induced lesions has thus far not been reported. Recombinational repair of replication-associated DSBs involves the Fanconi Anemia (FA) pathway and HR factors [23]. Recognition of DSBs by either KU70/KU80 or PARP leads respectively, to canonical NHEJ and alternative NHEJ (A-NHEJ, itself composed of sub-pathways), a backup pathway that has been described in GBM cells [24,25]. Finally, translesion synthesis polymerases (not represented) that allow bypass of TMZ-induced lesions during DNA replication have been implicated as a mechanism to tolerate TMZ-induced DNA lesions [26–28].

and characterization of the DNA repair factors and molecular mechanisms underlying the cellular response of GBMs to IR and TMZ (hereby referred to as chemoradiation).[3]

3. Experimental and pre-clinical models for the study of DNA repair in GBM

3.1. Cellular models

Established GBM cell lines (such as U87, U251 etc.) and low-passage primary cell lines grown in monolayer cultures in serum–complemented media have commonly served as in vitro models of GBM. More recently, GBM cell cultures have also been established from tumor-derived single-cell suspensions grown in serum-free medium containing EGF and FGF (neurobasal medium). Under these conditions, the cells generate spheres and display stem cell properties, including self-renewal and the ability to differentiate into multiple cell types resembling central nervous system (CNS) cell lineages [41] and are often referred to as GBM stem cells (GSCs). GSCs are sometimes isolated based on the expression of specific markers (such as CD133, CD44 and A2B5) although expression of these markers in patients is highly heterogeneous [7] and their relevance as stem-cell markers is controversial. It remains to be determined whether GSCs represent a genetically defined, stable subpopulation of cells or an adaptive cellular state responding to microenvironmental changes. Yet, several studies suggest that GCSs are more resistant to DNA damage than their non-GSCs counterparts [10,42] and are able to mediate tumor cell repopulation during recurrence [43–45]. GSCs can be maintained in adherent cultures when the serum-free culture system is adjusted [46], which greatly facilitates their experimental study and in particular ensures the uniform distribution of genotoxics during cell cytotoxicity experiments or high-throughput genetic screens.

3.2. In vivo animal models

Transgenic and knockout mice for DNA repair functions have provided invaluable knowledge on spontaneous and genotoxic-induced carcinogenesis, as well as the deleterious consequences of defective DNA repair [47,48]. Mouse models have revealed the importance of DSB repair mechanisms during brain development and in neurological diseases [49]. Chemically-induced rodent models have been used to investigate glioblastomagenesis whereas genetically-engineered mouse models (GEMMs) harboring selected GBM driver mutations offer unique contexts to investigate GBM development driven by specific alterations and test potential therapeutics [47,50]. Unlike medulloblastoma [51], the impact of selective DNA repair gene knockout on tumorigenesis and DNA repair in GEMMs of GBM still awaits to be analyzed (see Section 6.2.1 for a description of the epigenetic factor SETD2).

Xenograft models developed from human GBM-derived cells provide another preclinical model to investigate glioblastomagenesis and test therapeutic strategies using genotoxics. Although a great number of publications report observations made on tumors grown from subcutaneous implants of GBM-derived cells, stereotactic intracranial implantation in immunodeficient rodents provides clinically more relevant and experimentally reproducible models. Notable in the context of treatment with genotoxics is that the BBB and also DNA repair-relevant features of the tumor microenvironment (including tumor-stroma interactions and the impact of hypoxia) are recapitulated in these models. Xenograft models can be generated from established GBM cell lines or, more relevantly, obtained from patient-derived xenografts (PDX) where human GBM tissue is transplanted in the animal following limited in vitro processing [47].

Currently, most animal experiments designed to assess the response of GBM cells to genotoxics expose the animal to the DNA-damaging agent for some time during tumor development, without prior tumor resection. The time selected for genotoxic treatment is usually chosen based on tumor mass analysis by in vivo imaging (e.g., magnetic resonance imaging or bioluminescence analysis) or predetermined time course of tumor appearence [7]. In such cases, the entire tumor mass is challenged with the drug (Fig. 2, panel A). Genotoxic treatment can also be carried out in vitro, prior to implantation of the surviving cells in the animal (Fig. 2, panel B). This latter strategy has been used, for instance, to investigate the effect of combined treatment with the PARP inhibitor olaparib and IR on tumor formation by GSCs [52]. One idea behind this approach is that the in vitro treatment might lead to the selection of a subpopulation of surviving cells with increased DNA repair capacity, that may reflect in part the features/properties of the invasive cells that have migrated away from the tumor mass at the time of resection and chemoradiation. Although neither strategy accurately reflects the clinical situation with regard to tumor recurrence, the development of surgical resection models for GBM is still in its infancy [53]. Thus, attempts to assess the effects of genotoxics in animals that have been subjected to tumor micro-resection, and possibly also IR, (Fig. 2, panel C) have to our knowledge not been reported.

4. Novel insights into the regulation of MGMT expression and its inhibition

MGMT provides the main mechanism for removal of the cytotoxic O^6-MeG lesion. Identifying the factors that orchestrate MGMT expression as well as therapeutic means to debilitate this line of defense against TMZ is thus the object of intense efforts. The main features of MGMT regulation and inhibition are recapitulated in Fig. 3.

A thorough review on the regulation of MGMT expression, including its epigenetic silencing and value/use as a biomarker and clinical target, has recently been published [36]. Here, we focus on the molecular progress in this field as well as aspects that are most relevant to genotoxic chemotherapy.

4.1. Transcriptional regulation of MGMT

The tumor suppressor gene TP53 encodes a transcriptional activator that orchestrates fundamental cellular responses to a variety of stress signals including DNA damage [65]. TP53 mutations and loss of heterozygozity are frequently observed in GBM [66], as is the case with the amplification or overexpression of its negative regulators MDM2 and MDM4 [67]. Overexpression of p53 has been proposed to negatively regulate MGMT transcription by sequestering Sp1 transcription factor [63], thereby sensitizing tumor cells to alkylating agents in vitro. In glioma cells, p53 overexpression can be induced by interferon beta (IFN-β), which was shown to sensitize cells to TMZ [68]. However, IFN-β has also been reported to induce sensitization of GSCs to TMZ, independent

[3] The chloroethylnitrosourea alkylating agents 1,2-bis[2-chloroethyl]-1-nitrosourea (BCNU, carmustine) and 1-[2-chloro-ethyl]-3-cyclohexyl-1-nitrosourea (CCNU, lomustine) are also part of the arsenal against GBM although, since the advent of TMZ they have been more generally administered as second line agents, when recurrence occurs. Removal of DNA lesions induced by these compounds involves most of the mechanisms mediating repair of TMZ-induced lesions, including MGMT. However, as bi-functional agents, BCNU and CCNU are also able to induce interstrand crosslinks [16] whose removal also involves factors from the FA and nucleotide excision repair pathways [38,39]. Other genotoxics that have been considered for the treatment of GBM, such as the DNA topoisomerase inhibitor irinotecan [40] are not addressed here.

