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GBM Radiosensitizers: Dead in the Water…or Just the Beginning?

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Abstract:	The finding that most GBMs recur either near or within the primary site after radiotherapy has fueled great interest in the development of radiosensitizers to enhance local control. Unfortunately, decades of clinical trials testing a wide range of novel therapeutic approaches have failed to yield any clinically viable radiosensitizers (1). However, it is well-recognized that temozolomide chemotherapy is administered concurrently with radiotherapy specifically as a radiosensitizer. Furthermore, it can be argued that many of the previous radiosensitizing strategies, for GBM and many other tumor types were not backed by firm pre-clinical evidence supporting a synergistic or additive interaction (2). Another poorly understood variable is lack of blood-brain barrier penetration as potential limitation for treatment efficacy, as until recently most clinical trials did not assess the actual drug levels in GBM tumors. Finally, despite an understanding that DNA repair status was an important variable for radiotherapy treatment responses dating back to the 1970's, the actual DNA repair pathways and proteins were only fully elucidated in the last decade. Here, we present recent progress in the use of small molecule DNA damage response inhibitors as GBM radiosensitizers. In addition, we discuss the latest progress in targeting hypoxia and oxidative stress for GBM radiosensitization.

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Ranjit S. Bindra, Anthony J. Chalmers, Sydney Evans, and Mark Dewhirst

Introduction. The finding that most GBMs recur either near or within the primary site after radiotherapy has fueled great interest in the development of radiosensitizers to enhance local control. Unfortunately, decades of clinical trials testing a wide range of novel therapeutic approaches have failed to yield any clinically viable radiosensitizers (1). However, it is well-recognized that temozolomide chemotherapy is administered concurrently with radiotherapy specifically as a radiosensitizer. Furthermore, it can be argued that many of the previous radiosensitizing strategies, for GBM and many other tumor types were not backed by firm pre-clinical evidence supporting a synergistic or additive interaction (2). Another poorly understood variable is lack of blood-brain barrier penetration as potential limitation for treatment efficacy, as until recently most clinical trials did not assess the actual drug levels in GBM tumors. Finally, despite an understanding that DNA repair status was an important variable for radiotherapy treatment responses dating back to the 1970's, the actual DNA repair pathways and proteins were only fully elucidated in the last decade. Here, we present recent progress in the use of small molecule DNA damage response inhibitors as GBM radiosensitizers. In addition, we discuss the latest progress in targeting hypoxia and oxidative stress for GBM radiosensitization.

SECTION I. TARGETING DNA DAMAGE RESPONSE PATHWAYS FOR GBM RADIOSENSITIZATION

A. Overview of the Key DNA Damage Response Pathways

DNA repair pathways are critical for tumor cell survival after exposure to DNA damaging agents such as ionizing radiation (IR) and a range of cytotoxic chemotherapies, and as such they have emerged as excellent targets for radiosensitization. Double-strand breaks (DSBs) are the most lethal threats to genomic integrity, and as such mammalian cells have developed complex mechanisms to detect and repair these lesions. With regard to proximal DNA damage sensing, DSBs activate ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) proteins, which in turn activate two downstream key kinases, checkpoint kinases 1 (Chk1) and 2 (Chk2) (3). The end result is cell cycle arrest and DNA repair, which are closely intertwined. By transiently arresting or delaying the cell cycle, they provide the necessary time for the repair of a lesion prior to DNA replication and mitosis, where unrepaired lesions can lead to mitotic cell death. Once recognized, there are two main pathways to repair DSBs: homologous recombination (HR) and non-homologous end joining. While HR utilizes homologous DNA sequences as a template for repair, NHEJ processes and re-ligates the ends of the breaks (4). Examples of key HR proteins include BRCA1 and BRCA2, which are mutated in both hereditary and sporadic forms of breast and ovarian cancer (5). DNA-PK plays a critical role in NHEJ, by forming the initial bridge across DSBs to initiate repair (6).

