New chemotherapy options for the treatment of malignant gliomas

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This review focuses on the recent advances in chemotherapy of malignant gliomas, with special emphasis on the most common primary brain tumor in adults, glioblastoma. The demonstration of the superiority of concomitant and adjuvant temozolomide with standard radiotherapy over radiotherapy alone in patients with newly diagnosed glioblastomas by means of phase III international trial has been the major advance in the care of these patients so far. Moreover, patients whose tumors display the hypermethylation of the promoter of the gene for the repairing enzyme O^6-methylguanine-DMA methyltransferase are most likely to benefit from the combination regimen. The advantage of a postsurgical local administration of carmustine by slow-release polymers ('gliadel wafers') is more modest, and the efficacy and safety of a sequence of carmustine wafers followed by temozolomide combined with radiotherapy remain to be defined. Different DNA repair modulation strategies are being investigated to further improve the results: dose-dense regimens of temozolomide, combination of temozolomide with specific inhibitors of O^6-methylguanine-DMA methyltransferase and combination of temozolomide with specific inhibitors of base excision repair [poly(ADP-ribose) polymerase inhibitors]. Other developments include the combination of cytotoxic, cytostatic and targeted therapies. Multitargeted compounds that simultaneously affect multiple signaling pathways, such as those involving epidermal growth factor receptor, platelet-derived growth factor receptor and vascular endothelial growth factor receptor, are increasingly employed. In the future, innovative trial designs (factorial and adaptative designs), pretreatment molecular profiling of individual tumors and the adoption of biological end-points (changes in serum tumor markers, measures of target inhibition), in addition to the traditional clinical and radiographic end-points, will be needed to achieve further advances. *Anti-Cancer Drugs* 18:621–632

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Introduction

Despite advances in neuroimaging, neurosurgery, radiation and medical oncology, the prognosis for patients with malignant gliomas [glioblastomas (GBMs) and anaplastic astrocytomas (AAs)] has changed little in the last 30 years. Median survival of patients with GBM, the most frequent tumor type, is 9–12 months, with the vast majority of patients dying within 2 years and less than 5% surviving at 5 years. Young age and good Karnofsky performance status have been identified as major prognostic factors.

The clinical management of these tumors includes corticosteroids to reduce peritumoral edema, anticonvulsants to control epileptic seizures, maximally safe surgical debulking, and fractionated external-beam radiation therapy to the tumor and surrounding margins.

The treatment of brain tumors with antineoplastic drugs poses several unique challenges. The blood–brain barrier (BBB) allows a rapid diffusion into the nervous tissue of highly lipid-soluble and low-molecular-weight compounds, although restricting the passage of water-soluble compounds and macromolecules. As demonstrated by enhancement on computed tomography or magnetic resonance imaging (MRI) BBB disruption in GBM is variable and minimal or absent in the brain adjacent to tumor in which infiltrating neoplastic cells are present. Thus, for chemotherapeutic drugs with poor BBB penetration, it is difficult to achieve therapeutic concentrations in all tumor-bearing areas. Moreover, patients with brain tumors frequently receive corticosteroids and this can reestablish the BBB integrity. The second challenge concerns the concurrent use of antiepileptic drugs that can profoundly impact the pharmacokinetics of several antineoplastic agents. Phenytoin, carbamazepine and phenobarbital induce hepatic cytochrome P450 enzymes, markedly accelerating the metabolism and clearance of anticancer drugs [nitrosoureas, procarbazine, paclitaxel, irinotecan (CPT-11), topotecan, cyclophosphamide, ifosfamide, doxorubicin, vinca alkaloids, etoposide, teniposide, thiotepa, gefitinib,

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erlotinib, imatinib, etc.] that are metabolized by the same system [1,2].

Adjuvant chemotherapy

During the past three decades, thousands of patients with malignant gliomas have been entered on adjuvant chemotherapy trials in North America and Europe. The most commonly used drugs have been nitrosoureas, either alone [bis-chloroethylnitrosourea (BCNU)] or in combination with other agents [PCV: procarbazine, lomustine [1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU)], vincristine]. Studies of BCNU originally documented a modest 10–15% benefit in survival at 18 months, but no difference in median survival or survival at 12 or 24 months [3,4]. A randomized study of 674 patients with newly diagnosed high-grade gliomas compared radiotherapy alone versus radiotherapy with PCV [5]. This study found that PCV provided no additional survival advantage either for the overall patient population or for patients with GBM or AA. PCV has been suggested to be superior to BCNU alone in the adjuvant treatment of AAs [6], but this finding was not confirmed in a retrospective review of patients who had participated in Radiation Therapy Oncology Group (RTOG) trials [7].