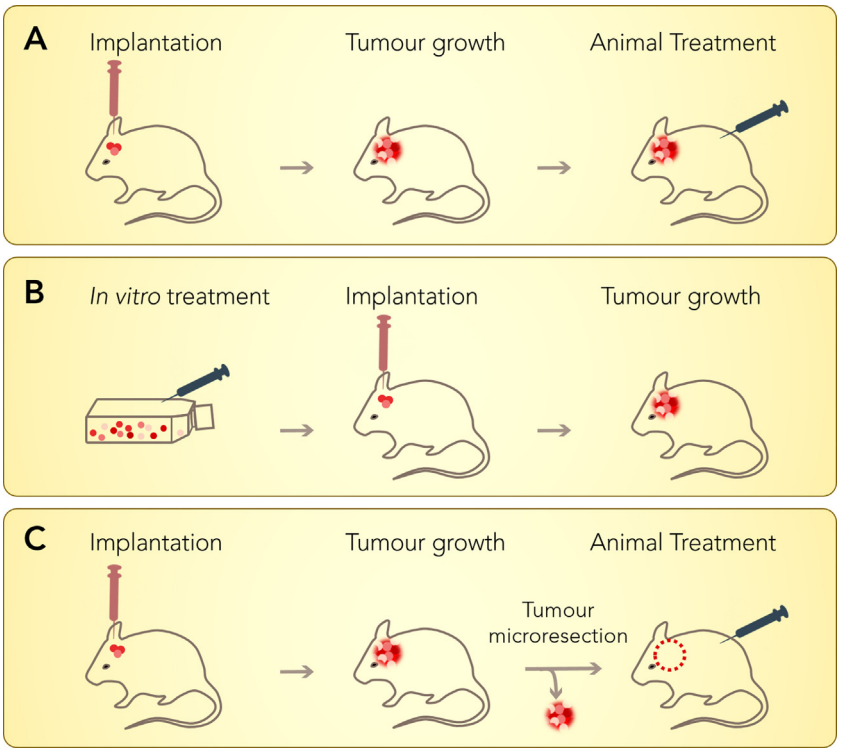

Fig. 2. Assessing GBM tumor response to genotoxics using orthotopic xenograft models. The response of GBM tumors (derived from established cell lines/GSCs/patient-derived GBM cells) to genotoxics can be tested in various xenograft settings. A. Xenotransplantation followed by treatment is the most commonly used orthotopic model. In this case, tumor growth can be monitored by MRI or bioluminescence analysis, followed by treatment and its evaluation. B. Cells are treated in vitro and the surviving cell population is implanted, followed by monitoring of tumor formation. C. Same as in A., except that tumor formation is followed by surgical resection, leaving resection margins as well as infiltrating cells that have migrated from the tumor mass. The animal is then treated and tumor recurrences are monitored. This model best reflects the current clinical situation in the setting of GBM.

of MGMT and p53 status [69]. Although uncertainties remain about the underlying mechanisms, Motomura et al. [70] have proposed IFN-β as a good candidate for adjuvant GBM therapy, especially for patients with an unmethylated MGMT promoter, based on a retrospective study of 68 newly diagnosed primary GBM patients showing that treatment with IFN-β increased the median survival time from 12.5 (TMZ alone) to 17.2 months (TMZ + IFN-β).

A long list of microRNAs (miRs) has been shown to target MGMT for downregulation (Fig. 3). Some miRs modulate TMZ sensitivity in vitro [59,64,71–73] and have been proposed to predict either the response to chemoradiation in GBM patients [73,74] or patient outcome in different GBM subtypes [75].

MGMT expression is induced by c-MYB and appears to be positively regulated by the transcription factor ZEB1 (zinc finger E-box binding homeobox 1), which operates through inhibition of miR-200c, a negative regulator of c-MYB [59]. Thus, ZEB1 was found to modulate TMZ chemoresistance in GBM cells and the authors have suggested that it could be a target for future therapeutic approaches.

4.2. MGMT inhibition: pseudosubstrates and genetic therapy

MGMT operates by stoichiometrically transfering the methyl group of O^6-MeG to a cysteine residue within its active site. This reaction is irreversible as it is followed by the ubiquitination and degradation of MGMT. Such a suicide reaction has been exploited in therapeutic strategies aiming at decreasing the pool of MGMT molecules in MGMT-positive patients (Fig. 4, panel A). However, attempts to sensitize GBMs and other solid tumors to TMZ through pharmacological depletion of MGMT levels using the pseudosubstrates O^6-benzylguanine (O^6-BG) or O^6-(4-bromothenyl) guanine (O^6-BTG, also known as lomeguatrib or PaTrin-2) have been faced with severe myelosuppression toxicity, forcing the use of decreased doses of TMZ which proved inefficient in clinical trials [76,77]. A number of approaches have been considered to overcome these adverse effects whilst targeting tumor cells more efficiently, including local administration of O^6-BG [78] or the use of glucose- [79] or folate-conjugates [80] of O^6-BG and O^6-BTG that target tumor cells through highly-expressed transporters or receptors. However, these early efforts have not been fruitful.

More recently, expression of the O^6-BG-resistant MGMT (P140K) mutant by hematopoietic cells was shown to provide significant protection against toxicity from O^6-BG/alkylator chemotherapy [81], leading to a prospective phase I/II clinical trial in which newly diagnosed MGMT-positive GBM patients were transplanted with autologous MGMT(P140K) gene–modified hematopoietic $CD34^+$ cells [82]. Gene therapy increased tolerance to the O^6-BG/TMZ combination as well as patient survival, supporting further development of chemoprotective gene therapy in GBM patients treated with O^6-BG and TMZ [83].

Other molecules that might in the future be considered to sensitize MGMT-positive patients to TMZ, include the direct inhibitor disulfiram [61,84], as well as various epigenetic drugs that sensitize MGMT-positive GBM cells to TMZ by down-regulating MGMT gene expression. Examples include the histone deacetylase inhibitors levetiracetam and valproic acid [85], and the DNA methylation inhibitor decitabine [56].

5. Inhibition of IR- and TMZ-induced DNA damage repair in GBM

5.1. DNA damage signaling and its inhibition

Various forms of DNA damage activate key sensor kinases such as ATM and ATR, triggering the phosphorylation of downstream