It is well established that other DNA repair pathways also are important for cell survival after IR, including base excision repair (BER; (7)). Poly (ADP-Ribose) Polymerase (PARP) proteins play important roles in BER, and down-regulation of these factors induce radiosensitivity. PARP inhibitors recently have emerged as potentially useful radiosensitizers and are now being tested in clinical trials. PARP inhibitors lead to increased rates of DSBs by at least two known mechanisms: (A) BER inhibition leads to increased single-strand breaks (SSBs) in the genome which are eventually converted to DSBs in S-phase at replication forks; and (B) these inhibitors "trap" PARP at sites of unrepaired DNA damage, which has a dominant negative effect on DSB repair by limiting access to these lesions. These two mechanisms provide the basis for why PARP inhibitors preferentially target actively replicating cells (8). This particular feature makes these drugs attractive as radiosensitizers, since tumors typically contain large numbers of replicating cells and have defective cell cycle checkpoints (9).

Finally, the Wee1 kinase has also emerged as an important mediator of the radiation-induced G2/M checkpoint (10). Wee1 is phosphorylated by Chk1 in response to DNA damage. This checkpoint plays a key role in repairing DNA damage prior to entry into mitosis. Inhibition of Wee1 has been shown to abrogate G_2/M arrest and propel cells into premature mitosis which can ultimately lead to cell death via mitotic catastrophe or apoptosis (11).

Below, we review recent progress in the development of small molecule-based strategies to inhibit the targets presented above for glioma radiosensitization. Numerous inhibitors have been developed for DNA damage response proteins over the last decade, and many of them have been tested, or are currently being tested, in clinical trials (summarized in Table 1). As a full description of this work is beyond the scope of this brief review, we present the most promising developments here.

B. Targeting PARP Proteins

Several small molecule inhibitors of PARP are in clinical development; most of these have activity against both PARP-1 and PARP-2, which are the 'DNA repair' members of the PARP family. The PARP inhibitors in clinical use function by competing with NAD at PARP's catalytic site, however different compounds are associated with different levels of 'PARP trapping'. Whether these differences have any clinical significance is not yet known. Extensive clinical experience with olaparib (AstraZeneca), veliparib (Abbvie), rucaparib (Clovis), niraparib (Tesaro) and talazoparib (Medivation) as single agents, mainly in the treatment of HR deficient cancers, has shown these agents to be extremely well tolerated (12). GBM do not generally exhibit HR deficiency but there is a large body of pre-clinical data to support the use of PARP inhibitors in combination with both radiotherapy and temozolomide (13). The observation that the radiosensitising effects of these agents are observed only in replicating cells supports the exciting hypothesis that tumour control will be increased without adverse effects on the normal brain (14).

Unfortunately, clinical evaluation of these combinations in GBM has been delayed by uncertainties over blood-brain barrier penetration and the well-established propensity of PARP inhibitors to exacerbate the hematological toxicity of temozolomide. For example, one study reported that rucaparib had poor penetration into the CNS, suggesting it would have limited activity as a temozolomide sensitizer in GBM (15). Recently, however, the field has been revitalized by the demonstration that olaparib penetrates GBM at therapeutic levels *in situ* in patients (16), and also by promising toxicity and efficacy data arising from a phase I study of veliparib in combination with whole brain radiotherapy for brain metastases (17), and several phase I studies are now underway.

The risk of excess bone marrow toxicity can be side-stepped by combining a PARP inhibitor with radiotherapy alone, an approach that has been adopted in two different UK phase I studies: the PARADIGM trial, in which olaparib is combined with short course radiotherapy (40 Gy in 15 fractions) in elderly patients, and PARADIGM-2, in which younger patients with MGMT unmethylated tumors receive standard radiotherapy (60 Gy in 30 fractions) together with olaparib instead of temozolomide. PARADIGM-2 is also recruiting patients with MGMT methylated tumors to a parallel arm in which olaparib is added to both radiotherapy and temozolomide in a cautious dose escalation design. The aim of this arm is to identify an intermittent olaparib dosing schedule that can be safely combined with temozolomide. These studies incorporate translational research programs aimed at identifying molecular biomarkers to identify which patients will benefit from the addition of the PARP inhibitor to conventional treatment.