Two metaanalyses of clinical trials for high-grade gliomas have demonstrated therapeutic benefits from the addition of nitrosourea-based chemotherapy to radiotherapy [8,9]. The study of the Glioma Metaanalysis Trialist Group [9] was performed on individual patient data from 12 randomized trials and showed a modest, but significant prolongation of survival associated with the addition of chemotherapy: an absolute increase in 1-year survival of 6% (from 40 to 46%) and in 2-year survival of 5% (from 15 to 20%). No difference was found by age, sex, histology, performance status, extent of resection and single versus combination chemotherapy.

Similarly, two moderately sized, prospective, randomized, double-blind, placebo-controlled trials of locally administered BCNU have demonstrated a small, but statistically significant improvement in survival. Biodegradable polymers, loaded with 3.85% BCNU ('gliadel wafers') and designed to release the drug slowly over a 2–3-week period, were placed along the surface of the tumor resection cavity. The first study enrolled 222 patients with recurrent high-grade gliomas who were candidates for resection [10]. The median survival after BCNU wafers was 31 weeks compared with 23 weeks for placebo wafers, with a 6-month mortality of 44 versus 64% ($P = 0.02$). On the basis of this study, the US Food and Drug Administration (FDA) approved BCNU wafers for the treatment of recurrent high-grade gliomas. A similarly designed adjuvant study was conducted in 240 patients with newly diagnosed high-grade gliomas who had initial total or subtotal resection followed by conventional radiotherapy starting 2 weeks after wafer implantation [11]. The median survival of patients receiving BCNU wafers was 13.9 versus 11.6 months for placebo-treated patients ($P = 0.03$), with a risk reduction of 29%. For the subset of patients with GBM, the median survivals were 13.5 and 11.4 months ($P = 0.10$), with a 24% reduction in the risk of death. The survival advantage after BCNU wafers noted on earlier analysis persists through longer follow-up: the 2- and 3-year survival rates were 15.8 and 9.2% compared with 8.3 and 1.7% after placebo wafers [12].

Temozolomide (TMZ), an oral alkylating agent with ability to cross an intact BBB and excellent toxicity profile [13], has demonstrated antitumor activity as a single agent in the treatment of recurrent malignant gliomas after conventional therapies [14–16], with response rates (complete + partial) ranging from 35% for AAs to 5% for GBMs. Regarding recurrent GBMs, a progression-free survival (PFS) at 6 months of 21% [15] compared favorably with a rate of 15% considered as the cutoff-point to define an ineffective regimen in this setting [17]. The FDA approved TMZ for the treatment of recurrent AAs only, whereas the European authorities approved the drug for both AAs and GBMs. The approved schedule ('standard regimen') was a daily dose of 150–200 mg/m^2 for 5 days of every 28-day cycle. On the basis of experimental data showing a synergism between TMZ and radiation [18,19], and the availability of an extended continuous schedule of the drug for up to 7 weeks [20], Stupp *et al.* [21] conducted a pilot phase II trial for newly diagnosed GBMs with concomitant administration of TMZ (75 mg/m^2/day) and conventional fractionated radiotherapy, followed by up to six cycles of adjuvant TMZ using the standard regimen. As this regimen showed promising clinical activity (median survival of 16 months and 2-year survival rate of 31%), the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada Clinical Trials Group launched a randomized multicenter phase III trial to compare this regimen with radiotherapy alone. From August 2000 to March 2002, 573 patients with newly diagnosed GBM from 85 institutions in 15 countries were enrolled, and the results were published in the *New England Journal of Medicine* in 2005 [22]. The median survival was 14.6 months after radiotherapy and TMZ compared with 12.1 months after radiotherapy alone ($P < 0.001$), with a 37% reduction in the risk of death. The 2-year survival rate was 26.5% in the group given the combined treatment as compared with 10.4% in the group given radiotherapy alone. The combination of radiotherapy and TMZ was associated with a significant improvement in median survival in all subgroups of patients with the exception of those who had a biopsy only and those with a poor performance status. The combined treatment was well tolerated: grade 3 or 4 hematologic toxicity was observed in 7% of patients

during the concomitant part and in 14% during the adjuvant part; 88% of patients received more than 90% of the planned dose of TMZ and interruptions or delays were generally brief. At disease progression, many patients were retreated: 68% of the patients in the radiotherapy only arm had additional chemotherapy, with 56% receiving TMZ; in the radiotherapy and TMZ arm 49% had additional chemotherapy, and 25% received further TMZ. At a median follow-up of 28 months, there was no evidence of an increase in treatment-induced late toxic effects. Accordingly, the same group of investigators reported that the combination regimen does not negatively affect the health-related quality of life in comparison with radiotherapy alone [23].

Why did this trial show a greater benefit with TMZ than all the previous trials with nitrosoureas? Several explanations can be put forward [24]. First, most patients had favorable prognostic factors (i.e. age under 70 years, good performance status, total or partial resection). Second, TMZ is better tolerated and may be more active than nitrosoureas. Third, the concurrent administration of the drug during radiotherapy, by exploiting the radiosensitizing properties, may have played an important role in improving the outcome. This latter hypothesis is supported by the fact that the adjuvant part of chemotherapy (six cycles) was relatively short and the same regimen of TMZ was of limited efficacy when employed at relapse.