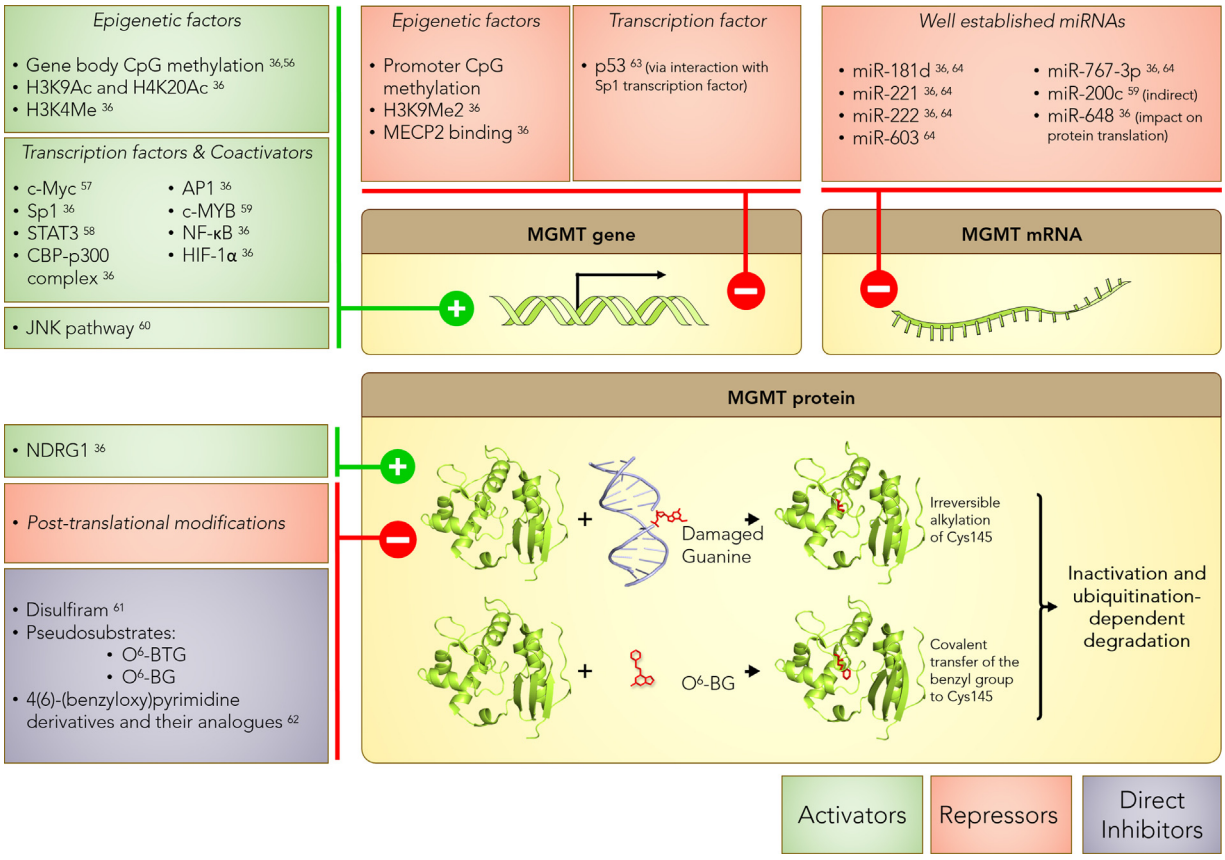

Fig. 3. MGMT regulation and inhibition. Depicted are the activating factors (green boxes) and repressing factors (peach boxes) of MGMT expression as well as direct inhibitors that affect MGMT activity (violet boxes). Various epigenetic mechanisms have been shown to modulate MGMT expression, including CpG methylation of MGMT promoter and gene body, histone H3K4 methylation and H3K9 dimethylation, acetylation of histones H3K9 and H4K20, and binding by the methyl-CpG binding protein MeCP2. MGMT Expression can also be controlled by a variety of transcription factors, as well as numerous miRs. Note that miR-648 interferes with MGMT protein translation whereas miR200 impacts MGMT through targeting of cMYB (see Section 4.1). At the post-translational level, MGMT can be stabilized by binding of the NDRG1 protein. Following binding of a methylated guanine or O^6-BG, MGMT becomes a substrate for ubiquitin conjugation, leading to its subsequent degradation by the proteasome. Direct inhibitors of MGMT include the pseudosubstrates O^6-BG and O^6-BTG, as well as disulfiram. PDB structure references: 1EH6 (native MGMT), 1EH7 (O^6-MeG-bound MGMT) and 1EH8 (O^6-BG-bound MGMT) [54]; 1T38 (O^6-MeG-DNA) [55]. This figure is inspired from [36,57,58,60,62].

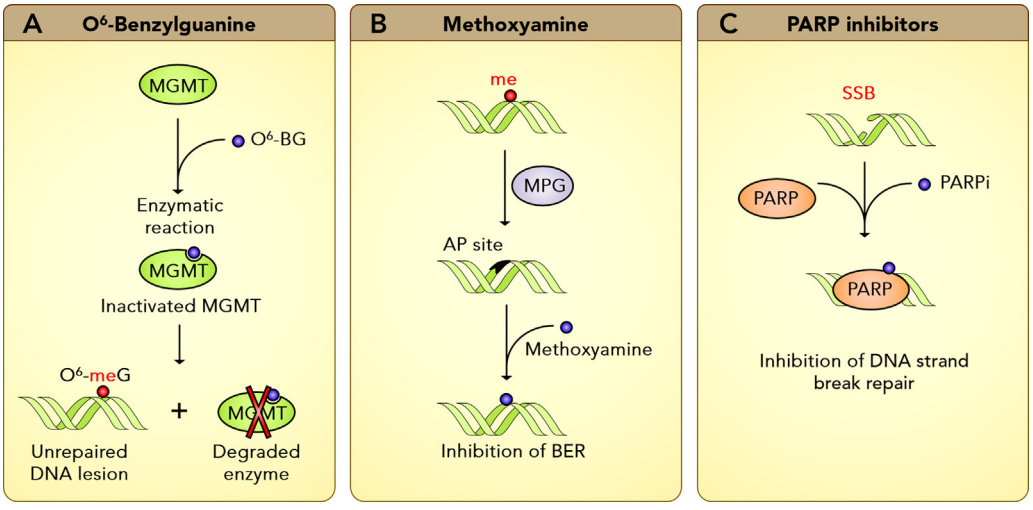

Fig. 4. Molecular basis of DNA repair inhibition by O6-benzylguanine, methoxyamine and PARP inhibitors. See text for details.

targets involved in cell-cycle arrest, DNA repair and apoptosis. ATM and ATR are activated, respectively, by DSBs and single-stranded DNA (ssDNA) structures. Key targets of ATM include p53 and the effector kinase CHK2, resulting in the control of the G1/S and intra-S checkpoints, whereas ATR acts primarily through CHK1 and controls the intra-S and G2/M checkpoints. However, there are overlaps between ATM- and ATR-dependent signaling [86].

Based on the characterization of genetically-engineered mouse models, Squatrito et al. [87] provided evidence that the ATM/CHK2/p53 pathway operates as a tumor suppressor in the brain. Chk2-/- mice exhibited increased GBM resistance to IR and failed to activate DNA-damage-induced cell-cycle checkpoints. The authors also showed that crucial DDR components were constitutively activated in primary GBM biopsies from patients not previously exposed to chemoradiation. Robust activation of the ATM/CHK2/p53 cascade in GBM specimens was also observed by Bartkova et al. [88] who identified oxidative DNA damage and replication stress as the underlying sources of endogenous genotoxic stress. Constitutive activation of the DDR has led to the suggestion that its inhibition might have potential application in the management of GBM. Thus, in contrast with the observations made in the Chk2 null glioma mouse model [87], dual inhibition of CHK1 and CHK2 [43] or ATM [89] sensitized GSCs to IR in vitro. Likewise, the ATM inhibitor KU-60019 has been described as an efficient radiosensitizer of GBM cells both in vitro [90] and, when administered intratumorally by convection-enhanced delivery or osmotic pump, in orthotopic GBM mouse models [91]. Importantly, the authors showed that p53 mutant GBM cells were much more sensitive to KU-60019 radiosensitization than their isogenic wild-type counterparts. The IR-induced synthetic lethality between p53 and ATM likely reflects the inability of DNA damage-ridden cells to execute functional cell cycle checkpoints, leading to mitotic catastrophe [92]. Other molecules that may show promise include AZ32, an orally-bioavailable, BBB-penetrant ATM inhibitor which has been reported to radiosensitize GBM cells in xenograft models [93].

The stimuli that activate ATM and ATR can also be generated during processing of the major lesions elicited by TMZ. BER-mediated removal of N^7-meG and N^3-meA – a process that is independent of MGMT and MMR, leads to the early phosphorylation/activation of CHK1 driven by ssDNA repair intermediates [94]. Although ssDNA and DSB stimuli are not produced when the O^6-MeG lesion is processed by MGMT, its handling by MMR in the absence of MGMT results in SSBs and DSBs that trigger the DDR and late activation of CHK1 and CHK2 [94,95]. Eich et al. [95] have shown that both ATM and ATR contribute to the resistance of GBM cells to TMZ in vitro; however, cells depleted of ATR by small interfering RNAs (siRNA) showed a more pronounced sensitivity to the drug compared to ATM-depleted cells, and knockdown of ATR, but not ATM, abolished phosphorylation of the DSB marker H2AX, as well as CHK1 and CHK2.

Litman-Flynn et al. [96] have shown that GBM cells relying on the alternative lengthening of telomere (ALT) pathway to overcome cell senescence (about 11% of adult GBM and 44% of pediatric GBM [97], see Section 6.1) were hypersensitive to ATR inhibitors. Such inhibitors appeared highly specific for ALT cells and may therefore offer a useful treatment approach in ALT-positive GBMs.