C. Targeting the ATM and ATR Signaling Nodes

As the chief 'proximal' DNA damage signaling molecule, ATM regulates a multitude of cell cycle checkpoint processes and also plays an important role in facilitating DNA repair. Consistent with this, ATM inhibitors are highly potent radiosensitizers. Exciting pre-clinical data show that the constitutive upregulation of ATM signaling that is associated with the impressive radioresistance of GBM stem-like cells renders them particularly sensitive to the radiosensitising effects of ATM inhibitors (18). This *in vitro* phenomenon translates into marked improvement in tumour control and mouse survival in orthotopic xenograft models of GBM (19). In this study the radiosensitizing effects of ATM inhibition were reported to be dependent upon the presence of p53 mutations in the target tumor, but other studies have yielded different results and it is not yet clear whether p53 status per se or G1/S checkpoint proficiency in general is the key factor. Of note, G1/S checkpoint defects are observed in the vast majority of GBM whereas p53 mutations or deletions are observed in approximately one third of cases. ATM inhibitors are in pre-clinical development and GBM has been identified as a key tumour site for early phase clinical testing. While their mechanism of action predicts tumor-specific sensitization (20), the magnitude of the radiosensitizing effects of these compounds mandates a cautious approach in the clinic.

ATR inhibitors are less potent radiosensitisers than ATM targeting drugs, but they show exciting combination activity in a variety of preclinical models (21) and have similarly promising activity against GBM stem-like cells. Mechanistic cellular studies indicate that dual inhibition of cell cycle checkpoints and DNA repair mechanisms yields the most effective radiosensitization of GBM stem-like cells and there is exciting potential to combine small molecule inhibitors of ATR with the PARP inhibitor compounds described above (22). Systemic toxicity is likely to be an issue in this context, especially if temozolomide is included in the therapeutic cocktail, but intelligent scheduling of DNA damage response inhibitors, along with judicious combination with targeted radiotherapy, should enable a beneficial therapeutic index to be achieved. The AstraZeneca ATR inhibitor, AZD6738, is currently undergoing 'first in human' evaluation in combination with radiotherapy in the UK PATRIOT trial but, to the authors' knowledge, studies in GBM are not currently in development.

Many of the functions of ATM and ATR are mediated via the downstream signaling proteins Chk1 and Chk2. Small molecule inhibitors of Chk1 exhibit similar radiosensitising effects to ATR inhibitors in the preclinical setting, but clinical development has been hindered by the cardiotoxicity that was observed in phase I studies of the first clinical candidates (23). Chk2 inhibitors programs are currently at the pre-clinical stage, with early data indicating an interesting synergy with PARP inhibitors rather than potentiation of genotoxic agents. Phase I/II studies of the Eli Lilly Chk1 inhibitor, LY2606368, are underway but the portfolio does not currently include GBM.

D. Targeting NHEJ and HR Repair

The HR pathway is an attractive potential target for GBM because this pathway is most active in S/G2-phase cells, which suggests a favorable therapeutic index. There are very few specific HR inhibitors in pre-clinical development at this time, and most of them appear to inhibit Rad51 (24). Recent studies have suggested that these molecules may be sensitize the effects of alkylators (25) and radiotherapy (26) specifically in glioma. Small molecule inhibitors of canonical NHEJ proteins, such as DNA-PK, have been developed and are now being tested as radiosensitizers in clinical trials, although currently there are no studies specifically in GBM (27). One concern regarding the clinical utility of these agents is the lack of a therapeutic index, since normal tissue typically relies on canonical NHEJ repair to address DNA damage induced by radiotherapy (28).