Neoadjuvant TMZ has not been proven to be superior over the conventional treatment [25–27].

Mechanisms of chemoresistance

A number of mechanisms of resistance to chemotherapeutic drugs have been reported in brain tumors [28–30], but to date only a few have known clinical relevance.

The most important mechanism of resistance in malignant gliomas is based on the activity of the cellular enzyme O^6-methylguanine-DNA methyltransferase (MGMT). This enzyme removes methyl adducts at the O-6 position of guanine, thus repairing the damage to DNA induced by alkylating agents. A correlation of tumor MGMT levels (evaluated by immunohistochemistry) with outcome of malignant glioma patients has been reported with both BCNU [31] and TMZ [25]: patients with low MGMT levels (i.e. those with a reduced capacity of repair of DNA damage) displayed significantly longer survivals and/or higher response rates. The MGMT function is frequently lost in the presence of CpG island hypermethylation in the promoter region of certain types of human tumors, including brain tumors [32], and the inactivation of genes by methylation is a common epigenetic event during malignant progression [33]. The methylation of the promoter of the MGMT gene turns off its transcription, thereby reducing the intracellular level of MGMT. Recent studies reported that the methylation of the MGMT promoter predicts a better outcome in patients with malignant gliomas (both AAs and GBMs) who were treated with nitrosourea-based regimens [34–37] or TMZ [38,37]. Hegi *et al.* [39] assessed the methylation status of the promoter of MGMT in 307 of 573 (53.6%) GBM patients enrolled in the EORTC trial. The MGMT status could be determined for 206 patients and in 45% the promoter of the gene was methylated. This study confirmed MGMT promoter status as an independent prognostic factor: the median survival for the entire population irrespective of treatments was 18.2 months among patients with methylated MGMT promoter versus 12.2 months among patients with unmethylated MGMT promoter ($P < 0.001$). When treatment modalities were taken into account, among patients with MGMT promoter methylation median survival was 21.7 months for those receiving TMZ and radiotherapy versus 15.3 months for those receiving radiotherapy alone ($P < 0.007$), with 2-year survival rates of 46 and 22.7%, respectively. By contrast, among patients with unmethylated MGMT promoter, the difference in survival favouring the group with TMZ and radiotherapy was less significant ($P = 0.06$): the median survival was 12.7 months versus 11.8 months, with 2-year survival rates of 13.8% and less than 2%, respectively. The predictive value of MGMT promoter status was confirmed by analysis of progression-free survival according to the treatment received: among patients with methylated MGMT promoter, those who received the combined treatment had a progression-free survival of 10.3 versus 5.9 months for those who received radiotherapy alone; among patients with unmethylated MGMT promoter, those who received the combined treatment had a progression-free survival of 5.3 versus 4.4 months for those who received radiotherapy alone.

Despite the survival benefit after combination of radiotherapy with TMZ or, to a lesser extent, with nitrosoureas, the survival curves for combined treatments and radiotherapy alone remain similar for the first 9–12 months. This means that a proportion of patients, even if they have MGMT promoter methylation, fails early when on chemotherapy and other factors/mechanisms are likely to be relevant for the outcome, and need to be identified. Some data suggest that epidermal growth factor receptor (EGFR) overexpression/amplification could be associated with resistance to alkylating agents [40].

The methylation status of the MGMT promoter in the initial tumor does not correlate with clinical response to TMZ when the drug is administrated at relapse [37]. This suggests that the methylation status, and in general

the genetic patterns, may change from newly diagnosed to recurrent tumors, thus rendering the analysis of biomarkers in the original tumor as rather unreliable to predict the response to treatment or outcome at relapse.

A number of issues exist regarding MGMT testing. First, the determination of the methylation status of MGMT promoter by polymerase chain reaction (PCR), as compared with analysis of gene or protein expression levels or enzyme activity, has the advantage that it is not susceptible to contamination by tumor-infiltrating lymphocytes or normal tissue (that may express considerable amounts of MGMT protein). Second, cryopreserved tumor specimens yield the best results with methylation-specific PCR. On the other hand, MGMT expression is inducible by corticosteroids, ionizing radiation and genotoxic agents, the MGMT promoter being unmethylated [41]: thus, studies are needed comparing the methylation status of the promoter and MGMT expression in the tissue.

The DNA-mismatch repair pathway influences the cytotoxicity of alkylating agents, including TMZ. A defective mismatch repair confers resistance to TMZ, even in the absence of MGMT [42,43]. In a clinical trial, patients with newly diagnosed GBM and AA had their tumors assessed for mismatch repair proteins before commencing TMZ therapy [25]: all three of the complete responders had high levels of mismatch repair proteins, as did 12 of the 14 partial responders.