Aghinotri et al. [98] recently uncovered a novel function for ATM as an important regulator of BER in pediatric GBM, through modulation of MPG (3-methylpurine-DNA glycosylase, also known as alkylpurine–DNA-*N*-glycosylase (APNG)) glycosylase activity. The authors demonstrated direct phosphorylation of MPG by activated ATM, and showed that combined depletion of ATM and MPG resulted in increased TMZ-induced cytotoxicity in vitro and prolonged survival in mice with intracranial GBM xenografts. It was therefore suggested that ATM inhibition, in addition to preventing DSB repair, may sensitize cancer cells to TMZ through inhibition of BER, and that the combination of ATM and BER inhibitors may improve GBM treatment. The nature of the signals triggering ATM-mediated phosphorylation of MPG is currently unknown.

In summary, ATM and ATR appear to be attractive targets in combination with chemoradiation treatment.

5.2. Strategies to inhibit base excision repair

In 2012, Agnihotri et al. reported a positive correlation between the expression levels of the BER factor MPG and the TMZ IC50 in adult GBM cells, where MPG was found to promote TMZ resistance [99]. Later, the authors identified a similar correlation in pediatric GBM lines and also uncovered MPG and the apurinic/apyrimidinic (AP) endonuclease 1 (APEX1) amongst TMZ sensitizers in a siRNA screen with TMZ-resistant pediatric GBM cell lines [98]. Targeting MPG together with non-BER genes, or multiple targeting of BER genes in pediatric cells caused additive sensitivity to TMZ, whereas re-introduction of MPG in TMZ-sensitive cells conferred resistance to TMZ in orthotopic mouse models of pediatric and adult GBM [98].

MPG catalyzes the cleavage of N^7-meG and N^3-meA, generating abasic sites that are substrates for APEX1, and the MPG-APEX1 axis in GBM cells could serve as target for therapeutic strategies with methoxyamine (Fig. 4, panel B). This small molecule inhibitor binds the abasic sites generated by MPG, thereby preventing further processing. Compared to treatment with either molecule alone, the combination of methoxyamine plus TMZ dramatically increased survival of mice injected intracranially with SJG2 cells, as well as in an orthotopic PDX mouse model generated from pediatric GBM primary cells [98,99].

Other strategies targeting BER to sensitize GBM cells to TMZ include inhibition of DNA polymerase β whose lyase activity is required for excising 5′-deoxyribose phosphate (5′dRP) residues generated by APEX1 (Fig. 1) [100].

5.3. DSB repair pathways and their inhibition

Little or no sensitization towards IR was observed when the RAD51 recombinase was inhibited in LN-229 cells, whereas inhibition of the NHEJ factor DNA-dependent protein kinase (DNA-PK) resulted in increased sensitivity to IR irrespective of RAD51 knockdown [101]. Hypersensitization of GBM cells to IR was also observed following depletion of 53BP1, a positive regulator of NHEJ, in U87, U251 and T98G cells [102]. However, targeting HR in T98G [103] and GSCs [104] resulted in sensitization to IR, suggesting that HR can also contribute to IR-induced DSB repair in GBM.

The importance of the major DSB repair pathways in the response of GBM cells to alkylating agents was addressed by Quiros et al. [101], who described the impact of inhibiting HR or NHEJ on the response of LN-229 to TMZ. RNA interference (RNAi)-mediated depletion of the HR factors RAD51 or BRCA2 specifically sensitized GBM cells to TMZ in MGMT-negative backgrounds, underlining O^6-meG as the crucial lesion leading to DSBs in the absence of MGMT. That RAD51 knockdown resulted in increased sensitivity to TMZ in the absence of MGMT was confirmed by Short el al [103]. In contrast, hampering NHEJ through pharmacological inhibition of DNA-PK did not sensitize LN-229 cells significantly to TMZ [101]. Likewise, depletion of 53BP1 had no significant effect on the response of U87, U251 and T98G cells to TMZ [102]. It should be mentioned, however, that the NHEJ factors LIG4 and XRCC4 were identified as TMZ-sensitizers in pediatric GBM cell lines [98] whilst siRNA-mediated depletion of LIG4 sensitized A172 cells to TMZ [105]. Several alternative NHEJ pathways (A-NHEJ) have been described [22]. LIG4 and XRCC4 mediate the ultimate step of the

canonical NHEJ pathway. It is therefore possible that NHEJ intermediates are repairable by several alternative routes until the final ligation step which, once engaged through canonical NHEJ, can only be carried out by LIG4/XRCC4.

Recently, gene expression analysis using The Cancer Genome Atlas (TCGA) database by Rivera et al. [106] showed that components of the meiotic HR machinery are expressed in GBM. In addition, increased levels of the meiotic recombinase DMC1 were found in a battery of GBM cell lines compared to non-neoplastic brain. In vitro, siRNA-mediated depletion of DMC1 resulted in increased genomic instability and replication stress, and decreased proliferation in the absence of exogenous genotoxic stress, whereas it inhibited IR-induced activation of the DDR and radiosensitized GBM cells. Intracranial implantation of DMC1-depleted U87 in mice decreased tumor growth and prolonged survival. Pharmacological inhibition of DMC1 has hitherto not been reported.

5.4. Endogenous and chemoradiation-induced oxidative DNA damage in GBM: extra burden of DNA damage

Elevated rates of reactive oxygen species (ROS) production are a hallmark of most cancer cells where they are associated with tumor development and progression [107]. In GBM, increased ROS levels, DNA damage and genetic instability were observed in cells overexpressing EGFRvIII, a common oncogenic variant of EGFR amongst GBM patients [108], leading to a DNA repair pathway addiction phenotype that was exploited by Nitta et al. [109]. In this textbook illustration of the framework of non-oncogene addiction [110], the authors carried out a siRNA screen of 240 DDR genes, identifying BER genes involved in oxidative DNA damage repair, as well as poly(ADP-ribose) polymerase 1 (PARP1) (see Section 5.5) amongst those gene silencings that sensitized U87MG cells overexpressing EGFRvIII, but not the parental U87MG cell line, to IR. Of note, overexpression of EGFRvIII has also been linked to increased radioresistance in U87 cells, through the upregulation of DNA-PK [111].

In addition to causing base alkylation, TMZ, like IR, also induces oxidative DNA damage [112,113] which further mobilizes the BER machinery. Thus, Svilar et al. [114] showed that TMZ induced the production of ROS in GBM and validated the involvement of oxidative DNA glycosylases that do not recognize alkylated bases, but instead act upon oxidative DNA lesions, in a siRNA screen targeting “druggable” targets (5520 genes) for depletions conferring TMZ hypersensibility in T98G cells.

It therefore appears that BER and DSB repair mechanisms play a crucial role in the repair of oxidative DNA damage from endogenous and exogenous sources, which could be exploited in therapeutic approaches.

5.5. Strategies using PARP inhibitors

PARP1 is a sensor of SSBs involved in several forms of DNA repair (Fig. 1) [115]. Small molecule PARP inhibitors (PARPi), administered as monotherapies or in combination with genotoxic chemotherapeutics, have showed encouraging results against tumors harboring DNA repair defects, in which they induce synthetic lethality (a thorough review of the use of PARP inhibitors in the treatment of cancer is provided by [116]). Well characterized examples include the sensitivity of BRCA1 and BRCA2 deficient breast cancer cells defective in homologous recombination, to inhibition of PARP via small molecule inhibitors [117].

PARPi are thought to mediate their cytotoxic effects by trapping PARP1 enzymes (as well as the other PARP family member PARP2) on SSBs formed by endogenous cellular metabolism or as intermediates of crucial DNA repair pathways (Fig. 4, panel C). In addition, they prevent auto-PARylation (an activity required for dissociation of PARP from DNA and completion of repair) as well as PARylation of chromatin proteins that mediate the recruitment of DNA repair factors. Thus, by blocking pathways such as SSB repair, BER and A-NHEJ, persistent PARP-SSB complexes ultimately lead to replication collapse and the formation of DSBs that require HR for accurate repair.