Non-canonical NHEJ repair has recently emerged as a potentially viable target for tumor cell radiosensitization, as recent studies suggest this may be an active pathway that competes with HR, or serves as a back-up pathway, in replicating tumor cells (29). We recently identified a novel inhibitor of this pathway, mibefradil dihydrochloride, which was previously FDA-approved for the treatment of hypertension (30). We subsequently demonstrated substantial radiosensitization by mibefradil in glioma cell lines, which also was independently confirmed by another laboratory *in vivo* using a rat C6 glioma model (31). Based on these findings, we recently designed a single center, open-label Phase I trial testing whether we could repurpose this drug as a radiosensitizer in recurrent GBM (NCT02202993). A subset of eligible subjects are offered the option to participate in a translational research sub-study that administers mibefradil for 5 days prior to planned surgery, and a sample of resected tissue is sent for analysis of mibefradil brain tissue concentrations. Preliminary results in the first 13 patients show that mibefradil can be safely combined with RT, and several intriguing cases of local control were observed in our study patients, including complete responses (CRs) at the treated sites. Moreover, results from 2 translational-surgical sub-study subjects have demonstrated mibefradil brain tumor tissue concentrations up to 3.5 micromolar in contrast-enhancing tumor tissue, and up to 0.788 μM in non-enhancing tissue. These levels are well within the range of concentrations required for radiosensitization *in vitro* (32,33). While this is a Phase I trial with primary safety endpoints, these data are nonetheless promising for potential efficacy, and the highlight the potential for targeting non-canonical NHEJ for GBM radiosensitization.

E. G2/M Checkpoint Targeting

The preclinical efficacy of the Wee1 inhibitor, MK-1775, as a radiosensitizer first was studied in p53-deficient mouse xenograft models (34), and others subsequently demonstrated that inhibition of Wee1 is also effective in p53 wild-type tumors, including a pediatric high-grade glioma xenograft model (35). The successful pre-clinical studies led to clinical trial testing in humans. Recently, Do *et al* published a phase I trial which examined the safety of MK-1775 monotherapy (36). The drug exhibited a half-life of approximately 11 hours and common toxicities included myelosuppression and diarrhea. There are currently two ongoing clinical trials that are studying the efficacy of Wee1 inhibitors with radiation in CNS tumors. In the ABTC1202 trial, MK-1775 is being combined with radiation and temozolomide for newly diagnosed or recurrent GBM (NCT01849146). In another ongoing Phase I trial, MK-1775 is being studied together with local radiation for pediatric patients with diffuse intrinsic pontine glioma (NCT01922076). Whether the combination of radiation and MK-1775 is effective in humans remains to be seen, since MK-1775 was recently reported to have limited blood brain barrier penetration in mouse xenograft models of GBM (37). In contrast, Sanai and colleagues recently reported excellent uptake in GBM tumors *in situ*, in a Phase 0 trial in patients with recurrent GBM (38). This particular finding highlights the need to analyze drug penetration in clinical trials, since mouse models may not always accurately predict blood-brain-barrier and GBM tumor penetration in humans.

SECTION II. A critical assessment of failure to effectively eliminate hypoxia as a part of multimodal therapy.

A. Overview and Rationale for Targeting Hypoxia

Classical radiobiology dictates that hypoxic tumor cells are approximately 3 times more resistant to radiotherapy

and therefore could dictate the overall radioresponse of tumors if they are not eliminated (39). With the discovery of the hypoxia-inducible transcription factor (HIF) family (40,41), we now know that hypoxia drives angiogenesis, promotes cell motility and self-renewal of stem cells in GBM (42). The global effects of hypoxia in driving a more malignant phenotype dictate the need to selectively eliminate these cells.

B. Evidence for Hypoxia and the Need to Measure it for Hypoxia Modification Trials in Glioblastoma

If one is going to use a method to target hypoxic tumor cells, it is important to verify that hypoxia is present. This principle has been painfully recognized in the aftermath of many failed randomized trials (43). Having a mixture of tumors, where only a proportion are hypoxic increases trial size by 8-10 fold to have the power to show a therapeutic benefit (44). Patient selection is thus a requirement. There is evidence that patient selection can be achieved. In three clinical trials better outcome was found in patients who exhibited evidence for tumor hypoxia, prior to initiation of hypoxia mitigation + radiotherapy compared with radiotherapy alone (45). Thus, modification of hypoxia should not be attempted unless its presence can be verified in each subject's tumor.