The 'multidrug resistance' system, codified by several genes, leads to a reduced intracellular uptake of drugs, such as epipodophyllotoxins, anthracyclines, vinca alkaloids, cisplatin and taxanes, due to an increased activity of transport proteins (P-glycoprotein, BCRP, MRP) across the cellular membranes of endothelial and tumor cells [28]. Molecular agents such as imatinib and gefitinib have been employed in clinical trials for GBM, and have shown high affinity for the multidrug transporters, suggesting that their efficacy might be altered by these mechanisms.

New standard of care for first-line therapy and new questions

Following the publication of the EORTC trial results, both the FDA and European Medicines Agency (EMEA) approved TMZ and radiotherapy for the treatment of newly diagnosed GBMs. Most European and North American clinical research protocols have been revised to accommodate the need for concomitant and adjuvant TMZ and radiation rather than radiation alone as their standard treatment arm. Similarly, one can expect that the new standard of care will be increasingly adopted both in academic and community practice, making adjuvant chemotherapy more popular than in the past when nitrosoureas only were available [44].

The therapeutic benefit of the combined treatment is largely confined to patients with silencing of the DNA repair gene MGMT by promoter methylation. This implies that in clinical trials the MGMT promoter methylation status will be a stratification factor and could even lead to a separate design depending on the presence/absence of the molecular alteration. In clinical practice, it is reasonable to offer TMZ with radiotherapy to all patients with newly diagnosed GBM, with the exception of those with poor performance status whose life expectancy appears short. This approach is based on different reasons: the assay for MGMT promoter methylation needs to be validated in additional studies, it is not informative in a number of patients and it is not widely available to most practicing clinicians. Moreover, there is a lack of active drugs to be offered in alternative to TMZ.

Two important questions have been raised by the results of the EORTC trial. First, could a prolongation of TMZ in the adjuvant phase (i.e. for 12 instead of six cycles) further improve the outcome of GBM patients? Second, should concomitant and adjuvant TMZ and radiation be the standard treatment for grade III gliomas (AA1 and anaplastic oligodendroglial tumors) as well? An argument in favor of the latter is that the most recent metaanalyses [9] did not show any difference between GBM and AA with respect to adjuvant chemotherapy, and that high response rates were observed in recurrent AAs and AOAs after TMZ [14]. Arguments against this approach are the potential risk of neurotoxicity in long-term survivors and the lack of benefit in terms of overall survival after adjuvant chemotherapy with PCV in anaplastic oligodendroglial tumors [45,46], even if PCV is different from TMZ and TMZ could be superior to PCV. In this regard, there is general agreement among the different cooperative groups in the US and Europe to develop new randomized trials to validate the generalization of the combined regimen.

The role of locally administered adjuvant BCNU is more controversial. The FDA and EMEA have approved 'gliadel wafers' for the treatment of newly diagnosed GBMs, on the basis of a benefit in survival that was statistically significant for the whole population of malignant gliomas, but not for GBMs. In the US, this has led to an increasing use of 'gliadel wafers' after extensive resection of malignant gliomas outside of the clinical trial setting [47]. The adverse events after 'gliadel wafers' (seizures, cerebrospinal fluid leaks, healing abnormalities, intracranial infections) have been modest in the Westphal's study [11,12], but a recent retrospective review of GBM patients receiving gliadel

followed by radiotherapy [48] reported that up to 50% of patients had neurologic symptoms during radiation requiring steroids and 33% of those undergoing reoperation for suspected tumor recurrence on MRI had pathologic findings of necrosis or treatments effects without active tumor. Thus, more data are needed regarding safety and quality of life of patients treated by wafers containing both low (3.85%) and high (up to 20%) BCNU doses [49]. BCNU wafers are not indicated in patients with GBM who have deep or multifocal disease, tumor in eloquent areas or extending to the ventricles, or who are not surgical candidates.

From population-based studies, it is well known that there is a proportion (1–2%) of GBM patients who survive 3–5 years [50,51], and this proportion may increase after TMZ and radiotherapy (up to 25% at 3 years) [21]. Long-surviving patients are generally young, have a good performance status at diagnosis and have undergone aggressive multimodality treatment (gross total resection, radiotherapy and adjuvant chemotherapy). These favorable prognostic factors, however, cannot entirely explain the long-term survival as most other patients with similarly favorable factors survive only for a few months. Thus, another question to be answered is whether long-term survivors of GBM represent a peculiar molecular subtype [52]: in addition to MGMT promoter methylation, 1p deletion [53], overexpression of p53 [54] and low EGFR expression [55,56] have been reported in these patients. Overall, the molecular factors investigated so far in GBM seem of limited prognostic value [57]. Possibly, multimarker profiling, using genomic, transcriptomic and proteomic techniques, will better identify subsets of GBMs who are likely to be long-term survivors or/and respond to radiotherapy and chemotherapy.