The therapeutic potential of PARPi is not confined to cells deficient in HR. Thus, synthetic lethality was uncovered between deficiency in XRCC1 (involved in SSB repair, BER and A-NHEJ, Fig. 1) and PARP inhibition [118,119]. More recently, Horton et al. [120] showed that Polβ-/- and Xrcc1-/- mouse fibroblasts were hypersensitive to PARPi. In these defective cells, endogenous DNA damage led to unrepaired BER intermediates sufficient to trigger increase in PARP binding sites in the presence of PARPi, leading to replication fork disruption and DSB. The authors therefore suggested that BER deficiency could represent a therapeutic opportunity for PARPi single-agent therapy.

Several reports suggest that PARPi may also synergize with genotoxic therapeutics, including IR and TMZ, two treatments which bear direct relevance to GBM. Thus, Quiros et al. [101], presented evidence that MGMT-deficient LN-229 cells were hypersensitive to TMZ-induced DNA damage following treatment with the PARPi olaparib, which was exacerbated upon depletion of RAD51 recombinase. Dungey et al. [121] reported that olaparib sensitized the GBM cell lines T98G, UC373-MG, UVW and U87-MG to IR in vitro. A similar observation was made by Russo et al. [122] with U251 cells and the PARPi E7016. In both cases, treatment with PARPi was found to inhibit the repair of IR-induced DSBs. In addition, compared to IR plus TMZ, the trimodal combination of IR, TMZ and E7016 resulted in delayed tumor growth of U251 subcutaneous xenografts [122]. The sensitizing effect of another PARPi, ABT-888, in combination with IR and TMZ was also observed in various GBM cell lines [123].

Understanding the determinants of PARPi sensitivity and the mechanisms of resistance to PARPi is crucial to identify the cancer patient populations that may benefit from treatment with PARPi. Resistance to PARPi can occur via i) secondary mutations restoring BRCA1/2 open reading frame, ii) overexpression of the RAD51 recombinase and/or loss of PARP1 expression, iii) upregulation of drug efflux pumps, and iv) loss of the DNA repair factor 53BP1 [124]. In a recent study, Venere et al. [52] provided additional support for GBM clinical trials with PARPi, showing that, compared with non-GSCs, GSCs exhibited higher levels of ROS, increased oxidative base damage and SSBs, and exacerbated dependence on PARP1 activity, which could be exploited in vitro and in vivo. Thus, inhibition of PARP sensitized GSCs to IR, hampered growth, self-renewal and DNA repair, and inhibited tumor initiation in orthotopic xenotransplantation experiments [52]. The sensitivity of GSCs to PARPi was also reported by Majuelos et al. [125] using olaparib. In addition, the authors tested the effect of PARPi in U87 (PTEN-deficient) and LN229 (PTEN-proficient) GBM cells. PTEN-deficient cells were more sensitive to olaparib than the proficient cells, and this sensitivity was not increased in combination with IR or TMZ. Microarray analysis of U87 cells following treatment with PARPi revealed the down-regulation of HR factors, which was corroborated by the observation that HR is impaired in PTEN-deficient cells treated with PARPi.

Taken together, these studies suggest that conditions of increased ROS levels (e.g., cells expressing EGFRvIII), as well as defective BER or HR may provide contexts in which GBM patients might be amenable to PARPi-based therapeutic strategies. Single-agent and combination clinical trials involving PARPi have been reviewed [116]. A phase I clinical trial is underway to test the combination of olaparib and TMZ in patients with relapsed GBM (clinicalTrials.gov identifier: NCT01390571).

Table 1
List of abbreviations.

Abbreviation	Definition
2-HG	2-hydroxyglutarate
ALT	alternative lengthening of telomeres
A-NHEJ	alternative non-homologous end-joining
AP site	apurinic/apyrimidinic site
BBB	blood brain barrier
BCNU	bis-chloroethylnitrosourea, carmustine
BER	base excision repair
CCNU	1-(-2-chloroethyl)-3-cyclohexyl-1-nitrosourea, lomustine
CED	convection-enhanced delivery
CNS	central nervous system
CRISPR	clustered regularly-interspaced short palindromic repeats
DDR	DNA damage response
DSB	double-strand break
FA	fanconi anemia
GBM	glioblastoma
G-CIMP	glioma CpG island methylator phenotype
GEMM	genetically-engineered mouse model
GSC	glioma stem cell
HDAC	histone deacetylase
HR	homologous recombination
IR	ionizing radiation
MMR	mismatch repair
N^1-meA	N^1-methyladenine
N^3-meA	N^3-methyladenine
N^3-meC	N^3-methylcytosine
N^7-meG	N^7-methylguanine
NHEJ	non-homologous end-joining
O^6-BG	O^6-benzylguanine
O^6-BTG	O^6-bromothenylguanine, lomeguatrib
O^6-meG	O^6-methylguanine
PARPi	PARP inhibitor
PDB	protein database
PDX	patient derived xenograft
PRC2	polycomb repressive complex 2
RNAi	RNA interference
ROS	reactive oxygen species
RT	radiotherapy
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNP	single nucleotide polymorphism
SSB	single strand break
ssDNA	single-stranded DNA
TALEN	transcription activator-like effector nuclease
TCGA	The Cancer Genome Atlas
TMZ	temozolomide
α-KG	α-ketoglutarate

6. Novel DNA-damage response-related biomarkers of GBM

Table 2 summarizes the DDR- and DNA repair-relevant features of well-established molecular alterations that have been used in clinical diagnosis and prognosis of adult and pediatric GBMs, as well as recently-described alterations that impact the DDR, some of which are proving valuable as markers to improve the classification of gliomas and/or as therapeutic targets [155,156]. Here, we focus on telomere-maintenance mechanisms and DNA repair changes associated with epigenetic alterations in pediatric and adult GBM. Specifically, we address alterations affecting histone H3 variants, the histone chaperones ATRX and DAXX, the histone methyltransferases EZH2 and SETD2, and also isocitrate dehydrogenase IDH1 (including its impact on the glioma-CpG island methylator phenotype (G-CIMP)).

6.1. Telomere-maintenance mechanisms

Telomeres are nucleoprotein structures that protect the ends of chromosomes and prevent their recognition as DSBs. Although maintenance of telomeres requires the taming of DSB repair pathways (NHEJ and HR), other DNA repair factors collaborate with telomere-specific factors and epigenetic mechanisms to protect chromosome ends [157]. Central to the synthesis of telomeric DNA sequences is telomerase, a specialized ribonucleoprotein reverse transcriptase. In most cells, telomerase activity decreases as cells differentiate and telomeres gradually shorten after each round of cell division, resulting in DNA instability, cellular senescence and ultimately cell death. In order to maintain viable telomere length, tumor cells rely either on the re-expression of telomerase or a telomerase-independent, HR-dependent mechanism called alternative lengthening of telomeres (ALT). Reactivation of telomerase is largely limited to adult GBMs (80% vs 3–11% in pediatric GBM), whereas, a reverse pattern is observed for ALT (pediatric GBMs (44%), adult GBMs (11%)) [158].

In recent years, several studies have examined the prognostic value of telomerase-associated parameters like telomere length, telomerase activity and expression of its catalytic subunit, hTERT, in relation to GBM. Expression of hTERT is associated with high telomerase activity and adverse prognosis in GBM patients [146]. Induction of hTERT expression through mutations in the promoter core region of hTERT is responsible for the reactivation of the telomerase in various cancer types including GBM. In a cohort of GBM patients, where 80% were found to carry hTERT promoter mutation, a bad prognostic value was confined to those patients who also harbored the G-allele of a SNP in the promoter of hTERT (rs2853669) [149,159].