The polarographic needle electrode was used extensively to define the presence and clinical relevance of hypoxia in peripherally accessible cancers (46). However, Eppendorf electrode studies in patients with GB required anesthesia, which influenced the level of hypoxia detected (47). Evans and Koch, used the hypoxia marker drug, EF5, administered prior to surgical resection of GB, to better define extent of hypoxia. EF5 binding was calibrated to a maximum binding control determined *in vitro* with tissue from the same patient; this technique uniquely allows quantification of the actual pO_2 levels, based on quantitative analysis of immunohistochemistry. GB, but not lower grade gliomas, contained regions of severe to moderate hypoxia (pO_2= 0.76-3.8 mm Hg; (48); Figure 1**)**. The overall percent of moderately to severely hypoxic cells was low, so the pO2 status of a whole GB was moderately-mildly hypoxic (pO2= 3.8-19 mm Hg). This has ramifications for hypoxia imaging and hypoxia-targeted drug development. Imaging methods must be capable of identifying small regions of hypoxia and drugs must be able to penetrate into such regions in order to be effective.

C. Tumor Hypoxia Imaging.

The most commonly studied non-invasive oxygen imaging techniques are positron emission tomography (PET) and magnetic resonance imaging (MRI; (49)). Both MRI and PET are susceptible to partial volume effects related to variation in extent of hypoxic subregions within and surrounding an imaging voxel. Hypoxic cords surrounding a microvessel have dimensions of a few 10's of microns – far below the spatial resolution of either MRI (mm^3) or PET (cm^3). However, groups of hypoxic cords tend to occur together. These clusters of hypoxic zones, in aggregate, can be seen with imaging. MRI is more likely to detect location and extent of hypoxia than PET, because voxel sizes with MRI are closer to the size of hypoxic aggregates (45). These two imaging modalities are briefly summarized below.

i. Magnetic resonance imaging: Several MRI techniques have been associated with hypoxia in human tumors(49). Here, we focus on Blood Oxygen Level Detection (BOLD) and Oxygen Enhanced MRI (OE-MRI)(45,49). These methods are based upon measurement of MR signal change when switching from air to hyperoxic gas breathing. BOLD imaging is based on changes in T2*, which are governed by hemoglobin-saturation and -concentration. This MRI method is subject to artifact, because hyperoxic gases are vasoactive; the vasoactivity can change microvascular hematocrit, independent of change in hemoglobin saturation (50). OE-MRI is based on measurement of T1 relaxation, which is sensitive to dissolved oxygen in tissue (51). It is not subject to hemodynamic artifacts caused by high oxygen content gas breathing. OE-MRI has been evaluated in pre-clinical models of GB as well as in patients with GB(52). Boxerman and Ellingson (53) emphasize the measurement variability of MRI techniques and the importance for standardizing image acquisition parameters.

ii. Positron Emission Tomography (PET). PET hypoxia radiotracers are primarily labeled 2-nitroimidazoles, which bind to hypoxic tumor regions. The 2-nitroimidazole-based PET agents that have been tested in human brain studies include ^{18}F-MISO (54) and ^{18}F-EF5 (55,56). CuATSM, which has a different mechanism of binding, has also been studied in GB (57).

D. Clinical Trials Involving Hypoxia Mitigation in GBM

The most extensively studied hypoxia mitigators in GBM were the hypoxic radiosensitizer family of nitroimidazoles

(58). The first positive randomized study came from Canada using metronidazole as a hypoxic radiosensitizer. The encouraging results of this study stimulated several additional trials, including two large cooperative group randomized trials. However, none of the follow up trials was significant. Unexpected toxicities necessitated that the drugs could not be given with every radiation dose fraction. The altered drug dose schedule may have compromised any chance of seeing benefit.

Hyperbaric oxygen breathing has been tested in combination with radiotherapy for GB. The most promising results involved having patients breathe hyperbaric gas prior to radiotherapy. Although this seems counterintuitive, the tumors remain hyperoxygenated for several minutes after decompression, allowing time to deliver the radiotherapy dose (59). However, no properly powered randomized studies have been conducted. Further, to be contemporary, future studies should include standard of care, temozolomide.