DNA repair modulation strategies

MGMT is a suicide enzyme, which is irreversibly inactivated after accepting a methyl group from DNA onto an internal cysteine residue within its active site; thus, the restoration of the protective mechanism requires 'de-novo' protein synthesis. MGMT levels fall after TMZ dosing as DNA damage is repaired. The timing of this depletion and subsequent recovery of the repair protein levels after 'standard schedule' have been measured in peripheral blood mononuclear cells (PBMCs) [58]. MGMT depletion was seen consistently within 4 h of the first dose, and with further doses over subsequent days this depletion was shown to be cumulative and progressive, whereas a recovery of MGMT levels was observed from 48 h after the last drug dose. These data suggest that a schedule extension of TMZ ('dose-dense regimens') may enhance MGMT depletion and hence be useful to overcome cellular resistance to TMZ associated with overexpression of MGMT [59]. Tolcher *et al.* [60] have reported the results of MGMT depletion in PBMCs after two different extended schedules of TMZ. A schedule of 50–175 mg/m^2/day every day for 7 days on alternate weeks reduces MGMT activity by 72% on day 7 and by 55% on day 14, whereas a schedule of 50–150 mg/m^2/day for 21 days followed by 1 week of rest reduces MGMT activity by 63% on day 14 and by 73% on day 21. Overall, these extended schedules allow just over twice the TMZ dose intensity of the 5-day regimen. In recurrent GBMs treated with the 7 days on/7 days off schedule at 150 mg/m^2/day, an increase in progression-free survival at 6 months up to 48% has been reported [61]. By using an extended adjuvant schedule after combined modality treatment a further improvement of progression-free survival over the EORTC study (7.2 versus 5 months) has been reported [62]. An Intergroup (RTOG, EORTC, National Cancer Institute of Canada Clinical Trials Group) phase III, trial comparing standard TMZ with extended TMZ (21 days on/7 days off at 100 mg/m^2/day) in patients with newly diagnosed GBM treated with concomitant TMZ and radiation, is ongoing at the present time.

Other dose-dense TMZ regimens have been explored in phase I/II studies [63–67]: the major problem when employing extended schedules can be a lymphopenia, with a preferential effect on CD4$^+$ T cells, leading to a higher incidence of opportunistic infections (including *Aspergillus* or *Pneumocystis pneumonia*) and thus raising the question of a prophylaxis during treatment.

It is still to be proven whether resuming TMZ at disease progression ('rechallenge'), either in the standard or a dose-dense regimen, is safe and effective [68,69].

The resistance associated with MGMT overexpression could be overcome by combining alkylating agents with specific inhibitors of MGMT, such as O^6-benzylguanine (O^6-BG) or O^6-4-bromothenylguanine. O^6-BG has been shown to suppress MGMT activity to undetectable levels in 94% of patients with malignant gliomas when given shortly before surgery (6 h); nonetheless, a phase II trial of BCNU and O^6-BG in a cohort of patients with nitrosourea-resistant malignant gliomas failed to induce complete or partial responses and myelotoxicity was notable [70]. O^6-BG is now being investigated in association with gliadel and with TMZ. Another approach to reduce the DNA repair is to use specific inhibitors of the base excision repair [71]. This pathway repairs the lesions owing to N^7-methylguanine and N^3-methyladenine, that represent the majority of DNA adducts produced by nitrosoureas or TMZ. It depends on a key enzyme, poly(ADP-ribose) polymerase-1 (PARP-1) and assumes particular importance in cells deficient in the mismatch repair system [72]. Systemic administration of PARP-1 inhibitors significantly increased antitumor

activity of TMZ, CPT-11 and radiation in a variety of human xenograft from different tumors [73,74]. Potent nontoxic PARP inhibitors have been developed and are being investigated in association with TMZ in clinical trials [75].

Combination of cytotoxic agents ('cytotoxic synergy')