Overexpression of hTERT in normal fibroblasts led to enhancement of DNA repair capacity [148]. Whether a similar situation occurs in GBM tumors might explain why increased hTERT expression confers resistance to genotoxics and poorer patient survival. In line with the observation that RNAi-mediated depletion of hTERT resulted in impaired DNA damage response and sensitivity to IR [160], direct inhibition of telomerase activity in GSCs using the antagonist imetelstat [162], or treatment of telomerase-reactivated GBM cells with quadruplex-selective ligands that stabilize G-quadruplex structures present in telomeric DNA [161,162], inhibited cellular proliferation and sensitized cells to IR in vitro. A similar observation was made with ALT-positive GBM cells [163], although these cells were naturally more resistant to IR than GSCs with reactivated telomerase [164].

6.2. DNA repair changes associated with epigenetic alterations in pediatric and adult GBMs

6.2.1. Driver mutations in histone H3 variants and histone chaperones

Recent analyses have underlined the unique molecular features of pediatric GBMs [5,165,166]. Specifically, driver mutations in the H3F3A gene encoding the histone variant H3.3 as well as in the H3.3 histone chaperones ATRX and DAXX were uncovered at high frequency in pediatric GBMs [136,137]. The point mutations in H3.3 resulted in the amino acid substitutions K27M or G34R/V, which were mutually exclusive and heterozygously expressed. K27M substitutions were also observed in the HIST1H3B gene encoding H3.1. Residues K27 and K36 (close to H3G34) in H3/H3.3 are critical sites for post-translational modifications, and the epigenetic impact of the observed mutations was seen at the level of gene expression and chromatin dynamics. Thus, K27M and G34R/V mutations showed distinct gene expression profiles and DNA methylation patterns. Importantly, nearly all tumors bearing G34R/V mutations also exhibited mutations in ATRX/DAXX and displayed the ALT-phenotype [136,167], in line with the documented requirement of these chaperones for the deposition of H3.3 at normal telomeres [168,169]. The H3.3K27M mutation resulted in reduced levels of H3K27me3, a repressive mark deposited by EZH2, the catalytic subunit of polycomb repressive complex 2 (PRC2). In

Table 2
Established and newly described alterations affecting DNA repair and chromatin factors in primary adult and pediatric GBMs.

Gene	Function	Alteration type*	General effect	Impact on DDR	Drug**	Ref.
Established markers in primary adult GBMs						
MGMT	Removal of O^6-meG in DNA	CpG-island promoter methylation	Increased overall survival in patients presenting alteration	Reduced O^6-meG removal; increased response to TMZ treatment	O^6-BG	[4,31,126]
IDH1	TCA cycle enzyme	Gain-of-function mutation: R132H (±5%)	Alteration of metabolic, DNA methylation and gene expression profiles	Increased RAD51-mediated HR; 2-HG mediated inhibition of ALKBH2/3	AGI-5198	[4,19,126–128]
EGFR	Cell surface tyrosine kinase receptor	Focal amplification (±50%) as well as activating, intragenic deletions (i.e: EGFRvIII variant)	Tumor growth stimulation by activation of downstream RAS/MAP, mTOR and PI3K/Akt signaling pathways	EGFRvIII variant is associated to increased ROS production and DNA damage, as well as activation of DNA-PKCs and NHEJ-mediated DSB repair	Erlotinib Cetuximab	[4,109,111,126]
PDGFRA	Cell surface tyrosine kinase receptor	Focal amplification (11%) and activating, intragenic deletion (Δe8-9)	Stimulation of tumor growth	***	Sorafenib Imatinib	[4,126,129]
PTEN	Tumor suppressor, inhibitor of oncogenic Akt signaling pathway	Focal deletion (10%), mutation (30%)	Re-activation of Akt pathway signaling	Impaired DNA repair in GBM cells; compromised HR in normal astrocytes	/	[4,126,130–132]
TP53	Tumor suppressor, DDR factor involved in cell cycle regulation and apoptosis modulation	Mutation and LOH	Loss of cell cycle checkpoint control, tumor proliferation	See Section 4.1	/	[4,126,133]
CDKN2A/B	Tumor suppressor, negative regulator of cell cycle, regulator of p53	Focal/Homozygous deletion (60%), promoter hypermethylation	Dysregulation of cell cycle control	***	/	[4,126]
Established alterations in primary pediatric GBMs						
H3F3A/B	Histone variant H3.3	Mutations (K27M, G34V/R, K36M)	Epigenetic changes and alteration of gene expression profiles	See Section 6.2.1	/	[134–136]
HIST1H3B	Histone variant H3.1	Mutation (K27M)	Epigenetic changes and alteration of gene expression profiles	See Section 6.2.1	/	[137]
ATRX	Histone H3.3 chaperone	Mutation	Decreased H3.3 deposition at telomeres, epigenetic changes, ALT-phenotype	Increased sensitivity to DNA damaging agents; increased DNA damage	/	[136,138]
SETD2	H3K36 specific tri-methyltransferase	Mutation (15%)	Defective H3K36 tri-methylation	Defective HR-mediated DSB repair; impaired MMR	/	[139–142]
Recently described alterations in primary GBMs						
EZH2	Mono-, di- and tri-methylation of H3K27	Up-regulation	Epigenetic changes and alteration of gene expression profiles; tumor proliferation	Inhibition associated with increased expression of HR genes, including RAD51	GSK126 DZNeP	[143–145]
hTERT	Catalytic subunit of telomerase	Overexpression through activating mutation in promoter core region (80%)	Cellular transformation, immortalization	Improved DNA repair capacity	GRN163L Telomestatin	[146–149]
ID1	Transcription factor	High expression (50%)	Increased tumor growth	Deficient DDR; downregulation of LIG4 and ATM	Cannabidiol	[150,151]
PGRN	Secreted growth factor	Overexpression	Tumor progression	Increased DDR and resistance to TMZ	/	[152]
XRCC6BP1	DNA –dependent protein kinase	Amplification (14%)	Increased proliferation	Improved DSB repair	/	[153,154]

Abbreviations: ATRX, α-thalassemia/mental retardation syndrome X-linked; CDKN2A/B, Cyclin-dependent kinase inhibitor 2A/B; EGFR, Epidermal growth factor receptor; EZH2, Enhancer of zeste homolog 2; H3F3A/B, H3.3 histone; HIST1H3B, histone cluster 1, H3b; hTERT, human telomerase reverse transcriptase; ID1, Inhibitor of DNA binding 1; IDH1, Isocitrate dehydrogenase 1; MGMT, O6-methylguanine-DNA methyltransferase; PDGFRA, platelet-derived growth factor receptor; PGRN, Progranulin; PTEN, phosphatase and tensin homolog; SETD2, SET domain containing 2; TP53, tumor protein p53; XRCC6BP1, XRCC6 binding protein 1; 2-HG, 2-hydroxyglutarate; α-KG, ketoglutarate.

* Incidence when available, unless quoted in the text.

** Example of drug.

*** These important markers have been included although the association between the indicated alterations and DNA repair has not been reported in GBM.

addition, mutant H3.3K27M was shown to interact with EZH2 and inhibit PRC2 activity [170].

The oncogenic mechanisms associated with the H3.3K27M and H3.3G34R/V mutations in pediatric GBMs have been reviewed by Yuen [166]. Remarkably, a recent report by Gallo et al. [171] identified mixed lineage leukemia 5 (MLL5)-mediated repression of H3F3B, the second gene encoding human H3.3, as a mechanism that phenocopies the DNA methylation profiles of pediatric GBMs with H3.3 mutations, in adult GSCs. Moreover, the authors demonstrated that MLL5 action impacts chromatin structure, thus modulating gene expression to maintain the tumorigenic and self-renewal properties of adult GSCs.