Two randomized trials were recently compared the combination of the VEGF inhibitor, bevacizumab + radiotherapy + temozolomide vs. radiotherapy + temozolomide for treatment of newly diagnosed GBM (60,61). Since it has been reported that inhibition of VEGF signaling can lead to at least transient vascular "normalization", one might conclude that this therapy could improve GBM oxygenation and increase radiosensitivity (62). Both trials exhibited a slight prolongation of progression free survival, but no significant effect on overall survival.

Importantly, no measurements of hypoxia have been made in any of the trials reported above, so we do not know if tumors that were relatively more hypoxic may have benefitted more from the mitigation strategies.

E. Targeting Oxidative Stress

It is well established that the level of oxidative stress in tumors exceeds that in normal tissue. This occurs because of downregulation of catalase and periredoxins, in the face of upregulated superoxide dismutase (63). This difference in redox balance between tumor and normal tissue has been exploited using a series of cationic Mn(III) N-substituted pyridyl porphyrins (MnPs; Figure 2, panel A). Interestingly, MnPs, which exhibit catalytic reducing capacity, have been shown to be potent protectors against late radiation damage in normal tissue (64,65). In the brain, MnPs protect against white matter loss and preserve neurocognition after radiotherapy or radiotherapy plus temozolomide ((65); Figure 2, panel B).

In tumors, on the other hand, MnPs are radiosensitizers. We reported recently that these drugs prolong GB xenograft growth delay when combined with radiotherapy or radiotherapy+temozolomide ((65,66); Figure 2, panel C). In a head and neck cancer model, there is a significant shift downward in TCD_{50} (radiation dose to cure 50% of animals), with a dose modifying factor of 1.3 (64). We have shown previously that oxidative stress increases over several days after radiotherapy, and that this increase in oxidative stress is accompanied by upregulation of HIF-1 transcriptional activity as a result of stabilization of the oxygen sensitive HIF-1α subunit (67,68). HIF-1 drives production of VEGF and anaerobic metabolism, both of which protect tumor from radiation damage. MnPs have been shown to block the upregulation of HIF-1 transcription, thereby resulting in radiosensitization (67).

The lead Mn(III) N-substituted pyridyl porphyrin compound, MnTnBuOE-2-PyP^{5+} (MnBuOE) has entered into a first in man phase I/II trial in patients with newly diagnosed GBM (NCT02655601; Trial of newly diagnosed high grade glioma treated with concurrent radiation therapy, temozolomide and MnBuOE). Secondary outcome variables included whether the drug preserves neurocognitive function and improves progression free survival.

SECTION III. Conclusions and Future Directions.

The myriad failures of radiosensitizers in GBM have prompted many to conclude that this therapeutic approach has little potential to improve treatment efficacy for this disease. However, we would argue that more rationally designed strategies, with small molecule inhibitors of targeting specific DNA repair proteins, has great potential to enhance local control in GBM. In addition, while most GBM studies targeting hypoxia did not yield improved treatment efficacy previously, better screening and selection of patients with tumors that have high levels of hypoxia would be critical for success. In addition, targeting oxidative stress may represent a unique approach to enhance GBM tumor cell kill while simultaneously reducing normal tissue toxicity.

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Table

Examples of Clinically Relevant Radiosensitizers Targeting DNA Damage Response Pathways			
Molecular Pathway	**Target(s)**	**Drugs in Clinical Trials Testing**	**Clinical Development Stage (as a radiosensitizer)**
Base Excision Repair	PARP Proteins	Olaparib, Veliparib	Phase I/II Clinical Trials; Glioma
Proximal DDR Sensors	ATM	AZD0156	None
	ATR	VX-970, AZD6738	Phase I Trials; H&N cancers
	CHK1/2	LY2606368, CCT245737	None
Non-homologous End Joining	DNA-PKcs	MSC2490484A	Phase I Trials; Metastatic Cancer
G2/M Checkpoint	Wee1	AZD1775	Phase I Trials; Glioma

Table 1