One can combine drugs with either similar or different mechanism of action. Nitrosoureas (BCNU, CCNU, fotemustine) and TMZ are both alkylating agents, with independent activity in malignant gliomas: when combined, MGMT depletion could be maximized [76,77]. Preclinical models have observed a synergistic activity of BCNU and TMZ, but have not identified the optimal sequence and schedule to maximize antitumor activity and minimize toxicity [78]. Different schedules have been employed clinically, including administration of both drugs on day 1, as proposed by Plowman *et al.* [78] in preclinical models, but this regimen resulted in significant myelotoxicity, without any additive antitumor activity in phase I and II studies [79–81]. The best-tolerated sequence seems represented by BCNU (on day 1) followed by TMZ (days 1–5), that results in a three-fold decrease in MGMT activity in PBMCs [82]. As neoadjuvant therapy in inoperable GBMs this combination has exhibited promising activity [83]. To avoid the overlapping toxicities, the combination of locally administered BCNU ('gliadel wafers') and TMZ seems attractive as serum BCNU levels are undetectable after gliadel implant [49] and the results of a phase I study are encouraging [84]. Moreover, there seems to be a rationale for sequencing gliadel wafers and TMZ + radiotherapy in resected patients with newly diagnosed GBMs: first, to kill with BCNU-resistant tumor cells in the postoperative period, during which an early tumor repopulation can occur; second, to deplete MGMT by BCNU, so that TMZ could be more effective, and, finally, to exploit a synergism between BCNU and radiation (inhibition of sublethal damage) during the first weeks of radiotherapy. A phase II study of radiation with concomitant and adjuvant TMZ in patients with newly diagnosed supratentorial malignant glioma who have undergone surgery with BCNU wafer insertion is ongoing in the US; preliminary results have been reported [85], and include a 1-year survival rate of 63% and an incidence of deep-venous thrombosis and pulmonary embolism that needs careful surveillance. If the final results confirm the efficacy and safety of this combination, a phase III study comparing gliadel and TMZ + radiotherapy versus TMZ + radiotherapy would be warranted.

The combination of CCNU, TMZ and radiotherapy has yielded promising survival data (2-year survival rate of 44.7%), and acceptable toxicity in patients with newly diagnosed GBM [86].

Both procarbazine and cisplatinum have been explored as MGMT modulators before nitrosoureas or TMZ with contradictory results [87–89].

BCNU and cisplatin have been suggested to be synergistic, and moreover cisplatin has a well-known radiation sensitizer effect that can persist long after administration, thus being attractive to be incorporated in chemotherapy regimens in the preradiation setting. Despite these premises and impressive response rates in phase II trials [90], continuous infusion of BCNU and cisplatin before radiotherapy in newly diagnosed malignant gliomas has not led to improvements in survival and having relatively high toxicity [91].

Alkylating agents are ideal candidates for combination therapy with CPT-11, because they have different mechanisms of action and different systemic toxicities. Irinotecan (a camptothecin derivative) is a topoisomerase I inhibitor with substantial activity in glioma cell cultures and human GBM xenografts [92]. After intravenous injection, it is metabolized in the liver into the active metabolite SN-38 that has some ability to cross the BBB. Clinical trials using CPT-11 in patients with recurrent malignant gliomas have reported response rates ranging from 2 to 15%, and extended progression-free survival at 6 months as high as 56% [93–96]. As the concurrent administration of enzyme-inducing antiepileptic drugs enhances the hepatic metabolism of irinotecan and SN-38, the maximum tolerated dose for patients who are taking enzyme-inducing antiepileptic drugs is higher.

BCNU and irinotecan display a schedule-dependent synergistic activity against glioma cell lines, with maximal activity achieved when administering BCNU before irinotecan [97]. To date, BCNU and irinotecan for patients with malignant gliomas have not been proven to be superior to irinotecan alone, and may be associated with increased pulmonary toxicity [98]. To enhance the BBB penetration and reduce the systemic toxicity, a locally delivered camptothecin (by biodegradable wafers) could be coupled with systemic BCNU, as suggested in an intracranial brain tumor model [99].

Another alternative option is represented by TMZ combined with irinotecan, that has demonstrated encouraging preclinical activity [100]. A phase I trial with escalating doses of irinotecan and a standard dose of TMZ in recurrent GBMs has shown a response rate of 14%, with a progression-free survival at 6 months of 27% [101]; a number of phase II studies are ongoing.

The combination of TMZ and pegylated-liposomal doxorubicin has been suggested as a potentially active regimen [102]: doxorubicin has in fact some antiglioma

activity [103] and pegylation increases the penetration through the BBB.

Combination of cytotoxic, cytostatic and targeted molecular agents

The rationale for combining cytotoxic and cytostatic agents lies in the different mechanism of antitumor activity and nonoverlapping side effect profiles. Cytostatic agents may have antiangiogenetic, antiinvasion/migration and differentiating properties [104].

Thalidomide, a synthetic compound initially used as a sedative, inhibits endothelial cell proliferation and basic fibroblast growth factor-induced angiogenesis, and has immunomodulatory effects. To enhance its modest activity as single agent in malignant gliomas, thalidomide has been combined with cytotoxic agents such as BCNU and TMZ. Fine *et al.* [105] evaluated the combination of BCNU and thalidomide in a cohort of patients with predominantly recurrent GBMs. The response rate was 24% and the 6-month progression-free survival 27%: these results compared favorably with historical data for BCNU alone. The combination of TMZ and thalidomide (both oral agents) as adjuvant therapy (concurrent or following radiotherapy) has yielded interesting results [106,107]: in the study of Chang *et al.* [107] the median survival of newly diagnosed GBM patients (73 weeks) appeared to be better than that after radiation therapy alone, but similar to that after radiotherapy and adjuvant nitrosoureas. Common side effects of thalidomide were sedation, constipation, peripheral neuropathy and skin rash. Lenalidomide (CC-5103) is an analog of thalidomide, which has increased antiangiogenetic activity and fewer side effects, and is under investigation.