Whether H3.3 mutations or expression alterations result in altered DNA repair capacities in pediatric GBM remains to be investigated. However, studies of the phenotypes associated with EZH2 depletion suggest a strong connection between epigenetic remodeling and DNA repair in pediatric as well as adult GBM. EZH2 is overexpressed in adult and pediatric GBM [172]. This observation led de Vries et al. [143] to target EZH2 via inducible short hairpin RNAs (shRNA) in mouse-derived GSCs. Prolonged EZH2

depletion led to global reformatting of gene expression profiles yielding highly proliferative and undifferentiated tumors and resulting in increased tumor progression. Importantly, gene ontology analysis identified DNA Repair as a major enriched term in EZH2-depleted GBMs where key HR factors including RAD51 were upregulated. In vitro and in vivo experiments indicated that enhanced DNA repair resulting from EZH2 inhibition decreased the sensitivity of GBM cells to TMZ, highlighting a crucial role for RAD51-mediated DSB repair in this process. The authors suggest that concomitant inhibition of EZH2 and HR (e.g., RAD51) might potentiate TMZ toxicity in GBM [143].

Although they may operate more indirectly, the H3.3G34R/V mutations, like H3K27M, could also affect DNA damage response activities in GBM. Indeed, point mutations in H3.3G34 decrease the levels of H3K36me3 on the same and nearby nucleosomes, and the evidence suggests that the G34R/V mutant histones disrupt the activity of SETD2, the only methyltransferase that mediates trimethylation of K36me2 in H3 and H3.3 [166]. H3.3 variant histones are enriched at telomeres and pericentromeres. Thus, it remains to be seen how H3.3G34R/V-induced local perturbations of H3K36me3 and H3.3K36me3 might affect DNA repair and genetic instability. However, the documented role of H3K36me3, a mark associated with transcription elongation in active genes, as an important modulator of MMR [141] and HR-mediated DNA repair [142,173] suggests that depletion of SETD2 may impact the response of GBM cells to genotoxics. Importantly, point mutations in SETD2 have been identified in pediatric and adult GBMs [174].

6.2.2. IDH1 and the glioma-CpG island methylator phenotype (G-CIMP)

Mutations in isocitrate dehydrogenase (IDH) 1 and 2 (metabolic enzymes which convert isocitrate into α-ketoglutarate (α-KG, also called 2-oxoglutarate)) occur predominantly as driver mutations in low grade gliomas and secondary high grade gliomas, although IDH1 mutations have been observed in 6% of GBM [4,175–177]. IDH1 mutations are associated with longer patient survival and improved response to TMZ. The most frequent mutation in IDH1 is R132H, a gain-of-function mutation that confers the ability to convert α-KG into 2-hydroxyglutarate (2-HG). Because 2-HG inhibits Fe(II)- and 2-oxoglutarate-dependent oxygenases such as histone lysine demethylases [178] and DNA demethylases [179], its accumulation in IDH1R132H tumors has the potential to generate global epigenetic defects and alter gene expression [180]. The R132H mutation in IDH1 is closely associated with the glioma-CpG island methylator phenotype (G-CIMP) defined as the concerted hypermethylation of a large number of loci [181]. Although it is more prevalent among low- and intermediate-grade gliomas, this phenotype appears to be also enriched in a subset of primary GBMs belonging to the proneural subtype (one of 4 GBM subtypes – proneural, neural, classical, mesenchymal – proposed by Verhaak et al. [66]) where it is associated with a younger age at diagnosis and better survival [181]. Expression of a G-CIMP phenotype, which could be obtained by the sole introduction of exogenous IDH1R132H into primary astrocytes was suggested to result from 2-HG-mediated inhibition of DNA demethylating enzymes such as TET2, and probably also from DNA-methylation promoting alterations in H3K9me2 and H3K27me3 [182]. Recently, a refined classification of gliomas based on G-CIMP status, DNA methylation profiles and IDH mutation has been proposed [183].

The impact of mutant IDH1 on the cellular response to TMZ was investigated by Ohba et al. [127], using MGMT-deficient human astrocytes immortalized by expression of virally-encoded E6, E7 and hTERT, and infected with lentiviral constructs encoding wild-type or mutant IDH1 (which in both cases led to cellular transformation). Compared with wild-type cells, IDH1R132H cells displayed more efficient processing of TMZ-induced DSB lesions and increased survival. Furthermore, mutant IDH1-driven transformation resulted in enhanced RAD51-mediated HR, and siRNA-mediated depletion of RAD51 reduced the TMZ resistance conferred by overexpression of mutant IDH1. Thus inhibitors of HR may offer an efficient therapeutic options in IDH1 mutant GBM tumors.

In addition to an indirect effect resulting from the impact of global epigenetic and gene expression alterations, mutations in IDH1 have recently been shown to also exert a direct effect on alkylated DNA repair enzymes in GBM cells. Thus, Wang et al. [19] found that 2-HG produced by IDH1 mutants inhibited, in vitro, purified recombinant forms of the Fe(II)- and α-KG-dependent DNA demethylases ALKBH2 and ALKBH3, the major enzymes involved in the removal of N^1-meA and N^3-meC (Fig. 1). Furthermore, characterization of GBM cells engineered to express wild-type or R132H mutant IDH1, together with manipulation of heterozygous $IDH1^{+/R132C}$ fibrosarcoma cells and ALKBH2/3-overexpression experiments indicated that mutant IDH1 sensitize cells to DNA alkylating agents through 2-HG-mediated inhibition of ALKBH2 and ALKBH3. The clinical relevance of these findings are supported by a previous report that ALKBH2 confers resistance to TMZ in GBM cells [20].

IDH1-mediated oxidative decarboxylation of isocitrate to α-KG is accompanied by production of NADPH from NADP+. IDH1 mutant cells are thought to display lower production of NADPH, an important player in the cellular defense against oxidative damage, which was proposed to sensitize GBM to chemoradiation [184]. Shi et al. [185] showed that the decreased intracellular NADPH levels elicited by overexpression of the IDH1R132H mutation were associated with glutathione (GSH) depletion and ROS generation. However, conflicting data have been obtained when the impact of IDH1R132H overexpression on the response of GBM cells to genotoxics was tested. Thus, one study found that, compared with control cells and cells overexpressing wild-type IDH1, overexpression of IDH1R132H sensitized U87 and U251 cells to TMZ [186]. Another study found that overexpression of IDH1R132H conferred sensitivity to IR, but not TMZ in U87 and U373, whereas overexpression of wild-type IDH1 increased resistance to TMZ compared to control cells [141]. It should be noted that IDH1-overexpressing cell lines most likely do not recapitulate the endogenous IDH1 mutant status of glioma cells (see Section 7.2).

Although MGMT promoter methylation is more prevalent in G-CIMP GBMs than in non-G-CIMP GBMs (79% vs 46%, respectively), MGMT promoter methylation correlated with patient response only in the classical subgroup but not in the proneural subgroup [4]. Furthermore, analysis of hypermethylated and downregulated genes within proneural G-CIMP positive tumors [181], did not show significant enrichment in DNA damage signaling and DNA repair. These observations suggest that the impact of the G-CIMP phenotype on DNA repair in GBMs might be limited.

7. Discussion

7.1. Integrative strategies that must be considered for genotoxic-based therapeutic management of GBM

Elucidating the DNA repair pathways and factors activated by GBM cells in response to IR and TMZ-induced lesions, identifying selective DNA repair inhibitors that can be used as monotherapy or in combination with chemoradiation, and defining the (epi)genetic features that shape the DNA-repair makeup of GBM cells, are crucial steps to tackle the development of resistance currently seen in GBM patients whilst tailoring treatment to the patient's tumor.