TMZ and cilengitide, an integrin antagonist, could be synergistic in inhibiting the increased expression of $\alpha_v\beta_3$ integrin after radiotherapy [108], thus interfering with the radiation-induced upregulation of angiogenesis.

Cyclooxygenase-2 is often upregulated in gliomas and there is increasing evidence that COX-2 inhibitors may block angiogenesis and growth [109]. Clinical trials combining Cyclooxygenase-2 inhibitors, such as celecoxib or rofecoxib, with irinotecan [110] or TMZ [111,112] have been performed with some activity.

High-dose tamoxifen, a protein kinase C inhibitor, has shown some activity both alone [113] and in combination with carboplatin [114] or procarbazine [115]. Protein kinase C inhibitors (tamoxifen, staurosporine, hypericin and calphositin C) act as chemosensitizers by decreasing the antiapoptotic protein bcl-2 and increasing the proapoptotic protein Bax, and thus could enhance the cytotoxicity of irinotecan [116]. The association of high-dose tamoxifen and continuous TMZ in recurrent malignant gliomas is not effective and relatively toxic [117].

Enzastaurin is an inhibitor of protein kinase-β2 with potent antiangiogenetic activity in preclinical models. A phase II trial in patients with recurrent malignant gliomas has shown promising activity (14 objective radiographic responses out of 79 evaluable patients, i.e. 18%) [118]. An ongoing phase III study is comparing enzastaurin to CCNU in patients with GBM at first relapse and a trial of enzastaurin with TMZ is planned.

Marimastat is a low-molecular weight peptide, which inhibits the family of enzymes known as matrix metalloproteinases. matrix metalloproteinases degrade the extracellular matrix, and thereby enable tumor invasion and migration. In-vitro studies of marimastat demonstrated a significant inhibition of invasion of glioma cell lines [119]. Single-agent marimastat has failed to improve survival in patients with GBM or gliosarcoma following surgery and radiotherapy [120]. Two phase II trials tested marimastat in combination with TMZ in patients with recurrent GBM and anaplastic gliomas, respectively. In the first study [121], the combination resulted in a progression-free survival at 6 months that exceeded the historical control group by 29%. Joint and tendon pain were the major therapy-related side effects, occurring in 47% of patients and leading to interruption of treatment in 11%. In the second study [122], the regimen was roughly equivalent to single-agent TMZ and was associated with additional toxicity.

Differentiating agents, such as retinoids, induce the differentiation of malignant cells into mature cells, and can also suppress cell proliferation and induce apoptosis. Retinoids have been studied in clinical trials with good tolerance but modest success [104]. The combination of 13-*cis*-retinoic acid with TMZ in malignant gliomas, both at recurrence [123] and adjuvantly with concurrent radiotherapy [124], has yielded results not superior to that obtained with conventional therapy.

Many targeted molecular agents are currently being evaluated in the treatment of malignant gliomas [125,126]. Studies in colorectal cancer have shown that targeted therapeutics can enhance the activity of cytotoxic chemotherapy, being the combinations far more active than single agents alone [127,128].

The EGFR is an attractive therapeutic target in GBM, as it is frequently amplified, overexpressed and mutated, and is associated with resistance to treatment with radiation and chemotherapy [129]. Several EGFR tyrosine kinase inhibitors are available. Gefitinib (ZD 1839), alone or in combination with TMZ, has been investigated

in malignant gliomas, both at recurrence and adjuvantly (in association with radiotherapy): overall, the activity seems modest, but more definitive results are still awaited. Two aspects are noteworthy: first, there was no correlation between EGFR expression and response, and patients who experienced diarrhea and rash survived significantly longer than those without these complications, thus suggesting that patients with a higher drug exposure derive maximal benefit from the therapy [130]. Preliminary data with erlotinib (OSI-774) seem more encouraging than with gefitinib, both in terms of response and duration of progression-free survival. The advantage of erlotinib could derive from the inhibition of EGFRvIII mutant receptor, which is constitutively active and thus ligand-independent. Recent correlative studies suggest that the response to erlotinib could be restricted to the subgroup of patients with a specific molecular signature, i.e. with coexpression of EGFRvIII and PTEN [131] or with EGFR amplification/expression and low levels of protein kinase B/Akt phosphorylation [132]. The combination of erlotinib with TMZ in both recurrent and newly diagnosed (in association with radiotherapy) GBMs has yielded encouraging preliminary results [133,134], and is being now investigated in new trials.