Significant progress has been made in our understanding of the molecular details of the DNA repair mechanisms that operate in GBM, leading to the discovery of novel, druggable DNA repair axes,

including the ATM-BER axis reported by Agnihotri et al. [98]. Such studies are expanding the list of pathways that could be targeted by a given inhibitor. In addition, several studies have helped refine the molecular settings in which specific DNA repair inhibitors, such as PARPi, can be used. Most relevant in this respect is the fact that GBM cells have to cope with pervasive oxidative DNA damage resulting from ROS production, rendering them more dependent upon the BER machinery.

GBM driver mutations have recently been described in genes encoding (e.g., H3.3, ATRX, DAXX) or affecting (e.g., IDH1) epigenetic factors, raising the issue of whether drugs capable of reprogramming abnormal epigenomes may compose part of the therapeutic arsenal against glioblastoma [187]. Although driver mutations in histone H3 variants have been discovered in pediatric GBMs, the evidence suggests that at least some of their phenotypic consequences might also be recapitulated in adult GBMs, through MLL5-mediated repression of H3.3 expression. Moreover, it is notable that the resulting amino-acid changes in H3.3 mutants affect post-translational modifications that can also be impacted by alterations in epigenetic writers such as EZH2 and SETD2, as such alterations are also found in adult GBMs. Thus, future lessons learned from the characterization of pediatric GBMs might, to some extent, serve in the management of adult GBMs as well. The changes in gene expression and chromatin dynamics induced by such mutations/alterations appear to impact the DNA repair capacity and response of GBM cells to chemoradiation. Based on the documented effect of histone deacetylase (HDAC) inhibitors [188,189] and DNA methyltransferase inhibitors [190,191] on DNA repair, it is tempting to speculate that epigenetic drugs may help reverse the adverse effects (e.g., resistance to genotoxics) associated with altered DNA repair in GBM, in part by impacting the expression of DNA repair factors or perturbing DNA repair-associated chromatin modifications. Moreover, HDAC inhibitors have been shown to trigger not only widespread changes in histone acetylation but also, by mechanisms that remain obscure, the production of ROS [188], thus imposing an extra burden for DNA repair that might be exploited in therapeutic strategies.

How specific GBM driver mutations/alterations affecting other processes than epigenetics impact DNA repair remains a matter of investigation that would greatly benefit from the characterization of DNA repair genes and pathways in GEMMs. Likewise, knocking down selected DNA repair genes in these models may help elucidate the contribution of specific DNA repair defects (for instance, caused by downregulation of DNA repair factors) to glioblastomagenesis and response to genotoxics.

7.2. Considerations on the cellular and animal models used to study DNA repair in GBM

The great variety of GBM-derived cells currently used both in vitro and in preclinical studies with animal models raises issues that are of paramount importance in DNA repair and chemotherapy. Indeed, preservation of the genetic and epigenetic alterations of the original tumor is crucial to maintain the "DNA repair makeup" of the tumor cell and study its impact on DNA repair and cellular response to genotoxics. Although they commonly serve as in vitro model, established GBM cell lines (such as U87, U251, T98 etc.) suffer the limitation of having incurred genetic alterations, changes in DNA ploidy, clonal selection and novel gene–gene interactions during adaptation to and prolonged passages in monolayer cultures. The inability of these models to preserve genetic and epigenetic features of the original tumor is also reflected in their failure to recapitulate the pathohistological GBM phenotypes in xenografts [47,192]. Thus, tumor invasion, infiltration, necrosis, and vascular proliferation in these models are usually not comparable to a GBM in patients. The questionable pertinence of such cellular models for the study of DNA damage also stems from the conflictual results to which they can give rise, as illustrated, e.g., by two studies of the response to TMZ conferred by mutant IDH1 expressed in U87 cells ([141,186], see Section 6.2.2). Although the observed discrepancies might be due to inter-laboratory variations within U87 lines, it may be argued that U87 cells, which were established from an adult GBM, do not represent an adequate model to investigate the impact of mutant IDH1 since, unlike the astrocyte-derived model generated by Ohba [127], expression of the IDH1 mutation played no role in their genesis. Thus, extrapolating conclusions from studies involving established GBM cell lines must be done with caution. Organotypic spheroids formed from patient-derived GBM biopsies better preserve the original tissue architecture, the genomic profile and DNA ploidy of the parental tumor [7,193]. Importantly, such spheroids do not undergo passaging and selection in vitro and, instead, can be maintained by serial transplantation in vivo, which maintains the original genetic and phenotypic heterogeneity of the biospy [194]. Similarly, GSC sphere cultures partially reiterate the molecular features of the original tumor and we have recently shown that such cell lines establish similar histological phenotypes as organotypic spheroid-based xenografts [194]. Nevertheless, GSC cultures also undergo in vitro selection and changes in DNA ploidy [7].

GBM cell lines are often preferred tools because they can be engineered to express a transgene or subjected to RNA interference-mediated gene silencing using shRNAs. In the case of PDXs based on organotypic spheroids, efficient gene silencing is challenging but can be obtained with lentiviral vectors without selection, which minimizes the risks of genetic drift. In principle, xenograft models should also be amenable to gene knockout through genome-editing technologies such as TALEN (Transcription Activator-Like Effector Nucleases) or CRISPR (Clustered, Regularly Interspaced, Short Palindromic Repeats), although the cellular manipulations and time constraints associated with these techniques will lead to selection processes and possibly genetic drift.

7.3. Non-coding RNAs in the response of GBM tumors to chemoradiation

Targeting of miRs regulating DNA repair is associated with a therapeutic potential [195]. With the exception of the miRs that affect MGMT expression, this review has not addressed the roles played by miRs in glioblastomagenesis and response to genotoxics. Other non-coding RNAs known as long non-coding RNAs have emerged as essential elements involved in GBM development and progression [196]. The roles of non-coding RNAs in DNA repair as well as their potential as biomarkers and potential therapeutic targets in the management of GBM remains largely to be elucidated.

7.4. Improving the delivery of chemotherapeutic agents to the brain

The need for chemotherapeutic agents that cross the BBB efficiently and maintain an effective steady state concentration remains a serious challenge in the management of GBM. With the exception of TMZ, most of the anti-cancer agents that have been tested for the management of GBM fail to cross the BBB efficiently [197]. Due to a short half-life in plasma, TMZ itself must be administered in high systemic doses to achieve therapeutic levels in the brain, leading to undesired side-effects, whereas the TMZ/O^6-BG combination results in hematopoietic toxicity.

Local intracerebral administration of drugs or macromolecules has been regarded as desirable in order to bypass the obstacle of the BBB and eliminate the potential undesired toxicity. Delivery

technologies like drug-impregnated wafers (such as BCNU-loaded polymers (Gliadel® Wafer) [198]), Ommaya reservoir, or convection-enhanced delivery (CED) systems, make it possible to use drugs that do not necessarily cross the BBB or whose adverse systemic effects are too unsafe for the patient [199]. CED, for instance, delivers drugs, macromolecules or nanoparticules through one to several catheters placed stereotactically directly within the resection cavity. Its safety was established in a study investigating the delivery of a MGMT-siRNA/cationic liposome complex in normal rat and pig brains treated or not with TMZ [200]. Animal studies have also shown that CED-mediated distribution of O^6-BG via nanoparticles, in parallel with TMZ treatment resulted in a 3-fold increase in median overall survival compared with TMZ-only control animals [201].

Although such delivery techniques should greatly widen the pipeline of genotoxic agents and DNA inhibitors that could be used to treat GBM, the invasive nature of these procedures remains an obstacle. Thus, the design of novel anti-cancer agents, including DNA repair inhibitors, with better CNS bioavailability and the ability to be administered intravenously or orally remains a crucial challenge in the management of GBM.

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