Imatinib mesylate (STI 571) is a small molecule acting as a tyrosine kinase inhibitor of platelet-derived growth factor receptors (PDGFRs). PDGFRs are overexpressed in up to two-thirds of GBM. This agent has demonstrated significant antitumor activity in chronic myelogenous leukemia by inhibiting the Abl tyrosine kinase and in gastrointestinal stromal tumors by inhibiting the c-kit. Several phase II studies in recurrent gliomas have been conducted by the North American Brain Tumor Consortium and the EORTC [135,136], showing a minimal–modest activity when used as monotherapy. Two papers have recently suggested the efficacy of the combination of imatinib and hydroxyurea (both administered continuously) in recurrent GBMs, with a response rate of 9–20% and a progression-free survival at 6 months of 27–32% [137,138]. Different explanations exist for the combined activity of imatinib and hydroxyurea. First, imatinib may enhance hydroxyurea cytotoxicity, either by improving its delivery into the tumor microenvironment (acting as a substrate for the multidrug transporter protein ABCG2/BCRP at the BBB and tumor cellular levels) or diminishing tumor cell DNA repair after chemotherapy. Second, the inhibition of angiogenesis by imatinib, primarily obtained by targeting perivascular cells, could have been potentiated by metronomic chemotherapy (continuous hydroxyurea administration). Last, hydroxyurea, by virtue of its ability to cross the BBB, could enhance the delivery of imatinib by inhibiting the activity of multidrug transporters. A phase II trial of imatinib with TMZ is ongoing. For future studies, the analysis of PDGFR expression and/or mutations in tumor samples, as possible determinants of response to therapy, will be critical.

Vascular endothelial growth factor receptors (VEGFRs) are commonly overexpressed in GBMs and VEGF inhibitors are promising agents to interfere with angiogenesis and reduce peritumoral edema. PTK 787/ZK222584, an oral VEGFR tyrosine kinase inhibitor, has been shown to decrease glioma growth and vascularization in rats [139] and is undergoing phase I/II studies in malignant gliomas, alone or in combination with CCNU or TMZ [140]. A phase II trial investigating the addition of PTK 787 to standard TMZ/radiotherapy in newly diagnosed GBM patients has been launched by the EORTC. In combination with irinotecan, bevacizumab, the monoclonal antibody against vascular endothelial growth factor has shown activity in patients with recurrent high-grade glioma (response rate of 63% and median progression-free survival of 24 weeks) [141]: confirmatory trials are ongoing or planned.

Farnesyltransferase inhibitors inhibit the activity of an enzyme implicated in the Ras/PAPK signaling pathway. Synthetic farnesyltransferase inhibitors, such as tipifarnib (R111577) and lonafarnib (SCH66336), are being tested in clinical trials in GBMs, either alone [142] or in association with radiation and/or TMZ [143].

Compounds such as mummalian target of rapamycin inhibitors could interfere with the activation of the phosphatidylinositol-3-kinase/Akt pathway, both alone and in combination with radiotherapy, cytotoxic chemotherapy and other molecular agents [126,144].

The TP53 status seems to influence the resistance to TMZ [145]: both inhibitors [146] and agonists [147] of p53 function have been reported to sensitize different glioma cell lines to TMZ.

Conclusions and future advances

The history of medical treatment of malignant gliomas has demonstrated that methodological issues are critical to evaluate new treatment agents and, ultimately, to achieve further advances. The identification of homogeneous subsets of patients with high-grade gliomas on the basis of pretreatment prognostic factors (recursive partitioning analysis classes) [148], has proven useful for correctly interpretating phase II or III studies results and determining if a particular category of patients would benefit most from newer approaches [149,150]. The adoption of a progression-free end-point (i.e. progression-free survival at 6 months for recurrent GBMs) instead of a response end-point in terms of tumor reduction on neuroimaging can address concerns owing to previous surgery or radiotherapy, or to treatment with targeted compounds that inhibit tumor progression rather than

cause tumor regression. Innovative trial designs (factorial and adaptative designs) now allow multiple treatment combinations to be evaluated simultaneously although requiring far fewer patients than would be necessary by conducting an equivalent number of sequential phase II studies. This permits rapid elimination of ineffective regimens and replacing ineffective or toxic arms to maintain accrual so that patients move seamlessly into a definitive comparative testing of the most promising options. Common to all future trials is the need for tumor tissue to stratify for known prognostic molecular markers and to conduct associated translational research. Two aspects are noteworthy in regard to molecular agents. Pretreatment molecular profiling of tumors will be increasingly needed to determine if the mechanism of a drug is appropriate to the genetic alterations found within individual tumors. In addition to traditional clinical end-points, biological end-points (change in serum tumor markers, measures of target inhibition) seem to be appropriate, and in particular dosing schedules in phase I trials that focus on the determination of an optimal biological dose rather than the maximum tolerated dose must be explored [151]. Finally, multitargeted agents are needed to target simultaneously multiple signaling pathways that concur at the same time or sequentially, as a compensatory activation, to tumor growth and resistance to treatments.

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